http://researchcommons.waikato.ac.nz/

Research Commons at the University of Waikato

Copyright Statement:

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

The thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of the thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from the thesis.

Characterization of an Alkaliphilic Thermophilic *Bacillus* isolate TA2.A1

A thesis submitted in partial fulfilment
of the requirements for the degree
of
Doctor of Philosophy
in Biological Sciences

by

Catherine Joan Peddie

The University of Waikato
Te Whare Wānanga o Waikato

The University of Waikato
Hamilton, New Zealand
1999

Abstract

A novel aerobic, alkaliphilic, thermophilic *Bacillus* species was isolated from a thermal bore in Te Aroha, New Zealand with a source temperature and pH of 77°C and pH 8.28. The strain designated TA2.A1 was a Gram positive spore-forming rod with a filamentous morphology that grew optimally at pH 9.2 and 70°C. The organism grew over a temperature range of 45°C to at least 75°C and a pH range of 7.7-10.5. The substrate range that supported growth was unusual and included glycerol, sodium pyruvate, trehalose and sucrose. The monosaccharide components of sucrose, glucose and fructose, did not support growth. Membrane lipid analysis was typical of that of many thermophilic bacteria with a very high proportion of saturated branched chain fatty acids. The DNA base composition of isolate TA2.A1 was 47.3 mol% guanine plus cytosine. When the 16S rDNA sequences of *Bacillus* isolate TA2.A1 and other *Bacillus* strains are aligned, six sequences including *Bacillus* isolate TA2.A1 contained a five base pair insert positioned at bases 36-40 from the 5′ end of the isolate TA2.A1 sequence. The five base pair insert was the same for isolate TA2.A1 and fragment ab009 (complete 16S rDNA sequence not currently available) from a clone library from mesophilic sewage sludge. A phylogenetic dendogram indicated that isolate TA2.A1 was a deeply branching member of the *Bacillus* genus.

Bacillus strain TA2.A1 utilized glutamate as a sole carbon and energy source for growth and sodium chloride (> 5mM) was an obligate requirement for growth. Growth on glutamate was inhibited by monensin and amiloride, both inhibitors that collapse the sodium gradient (ΔpNa) across the cell membrane. N,N- dicyclohexylcarbodiimide inhibited the growth of *Bacillus* strain TA2.A1 suggesting that an F_1F_0-ATPase (H type) was being used to generate cellular ATP needed for anabolic reactions. Vanadate, an inhibitor of V-type ATPases, did not affect the growth of *Bacillus* strain TA2.A1. Glutamate transport by *Bacillus* strain TA2.A1 could be driven by an artificial membrane potential ($\Delta\Psi$), but only when sodium was present. In the absence of sodium, the rate of $\Delta\Psi$-driven glutamate uptake was fourfold lower. No glutamate transport was observed in the presence of ΔpNa alone (i.e., no $\Delta\Psi$). Glutamate uptake

was specifically inhibited by monensin, and the K_m for sodium was 5.6mM. The Hill plot had a slope of approximately 1 suggesting that sodium binding was non-cooperative and that the glutamate transporter had a single binding site for sodium. Glutamate transport was not affected by the protonophore carbonyl cyanide m-chlorophenylhydrazone, suggesting that the transmembrane pH gradient was not required for glutamate transport. The rate of glutamate transport increased with increasing glutamate concentration; the K_m for glutamate was 2.90 μM, and the V_{max} was 0.7 nmol.min^{-1} mg of protein. Glutamate transport was specifically inhibited by glutamate analogues.

Sucrose uptake by isolate TA2.A1 was also evaluated and was actively transported by the presence of sodium ions, probably by a sucrose/Na^+ symport. A number of thermophilic microorganisms use sodium-coupled uptake for amino acid utilization. However, the fact that both sucrose and glutamate uptake in isolate TA2.A1 were sodium dependent was unusual. [^{14}C]sucrose uptake was inhibited by cold trehalose which suggested that both carbohydrates may be competing for the same uptake system. Sucrose grown cells could transport [^{14}C]glucose at low concentrations but the rate was 10 fold lower than for [^{14}C]sucrose transport. No transport of [^{14}C]fructose by sucrose grown cells could be demonstrated.

Acknowledgements

I wish to thank my supervisor Prof. Hugh Morgan for his advice and help throughout this project and in the writing of this thesis.

Thanks to Colin Monk for his technical assistance (and reliability in many different situations) and to Lynne Parker for her technical assistance and reminding me to "go and buy a hat" occasionally.

I am thankful to Dr Greg Cook for his advice and enthusiasm with respect to the bacterial energetics work. I am also grateful to Dr Merilyn Manley-Harris for the helpful discussions on carbohydrate chemistry and to Dr Dave Saul for his assistance with phylogenetic dendrograms.

Thanks to the many people who have made the thermophile lab an enjoyable place to work: Dion, Helen, Judy, Rachel, Richard, Ron and to Dorothee and Peter for their help and humour.

Thanks to Rosemary, Rachel, Diane and other staff in the science department at Waikato Polytechnic for their support and understanding.

Thanks to all the friends who have supported me throughout this project in many different ways (you know who you all are!)

This thesis is dedicated to the memories of:
Bessie and Fred Jones, Hilary and Vivian (Wib) Skilton, Ada Turton and Brian Skilton, all of whom continue to be an inspiration to me.

Last but not least, thanks to my family for all their love, support and encouragement.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

T_{min}	minimum temperature
T_{opt}	optimum temperature
T_{max}	maximum temperature
$\Delta\Psi$	membrane potential
ΔpH	pH gradient
Δp	proton gradient / proton motive force
$\Delta\mu$	electrochemical energy
$\Delta\mu H^+$	proton electrochemical energy
ΔpNa	sodium gradient / sodium motive force
ATP	adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
P_i	inorganic phosphate
3-0-β-D-G	3-0-β-D-glucopyranosylfructose
1-0-β-D-G	1-0-β-D-glucopyranosylfructose
MOPS	3-[N-Morpholino]propane-sulfonic acid
TAPS	N-tris[Hydroxymethyl]methy-3aminopropanesulfonic acid
CAPS	3-[Cyclohexylamino]1-propanesulfonic acid
CCCP	carbonyl cyanide -chlorophenylhydrazone
DCCD	N,N-dicyclohexylcarbodiimide
[U-^{14}C]	universally labeled carbon 14
pNP	p-nitrophenol
TVA	teichuronic acid
TUP	teichuronopeptide
ND	not done / not determined
ANO_2	anaerobic
G+C	guanine + cytosine

Chapter 1 - Introduction

From a human perspective, habitats with a pH close to neutral and a temperature range from 20-35°C are regarded as non-extreme. When compared to the number of organisms found living in these "normal" conditions, relatively few have been isolated at extremes of temperature (high and low), pH (acid and alkali), osmolarity or inhibitor concentration (e.g. metal ions). This may, however, reflect the relative infancy of study into the organisms living in these environments, rather than the number that actually exist. Certainly the thermophilic organisms discovered to date are extremely phylogenetically diverse.

Because microorganisms grow in a greater range of extreme conditions than any other organisms they are excellent models for understanding adaptation to extreme environments. The subject of this thesis is an investigation of a bacterium displaying adaptation to two extremes, those of high pH and high temperature.

Alkaliphilic organisms have been investigated for two principal reasons. Firstly, because the extracellular enzymes that they produce are generally stable at high pH and so have industrial and biotechnological applications (for example, in detergents and washing powders). Secondly, there is interest in the energetics of these organisms and the mechanisms used for survival at high pH. Bacteria that grow at alkaline pH have to overcome bioenergetic problems in terms of chemiosmotic energy generation and solute transport driven by the proton motive force (Krulwich *et al.* 1996). It is generally accepted that alkaliphiles use sodium/proton antiporters to acidify the cytosol and generate an inwardly directed sodium motive force (Krulwich and Guffanti 1989). The use of sodium as a coupling ion circumvents the problem of a low proton motive force. Growth at extremes of temperature also poses the additional problem of a cytoplasmic membrane that is 'leaky' to protons (van de Vossenburg *et al.* 1995). Some thermophilic bacteria overcome this problem by using sodium as a coupling ion for solute transport.

Thermophilic organisms have been studied not only in a quest to ascertain the upper temperature limit for life, but in an attempt to understand the adaptations necessary to survive under conditions of high temperature. Like other extremophilic organisms there has been interest in the enzymes that thermophilic bacteria produce since they are often more thermostable than those from mesophilic isolates.

When the adaptations necessary for survival at both high pH and temperature are considered, the study of alkaliphilic thermophiles presents a unique opportunity to understand adaptations, both in terms of energetics and bacterial structure, to an environment that imposes many challenges on its inhabitants.

Compared to the number of species capable of growth at either high pH or high temperature few species or strains capable of growth under both alkaliphilic and thermophilic conditions have been described (Li *et al.* 1993, 1994; Prowe *et al.* 1996; Wiegel 1998).

Chapter 2 – Literature Review

2.1 Thermophilic organisms

2.1.1 What defines an organism as thermophilic

The temperature range over which an organism can grow and reproduce begins at T_{min} where growth starts and then the growth rate increases almost linearly for about 15 to 25°C, up to the optimum growth temperature (T_{opt}), after which it usually falls abruptly for the next 5 to 10°C to T_{max}, above which no growth occurs (Kristjansson and Stetter 1992; Russell and Fukunaga 1990). These three "cardinal temperatures" are characteristic for each microbial species or strain. Clarifying bacterial groups according to cardinal temperatures is difficult since there are always exceptions and overlaps. Microorganisms have traditionally been divided into three main groups in this respect, i.e. psychrophiles, mesophiles and thermophiles. There has been a dramatic evolution in the notion of extreme thermophile, which, in the 1960s, was applied to bacteria with temperature maxima of 65-70°C, whereas the present (and perhaps not definitive) limit is above 110°C. Aragno (1992) proposed that a moderate thermophile was any organism with a temperature optimum between 45 and 60°C, and that strict or extreme thermophiles had temperature optima between 60-90°C and greater or equal to 90°C respectively. Kristjansson (1989) suggested the definition of thermophilic and extremely thermophilic procaryotes as those which can grow from 55-60°C to 80-85°C and hyperthermophiles as those which can grow optimally above 80-85°C. A broader definition of what constitutes a thermophilic organism states that organisms with a minimum temperature above 25°C, an optimum temperature above 45°C, and a maximum temperature above 50°C should be considered thermophilic (Wiegel 1990).

The definitions used in this thesis were chosen as they gave a general overview of three descriptive groups of thermophilic organisms most relevant within the scope of this thesis and were based on Farrell and Campbell (1969).

1. Strict or obligate thermophiles show optimal growth at 65-70°C, and do not grow below 40-42°C.
2. Facultative thermophiles have a maximum temperature for growth between 50°C and 65°C and are capable of growth at room temperature.
3. Thermotolerant organisms have a maximum growth temperature of 45-50°C and also grow at room temperature.

Thermophilic organisms are often listed with their maximum temperature as their main characteristic. Due to the quest for the upper temperature limit of life and the interesting biochemistry at high temperature this is not surprising. However, ecologically the optimum growth conditions of an organism are more important. Thermophilic bacteria will survive best in micro-environments where the temperature is closer to the growth optimum (Kristjansson and Stetter 1992) than either of the other cardinal temperatures. This gives them greater flexibility to withstand fluctuations in temperature.

2.1.2 Where are thermophilic organisms found

Although thermophiles are obviously adapted to growth at high temperatures, they are frequently found where high temperatures do not occur. In fact their distribution is quite ubiquitous, having been isolated from soils of both temperate and tropical regions, snow and permafrost, air, both cold and thermal salt and fresh water, from foods and grain, raw and pasteurized milk, composting vegetation, sewage digesters, deep ocean basin cores and deep sea hydrothermal vents.

A. Nongeothermal habitats

1. Organic compost piles

Composting involves a rapid succession of microorganisms which are successively more thermophilic, where the climax thermophilic microflora comprise mainly facultative thermophiles or thermophilic spore-formers, likely to have a widespread distribution through transport by air. The high temperatures (60–80°C) of compost might last for

several weeks, and eventually decrease when the most easily metabolisable materials are exhausted (Beffa *et al.* 1996; Blanc *et al.* 1999). Beffa *et al.* (1996) reported the presence of highly thermophilic chemolithoautotrophic bacteria related to the genus *Hydrogenobacter* in hot compost. Other bacteria isolated from compost include *Bacillus schlegelii* (Beffa *et al.* 1996) and *Bacillus stearothermophilus* strains (Strom 1985a, 1985b).

2. Leach dumps of sulfide minerals

Sulfide mineral piles, which often have temperatures as high as 50-80°C, are the typical habitats of highly acidophilic, sulfur- and iron-oxidizing bacteria. By enrichment at 65°C *Sulfolobus*–like organisms were isolated (Marsh and Norris 1983a) from these leach dumps. Several authors have obtained cultures of moderately thermophilic (optimum temperature around 50°C), strongly acidophilic, iron and sulphur oxidizers (Brierley 1978; Brierley and Lockwood 1977; Hendy 1987; Marsh and Norris 1983b) among them the spore-former *Sulfobacillus thermosulfidooxidans* (Golovacheva and Karavaiko 1978; Kovalenko and Malakhova 1983; Vartanyan *et al.* 1988).

3. Other man-made sites

Several man-made, constant hot environments have been created. These include hot tap water (Pask-Hughes and Williams 1975), hot water heaters (Brock and Boylen 1973; Brock 1978), waste treatment plants, heat exchangers, water discharged from steel mills or nuclear power plants (Stramer and Starzyk 1981) and sugar refinaries (Nystrand 1984). Many processes in the food and chemical industries use evaporation or extraction run at high temperatures creating a suitable environment for thermophilic microorganisms. A wide variety of thermophilic microorganisms have been isolated from sugar refinaries including: *Saccharococcus thermophilus* (Nystrand 1984), *Bacillus stearothermophilus*, *Thermoanaerobacter thermohydrosulfuricus* (*Clostridium thermohydrosulfuricum*), *Thermoanaerobacterium thermosaccharolyticum* (*Clostridium thermosaccharolyticum*), *Desulfotomaculum nigrificans* and *Bacillus thermoaerophilus* (Meier-Stauffer *et al.* 1996; Hollaus and Klaushofer 1973). In contrast, other sites for

example hot tap water, have yielded a less diverse range of thermophilic organisms (Kristjánsson *et al.* 1994).

B. Geothermal habitats

Hot environments are found in volcanic areas above and below sea level and in deep rock formations heated by the earth's interior. The highest habitat temperature for a bacterial species is 95°C for *Aquifex pyrophilus* (Huber *et al.* 1992). However, archaeal species can grow to temperatures of 113°C, (Blöchl *et al.* 1997) in marine and volcanic areas. Geothermal sites are flushed by anoxic gases from underground and this combined with the limited availability of oxygen at higher temperatures means that anaerobic organisms predominate. The temperature, pH, presence of potential inorganic electron donors, presence of carbon dioxide, and limitation of oxygen all vary in geothermal sites. These sites generally have a very low organic matter content and high concentrations of dissolved mineral salts. Brock (1971) in a survey of hot springs in New Zealand, Iceland and the USA noted that most of the pools sampled either had a pH in the range of 2-4 (acid sulphate) or 7-9 (neutral bicarbonate). Thermophilic bacteria have been isolated from other sites including steam-warmed soil in the antarctic (Hudson and Daniel 1988; Hudson *et al.* 1989), deep-sea hydrothermal vents (Marteinsson *et al.* 1995, 1996) and hot North Sea oil field water (Beeder *et al.* 1995).

2.1.3 How did thermophilic organisms originate and adapt to high temperature

Thermophilic bacteria are distributed among all the main groups in the bacterial tree of life. Interestingly hyperthermophiles are usually the deepest branching types in their respective groups. This is interpreted as thermophily being very early in the evolution of bacteria and even that the ancestral bacterium might have been a thermophile (Achenbach-Richter *et al.* 1987). It has been suggested that both *Archaea* and *Bacteria* evolved from thermophilic ancestors (Woese 1987; Stetter 1992). The rooting of the tree of life in the bacterial branch (Woese *et al.* 1990; Stetter 1992) supported this theory. Supporters of a thermophilic origin of life argue that high temperatures in highly pressurized environments could create, instead of destroy, organic compounds (Holm

1992). Shock *et al.* (1998) reported on the potential for organic synthesis in submarine hydrothermal systems on the early Earth and that the formation of organic compounds would lower the free energy of these systems, increasing their likelihood as environments for the emergence of metabolism. This report suggested that the synthesis of amino acids required for life may be considerably easier at high temperatures than at temperatures conventionally considered 'hospitable'. Baross (1998) stated that the best evidence to support a hyperthermophilic (thermophilic) ancestor was in the geological record particularly since hydrothermal vents are primordial and their emergence coincided with the accumulation of liquid water on Earth. López-García (1998) suggested that before domain diversification a thermophilic ancestor existed that only had relaxing topoisomerases and whose optimal DNA structure was generated spontaneously at high temperature. It was suggested that evolution of supercoil-introducing activities (reverse gyrase and gyrase) would have facilitated adaptation to hyper-thermophily and mesophily in archaea and bacteria respectively.

Against the proposal of a thermophilic origin of life it has been suggested that the adaptation to thermophily probably occurred after DNA was the repository of genetic transfer from one generation to the next (Forterre 1995a, 1995b), since double-stranded DNA is much more resistant to thermodegradation than RNA (Forterre *et al.* 1992; Miller and Bada 1988). Forterre proposed that the universal ancestor was a mesophile and that adaptation to thermophily occurred after divergence between the ancestors of eucarya and the two procaryotic lineages. The ubiquitous occurrence of thermophiles in nonthermophilic environments might suggest that they evolved from mesophiles, either by adaptation or mutation. However this is a matter of debate, it has also been proposed that thermophilic life forms adapted to lower temperatures and still contain many 'thermostable characteristics' (i.e. "cryptic thermophiles) (Wiegel 1990, 1998b). The use of adaptive devices for life at high temperatures such as: positive super coiling (Bouthier de la Tour *et al.* 1991), specific tRNA modifications (Edmonds *et al.* 1991) or formation of macrocyclic diether or tetraether lipids from "classical " diether lipids (Sprott *et al.* 1991) have been cited as evidence that hyperthermophiles are more highly evolved. Studies on the transformation from mesophily to thermophily remain very controversial (Lindsay 1995). It has been argued that the large number of components that are

thermostable in thermophilic bacteria suggest that many genes are involved in specifying the trait of thermophily (Sundaram 1986; Brock 1986). It is also possible that relatively few genes allow for thermophilic growth, however, as Sundaram (1986) commented, there is little hard evidence to support this. While a single mutation may change the thermostability of a protein it would appear unlikely that the transition from mesophily to thermophily would result from the spontaneous mutation of a single gene. It is unlikely that a large number of genetic changes could occur simultaneously to allow thermophily. Lindsay (1988) conducted a study that added weight to the argument that thermophily was a transferable genetic trait, affecting two developmental stages in *Bacillus*. Another possibility is that thermophily may be specified by plasmid borne adapter genes (Stahl and Olsson 1977; Castenholz 1979). If thermophily genes are plasmid encoded, many of the arguments as to the number of mutations required for the conversion to thermophily, are negated. Hyper-thermophiles may appear at the base of some phylogenetic trees because they out competed older mesophiles when they adapted to lower temperatures, perhaps through enhanced production of heat shock proteins or more regulated biosynthetic pathways (Miller and Lazcano 1998). The concept that life originated from one (type of) progenote remains a matter for debate (Wiegel 1998b). Kandler (1998) suggested that an ancestor common to the prokaryote, eukaryote and archaeal domains never existed, and that in fact proto-cells from each domain were formed at different evolutionary stages.

Wiegel (1998b) suggested that early thermophilic and more mesophilic entities co-emerged and co-evolved more or less simultaneously. It was proposed that if different 'temperature variants' were present in the same vicinity, constantly disintegrating and reforming, that some entities took up genetic information which enabled them to cope better with the temperatures (hotter or cooler) that existed. Olendzenski and Gogarten (1998) suggested that a substantial portion of the eubacterial genome was transferred to archaea. They proposed that horizontal gene transfer explained the presence of archaeal-type H^+-ATPases in *Thermus* and *Enterococcus* and the close association between eubacteria and archaea for many different genes. Investigation into the gene translation apparatus of eukaryotes by Lake *et al.* (1998) suggested that the eukaryotic cell is a chimera between mesophilic eubacteria and hyperthermophilic prokaryotes. Wiegel

(1998b) hypothesized that horizontal (interspecies) gene transfer might be a general mechanism in the evolution of microorganisms when changing temperatures impose an evolutionary pressure. Obviously microorganisms that are able to adapt to grow over a wider temperature range have an advantage in competing for nutrients or in survival. Irrespective of how thermophilic organisms originated; what is certain is that a number of their cell components need to be thermostable to ensure their survival.

Growth at high temperatures places a burden on cellular metabolism and means that enzymes, and other macromolecules, need to be more stable (Adams 1993). The intrinsic heat resistance of proteins of obligate thermophiles is often the result of subtle changes in hydrophobic interactions, hydrogen bonds, sulphur-sulphur bonds and ionic bonds within these proteins. Macromolecules (proteins/nucleic acids) and supramolecular structures (ribosomes/membranes) are irreversibly inactivated in non-thermophilic microorganisms by temperatures between 60 and 100°C. In thermophiles these components are usually stable at these temperatures for longer periods of time. Catalytic activities of thermophilic enzymes can be low or absent at moderate temperatures at which conventional enzymes of similar function are optimally active. The main focus of this section is on membrane lipids because of their importance in energy generation and substrate transport systems.

(1) Membrane-phospholipid composition

Membrane structure is vital for a cell because the cell must be separated from its surrounding environment. Archaea are distinctive from bacteria and eukaryotes in the nature of their membrane polar lipids, the stereochemistry of the glycerol moiety of polar lipid in archaea is the opposite of that of glycerol ester membrane lipids in bacteria and eukaryotes (Nishihara *et al.* 1998). Because of the greater physical strength of the archaeal cytoplasmic membranes containing L-glycerol isopranyl ether lipids (Kates 1993), instead of the bacterial D-glycerol acyl ester lipids, the invention of a further stabilising rigid cell wall was not essential for archaea as it was for bacteria (Kandler 1998). Growth at high temperatures requires not only adaptation of (membrane-bound) enzymes with respect to activity and stability but also at the level of membrane

phospholipid composition. The lack of thermophilic eukaryotes has been attributed to the thermolability of their intracellular membrane systems (Brock 1978). Archaea respond to high temperatures by a cyclization of the fatty acyl chains and a transition from diether to tetraether lipids (van de Vossenberg *et al.* 1998; De Rosa *et al.* 1991; Sprott *et al.* 1991; Yamauchi and Kinoshita 1995). As this thesis describes a bacterial, rather than archaeal, isolate the remainder of this paragraph describes membrane composition in thermophilic bacteria with an emphasis on *Bacillus* species. The composition of phospholipid membranes of thermophilic bacteria differs distinctly from mesophilic bacteria. This has been studied in some detail (Reizer *et al.* 1985) in *B. stearothermophilus*, a moderate thermophile where phosphatidyl-glycerol (PG) and cardiolipin (CL) form 90% of the cellular phospholipids. At increasing growth temperature the PG-content increases at the expense of the CL-content. Furthermore, with increasing growth temperature the acyl-chain composition of all membrane lipids changes. There was a gradual depletion of unsaturated fatty acids, a decrease in *iso* and *anteiso* fatty acids (Nicolaus *et al.* 1995) and an increase in abundance of longer saturated-linear fatty acids with increasing growth temperature (Reizer *et al* 1985; Prado *et al.* 1988; Svobodová *et al.* 1988; de Vrij *et al.* 1990). Nicolaus *et al.* (1995) examined the effects of growth temperature on the polar lipid pattern and fatty acid composition of Antarctic thermophilic *Bacillus* isolates. Significant differences in the lipid pattern of each isolate were observed under physiological stress, such as temperatures lower or higher than the optimum growth temperature. Over the entire temperature range of bacterial growth there was a general increase in lipids with a sugar-head from psychrophiles to moderate and extreme thermophiles. It is possible that the sugar-containing lipids increase the hydrogen-bonding capacity of the lipid bilayer surface, thus stabilizing the membrane at high temperatures, perhaps through additional interactions with cell components immediately exterior to the membrane (Russell and Fukunaga 1990). A change in fatty acid composition has been considered to be a prerequisite for keeping the fluidity of the membrane nearly constant over the entire temperature range of growth (homeoviscous adaptation) (Sinensky 1974; Reizer *et al.* 1985; Nicolaus *et al.* 1995; Driessen *et al.* 1996). Thus most of the lipid acyl chains in membranes of thermophilic bacteria are long (C_{18}-C_{24}) and saturated lipids (Driessen *et al.* 1996). For example, examination of Antarctic thermophilic *Bacillus* isolates revealed

branched C17 present in very high amounts compared to the content in the fatty acids of other *Bacillus* species (Kampfer 1994). Differences in membrane fatty-acid composition of thermophilic and mesophilic bacilli have been used to explain similar apparent membrane-microviscosities of these bacteria at their respective growth temperatures (de Vrij *et al.* 1990; Van de Vossenberg *et al.* 1995). The maintenance of appropriate membrane fluidity is of particular importance to thermophiles since membrane stability is one of the major factors allowing the survival of thermophiles in hot environments.

(2) Efficiency of energy-generation at elevated temperatures

Membrane stability however, is not the only factor that must be considered when discussing the adaptations of thermophilic microorganisms to high temperature. For example significant differences in turnover capacity of the respiratory chain, thermostability of enzymes and ion permeabilities of the membranes in membrane vesicles of the mesophilic *B. subtilis* and the thermophilic *B. stearothermophilus* have been recognized. In membranes from *B. stearothermophilus* extremely high oxidation rates are required to maintain a significant electrochemical proton gradient (Δp) at the growth temperature of the organism, indicating the high ion-permeability of the membranes and consequently inefficient energy-transduction. In thermophilic bacteria the homeo-proton permeability adaptation cannot be maintained, so in order to maintain their proton motive force at a viable level these organisms must use other methods. For example, increasing respiration rate with increasing temperatures (De Vrij *et al.* 1988) or shifting to the less permeable sodium ions as the main coupling ions for energy transduction.

Chemical reactions and diffusion proceed faster at higher temperatures. If coupling ions permeate through the membrane too fast the organism will be unable to establish or maintain a proton or sodium motive force. Because of this, proton permeability is a major factor in determining the maximum growth temperature. For most bacteria the physical properties of the cytoplasmic membrane are optimized to ensure low proton permeability at the respective growth temperature. Membranes are highly permeable when temperature is close to the maximal growth temperature of the organism (Van de

Vossenberg *et al.* 1995). At the growth temperature of thermophilic bacteria the permeability of the cytoplasmic membrane to protons is high compared to sodium ions. The lower permeability of sodium ions is thought to be due to the fact that sodium has a bigger hydration shell, which it must remove itself from before diffusing through the membrane, this process requires energy and slows down the rate of diffusion. In some thermophilic organisms therefore, sodium is the sole coupling ion. Comparison of the proton and sodium permeability of the membranes of a variety of bacterial and archaeal species that differ in their optimal growth temperature reveals that the permeation processes of proton and sodium ions must occur by different mechanisms (Van de Vossenberg *et al.* 1995).

Proton permeability increases with temperature and has a comparable value for most species at their respective growth temperatures. For thermophilic alkaliphiles the increase in proton permeability with increasing temperature may be especially pressing since they need to maintain an intracellular pH that is lower than the external pH (Krulwich and Ivey 1990). Having defined what constitutes a thermophilic microorganism, where they are found and a number of adaptations necessary to survive at high temperatures, the characteristic of alkaliphily will be discussed next.

2.2 Alkaliphilic organisms

2.2.1 Where are alkaliphiles found

Alkaliphiles can be isolated from environments that are non-extreme and transiently alkaline due to biological activity (ammonification, sulphate reduction), for example, in garden soil (Kimura *et al.* 1994; Langworthy 1978). Often the organisms from transiently alkaline environments are *Bacillus* species. Not surprisingly a wider range of alkaliphilic organisms are found in environments of increased pH. The naturally occurring alkaline environments on Earth include: alkaline soda (Na_2CO_3) lakes (pH >11.5) (Rift Valley) (Jones *et al.* 1998; Duckworth *et al.* 1996), alkaline salt lakes (pH 11) (Grant *et al.* 1979), thermal springs (pH 8.5 and greater) (Iceland) (Mathrani *et al.* 1993), thermal bore (pH 8.28) (Te Aroha, NZ) (This study). In these natural alkaliphilic environments sodium carbonate is the major source of alkalinity. An overview of these naturally occurring alkaline environments is given below.

There are also man-made alkaline environments, for example commercial processes, such as cement manufacturing, electroplating, paper manufacturing, the lye treatment of animal hides (Grant *et al.* 1990) and bauxite-processing (Agnew *et al.* 1995) for aluminum production all generate alkaline conditions and waste products. Potato processing (Gee *et al.* 1980) and indigo dying (Takahara and Tanabe 1960, 1962) also generate alkaline wastes. Many man-made environments have been sources of new alkaliphilic species, for example, the denitrifying *Halomonas desiderata* which grows optimally at pH 9 – 10 (Berendes *et al.* 1996) was isolated from a sewage treatment plant.

(a) Alkaline soda lakes and soda deserts

These are widely distributed but until recently poorly investigated due to their inaccessibility. Soda lakes are highly alkaline extreme environments that form in closed drainage basins, characterized by the presence of large amounts of sodium carbonate (or complexes of this salt) formed by evaporative concentration. As this occurs, other salts,

especially NaCl, also concentrate leading to alkaline environments that are also a little saline (Grant *et al.* 1990). Because of the scarcity of Mg^{2+} and Ca^{2+} in the water chemistry the lakes become enriched in CO_3^{2-} and Cl^-, with pH values that range from 8 to above 12 (Jones *et al.* 1998). Soda lakes tend to have microorganisms predominating as permanent or seasonal blooms, consequently these lakes often appear coloured (Grant and Tindall 1986). Highly saline alkaline brines have a prokaryote population distinct from that in more dilute lakes. Very saline lakes are coloured red by large numbers of haloalkaliphilic archaebacteria (Grant and Tindall 1986; Imhoff *et al.* 1979). These obligately halophilic and alkaliphilic microorganisms seem to represent distinct evolutionary lineages within the general halobacterial line (Ross and Grant 1985; Grant and Larsen 1989). These organisms have pH optima between 9 and 10, salinity optima between 2.5 and 4.0M NaCl (Tindall *et al.* 1984) and growth at very low Mg^{2+} concentrations. Duckworth *et al.* (1996) studied the phylogenetic diversity of soda lake alkaliphiles. They reported that Gram negatives were confined to the *Proteobacteria*, Gram positives were found both in low and high % G+C categories, many relating to either *Bacillus* or *Arthrobacter* spp. Two genera of haloalkaliphilic archaebacteria are recognised. These are *Natronobacterium* and *Natronococcus* (*N. occultus*) (Grant 1989a, 1989b; Duckworth *et al.* 1996). Unlike other archaebacteria these have membranes that contain significant amounts of C_{25} isoprenoid chains in ether linkage to glycerol (De Rosa *et al.* 1982, 1983).

Many organotrophic bacteria, from a wide range of taxa (G+C mol% 30-70), have been isolated from soda lakes and unlike those from soil environments, spore-formers are rare. Very saline lakes usually only have haloalkaliphilic archaebacteria isolated from them, but alkaliphilic and halotolerant *Bacillus* -types are occasionally enriched from these environments (Weisser 1983). Sorokin *et al.* (1998) isolated an alkaliphilic *Nitrobacter* species (*N. alkalicus*) after enrichments at pH 10 from sediments of three soda lakes (Siberia and Kenya) and from a soda soil (Siberia). The isolates differed from representatives of the genus *Nitrobacter* by the presence of an additional S-layer in their cell wall and by their unique capacity to grow and oxidize nitrite under highly alkaline conditions. Duckworth *et al.* (1998) isolated *Dietzia natronolimnaios* an alkali-tolerant aerobic organotrophic bacteria from a moderately saline and alkaline East African soda

lake. Zhilina *et al.* (1998) isolated moderately haloalkaliphilic, obligately anaerobic, fermentative, motile, Gram-positive bacteria, from soda lake deposits in Kenya. These mesophilic alkaliphiles depended on sodium and bicarbonate ions for growth, utilized only two amino acids (histidine and glutamate) and were reported as the new genus and species *Natranoincola histidinovorans* (Zhilina *et al.* 1998). The obligately anaerobic alkali-tolerant new species and genus *Tindallia magadii* (Kevbrin *et al.* 1998) which can grow from pH 7.5 to 10.5 was also isolated from Kenyan soda lake deposits.

(b) Ground-water

Highly alkaline Ca^{2+}-bearing ground waters are extremely rare, however, they do exist. The interest in these environments is that their hydrochemical characteristics are similar to those in cement pore waters. Since concrete is a major structural component in many storage structures, not just for water but for dangerous substances such as nuclear waste, any microbial activity that might damage these structures, even slowly over a long time, warrants investigation. High Ca^{2+}-environments, for example the Oman springs, invesigated by Bath *et al.* (1987) showed low bacterial populations. Most isolates were facultatively anaerobic and grew at pH 10 or above, but only a few were obligately alkaliphilic. Bath *et al.* (1987) concluded that the microbial genera in these springs were similar to those in less extreme soil and water environments.

(c) Thermal springs and bores

Geothermal areas are mainly of two types: one type is typified by solfatara fields with much sulfur, acidic soils, acidic hot springs, and boiling mud pots; the other is characterized by fresh-water hot springs and geysers and neutral-to-alkaline pH (Brock 1971). Natural geothermal areas are found in all parts of the globe associated with tectonic activity, but usually concentrated in discrete locations. For example, Iceland, U.S.A. (Yellowstone National Park), New Zealand and Japan. Most thermal springs are either neutral or acidic, rather than strictly alkaline. Brock (1971) noted that most pools sampled either had a pH in the range of 2-4 (acid sulphate) or 7-9 (neutral bicarbonate).

Representatives from a number of different genera have been isolated from alkaline thermal springs and a number of these are outlined below.

Bacteria of the genus *Thermus* were first isolated from hot springs in Yellowstone National Park (USA) at temperatures of 53 to 86°C and pH values between 8 and 9 (Brock and Freeze 1969) and were allocated to the type species *Thermus aquaticus.* More recently in the same location *Thermus* isolates have been obtained from sources between 55 and 80°C and up to pH 10.5 (Munster *et al.* 1986). The new genus and species *Anaerobranca horikoshii* an alkali-tolerant thermophile (Engle *et al.* 1995) was also isolated from Yellowstone National Park. Studies of an alkaline (pH 8.7) thermal (80°C) spring in Iceland reported high numbers of facultatively (an)aerobic xylanolytic and cellulolytic *Thermus* species (Kristjánsson and Alfredsson 1983; Mathrani *et al.* 1993). Souza *et al.* (1974) described a *Clostridium* isolate from an alkaline spring with a pH range of 8–11.3 (optimum pH 9.5).

As has been illustrated alkaliphilic bacteria have been isolated from a range of environments and are members of many different genera. Reports describing bacteria living at high pH often differ in their definition of what constitutes an alkaliphilic organism. The differentiation between alkaliphilic and alkali-tolerant organisms is discussed next.

2.2.2 What constitutes an alkaliphilic organism

There is a range of definitions of what consitutes an alkaliphilic or an alkali-tolerant organism. Alkaliphilic has referred to any organism whose optimum growth rate is observed at least two pH units above neutrality. Similarly alkali-tolerant has been defined as an organism with a maximum pH of pH 9 or above but having optimal growth around neutrality or less, for example, *B. halodenitrificans* which can grow between pH 5.8 and 9.6 (optimum pH 7.4) (Denariaz *et al.* 1989).

Krulwich and Guffanti (1989a) defined alkaline-tolerant organisms as those which grow optimally in the pH range of 7-9, with a maximum of pH 9.5. In contrast, Kroll (1990) used the definition of growth or survival at pH values in excess of 10 with optimal growth around neutrality. Different pH optima values have been used to define alkaliphiles, for example, growth in excess of pH 8 (Grant *et al.* 1990), at pH 9 (Kroll 1990) and greater than pH 9 (Horikoshi 1996). Spanka and Fritze (1993) defined organisms that failed to grow at pH 7 but grew at higher pH values as obligately alkaliphilic. One of the most stringent definitions was given by Krulwich and Guffanti (1989a) who defined an alkaliphilic organism as one that showed optimal growth between pH 10 and 12. They also subdivided extreme alkaliphiles into facultative alkaliphiles, which show optimal growth at pH 10 (or above) but can grow well in the neutral pH range, and obligate alkaliphiles, which show growth above pH 10 but cannot grow below pH 8.5 -9. Alkaliphiles were described by Aono (1995) as growing at pH 10 or higher, while Jarrell *et al.* (1997) stated that they had a pH optimum for growth between 9 and 11.

One of the problems with all these different definitions of what constitutes an alkaliphilic organism is that no temperature details are given for the described pH measurements (Wiegel 1998). In other words it is uncertain whether a pH range for growth above 10 refers to the pH at the optimal temperature for growth of the organism or the pH at room temperature (20°C). Another problem with clearly defining organisms as alkaliphilic (or alkali-tolerant) is that some alkaliphiles exhibit more than one pH optimum. Gee *et al.* (1980) isolated strains of alkaliphilic bacteria from potato-processing effluent. These strains could grow aerobically and anaerobically to pH 11.5 and 43°C. When grown in complex media at 25°C strain BL77/1 exhibited two pH optima of 8.5 and 9.5. In addition some organisms are either alkali-tolerant or alkaliphilic depending on the presence or absence of sodium ions in their growth medium.

Often reports refer to bacterial strains or their enzymes as being alkaliphilic when a closer examination of the data reveals that they are probably more accurately classified as alkali-tolerant. The definition used in this thesis is that obligate or strict alkaliphiles

have a pH optimum of pH 8 or above and are incapable of growth at neutral pH (pH 7.0) or lower. Alkali-tolerant organisms (or facultative alkaliphiles) can survive exposure to pH above 7.0 but have an optimum pH at neutral pH or lower. The pH on which classification was determined was the pH recorded at the optimum growth temperature of the organism, where this data was reported.

Of the alkali-tolerant and truly alkaliphilic aerobic species described the most commonly isolated and best understood strains belong to the genus *Bacillus*. This genus is the focus of more detailed discussion later in this review. Most facultative alkaliphilic *Bacillus* species are Gram stain positive. An exception is *Bacillus horti,* an aerobic Gram stain negative species from soil that has optimum growth between pH 8 and 10, but is able to grow at pH 7 (Yumoto *et al.* 1998). Most studies into alkaliphily have been conducted on three facultative alkaliphiles (only a few of the more recent publications are mentioned here); *Bacillus lentus* (Aono 1995), earlier named *Bacillus* sp. strain C-125, (Aono *et al.* 1996; Hamamoto *et al.* 1994; Hashimoto *et al.* 1994; Kitada *et al.* 1994), *Bacillus firmus* OF4 (Gilmour and Krulwich 1997; Ito *et al.* 1997a) and *Bacillus firmus* RAB (Guffanti *et al.* 1984; Rhode *et al.* 1989). Of these three organisms only *Bacillus lentus* is described in Table 2.1, the two *Bacillus firmus* strains have been used in many alkaliphilic energetics studies where few growth characteristics are mentioned. An outline of described alkali-tolerant *Bacillus* or closely related species is given in Table 2.1. The alkaliphilic *Bacillus* or closely related species are outlined in Table 2.2. In many cases a pH range for growth was not reported, only the ability or inability to grow at pH 7 was described, and that is what is recorded in Tables 2.1 and 2.2.

There are two main areas of interest in alkaliphilic bacteria both of which are reviewed in the sections that follow. Firstly the energetics involved in their survival at high pH, which is discussed in the following section (Section 2.2.3). Secondly the enzymes that are produced by alkaliphiles which due to their stability at high pH often have industrial/biotechnological applications. Since no enzymes were purified in the course of this study, only an overview of the applications of enzymes from alkaliphilic organisms is presented in Section 2.2.4.

Table 2.1: Alkali-tolerant (facultative) *Bacillus* or closely related species

	temp. opt. temp. range	pHopt.[1] pH range	mol % (G + C) DNA	source	reference
B. horti JCM 9943	- 15 - 40°C	8 – 10 ≤ 7 [2]	40.2 – 40.9	soil	Yumoto *et al.* 1998
B. lentus (*Bacillus* sp. strain C-125)	- 20 – 57°C at pH 10	- 6.8 – 10.8 at 37°C	42.2	-	Aono 1995
B. halodenitrificans	-	7.4 5.8 and 9.6	-	-	Denariaz *et al.* 1989
B. clausii DSM 8716	- 15 – 50°C	8 ≤ 7 [2]	42.8 – 45.5	soil	Nielsen *et al.* 1995
B. gibsonii DSM 8722	- 10 – 37°C	8 ≤ 7 [2]	40.6 – 41.7	soil	Nielsen *et al.* 1995
B. halmapalus DSM 8723	- 10 – 40°C	8 ≤ 7 [2]	38.6	soil	Nielsen *et al.* 1995
B. halodurans DSM 497	- 15 – 55°C	9 – 10 ≤ 7 [2]	42.1 – 43.9	soil	Nielsen *et al.* 1995
B. horikoshii DSM 8719	- 10 – 40°C	8 ≤ 7 [2]	41.1 – 42.0	soil	Nielsen *et al.* 1995
Bacillus No. 221	- ≤ 55°C	- 7 - 11	-	soil	Horikoski 1971a
Bacillus sp. TTUR 2-2	25 – 37°C ≤ 43°C	10 7 - 11	-	soil	Kimura *et al.* 1994
B. pallidus	thermophile	8 – 8.5 ≤ 7 [2]	-	-	Scholz *et al.* 1988
B. licheniformis strain IC	55°C 37 – 58°C	- 7 - 10	44.4	soil	Kitada *et al.* 1987

[1] in many instances the temperature at which the pH optimum has been measured was not stated. It should be noted that pH is medium and temperature dependent

[2] complete pH range data not provided

Table 2.2: Alkaliphilic (obligate) *Bacillus* or closely related species

	temp. opt. temp. range	pHopt.[1] pH range	mol % (G + C) DNA	source	reference
B. alcalophilis	- 10 – 40°C	10 > 7 [2]	36.2 – 38.4	human feces	Vedder 1934 / Nielsen 1995
B. agaradhaerens DSM 8721	- 10 – 45°C	10 + > 7 [2]	39.3 – 39.5	soil	Nielsen 1995
B. clarkii DSM 8720	- 15 – 45°C	> 10 > 7 [2]	42.4 – 43.0	soil	Nielsen 1995
B. pseudalcalophilus DSM 8725	- 10 – 40°C	10 > 7 [2]	38.2 – 39.0	soil	Nielsen 1995
B. pseudofirmus DSM 8715	- 10 – 45°C	9 > 7 [2]	39.0 – 40.8	soil /animal manure	Nielsen 1995
Bacillus vedderi DSM 9768	40 ≤ 50	10 8.9 – 10.5	38.3	bauxite- processing waste	Agnew *et al.* 1995
B. cohnii DSM 6307	- 10 – 47°C	- > 7 [2, 3]	33.9 – 35.0	soil	Spanka + Fritze 1993
B. thermocloacae ATCC 49805	55 – 60°C 37 – 70°C	pH 8-9 > 7 [2]	41.7 – 43.7	sewage sludge	Demharter and Hensel 1989
B. haloalkaliphilus DSM 5271	> 50°C -	9.7 > 7 [2]	37 - 38	Egyptian mud	Fritze 1996
Paenibacillus *campinasensis* KCTC 0364BP	40°C 10 – 45°C	10 7.5 – 10.5 [4]	50.9	soil	Yoon *et al.* 1998

[1] in many instances the temperature at which the pH optimum has been measured was not stated. It should be noted that pH is medium and temperature dependant

[2] complete pH range data not provided

[3] some strains described as facultatively alkaliphilic (Yumoto *et al.* 1998)

[4] *Paenibacillus campinasensis* (Yoon *et al.* 1998) is only classified as obligately alkaliphilic by the definition that obligate alkaliphiles are incapable of growth at pH 7. Under a stricter definition it would be classified as a facultatively alkaliphilic species.

2.2.3 What makes bacteria alkaliphilic

2.2.3.1 Why cannot all microbes survive at high pH

It is not certain exactly what makes alkaline environments inhibitory to most (non-alkaliphilic) forms of life. In most natural environments organisms live close to neutral pH. It is an oversimplification to consider all the effects of pH to be due to the hydrogen ion alone. H^+ is unique among cations since it is a proton with no electrons, however, in aqueous solutions it becomes rapidly hydrated and exists as the hydronium ion, H_3O^+. At alkaline pH the hydroxyl ion (OH^-) predominates (Langworthy 1978). The pH of the environment may influence the balance of electrical charges on cell surfaces giving increasing net negative charge at high pH. Extreme alteration of pH will effect cellular stability, permeability and the ability to interact with wanted or unwanted metabolites. The state of available nutrients, concentration of toxic metabolites and cellular instability at extreme pH values limits the ability of most organisms to survive outside normal limits of pH (Langworthy 1978). At alkaline pH many metabolites and ions, e.g. Fe^{2+}, Ca^{2+}, Mg^{2+}, become insoluble and precipitate, as do the carbonate, hydroxide or phosphate salts. Many cell constituents, i.e. proteins, nucleic acids, lipids, carbohydrates, contain charged groups which ionize and complex with ions and water molecules at high pH (Kroll 1990). The circulation of protons (or hydroxide equivalents) across cytoplasmic membranes is an essential feature of cellular processes (Mitchell 1973). Because of this an excess or deficit of protons in an organism's surroundings make these processes more difficult. It is probable that the pH at which an organism starts to lose control of transmembrane proton movements is close to the inhibitory pH for that organism (Kroll 1990).

Alterations in environmental pH may induce compensatory enzymatic changes in many microorganisms. Most actively metabolizing alkaline-resistant organisms have a tendency to reduce the pH value of the medium during growth. Kushner (1964) suggested such a response is most likely a secondary mechanism of resistance to alkaline environments. It is known that pH has a profound effect on the biological and chemical activity of enzymes. For instance, enzyme structure is directly affected by alterations in

the folding of the molecule and/or dissociation of monomers at extremes of pH. This affects access of both substrates and coenzymes to the active site and thus directly affects the activity of the enzyme.

The ability to grow and reproduce at low or high pH values has survival advantages since competition from other organisms is reduced. In addition to an alkaline environment, almost all alkaliphilic *Bacillus* strains have been shown to require Na^+ ions for growth, germination and sporulation (Horikoshi and Akiba 1982). It is uncertain why all bacteria with primary Na^+ cycles, do not grow well under extremely alkaline conditions (Krulwich and Guffanti 1992). It is possible that the mechanisms for pH homeostasis in alkaline-tolerant aerobic bacteria that have primary Na^+ cycles are inadequate to allow growth at the extreme pH values at which the alkaliphiles thrive.

2.2.3.2 What gives alkaliphiles the ability to thrive at high pH

Alkaliphilic bacilli are able to grow in media similar to that of neutrophilic members of the same genus provided that the pH is increased to accommodate their requirements. This suggests that no particular media components give an organism the ability to survive at high pH. One of the most characteristic features of many alkaliphilic bacilli is a profound requirement for Na^+ ions, which cannot be replaced with K^+ ions, for both growth and pH control (Krulwich *et al.* 1985b, 1994). Use of Na^+ coupled ion/solute symport systems bypasses problems that arise directly from the constraints of cytoplasmic pH regulation. A Na^+/H^+ antiporter catalyses coupled Na^+ extrusion and H^+ uptake across the membranes of extremely alkaliphilic bacilli. This exchange is electrogenic, with H^+ translocated inward > Na^+ extruded (Krulwich *et al.* 1990).

Krulwich and Guffanti (1989a) stated several possible prerequisites for alkaliphily that were shared by all extreme aerobic alkaliphiles, each of which are discussed in more detail later in this review:

(a) composition of membrane lipids and the membrane lipid / protein ratio
(b) very high levels of respiratory-chain components in the membrane

(c) a generally more acidic amino acid composition of proteins that are exposed to or excreted into the external milieu

(d) a Na^+ cycle that facilitates solute uptake and pH homeostasis

The cytoplasmic membranes of all extreme alkaliphiles possess high concentrations of cardiolipin (an anionic phospholipid), have phosphatidylethanolamine and phosphatidyl-glycerol as the other major phospholipids, and squalene and squalene derivatives, as well as C_{40} and C_{50} isoprenoids, among the neutral lipids. Preliminary evidence indicates that they have a polar C_{50} isoprenoid fraction. A novel lipid, bis-(monoacylglycero)-phosphate (BMP), which has been reported on lysosomes of mammalian tissues (Yamamoto *et al.* 1971) is only found in alkaliphilic bacteria (Horikoshi 1991b). The role of BMP in bacteria is not known. It was suggested that obligate alkaliphiles fail to grow at neutral pH because their membranes become leaky. This explains observations that even though respiration proceeds well at neutral pH, the $\Delta\mu H^+$ generated is low. By contrast, closely related facultative strains have alterations in the membrane lipids that result in a tighter coupling membrane at neutral pH and they generate a substantial $\Delta\mu H^+$ (Krulwich and Guffanti 1989a). Alkaliphilic bacilli are sensitive to lysozyme treatment and to cefoxitin treatment which is consistent with the presence of a peptidoglycan cell wall layer. The turnover of phosphatidylglycerol in alkaliphiles is less extensive. Polar lipids, such as glycolipids and phospholipids, which are common in other Gram positive bacteria are notably absent (Horikoshi 1991b). Except for phosphatidyl-ethanolamine all of the phospholipid is anionic giving the membrane a high negative charge. Findings suggest that the membranes have low melting points and hence are rather fluid. This may be linked to the inhibition of growth of alkaliphiles at low pH, because of poor membrane integrity and apparent increase in K^+ permeability at acidic pH. Krulwich and Guffanti (1989a) suggested that it may also account for rarity of thermophilic eubacterial alkaliphiles.

2.2.3.3 The cytoplasmic pH vs external pH challenge

The challenge of maintaining a cytoplasmic pH that is lower than the external pH is central to the adaptation of aerobic alkaliphilic *Bacillus* species to growth at pH 8 or above. Aerobic bacteria growing actively at pH 10.5 and above maintain a cytoplasmic pH that is over two pH units below the external pH (Krulwich 1986; Krulwich *et al.* 1998b; Guffanti *et al.* 1978, 1980; Kitada and Horikoshi 1982).

Krulwich and Guffanti (1989a) suggested that proteins exposed to a very alkaline milieu would have specific properties, for example, a restriction on the content or exposed position of residues whose pK values were close to the pH of the medium. Generally 'alkaliphilic proteins' that are exposed to outside bulk phase liquid have a significant replacement of basic amino acids for neutral or acidic amino acids. This is particularly evident in polytopic membrane proteins whose extracellular hydrophilic segments exhibit a pronounced deviation from homologous segments of neutralophilic bacterial proteins (Van der Laan *et al.* 1991; Kang *et al.* 1992; Quirk *et al.* 1993; Ito *et al.* 1997b).

Over 70% of the proteins predicted from the genomic sequence of *Helicobacter pylori*, a gastric pathogen that grows in a highly acidic environment, have a calculated isoelectric point above 7.0, with a much higher lysine and arginine content than homologues from neutralophilic bacteria (Tomb *et al.* 1997). This suggests that at both extremes of pH, adapted extremophiles minimize the use in their proteins of amino acids whose free acid or base groups might be subjected to pH changes in a range near their pK values (Krulwich *et al.* 1998). There is a particular interest in the central physiological processes that are challenged at highly alkaline pH, i.e. cytoplasmic pH regulation and bioenergetic work that depends upon an electrochemical proton gradient (Δp) whose energetically productive orientation is acid and positive out (Ivey *et al.* 1998).

The cytoplasmic values of extremely alkaliphilic *Bacillus* species found at the alkaliphiles' optimal external pH values for growth are not very different from the values of cytoplasmic pH for optimally growing non-alkaliphiles (Guffanti and Hicks 1991; Sturr *et al.* 1994; Padan and Schuldiner 1996; Aono *et al.* 1997). A rapid fall-off

in the growth rate of alkaliphilic *Bacillus* species is observed when the cytoplasmic pH starts to rise above 8.5. At values of cytoplasmic pH nearing 9.5 growth of *B. firmus* OF4 is 20-fold slower than at optimal values of cytoplasmic pH, (i.e. below pH 8.5). (Sturr*et al.* 1994). There is no evidence so far of an alkaliphile that is enhanced in its capacity to survive at unusually high values of cytoplasmic pH. Thus, for known alkaliphiles, a major challenge of life at high pH is the maintenance of a cytoplasmic pH (Krulwich *et al.* 1997) that is little different from that of neutralophiles, while the external pH that is optimal for growth is in the range of pH 10-11. In other words alkaliphiles need to maintain a cytoplasmic pH that is typically at least 2 pH units below the external pH. There is a role for passive mechanisms of proton retention in or near the cell surface, e.g. a dependency on negatively charged cell wall polymers (Aono and Ohtani 1990; Aono *et al.* 1995). These mechanisms are not sufficient to support pH homeoestasis on the failure of active, antiporter-dependent mechanisms (Kudo *et al.* 1990; Hamamoto *et al.* 1994). It is quite likely that alkaliphile antiporters have developed Na^+ specificity to minimize the chance that cytoplasmic K^+ concentrations, which must be adequate for optimal cytoplasmic protein stability and activity, will be adversely depleted (Krulwich *et al.* 1997). Alkaliphiles may live in niches that are subject to large, rapid pH changes. This would require them to have protective adaptations for such transient, but significant, events.

The two energy-dependent, membrane associated, bioenergetic processes that require inward proton translocation in extremely alkaliphilic bacteria are: oxidative phosphorylation (with an exclusively proton-coupled F_1F_0-type of ATP synthase); and pH homeostasis (electrogenic antiport of cytoplasmic Na^+ in exchange for H^+). There are a number of components to the interlocking Na^+ and H^+ cycles that allow respiring alkaliphiles that extrude H^+ during respiration, to accumulate H^+ and maintain a cytoplasmic pH that is more acidic than the external pH (Figure 2.1).

The H^+ pathway involves primary extrusion by the respiratory chain. Respiration establishes an initial, conventionally oriented pH gradient (ΔpH, acid out), and transmembrane electrical potential ($\Delta\Psi$, positive out) (Mitchell 1961). Net proton accumulation is then achieved by rapid, secondary, electrogenic exchange of protons,

from the external medium, for cytoplasmic Na^+, by Na^+/H^+ antiporters (Krulwich 1983). The re-entry of Na^+ occurs by Na^+/solute symporters and Na^+ channel(s) such as the one associated with the Na^+ flagellar rotation that is exhibited by alkaliphilic bacilli (Hirota and Imae 1983; Atsumi *et al.* 1990) (Figure 2.1).

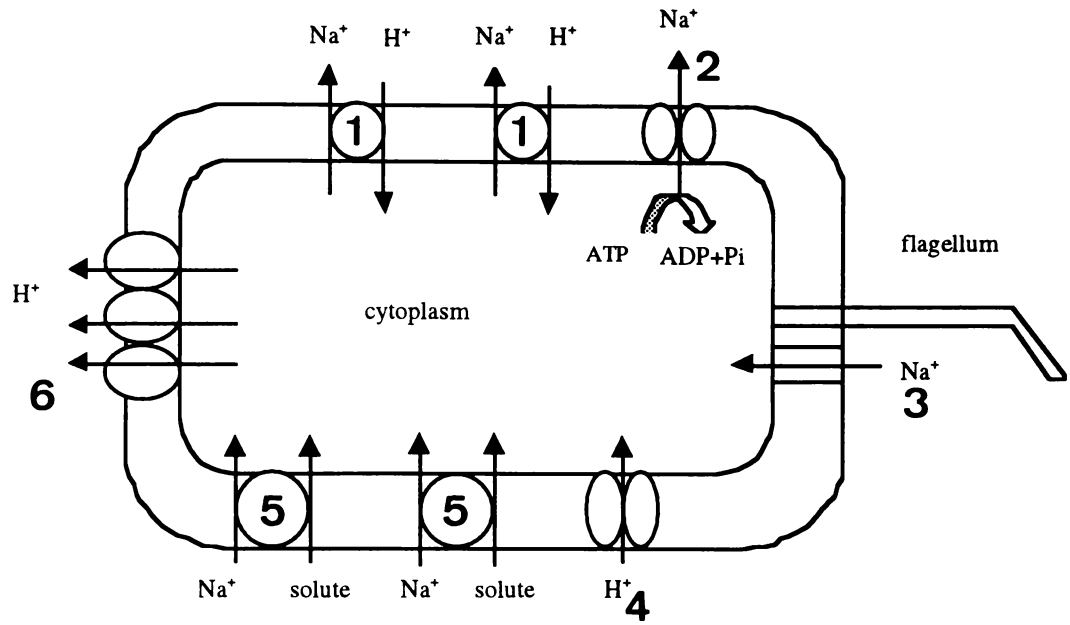


Figure 2.1: A representation of the H^+ and Na^+ cycles that may be the mechanism for the acidification of the cytoplasm during respiration for alkaliphilic *Bacillus* species.

Diagram modified from Krulwich *et al.* (1998). Components shown in this figure are discussed throughout section 2.2.3. Key: 1 = Na^+/H^+ antiporters (Nha), 2 = primary Na^+ pump(s), 3 = Na^+ channel associated with flagellum, 4 = proton-translocating element, 5 = Na^+/solute symporters, 6 = respiratory chain

Alkaliphilic bacilli can extrude H^+ by the proton extrusion-coupled hydrolysis of ATP by the F_1F_0-ATPase, under more fermentative conditions at which respiration and its associated H^+ extrusion might be insufficient to support requisite antiporter activity. Respiration is an essential part of the interlocking Na^+ and H^+ cycles for aerobically grown alkaliphilic *Bacillus* strains growing on non-fermentable carbon sources. Aerobic alkaliphilic bacilli all exhibit primary proton pumping out of the cell during respiration (Krulwich and Guffanti 1989a). Under normal cultivation conditions for *B. firmus* RAB

(an obligately alkaliphilic strain related to strain OF4) (Guffanti *et al.* 1986), all Na^+ extrusion was found to be inhibited by treatment of the respiring cells with agents that collapse the $\Delta\Psi$. Such inhibition would be anticipated for secondary, electrogenic antiporters whose energization depends on the $\Delta\Psi$. Thus, cells grown at pH 10.5, energized with malate under highly aerobic conditions with moderate concentrations of Na^+ in the medium (e.g. 25-200 mM) exhibit no Na^+ extrusion in the presence of valinomycin and sufficient K^+ to abolish any transmembrane potential developed during respiration (Krulwich and Guffanti 1989b). Quite possibly, primary Na^+ pumping would be inhibitory to extremely alkaliphilic bacteria that grow under conditions in which the Na^+ concentration is not especially high, e.g. in some conventional soils (Guffanti *et al.* 1986). While potentially contributing to the capacity to develop a productive $\Delta\Psi$, such Na^+ extrusion without concomitant H^+ uptake would reduce the cytoplasmic Na^+ concentration available for secondary antiporter activity that catalyzes net proton accumulation during respiration and sets the upper limit of pH to growth.

2.2.3.4 The antiporters

A detailed review on Na^+ /H^+ antiporters was published by Krulwich in 1983. Studies into the role of the Na^+ cycle of alkaliphilic *Bacillus* species (Ivey *et al* 1993; Krulwich and Guffanti 1992) postulated and showed (Kitada *et al.* 1994; Ito *et al.* 1997a) the presence of at least two Na^+/H^+ antiporters to exchange cytoplasmic Na^+ for H^+ from the growth medium. Guffanti *et al.* (1981) demonstrated that mutational loss of Na^+/H^+ antiport activity in the non-alkaliphilic mutant *B. alcalophilus* KM23 simultaneously led to a defect in at least three respiration-driven Na^+- dependent substrate symport systems. Monensin, an ionophore that catalyzes Na^+/H^+ exchange did not restore respiration-driven Na^+ /substrate symport. This suggests the possibility of a direct relationship between the Na^+-translocating antiport and symport systems. Guffanti *et al.* (1981) suggested that the systems may share a common Na^+-translocating subunit.

The specific importance of Na^+ for pH homeostasis, in bacteria that do not generally show a requirement for high concentrations of Na^+, is thus far a general feature of extreme alkaliphily. Several alkaliphilic *Bacillus* species possess K^+/H^+ antiporters

whose activity can be shown in membrane vesicles (Mandel *et al.* 1980; Kitada *et al.* 1997). A number of studies have suggested that a K^+/H^+ antiporter could be involved in the regulation of cytoplasmic pH at the lower end of the pH range for growth (Mandel *et al.* 1980; Koyama and Nosoh 1985; Koyama *et al.* 1987); however, the exact physiological role of the K^+/H^+ antiporter in alkaliphilic bacilli has not been established. Koyama *et al.* (1987) indicated K^+ uptake was electrogenic at neutral pH and electroneutral at high pH. They correlated this apparent difference with a greatly enhanced permeability of the alkaliphile membrane to K^+ at neutral compared with high pH. Matsukura and Imae (1987) found that the permeability to K^+, and K^+-dependent diminution of the $\Delta\psi$ of several different alkaliphilic bacilli at high pH, was greatly enhanced at pH 7.5 and when Na^+ was omitted. The mechanism of the Na^+ effect on the permeability to K^+ is unknown. Kitada *et al.* (1997) detected a K^+/H^+ antiporter in alkaliphilic *Bacillus* sp. No. 66 (JCM 9763) but were unable to elucidate its role. It has been reported that the presence or absence of Na^+ strongly affects the permeability of the alkaliphile membrane to K^+ (Koyama *et al.* 1987; Matsukura and Imae 1987). However in extreme alkaliphiles, there is no evidence that K^+/H^+ antiporters contribute to pH homeostasis (Krulwich *et al.* 1990). Krulwich *et al.* (1994) noted that, under comparable energetic driving force conditions, the aggregate Na^+/H^+ antiporter activity of *B. firmus* OF4 was about 10-fold higher than that of *B. subtilis*. This suggested that the specific requirement by alkaliphiles for Na^+ to achieve pH homeostasis, relates to avoiding losing too much K^+ from the cytoplasm. Loss of K^+ could create suboptimal cytoplasmic conditions for enzyme activity and protein stabilization especially if K^+ was used for pH homeostasis (Ito *et al.* 1997b). The Na^+-specificity of the pH homeostasis mechanisms of alkaliphiles, makes it likely that the cation specificity of the antiporters involved will be different from those of neutralophiles (e.g. *B. subtilis*), whose pH homeostasis mechanisms are less cation-specific (Krulwich *et al.* 1998, 1994; Cheng *et al.* 1994, 1996a, 1996b). However it is not yet known whether the K^+/H^+ antiporter activity is catalyzed by transporters that are distinct molecular species from the Na^+/H^+ antiporters. Na^+/H^+ antiporters might also have a low capacity for K^+/H^+ antiport activity that would be insufficient in itself to support pH homeostasis and would not occur in the presence of the requisite Na^+ concentration (Krulwich *et al.* 1998). Purification of antiporter proteins has been done for two of the Na^+/H^+ antiporters from *E. coli* (Padan and

Schuldiner 1996) and one of the antiporters involved in pH homeostasis in *B. subtilis* (Cheng *et al.* 1996b) but not yet for an alkaliphilic antiporter.

The critical importance of the H^+/Na^+ antiporter has been shown by the isolation of two alkali sensitive mutants of the alkaliphilic *Bacillus* sp. strain C-125. One of the mutants (38154) had a defective Na^+/H^+ antiport system (driven by $\Delta\psi$ membrane potential) but antiporter activity driven by ΔpH was normal (Hamamoto *et al.* 1994). This suggests that the alkali-sensitivity of the mutant was due to a defect in the electrogenic Na^+/H^+ antiporter. This mutant had lost its internal pH regulation, however, another alkali sensitive mutant (18224) retained low internal pH when exposed to alkaline pH (Kudo *et al.* 1990) which suggests that pH regulating functions were intact. Seto *et al.* (1995) suggested that the ORF3 region may be important in the connection of the respiratory chain complex and the Na^+/H^+ antiporter systems in the membrane of alkaliphiles. New host vector systems were developed using alkaliphilic *Bacillus* C-125 mutants that were alkaline sensitive, and the genes responsible for alkaliphily were investigated (Kudo *et al.* 1990; Aono and Ohtani 1990; Aono *et al.* 1992, 1993; Seto *et al.* 1995).

Monovalent cation/H^+ antiporters that catalyze net accumulation of protons during respiration are expected to be electrogenic. Evidence supporting this has been seen in various bacteria including alkaliphiles (Garcia *et al.* 1983; Bassilana *et al.* 1984; Cheng *et al.* 1996b). The stoichiometry of the antiport has been determined for two *E. coli* Na^+/H^+ antiporters to date, where H^+/Na^+ stoichiometries of 2/1 and 1.5/1 were found for NhaA and NhaB, respectively (Padan and Schuldiner 1996). Mechanistic stoichiometries have not yet been determined for any physiologically important alkaliphile antiporter (Krulwich *et al.* 1998). In extreme alkaliphiles, the available driving force, the total electrochemical proton gradient (Δp), for electrogenic antiporters that catalyze inward H^+ movement may limit the net proton accumulation. This could mean that a high coupling ratio of H^+/Na^+ is needed for the antiporters used. In addition to, or instead of this, there may be special mechanisms in the alkaliphile to maximize the antiport that can occur. For antiporters involved in pH homeostasis, it would be critical for alkaliphiles, as has been indicated for bacteria in general (Booth 1985), to possess a

significant complement of antiporters in order to adjust to sudden changes of pH (Krulwich *et al.* 1998b).

The first gene encoding an alkaliphile Na^+/H^+ antiporter, the *nhaC* gene from *B. firmus* OF4, was cloned and sequenced after partial functional complementation of a Na^+-sensitive mutant strain of *E. coli* that was deleted in its major antiporter-encoding gene *nhaA* (Ivey *et al.* 1991). Ito *et al.* (1997a) extended the sequence of the previously cloned *nhaC* gene *from Bacillus firmus*, which encodes an Na^+/H^+ antiporter, and the surrounding region. The full-length version of *nhaC* complemented the Na^+-sensitive phenotype of an antiporter-deficient mutant strain of *E. coli* but not the alkali-sensitive growth phenotypes of Na^+/H^+-deficient mutants of either alkaliphilic *B. firmus* OF4811M or *B. subtilis*. The functions of nhaC in *B. firmus* OF4 have been clarified in studies of the *nhaC* deletion strain N13 (Ito *et al.* 1997a). Since N13 (nhaC deletion strain) grew well on both fermentable and non-fermentable carbon sources at pH 10.5, NhaC is clearly not the major Na^+/H^+ antiporter, nor even a necessary one under most culture conditions. NhaC is virtually the only Na^+/H^+ antiporter in pH 7.5 grown cells that can carry out antiport at low concentrations of Na^+ (25mM at pH 7.5). In pH 10.5 grown cells, NhaC is an important part of the complement of antiporters that functions at Na^+ concentrations of 10mM and below (Ito *et al.* 1997a), but additional high-affinity antiporters are present in the high pH grown cells. During growth at pH 7.5, NhaC is a major component of the relatively high affinity Na^+/H^+ antiport activity available to extrude the Na^+ and to confer some initial protection in the face of a sudden shift upwards in external pH, i.e., before full induction of additional antiporters. Thus NhaC plays an important role when cells are shifted from pH 8.5 to pH 10.5.

Studies by Horikoshi and coworkers (Hamamoto *et al.* 1994; Hashimoto *et al.* 1994) in *B. lentus* C-125 were the first to use a non-alkaliphilic mutant strain of an alkaliphilic species as the host for cloning genes related to alkaliphily, presumably including antiporter-encoding genes. A pair of overlapping cloned fragments of *B. lentus* C-125 DNA were found to restore both Na^+/H^+ antiport activity and alkaliphily to mutant strains of the alkaliphile that were non-alkaliphilic and deficient in antiporter activity (Kudo *et al.* 1990; Hamamoto *et al.* 1994). Krulwich *et al.* (1998) suggested that perhaps

the Mrp antiporter of alkaliphiles will turn out to have a higher range of Na^+ concentrations for its optimal activity. This was hypothesised to account for observations in *B. lentus* C-125 that suggest a role in alkaliphily and pH homeostasis (Hamamoto *et al.* 1994). A feature of the mrp/pha-like operons that is not presently totally understood is the apparent need for the intact operon (or almost all of it) for any and all of the activities so far attributed to the operon to work (Ito *et al.* 1998). The studies of the *nha*C deletion strain of *B. firmus* OF4/N13, indicated that there are at least three physiologically important Na^+/H^+ antiporters, and probably more (Ito *et al.* 1997a). Studies with other alkaliphilic *Bacillus* species indicate the presence of multiple Na^+/H^+ antiporter species (Kitada and Horikoshi 1992; Kitada *et al.* 1994). More investigations will undoubtedly discover the presence of additional antiporters. Although this discussion has focused on alkaliphilic *Bacillus* species it is worth noting that alkaliphilic bacteria from other genera have been shown to have a Na^+/H^+ electrogenic antiporter for the maintenance of pH homeostasis, for example, an alkaliphilic cyanobacterium *Synechocystis* species isolated from soil (Buck and Smith 1995).

2.2.3.5 Hypotheses with respect to Na^+ re-entry

At moderate to high concentrations (e.g. >25mM) of Na^+, the re-entry of Na^+ into the cell may not be a major problem although it could still be rate limiting for the pH homeostatic mechanism (Krulwich *et al.* 1998). Clearly, at low concentrations of Na^+, the entry of the cation is rate-limiting (Krulwich *et al.* 1985b). The presence of a non-metabolizable amino acid analogue, whose uptake was coupled to Na^+ uptake, markedly improved pH homeostasis during an alkaline shift in the presence of sub-optimal concentrations of Na^+. Since the uptake itself would be $\Delta\Psi$-consuming, and so potentially competitive with the antiport, the positive overall effect on pH homeostasis strongly indicated that, at low Na^+ concentration the assistance given by the symport, to cation re-entry, was the dominant effect (Krulwich *et al.* 1998). It has been observed, however, that appreciable pH homeostasis during an alkaline shift is shown by alkaliphiles suspended in buffer without added solutes that enter with Na^+ (McLaggan *et al.* 1984; Krulwich *et al.* 1985b). This led to the suggestion that as the cytoplasmic pH

rises, alkaliphiles might have mechanisms triggered to greatly increase Na^+ entry to support the antiport that is necessary for pH homeostasis (Krulwich *et al.* 1998).

One possibility is that the Na^+/solute symporters themselves might have a mode whereby they allow Na^+ influx in the absence of solute above some threshold cytoplasmic pH (Krulwich *et al.* 1997; Ivey *et al.* 1998). McLaggan *et al.* (1984) suggested that there might be specific pH regulated channels for Na^+, for example the channel associated with flagellar rotation. Sugiyama (1995) has calculated that Na^+ flux through that channel would certainly be enough to support antiport at high pH. Motility, which is Na^+-coupled in many alkaliphilic *Bacillus* species (Hirota and Imae 1983), is appreciable only in the most alkaline range of pH for alkaliphile growth (Aono *et al.* 1992a; Sturr *et al.* 1994). Although many strains of alkaliphilic *Bacillus* have Na^+-dependent motility, several strains whose growth appears to have weak or no Na^+ dependence (Aono and Horikoshi 1983; Kitada and Horikoshi 1987; Kitada *et al.* 1989; Krulwich *et al.* 1982) also have weak or non-Na^+-dependent motility. The amiloride analog phenamil had a weak or almost non-inhibitory effect on the motility of these strains, which further suggests that the flagellar motors of these bacteria are different from typical Na^+ -driven flagellar motors. Atsumi *et al.* (1990) reported that phenamil inhibited Na^+ -driven flagellar motors without competing with the Na^+ in the medium. Thus, unlike amiloride, the interaction of phenamil on the motor is not identical to the Na^+ interaction site of the motor. The inhibitory action of amiloride is strongly affected not only by the Na^+ concentration but also the ionic strength of the medium (Sugiyama *et al.* 1988), however, Atsumi *et al.* (1990) found neither of these factors affected the inhibitory potency of phenamil on motility. Phenamil had no inhibitory effect on H^+-driven flagellar motors of neutrophiles but inhibited the Na^+ -driven motors of alkaliphilic *Bacillus* species. The motility of most alkaliphilic bacilli is energized by an electrochemical gradient exclusively of sodium. Neither substitution by Li^+ at high pH, nor energization by a ΔpH at near-neutral pH, could energize motility. This is unlike other organisms (e.g. *E. coli*) where the flagellar motor is driven by influx of protons. It is not surprising then that the swimming speed of some alkaliphilic bacilli has been shown to increase in a linear fashion with logarithmic increases in Na^+ concentration up to 100mM.

2.2.3.6 Outer cell wall layer involvement

Alkaliphilic bacilli can be divided into three groups based on differences in cell wall composition (Kroll 1990; Horikoshi 1991b):
(1) large amounts of glucuronic acid, teichuronic acid and hexosamine. These strains can not grow at pH 7 and have an essential requirement for Na^+.
(2) large amounts of glutamic acid with some aspartic, galacturonic, and glucuronic acid. These strain are capable of growth at pH 7 but have an essential requirement for Na^+.
(3) similar amounts of phosphorous and neutral sugars to neutrophilic walls (essentially identical to *Bacillus subtilis*). These strains are capable of growth at pH 7 and 10 in the presence of Na^+ and K^+.

Spanka and Fritze (1993) discovered *Bacillus cohnii*, an obligately alkaliphilic *Bacillus* species with ornithine and aspartic acid instead of diaminopimelic acid in the cell wall. Work by Aono and coworkers (Aono and Ohtani 1990; Aono *et al.* 1993a, 1995) on a variety of alkaliphilic *Bacillus* species grown on glucose, has shown that some groups of alkaliphiles produce two acidic cell wall-associated polymers in high quantity, a teichuronic acid (TUA) and a glutamate-rich teichuronopeptide (TUP). The concentration of these two acidic polymers, that bind to peptidoglycan, were higher than those of an organism grown at neutral pH (Aono and Horikoshi 1983; Aono 1987). Ito *et al.* (1994) reported that TUP was more important for the facultative alkaliphile *Bacillus* sp. C-125 to grow at alkaline pH than TUA. As stated by Krulwich and Guffanti (1989a) the bacterial cell wall appears to be important for the development and maintenance of alkaliphily. Mutational loss of these acidic polymers leads to poor growth on glucose at highly alkaline pH (Aono and Ohtani 1990; Aono *et al.* 1995). The investigators have suggested that alkaliphily in the parent strains of these mutants may depend upon a highly negatively charged cell surface to restrict entry of hydroxide ions. It has not yet been shown whether this sort of passive barrier is also important under other growth conditions, e.g. during growth on non-fermentable carbon sources. Possibly, an initial barrier of this type is the basis for the difference between the extent of Na^+-dependence of a pH homeostasis in glucose- versus malate-grown *B. firmus* OF4. This alkaliphile

does not possess significant or high pH-induced quantities of these polymers during growth on malate (Guffanti and Krulwich 1994), but glucose grown cells have never been examined. The sequestration of protons by acidic cell wall layers was suggested by Kemper *et al.* (1993). Aono and coworkers (Aono and Ohtani 1990; Ito *et al.* 1994; Aono *et al.* 1995) have described the possible involvement of the acidic cell wall polymers in binding divalent cations so that protons become trapped within the space between the coupling membrane and the polymers. It is clear that the acidic polymers (in those alkaliphiles where an important role has been found) do not replace the need for an active mechanism of H^+ accumulation. This is because single mutations that reduce energy-dependent antiporter activity produce a non-alkaliphilic phenotype (Hamamoto *et al.* 1994).

2.2.3.7 Other possible mechanisms of defence against pH change

There are other features of alkaliphilic cells that may contribute, at least transiently, to pH homeostasis after upward changes in pH. For cells growing on fermentable carbon sources these include the production of metabolic acids. Other possibilities include that the high buffering capacity of the alkaliphile cytoplasm (Krulwich *et al.* 1985a) might offer some transient protection against a change in pH. Furthermore, there may be specific membrane lipids required to produce a sufficiently ion-impermeable coupling membrane at high pH while also preserving optimal fluidity. Some polar isoprenoid lipids have been found in alkaliphilic *Bacillus* species that may have role in ion permeability (Clejan *et al.* 1986; Clejan and Krulwich 1988).

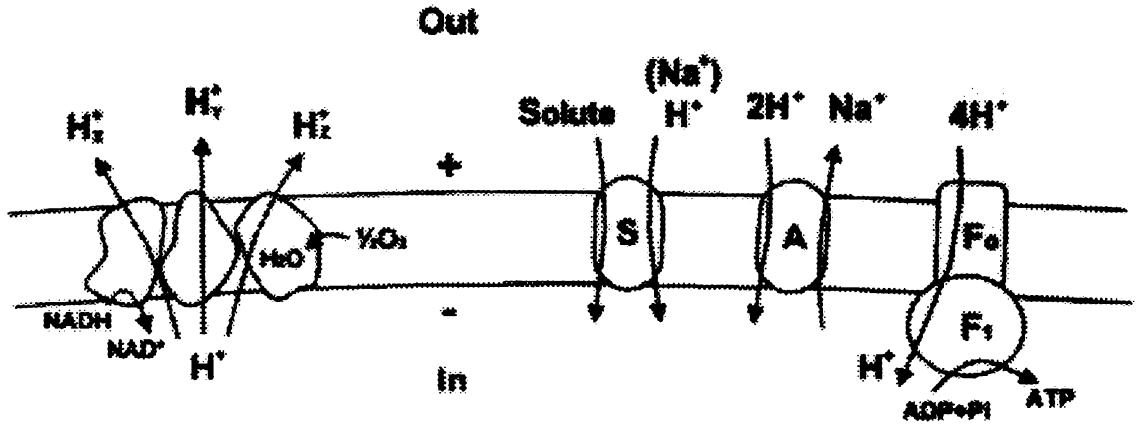
2.2.3.8 The energetics of oxidative phosphorylation

The chemiosmotic model for proton-coupled, membrane-associated bioenergetic work (energization of oxidative phosphorylation), states that the bulk Δp across the coupling membrane of mitochondria or respiring bacteria is the direct and complete source of energy for a variety of bioenergetic work functions (Mitchell 1961). Three examples of bioenergetic work that can be energised by the electrochemical proton gradient during bacterial respiration are illustrated in Figure 2.2. For alkaliphiles, the total driving force

(Δp) - the sum of the transmembrane pH gradient (ΔpH) acid out and the transmembrane electrical gradient ($\Delta\psi$) positive out - is adversely affected by a large ΔpH in the opposite orientation. Energy coupling to oxidative phosphorylation for alkaliphiles, is particularly difficult to fit in a strictly chemiosmotic model because of the low bulk electrochemical proton gradient that follows from maintaining a cytoplasmic pH just above 8 during growth at pH 10.5 and higher (Krulwich and Guffanti 1992). There are no bioenergetic problems with Na^+ -coupled processes because the energetically unfavorable ΔpH is not a component of the electrochemical Na^+ gradient, which is the chemiosmotic driving force in those instances. This force would consist of the ΔpNa^+, the inwardly directed chemical gradient of Na^+, and the $\Delta\psi$. For proton-coupled processes, however, there needs to be some accomodation of the low bulk Δp.

There is a consistently observed divergence of the rate and extent of ATP synthesis by alkaliphile oxidative phosphorylation from the anticipated, direct quantitative relationship to the magnitude of the putative driving force (the bulk Δp) (Krulwich *et al.* 1998). In carefully pH controlled continuous cultures of *B. firmus* OF4 the Δp drops substantially between pH 7.5 and 9.5 as a large ΔpH is generated to keep the cytoplasmic pH in the optimal range. There is a partially compensatory increase in the $\Delta\psi$ over this pH range but the fall in the Δp is still significant and continues as the growth pH is raised to pH 10.7 (Sturr *et al.* 1994).

Figure 2.2: Chemiosmotic energization of bioenergetic work: ion-coupled symport, antiport and F_1F_0 ATPase-mediated synthesis (from Krulwich *et al.* 1998)



The most extremely alkaliphilic bacteria are aerobic bacilli that grow optimally on non-fermentable carbon sources in a pH range in which their $\Delta\mu H^+$ levels are low. This represents a bioenergetic dilemma with respect to ATP synthesis. Although $\Delta\psi$ is substantial, total $\Delta\mu H^+$ is too low to account easily for the amount of ATP synthesized by oxidative phosphorylation. This quantitative dilemma is compounded by qualitative observations with respect to energization of ATP synthesis in extreme alkaliphiles. Energization of ATP synthesis by starved cells or ADP + P_i -loaded membrane vesicles from *B. firmus* RAB or OF4 can be initiated by addition of an electron donor e.g. malate. When starved cells are equilibrated at a pH up to 10.2 it is possible to initiate ATP synthesis by a sudden imposition of a ΔpH acid out, of 1-3 pH units. Under neutral or moderately alkaline conditions (up to pH 9.0) imposition of a valinomycin-mediated potassium diffusion potential is another way to energize ATP synthesis. However, establishment of this bulk transmembrane diffusion potential at very high pH (i.e. optimal pH for growth of the organisms) does not result in ATP synthesis. So far, methods using direct probes rather than indirect probing methods, have supported the conclusion that the total Δp at pH values above 10 is significantly lower, probably at least 3-fold, than at pH 7.5 (e.g. Aono *et al.* 1997). The work of Hoffmann and Dimroth (1991b) with *B. alcalophilus*, in which the Δp parameters were monitored in batch cultures whose pH declined from an initial value above 10 to near neutral, gave different

results. The values of the reversed ΔpH, measured on separate suspensions prepared at various intervals, were slightly lower than generally found. Using unconventionally low probe concentrations, they also reported somewhat higher values for the ΔΨ than have been reported by several groups working with logarithmically growing cells and more conventional measurements. ATP synthesis was highest at the initial high pH at which the Δp was lowest (Hoffmann and Dimroth 1991b). The inverse correlation between optimal ATP synthesis and the magnitude of the Δp was thus preserved. Hoffmann and Dimroth (1991b) suggested the use of a different coupling mechanism of protons to ATP synthesis.

If a non-chemiosmotic mechanism exists then this option of a direct proton pathway might offer a selective advantage to an organism (or organelle?) that is especially adapted to maximize the conservation of energy from respiration-dependent proton movements. Proposals of direct intramembrane pathways of energy transduction during oxidative phosphorylation, involving protein-protein interaction within the membrane, have been advanced by others for mitochondria (Gupte *et al.* 1991; Rottenberg 1990; Slater 1987; Westerhoff *et al.* 1984). The mitochondrial coupling membrane and that of the extremely alkaliphilic *Bacillus* species have in common a concentration of cytochromes that is higher than other bacterial species (Krulwich and Guffanti 1989a, 1992), a substantial cardiolipin concentration (Clejan *et al.* 1986), and the likelihood that energy transduction has been the focus of intense selective pressure. Krulwich (1986) suggested that an abundance of cytochromes may be an essential strategy to pump out protons with optimal efficiency. Several alkaliphilic *Bacillus* species have been shown to possess a high concentration of membrane-associated cytochromes (Lewis *et al.* 1980; Guffanti *et al.* 1986; Yumoto *et al.* 1991). Interestingly not all alkaliphiles show an increased concentration of cytochromes. A facultatively alkaliphilic aerobic soil *Corynebacterium* species (Ikeda *et al.* 1994) possessed less than one tenth the amount of cytochromes of *Bacillus alcalophilus*.

Other experiments suggest that this sort of flexible stoichiometry capacity is not one that alkaliphiles adopt. If a variable coupling stoichiometry was the reason for the increasing efficiency of a decreasing Δp in energizing ATP synthesis, an imposed transmembrane

electrical potential of about the same magnitude as that generated by respiration should support synthesis at the same coupling stoichiometry that occurs at any given pH during respiration (Krulwich *et al.* 1998). However, experiments indicate that artificially imposed, valinomycin-mediated K^+ diffusion potentials energize both ion-coupled transport and ATP synthesis at external pH values below about 9.2 but do not energize ATP synthesis above this pH, although the imposed gradients are still competent in energizing transport (Guffanti *et al.* 1984; Guffanti and Krulwich 1992).

2.2.3.9 The Respiratory Chain

Alkaliphilic bacilli contain high levels of respiratory chain components, particularly concentrations of cytochromes (Kitada *et al.* 1983), which explains the often intense pigmentation of these organisms (Krulwich and Guffanti 1989a). Kitada *et al.* (1987) investigated aerobic *Bacillus* strain IC, which they reported as a thermophilic alkaliphile. By the definitions used in this thesis *Bacillus* strain IC is thermotolerant (37–55°C) and alkali-tolerant (pH 7–10). Kitada and co-workers suggested that this strain might have been expected to have a very efficient respiratory system due to its ability to live under high temperature and high pH conditions. However, it did not have a high cytochrome content, but the cytochrome oxidase activity per heme aa_3 was about three times higher than that in other alkaliphiles (Kitada *et al.* 1987).

There is evidence that respiration-dependent Na^+-extrusion is entirely sensitive to abolition of the $\Delta\Psi$ under routine growth conditions at very alkaline pH (Krulwich and Guffanti 1989b). Those observations support the concept that primary Na^+-extrusion is not a normal function of the respiratory chain of extreme, non-marine alkaliphiles. In fact there is primary H^+-extrusion by the alkaliphile respiratory chain (Hicks and Krulwich 1995). It might be expected that the energetic costs of growth of extremely alkaliphilic *Bacillus* species (e.g. pH homeostasis) would force cells growing on non-fermentative carbon sources to maximize the energy conservation that can be achieved by H^+ during respiration. It is not yet clear whether alkaliphiles have a proton-translocating complex; for example, an energy conserving and non-energy coupled NADH dehydrogenase (Hicks and Krulwich 1995). Aono *et al.* (1996) suggested that

NADH dehydrogenation was one of the most important enzymes not only for an alkaliphile, *B. lentus* C-125, but also for a neutrophile, *B. subtilis* GSY1026, to grow in an environment that is more alkaline than the optimum pH for growth.

Other respiratory chain complexes that have been more completely characterized tend to be present in the membranes in high concentration, sometimes in a high pH-induced manner (Lewis *et al.* 1981; Qureshi *et al.* 1990, 1996; Yumoto *et al.* 1991, 1993; Quirk *et al.* 1993; Hicks and Krulwich 1995; Gilmour and Krulwich 1996). There is also an increase in the catalase content of high pH-grown alkaliphile cells (Yumoto *et al.* 1990; Hicks 1995). Both of the alkaliphilic *Bacillus* species whose respiratory chains have been studied extensively have been found to have a single major, high pH-induced terminal oxidase. In *B. firmus* OF4 that oxidase is a caa_3-type oxidase (Quirk *et al.* 1993). Disruption of the *cta* operon encoding this complex in *B. firmus* OF4 shows that, unlike *B. subtilis* (Lauraeus *et al.* 1991), the alkaliphile does not possess a second heme A-containing oxidase (Gilmour and Krulwich 1997). On the other hand, the alkaliphile does possess a cytochrome *bd*-type terminal oxidase that is generally expressed only during the stationary phase in high pH-grown cells (Hicks *et al.* 1991). The *bd*-type oxidase of *B. firmus* OF4, while over-produced upon disruption of the *cta* operon, did not support growth of the mutant strain on non-fermentable carbon sources even at near-neutral pH. The reason for this is not clear. Gilmour and Krulwich (1997) suggested that it may relate to an overall decrease in the levels of cytochromes of various types. It is interesting that even partial mutational loss of the *caa* -type oxidase, unaccompanied by significant decreases in other cytochromes, leads to a non-alkaliphilic phenotype (Krulwich *et al.* 1996). The single major terminal oxidase of alkaliphilic *Bacillus* YN-2000 is a cytochrome *aco*-type oxidase which has been purified and shown to be an oxygen and carbon monoxide binding oxidase that contains two copper atoms (Qureshi *et al.* 1990). Higashibata *et al.* (1998) found a branching respiratory system including two terminal components in an obligate alkaliphile, *Bacillus* YN-1. One of the two respiratory terminal components was identified as a usual caa_3-type heme-copper oxidase. However, the other respiratory terminal component was cyanide-insensitive and unique due to its non-proteinaceous nature and high oxygen-reducing activity. They discounted the possibility that the nonproteinaceous material was menaquinone (or a

menaquinone derivative) due to its much stronger oxygen-reducing activity, different hydrophobicity, absorption spectrum and molecular weight. Higashibata *et al.* (1998) proposed a novel respiratory oxygen-reducing mechanism involving a cyanide-insensitive pathway in which initially the non-proteinaceous component catalyzes the reduction of oxygen to produce hydrogen peroxide, which is then diminished by catalase. Azarkina (1995) reported studies of the facultatively alkaliphilic *Bacillus halodurans* which revealed that it contained terminal oxidases aa_3 (or caa_3) and bo_3 (bb_3). When the bacteria was cultured in alkaline medium, which induced a capacity for primary Na^+-transport, there was no detectable increase in the content of any cytochromes.

2.2.3.10 The ATP Synthase

The proton-translocating ATP synthase catalyses robust production of ATP under conditions in which the external proton concentration and the bulk chemiosmotic driving force are low. The absolute concentration of protons in the bulk, e.g. at pH 10-11, presents a problem in terms of proton capture by a membrane associated translocator, and also because the alkaliphile maintains a ΔpH such that the cytoplasmic proton concentration is higher than that in the medium (Krulwich *et al.* 1998b). A difference between the efficiency of artificially generated potentials across the alkaliphile membrane, and respiration-derived $\Delta\psi$ values of the same magnitude, in energizing ATP synthesis was noted in a number of studies (Guffanti *et al.* 1984; Guffanti and Krulwich 1992, 1994). It was originally thought, that like some anaerobic and other fermentative bacteria, extreme alkaliphiles would possess a sodium coupled F_0F_1 ATPase, that could in the alkaliphile be used in the synthetic direction to catalyze ATP synthesis in the same way that alkaliphilic *Bacillus* species would have resolved the problem of ATP synthesis at low Δp by utilization of a Na^+-translocating ATPase (Guffanti *et al.* 1978; Ivey *et al.* 1998). However, in both cases oxidative phosphorylation is carried out using an exclusively H^+-coupled ATPase (synthase) (Hicks and Krulwich 1990; Hoffmannn and Dimroth 1991a). This has been confirmed by studies in whole cells, isolated membrane vesicles (Ivey *et al.* 1998) and with purified reconstituted preparations of F_0F_1 ATP synthases from *B. firmus* OF4 (Hicks and Krulwich 1990) and *B. alcalophilus*

(Hoffmann and Dimroth 1991a). This F_1F_0-ATPase is distinct from true Na^+-translocating F_1F_0-ATPases that have been documented in a few non-alkaliphilic (especially anaerobic) bacteria (Kluge *et al.* 1992; Forster *et al.* 1995). The deduced amino acid sequence of the *a* and *c* subunits of the alkaliphile F_0 possess variations in regions that are thought to be important in terms of the path of protons through this part of the synthase assembly. These 'alkaliphile-specific motifs' have been found in *B. firmus* OF4 and *B. alcalophilus* (Ivey and Krulwich 1992). These differences include an unusual lysine residue in a putative transmembrane region of the *a* subunit and a second proline in the region of an important carboxylate-bearing residue of the *c* subunit. The importance, if any, of these deviations in sequence is yet to be determined. In alkaliphiles ATP synthesis is more rapid, and reaches a higher phosphorylation potential at highly alkaline pH, than at near-neutral pH, even though the bulk electrochemical protein gradient across the coupling membrane is lower at highly alkaline pH. Alkaliphiles appear not to utilize more substrate to generate ATP at high pH because the molar growth yeilds on malate are similar at both alkaline and near neutral pH (Guffanti and Hicks 1991; Sturr *et al.* 1994).

2.2.3.11 Possible models of alkaliphile oxidative phosphorylation

The discovery that the ion specificity of oxidative phosphorylation in *E. coli* can be converted to Na^+, by substitution of the F_0 and δ genes of the *atp* operon from the anaerobe *Propionigenium modestum,* shows that Na^+-coupling is capable of supporting aerobic growth of eubacteria (Kaim and Dimroth 1995). Bearing this in mind it is surprising that alkaliphiles like *B. firmus* OF4 do not utilize Na^+-coupling at the very alkaline growth pH at which the Δp is just a fraction of that observed at near-neutral growth pH, when the electrochemical Na^+ gradient is high. It is important when discussing how oxidative phosphorylation is energized at the upper end of the pH range for growth to realize that the molar growth yields are not compromised compared with growth at lower pH values (Krulwich *et al.* 1998). An energy-sparing aspect to oxidative phosphorylation, in the more alkaline part of the alkaliphiles' pH range for growth, has been suggested (Krulwich *et al.* 1998) because the energetic costs in maintaining pH-homeostasis and producing ATP from ADP and Pi increase as the pH rises. Experiments

by Krulwich and co-workers were conducted on (ADP + Pi)-loaded, right-side-out energized membrane vesicles (from pH 10.5-grown cells) in the presence of an electron donor with nigericin added in order to ensure that the Δp generated was all in the form of a $\Delta\Psi$ (positive out). Energization was carried out at either pH 7.8 or 9.5, i.e. below and above the pH at which an artificially imposed diffusion potential ceases to be effective in energizing ATP synthesis by the same preparations (Guffanti and Krulwich 1994). Downward titration of the transmembrane potential diminished the ATP synthesized at pH 7.8 even at levels of the $\Delta\Psi$ that were still well above -100 mV. In contrast at pH 9.5 the reduction of the $\Delta\Psi$ was comparatively without effect until it had been lowered beyond -100 mV. Krulwich *et al.* (1998) stated that these results supported the possibility that at the higher pH values, at which growth is as good or better than at near-neutral pH, there is some sparing component that is not reflected in the measured steady-state $\Delta\Psi$. They suggested that there may be some manner in which this system is not functioning at full equilibrium.

Sequestration of protons by acidic cell wall layers has been shown not to be necessary for alkaliphile oxidative phosphorylation in *Bacillus firmus* OF4 where ATP synthesis in wall-less membrane vesicle preparations was the same as in cells (Guffanti and Krulwich 1994). Several different models have been proposed where partial sequestration of the protons, that are translocated outwards by respiration, may allow that proton component to reach the ATP synthase before it can fully equilibrate with the bulk (Krulwich *et al.* 1998). In one model proton-pumping elements of the respiratory-chain polypeptides interact directly with the proton-binding elements of the F_0-ATPase within the membrane. Other protons do escape into the bulk, establishing a $\Delta\mu H^+$ that could serve as a parallel mode of energization. Importantly, this bulk $\Delta\mu H^+$ also makes localized coupling possible by forming a back pressure that inhibits the outward leak of protons from intramembrane binding sites on the pumps and sinks. The frequency with which the localized transfer occurs would depend upon the proximity of the pumps and sinks (Krulwich and Guffanti 1989a). Skulachev (1991) suggested that the low bulk Δp might not matter because the oxidative phosphorylation activity of the cell is sequestered in membrane-associated or subcellular organelles. This suggestion has not been

supported in a variety of electron microscope/structural examinations of cells and cell surfaces (Rhode *et al.* 1989; Sturr *et al.* 1994; Ivey *et al.* 1998).

The pattern of sequence diversion (i.e. the low basic amino acid composition) of short external loops of alkaliphile polytopic membrane proteins indicates that the surface must be strikingly different from that encountered, for example, in *B. subtilis* or *B. stearothermophilus* (Krulwich *et al.* 1998). It is possible that proton sequestration occurs with a fast movement between a respiratory chain complex and the ATP synthase on the surface of the membrane. This would mean that part of the driving force for synthesis is from protons that have never equilibrated with the bulk. In other words, protons move rapidly along the phospholipid or protein surfaces of the coupling membrane and so reach the synthase on emerging from a respiratory chain complex before moving out into the bulk (Krulwich *et al.* 1998b). Several groups have reported faster movements along the surface of membrane phospholipids and/or membrane protein surfaces, than in the extrusion of protons out into the bulk phase (Heberle *et al.* 1994; Scherrer *et al.* 1994; Alexiev *et al.* 1995; Gutman and Nachliel 1995; Gabriel and Teissie 1996). Whether these movements are of bioenergetic significance has still to be proven. Krulwich *et al.* (1998b) stated that there was recent evidence that membrane lipid 'remodeling' may be a feature of alkaliphile growth at the high end of the pH range, this is despite the fact that earlier studies failed to indicate major differences in the lipids at high pH.

Another possibility, suggested by Krulwich *et al.* (1998), is a model in which the proton pathway into the synthase above about pH 9.2 does not start at the external bulk phase or surface. They suggested that this could explain the efficacy of an imposed diffusion potential above such pH values by positing a pH-regulating closing of the outer entry point for protons. Above that gating pH, protons could move into the F_0 either by a direct hand-off from a respiratory chain complex (e.g. the cytochrome caa_3-type oxidase) or by the intermediary involvement of a coupling factor. In this scenario the 'alkaliphile-specific motifs' of the F_0 might be involved in modulating an important pK in connection with the gating and/or proton pathway and in making the conformation of the crucial *c* subunit capable of accepting protons from the 'alternate' intra- or perimembrane route from the interacting proton partner. Krulwich and co-workers

(1998) stated that models for the sequestration of protons translocated out by the respiratory chain during oxidative phosphorylation are currently under test. It will be of great interest to see what results these tests yield.

Of course it is possible that different alkaliphiles use strategies to support oxidative phosphorylation that do not depend on any sequestration of protons. Organisms may be discovered that generate a much higher steady-state $\Delta\Psi$. Perhaps bacteria will be discovered that have global cytoplasmic adaptations that enable them to grow well with cytoplasmic pH values greater than 9.5. If that was possible, a higher bulk Δp might be generated because the magnitude of the adverse pH gradient would not be as great as for the alkaliphilic *Bacillus* strains discovered so far.

2.2.3.12 Differences between obligate and alkali-tolerant alkaliphiles

The inability of obligately alkaliphilic strains to grow below about pH 9 is apparently related to the membrane lipid composition of these strains. This property may also correlate with a better ability to grow at the very upper edge of their pH range, than related facultative strains (Clejan and Krulwich 1988; Dunkley *et al.* 1991). Alkali-tolerant bacteria grow well at pH values around 9 but do not grow as well (or at all) at pH values above 10. Several members of this group, e.g. marine bacteria like *Vibrio* species, have been reported to have primary Na^+ pumps that increase the $\Delta\psi$ and the total electrochemical sodium gradient when the organisms are grown at pH 9-10. They also appear to have a Na^+-coupled ATPase that can synthesize ATP under at least some experimental conditions (Sakai *et al.* 1989; Avetisyan *et al.* 1991). As stated by Krulwich and Guffanti (1992) it is interesting that the possession of mechanisms that would be expected to bypass a low Δp is not sufficient to confer capacity for extreme alkaliphilic growth.

2.2.3.13 The genetic component of alkaliphily

Work on *B. firmus* OF4 offered the opportunity to discern whether there were features of chromosome and gene organization that were associated with alkaliphily. No clustering

of genes so far identified with roles in alkaliphily has been found (Grønstad *et al.* 1998). Direct repeat sequences (DRS) were reported (Ito *et al.* 1997a) upstream of a gene encoding a Na^+/H^+ antiporter that has a role when cells are shifted from pH 8.5 to pH 10.5. Grønstad *et al.* 1998 found that these sequences were present in multiple copies on the chromosome, most of which were in one 920-kb fragment. For the extremely alkaliphilic *Bacillus* species, only one partial physical map had been reported to date, that for the 3.7-Mb chromosome of alkaliphilic *Bacillus* strain C-125 (Sutherland *et al.* 1993), a strain of *Bacillus lentus* (Aono 1995).

An increasing number of gene loci that are implicated in alkaliphily are being identified in this species (Kudo *et al.* 1990; Aono *et al.* 1993; Hamamoto *et al.* 1994), but only one such locus was localized to a specific fragment on the initial map. Hamamoto *et al.* (1994) reported that a gene responsible for the Na^+/H^+ antiporter system was important in the alkaliphiliy of alkaliphilic microorganisms. Identification and localization of a larger number of genes that are critically involved in alkaliphily and comparative studies of several distinct alkaliphilic and nonalkaliphilic *Bacilus* species should allow assessment of whether there is a clustering of all or groups of genes related to alkaliphily, either on the chromosome or on extrachromosomal elements. It will also be interesting whether there are consistent differences in chromosomal organization of pH-inducible or housekeeping genes in alkaliphiles versus non-alkaliphilies

The physical map of the *B. firmus* OF4 chromosome shows that the genes involved in growth on non-fermentative carbon sources, such as the *atp* (F_1F_0-ATPase) (Ivey and Krulwich 1991) and *cta* (cytochrome oxidase) (Quirk *et al.* 1993; Gilmour and Krulwich 1997) operons are not closely aggregated on the chromosome. The two antiporter-encoding genes that are involved in alkaliphily in this and other alkaliphilic *Bacillus* species, *nhaC* and *mrp* (Hamamoto *et al.* 1994; Ito *et al.* 1997a; Krulwich *et al.* 1998), map to the same fragment but there is no evidence that they are close together within that fragment. A comparison of the localization of the genes common in *B. subtilis* and the *B. firmus* OF4 chromosome indicates a different gene organization for the nine genes analyzed. This may not be so surprising taking into consideration the random

organization of orthologous genes in other bacteria that are not very closely related (Kolstø 1997).

None of the genes that are thus far implicated as having a role in the energetics of alkaliphily or pH homeostasis were localized to the extrachromosomal element. Interestingly, Amano and Shishido (1995) noted direct repeat sequences in the regions of several *B. subtilis* strains that flank the tetracycline-resistance determinant. Grønstad and co-workers (1998) reported that this determinant encodes a multifunctional antiporter that has important physiological roles in Na^+ resistance and pH homeostasis in the moderately alkaline range, in addition to providing defense against low levels of the antibiotic (Cheng *et al.* 1996b). It would be of interest to determine the effects of introducing and deleting direct repeat sequences from *B. firmus* OF4 on the adaptability of this extremophile to extremely alkaline conditions at various concentrations of Na^+.

Another feature of pH homeostasis that may be special to the alkaliphiles is the generation of genetic variants that have an even greater capacity for antiport and maintenance of pH_{in} than the parent strains (Krulwich *et al.* 1986). Aono *et al.* (1994) developed conditions for fusing protoplasts from the facultative alkaliphile *Bacillus* sp. C-125 and reported the first successful use of cell fusion to produce genetically modified alkaliphilic bacteria. Cell fusion by protoplasts may provide a convenient method for genetic manipulation in alkaliphilic bacteria, which is essential for genetic studies and useful for industrial purposes.

2.2.4 Industrial Applications of Alkaliphiles

Since ancient times in Japan indigo has been naturally reduced from indigo leaves, in the presence of sodium carbonate, by bacteria that grow under alkaline conditions (Horikoshi and Grant 1991). Takahara and Tanabe (1960, 1961, 1962) isolated the indigo-reducing bacterium, *Bacillus* sp. No. S-8 from an indigo ball. They then improved the indigo fermentation process, improving product quality and process speed, by adding the alkaliphilic *Bacillus* sp. No. S-8 during fermentation under alkaline conditions. Thus indigo dyeing was the first industrial application of alkaliphilic bacteria

in the world. The first paper concerning an alkaline enzyme (protease) of an alkaliphilic *Bacillus* (number 221) was published by Horikoshi in 1971. This strain (Horikoshi 1971a) was actually, by the definition used in this thesis, alkali-tolerant since it was capable of growth at pH 7. In the same year details were published of an alkaline amylase produced by alkaliphilic *Bacillus* No.A-40-2 (Horikoshi 1971b). Alkaline proteases, alkaline amylases, and alkaline cellulases have been used on an industrial scale (Horikoshi and Akiba 1982; Horikoshi 1991b). Many more enzymes from alkaliphilic *Bacillus* species have been characterized (Horikoshi and Akiba 1982) but most have not met commercial criteria. The predominant industrial / biotechnological applications of enzymes from alkaliphiles are briefly presented in the following section.

(a) Detergents / washing powders

Since the early 1960's there have been pre-wash laundry detergents containing alkaline enzymes (e.g. alkaline proteinase–Alcalase® (Subtilisin Carlsberg) from *Bacillus licheniformis*.) (Horikoshi 1991b), however, poor cost effectiveness and quality of performance led to its withdrawal (Grant *et al.* 1990). More recently technical improvements, environmental concerns, and the desire to save energy have lead to the successful reintroduction of biological detergents. The most common enzymes in detergent formulations are proteinases (particularly serine proteinases from alkaliphilic *Bacillus* species) (Horikoshi 1971a; Aunstrup *et al* 1972; Yamagata and Ichishima 1993). Alkaline proteases have also been isolated from non-*Bacillus* alkaliphilic species, for example, from *Vibrio metschnikovii* (Kwon *et al.* 1994), the alkaliphilic thermophile *Thermoactinomcyces* (Tsuchiya *et al.* 1991, 1992) and *Streptomyces* sp. (Mizusawa *et al.* 1964, 1969; Yum *et al.* 1994).

Proteases used in detergents need to maintain activity under stringent washing conditions, for example, pH 10 and 50°C. For an enzyme to be an effective addition to a laundry detergent it needs to not only be active at high pH, but also to be compatible with the other components of the detergent, during both storage and the washing process. Liquid detergents, although usually less alkaline than powders, contain more surfactants and solvents (e.g. ethanol) which can alter enzyme stability and performance

(Grant *et al.* 1990). Not only do proteinases hydrolyse proteins and remove stains, they also ensure that coagulated protein is not redeposited on the fabric during the wash, which would give a grey rather than white appearance to clothes (Kalisz 1988a, 1988b).

Other alkaliphilic enzymes used in detergents and washing powders include:
(i) lipases which degrade fats (stains from oil based foods, cosmetics and body fats) into more hydrophilic fatty acid salts (Newmark 1988; Emanuilova *et al.* 1993).
(ii) cellulases which are used for fabric softening, colour brightening and the removal of sebum, soil and sweat (Hakamada *et al.* 1997; Ito *et al.* 1989; Shikata *et al.* 1990).

Two other types of enzymes isolated from alkaliphilic *Bacilli* have potential applications in detergents and washing powders:
(i) pullulanases (Nakamura *et al.* 1975; Ara *et al.* 1992, 1993; Igarashi *et al.* 1992; Lin *et al.* 1994, 1996).
(ii) amylases (Horikoshi 1971b; Boyer and Ingle 1972; Boyer *et al.* 1973; Kelly *et al.* 1983).

(b) Removal of hair from animal hide

Proteases from alkalophilic bacteria are highly effective in the de-hairing of animal hide and have brought about significant improvements in the leather tanning process. Several alkaline proteases from alkaliphilic actinomycetes are active against keratinous proteins e.g. hair, feathers, wool etc at alkaline pH. The removal of hair from hides traditionally involved the application of hydrated lime and sodium sulphide at high pH (pH 12), the addition of alkaline proteinases can reduce the need for sulphide and thus reduce the volume of toxic effluent produced. The enzymes used in this process need to be compatible with surfactants and oxidizing agents which are added to help clean and preserve the hides.

(c) The recovery of silver from X-ray films

The use of an alkaline protease to decompose the gelatinous coating of X-ray films, from which silver was recovered, has been described by Fujiwara and co-workers (Fujiwara and Yamamoto 1987; Fujiwara *et al.* 1991, 1993). X-ray film contains a great deal of silver, compared with other types of film, so it is worth recovering the silver for reuse. An alkaline protease from alkaliphilic *Bacillus* strain B21-2 (Fujiwara and Yamamoto 1987a) could rapidly break down gelatin layers on X-ray film in a continuous automatic process and enable the recovery of silver and the polyethylene terephthalate (PET) film base.

(d) Treatment of pulp and paper waste

Large quantities of xylan are released as effluents in paper and pulp industries. The use of thermophilic and alkaliphilic enzymes may allow many manufacturers to cut down on the amount of acid required for pH readjustment of the incoming pulp and the need for cooling and reheating of the large pulp mass, thus saving both time and money. Xylanases also play an important role when use of hazardous chlorine chemicals has to be decreased in the bleaching processes due to environmental issues (Viikari *et al.* 1994; Kulkarni and Rao 1996; Biely 1985). Yang *et al.* (1995) described a *Bacillus* strain (V1-4) isolated from hardwood kraft pulp, which was capable of growing in diluted kraft black liquor at pH 11.5 and produced xylanase when cultivated in alkaline medium at pH 9. Biobleaching studies showed that the enzyme would brighten both hardwood and softwood kraft pulp and release chromophores at pH 7 and 9. Because of the alkalinity of kraft pulps this enzyme could be used for prebleaching with minimal pH adjustment. Cellulase-free thermostable xylanases, active at alkaline pH, have been used for the selective hydrolysis of the hemicellulose components (biological debleaching) in pulp and paper (Viikari *et al.* 1994; Nakamura *et al.* 1993, 1994; Kulkarni and Rao 1996). Another potential application for alkalophilic xylanases is the utilization of rayon-containing waste. Horikoshi and Akiba (1982) described an alkaliphilic *Bacillus* isolate (C-11) from soil which could utilize rayon waste.

Many reports of xylanases from thermophilic or alkalophilic microorganisms are available (Nicolaus *et al.* 1996; Honda *et al.* 1985, 1986). Xylanases have been isolated from both mesophilic (Horikoshi and Atsukawa 1973a; Ikura and Horikishi 1977) and thermophilic (Okazaki *et al.* 1984, 1985; Akiba and Horikoshi 1988; Dey *et al.* 1992; Khasin *et al.* 1993; Nakamura *et al.* 1994) alkaliphilic *Bacillus* strains. Xylanases have also been isolated from other alkaliphilic Genera including *Aeromonas* species (Ohkoshi *et al.* 1985) and thermophilic and anaerobic *Dictyoglomus* strains (Mathrani and Ahring 1992).

(e) Food / Cosmetics / Pharmaceuticals

The main enzymes involved in the food, cosmetic and pharmaceutical industries are cyclodextrin glucanotransferases (CGTases) which are used for cyclodextrin manufacture from starch (Kitamoto *et al.* 1992; Horikoshi and Akiba 1982; Yim *et al.* 1997). Cyclodextrins are ring structures consisting of 6 to 8 glucose molecules linked by α-1,4 bonds which have interesting properties as artificial enzymes. α-cyclodextrin contains six glucose molecules, β-cyclodextrin seven and γ- cyclodextrin eight glucose molecules respectively. Because of their ring structure they have neither a reducing end-group nor a non-reducing end-group and are not decomposed by hot aqueous alkali (Horikoshi and Akiba 1982). Being soluble in water but having a hydrophilic interior, hydrophobic molecules with the right shape can bind two molecules so that they can react. They form a number of crystalline or "inclusion" complexes with organic substances, and inorganic complexes with other compounds. Cyclodextrin has been used in foodstuffs as emulsifiers, foaming agents or stabilizers; in cosmetics as colour or fragrance stabilizers or maskers; in pharmaceuticals for increasing the stability / solubility of antibiotics, vitamins or hormones; protecting against ultraviolet (UV) induced degradation and to inhibit oxidation in other areas, including pesticides and plastics. Many alkaliphilic microorganisms producing CGTases have been reported (Matsuzawa *et al.* 1975, Nakamura and Horikoshi 1976a, 1976b, 1976c, 1976d; Nomoto *et al.* 1984; Georganta *et al.* 1993; Kometani *et al.* 1994; Yoon *et al.* 1998).

(f) Other applications

Lichenases have specific applications in the brewing industry since their activity facilitates the access of the α–amlyases to the starch of endosperm cells. This aids the filtration process, reducing gels and haze formation in the finished products. Alkaliphilic (Tabernero *et al.* 1994) or thermophilic and alkaliphilic (Wang and Saha 1993) lichenases from *Bacillus* species have been described which, because of their stability at high pH, have promise for industrial application.

Pectinases are widely used in the fruit and vegetable processing industries. Alkaline pectinase-producing *Bacillus* sp. GIR-277 has been used in the macerating / retting of Mitsumata bast in the production of Japanese paper (Yoshihara and Kobayashi 1982). The treatment of waste water from the citrus processing industry by an extracellular endo-pectate lyase from alkalophilic *Bacillus* sp. GIR 621 was trialed by Tanabe *et al.* (1987). This proved to be a useful pretreatment to remove pectic substances.

Penicillinases (Sunaga *et al.* 1976, 1979) and β-lactamases (Akiba and Horikoshi 1980) have been isolated from alkaliphilic *Bacillus* species. These are important enzymes in antibiotic resistance mechanisms and chemotherapy since they can inactivate penicillins or cephalosporins by hydrolysis. β–Lactamase cleaves the amide bond of the β–lactam ring of penicillin or cephalosporin and is used in development of new semisynthetic antibiotics resistant to cleavage by the enzyme. β–Lactamase from alkaliphilic *Bacillus* strain 170 has been cloned into *E. coli*. Most of the plasmid-encoded penicillinase was excreted into the medium by *E. coli* along with other normally periplasmic proteins such as alkaline phosphatase (Kato *et al.* 1983; Kudo *et al.* 1983).

Invertase (β-fructofuranosidase) which hydrolyses sucrose into glucose and fructose is one of the most important enzymes in the food industry and is involved in the manufacture of invert-sugar which is used as an additive in confectionary. Sashihara (1991) isolated an alkaliphilic *Bacillus* strain No. NS-1018 from soil. This bacterium produced an extracellular invertase in an alkaline medium containing sucrose as a sole

carbon source. The optimum pH and temperature for enzyme action were 5.5–6.5 and 55°C.

2.3 The Genus *Bacillus* and closely related genera

2.3.1 Thermophilic or thermotolerant *Bacillus* and closely related genera

At present only a few genera of aerobic heterotrophic thermophilic bacteria have been described. In addition to the genus *Bacillus* and closely related genera which will be discussed in this section, only six other genera having a temperature optimum of 65°C or above have been described. These are *Thermus* (Brock and Freeze 1969; Brock 1978), *Thermomicrobium* (Jackson *et al.* 1973; Phillips and Perry 1976), *Rhodothermus* (Alfredson *et al.* 1988), *Saccharococcus* (Nystrand 1984), *Sulfolobus* (Zillig *et al.* 1980a, 1980b) and the facultative heterotroph *Thermothrix* (Caldwell *et al.* 1976). This last genus, however, is no longer available from culture collections.

Members of the genus *Bacillus* have been isolated from a diverse range of habitats including soil, hot-springs and refrigerated food, and exhibit a wide range of growth characteristics. With such a diverse genus it is not surprising that the first aerobic thermophilic microorganism isolated was a *Bacillus* species (Miquel 1888). This bacterium from the river Seine grew at 73°C and was incapable of growth at lower temperatures. Possibly the most widely known thermophilic bacterium is also a member of the *Bacillus* genus; *Bacillus stearothermophilus* has an optimum growth temperature of 50°C (Donk 1920). Smith *et al.* (1946) suggested that all strains growing at temperatures of 65°C or above belonged to the species *Bacillus stearothermophilus.* The first extensive examination of thermophilic isolates (Gordon and Smith 1949) resulted in the description of two main groups, *Bacillus coagulans* Hammer and *Bacillus stearothermophilus* Donk. The species *Bacillus coagulans* (Hammer 1915) was later recognised as thermotolerant rather than thermophilic. The original description of *B. stearothermophilus* (Donk 1920) was largely based on morphological and physiological characteristics and although the original strain was lost, Gordon and Smith (1949) considered the description typical of most of the obligately thermophilic species of *Bacillus*. There has been much discussion on the correct identification of *B. stearothermophilus.* Many investigators have given this species name to isolates if they

grew at 60°C or 65°C and possessed terminal spores; others have ignored the terminal spore characteristic and have used only growth at high temperature.

In 1971 Walker and Wolf studied the physiological and morphological characteristics of 230 *Bacillus stearothermophilus* strains and divided them into three major groups. A numerical analysis of thermophilic *Bacillus* strains was carried out by White *et al.* (1993). These species were later evaluated by DNA reassociation and by rRNA sequence analysis (Rainey *et al.* 1994). The genetic diversity of the genus *Bacillus* is shown by the fact that the G+C DNA content ranges from 32 to 69 mol%. 16S rRNA gene sequence analyses have identified a number of phylogenetic groups in the genus *Bacillus* (Ash *et al.* 1991; Farrow *et al.* 1992; Nielsen *et al.* 1994; Rainey *et al.* 1994; Suzuki and Yamasato 1994). Some of these groups have been reclassified as new genera; *Alicyclobacillus* (Wisotzkey *et al.* 1992); *Paenibacillus* (Ash *et al.* 1993); *Halobacillus* (Spring *et al.* 1996); *Brevibacillus* (Shida *et al.* 1996); *Aneurinibacillus* (Shida *et al.* 1996; Heyndrickx *et al.* 1997); *Amphibacillus* (Niimura *et al.* 1990) and *Virgibacillus* (Heyndrickx *et al.* 1998). Broad characteristics of the *Bacillus* genus and these seven closely related genera are shown in Table 2.4. Using PCR amplification of 16S rRNA fragments members of a number of these *Bacillus*–like genera have been able to be rapidly identified. For example, *Brevibacillus* and *Aneurinibacillus* strains have been identified with a specific primer (Shida *et al.* 1996) and *Paenibacillus* species have been identified using primer PAEN515F (Shida *et al.* 1997a, 1997b).

A number of the species in the *Bacillus*–like genera outlined in Table 2.4 have thermophilic or thermo-tolerant characteristics. The genus *Alicyclobacillus* is characterized by a cell membrane containing alicyclic fatty acids thought to confer stability under thermophilic acidophilic conditions. The three species in this genus have been isolated from soil (*A. acidoterrestris* and *A. cycloheptanicus* (Deinhard *et al.* 1987a, 1987b)) and a hot spring (*A. acidocaldaricus* (Darland and Brock 1971)). *Aneurinibacillus* species have been isolated from diverse environments including a sugar beet factory and human faeces. Their optimal temperature for growth is mesophilic but they are thermotolerant with a maximum growth temperature between 50 and 60°C depending on the species. Four species of the genus *Brevibacillus* have temperature

maxima of 50°C or above (*Brev. borstelensis* (Shida *et al.* 1995), *Brev. choshinensis* (Takagi *et al.* 1993), *Brev. parabrevis* (Takagi *et al.* 1993) and *Brev. thermoruber* (Manachini *et al.* 1985) further defining them as thermotolerant. Four *Paenibacillus* species have a maximum growth temperature of 50°C (*P. chibensis*, *P. illinoisensis*, (Shida *et al.* 1997b), *P. lautus and P. validus* (Nakamura 1984; Heyndrickx *et al.* 1995; Shida *et al.* 1997a). Currently there is only one species in the genus *Virgibacillus* (*V. pantothenticus*) and this is thermotolerant with a maximum growth temperature of 50°C.

The members of the genus *Bacillus* have a wide range of temperature optima from 15 to 55°C (Table 2.4). A number of *Bacillus* species or strains (Table 2.5a) have a temperature range for growth that includes 50°C or above, however, these are at least from the current data, thermotolerant strains. In some cases, it is difficult to determine whether a strain is thermotolerant or thermophilic when only optimal pH rather than pH range data is available, for example, *Bacillus* sp. strain TAR-1 and *B. thermoalcaliphilus*. A number of the species or strains outlined (Table 2.5a) have a maximum temperature for growth around 50°C but grow optimally at a much lower temperature, for example, *B. vedderi* and *B. alkaliphilus* S-8. The highest optimal temperature of the species listed is 80°C for *B. caldotenax* (Heinen and Heinen 1972).

2.3.2 Alkaliphilic or alkali-tolerant *Bacillus* and closely related genera

The first study of alkaliphilic *Bacillus* strains was carried out by Vedder in 1934. He isolated aerobic, endospore-forming bacteria which were obligately alkaliphilic from human and animal faeces, and proposed the name *Bacillus alcalophilus* for his strains. Chislett and Kushner (1961) isolated an alkaliphile from horse meadow soil that was initially regarded as a strain of *B. circulans* and later classified as *B. cohniii* (Spanka and Fritze 1993). Boyer *et al.* (1973) isolated halotolerant strains resembling *B. alcalophilus* which they regarded as a novel subspecies, *B. alcalophilus* subsp. *halodurans*.
Gordon and Hyde (1982) characterised 174 industrially important alkaliphilic *Bacillus* strains after adapting them to neutral pH. Based on a broad range of physiological and biochemical properties, they arranged the strains in a spectrum which they termed the *B. firmus-B. lentus* complex.

Fritze *et al.* (1990) divided the alkaliphilic *Bacillus* classified by Gordon and Hyde (1982) and other isolates into separate clusters on the basis of G+Cmol% content, viz. Group A 34.0-37.5%, Group B 38.2-40.8%, Group C 42.1-43.9%. They demonstrated that the clusters correlated with physiological properties that were stable under both alkaline and neutral conditions, making them valid characteristics for distinguishing alkaliphilic isolates. Considering the low DNA-DNA relatedness between strains in the same group, it was suggested that these strains could be assigned to numerous species. The wide variation in mol%G+C (34–44%) gave further support to the reassignment of these strains given the proposal that members of a species should not differ by more than ±1mol%G+C (De Ley 1978). Three strains previously assigned to the two subspecies of *B. alcalophilus* were shown to be genetically distinct, and were given specific status as *B. halodurans*. An amended description of *B. alcalophilus* was given by Nielsen *et al.* (1995).

Nielsen *et al.* (1994) conducted a phylogenetic analysis of alkaliphilic *Bacillus* species on the basis of 16S rDNA sequences. Nielsen *et al.* (1995) examined 119 alkaliphilic and alkalitolerant aerobic spore-forming bacteria by DNA reassociation and carbohydrate utilization profiles. This most recent examination of alkaliphilic and alkalitolerant *Bacillus* strains described eleven groups of strains, the existing species *B. alcalophilus* and *B. cohnii*, and nine other phenons whose major characteristics are in Table 2.3. The nine new groupings described included four obligate species (*B. agaradhaerens*, *B. clarkii*, *B. pseudalcaliphilus*, *B. pseudofirmus*) and five facultative (alkali-tolerant) species (*B. clausii*, *B. gibsonii*, *B. halmapalus*, *B. halodurans*, *B. horikoshii*).

B. agaradhaerens and *B. clarkii* were unusual among the alkaliphilic *Bacillus* strains described by Nielsen *et al.* (1995) in that they were unable to grow in the absence of sodium ions. These species were unable to grow at pH 7, grew optimally at pH 10 or above, and up to 45°C. The most thermotolerant species in this study were *B. clausii* (50°C) and *B. halodurans* (55°C).

Despite often having a dependence on the presence of sodium ions for growth few halophilic alkaliphilic *Bacillus* strains have been described. An exception is the mesophile *B. haloalkaliphilus* (Fritze 1996) which grows at pH 9.7 and has colonies which vary from crème-white to yellow with increasing NaCl concentration. Few *Bacillus* species that grow in the presence of 20% NaCl or more and are alkaliphilic have been described. *B. halophilus* (Ventosa *et al.* 1990) tolerates 30% total salts, grows from pH 6-8, but has an optimum around pH 7. Similarly, *B. halodenitrificans* (Denariaz *et al.* 1989) which can tolerate 25% NaCl, grows from pH 5.8 to more than 9.6, but has an optimum of 7.4. The highest NaCl concentration at which an alkaliphilic *Bacillus* has been reported to grow is between 16 and 18% (Nielsen *et al.* 1994).

Of the seven *Bacillus*-related genera outlined in Table 2.4, a number contain species that are capable of alkali-tolerant or alkaliphilic growth. *Aneurinibacillus* species have neutral pH optima but are alkalitolerant with pH maxima of between pH 8 – 9 depending on the species. Two members of the genus *Brevibacillus* (*Brev. choshinensis* and *Brev. parabrevis*) are alkali-tolerant growing to pH maximum of 9. Most species in the genus *Paenibacillus* have neutral pH optima, an exception is *P. campinasensis* (Yoon *et al.* 1998) with an optima of pH 10. Two members of the genus *Halobacillus* (*H. litoralis* and *H. trueperi* (Spring *et al.* 1996) are alkali-tolerant with a pH maximum for growth of 9.5. The sole member of the genus *Amphibacillus* (*A. xylanus*) (Niimura *et al.* 1990) grows optimally between pH 9 and 10.

Comparative growth characteristics of some species from *Bacillus* and closely related genera are outlined in Tables 2.5a and 2.5b. These species have been selected because of their ability to grow under either thermotolerant (thermophilic) or alkalitolerant (alkaliphilic) conditions. The highest optimal temperature of the species listed is 80°C for *B. caldotenax* (Heinen and Heinen 1972). A number of the species listed have pH optima above pH 10 in some cases, however, the published data only states that the pH optima are above 10 without stating a value. The highest stated pH optima for species listed in Table 2.5a is 11.5 for *B. alkaliphilus* strain S-8. The mol% G+C DNA content of these species and strains covers a broad range from 36.2 to 64.8% which illustrates the diversity in these related genera. Only six of the species were described as growing

at 65°C (Table 2.5a). A seventh species *B. caldotenax* grows optimally at 80°C and may also grow at 65°C, however, no lower temperature limit for growth was described. The range of salt concentratons (0 to 25%) tolerated and the utilization of different carbon sources varied between species (Table 2.5b).

It is clear from this overview of thermophilic (thermotolerant) or alkaliphilic (alkalitolerant) species of the genus *Bacillus* (or closely related genera) that few thermophilic alkaliphilic (alkalitolerant) *Bacillus* species have been described. Of the six described species that grew at 65°C (Table 2.5a), one had a pH optimum of 8-9 (*B. thermocloaceae*), one had a pH optimum from 6-9 (*B. flavothermus*) and the other species all had pH optima below pH 7. In the following section thermophilic alkaliphilic bacteria from different genera are described.

Table 2.3. Classification of alkaliphilic *Bacillus* species (based on Nielsen *et al.* 1995)

phenon	name	mol% G+C	optimum pH	temperature range (°C)	other characteristics
1	*B. pseudofirmus*[1]	39 – 40.8	9.0	10 - 45°C	from soil, high (16%) NaCl tolerance, deaminate phenylalanine
2	*B. agaradhaerens*	39.3 – 39.5	pH of 10 or above	10 – 45°C	from soil or mud, require sodium ions, hydrolyse celluolose + xylan, tolerate 16% NaCl
3	*B. clarkii*	42.4 – 43.0	above pH 10	15 – 45°C	from soil or mud, require sodium ions, good growth at 15% NaCl, unable to hydrolyse starch.
4	No specific name –heterogenous cluster	-	-	-	from soil and animal manure, two DNA-DNA hybridisation groups[2], no definitive features
5	*B. halodurans*[3]	42.1 – 43.9	pH 9 - 10[3]	15 - 55°C	animal manure and chicken yard soils, growth to 12% NaCl
6	*B. clausii*[4]	42.8 – 45.5	8[4]	15 - 50°C	reduced nitrate, unable to hydrolyse Tween or pullulan, sensitive to NaCl.
7	*B. cohnii*	33.9 – 35.0	above pH 7	10 – 47°C	characterized by Spanka and Fritze (1993)
8[5]	*B. halmapalus*	38.6	pH 8[6]	10 - 40°C	unable to grow in the presence of 5% NaCl
	B. horikoshii	41.1 – 42.0	pH 8[6]	10 - 40°C	growth at 8-9% NaCl
9	*B. pseudalcalophilus*	38.2 – 39.0	pH 10	10 - 40°C	moderately salt tolerant stains
10	*B. alcalophilus*[7]	36.2 – 38.4	pH 10	10 - 40°C	similar to *B. pseudalcalophilus* but less salt tolerant and lower G+C content
11	*B. gibsonii*	40.6 – 41.7	pH 8[6]	10 – 37°C	relatively low NaCl tolerance

[1]included strains classified as *B. firmus* by Gordon and Hyde (1982) but not closely related to authentic *B. firmus*, unlike *B. firmus* most strains are unable to grow at pH 7 and tolerate 16% NaCl.

[2]utilization of amygdalin, gentobiose and mannose gave some distinction between the two DNA-DNA hybriisation groups

[3]Growth possible at pH 7, strains had been previously assigned to *B. alcalophilus* subsp. *halodurans* (Boyer *et al.* 1973) and *B. lentus* (Gorson and Hyde (1982).

[4]Growth possible at pH 7, highest G+C content (42.8-45.5mol%) of the strains examined by Nielsen *et al.* (1994)

[5]two distinct groups of strains with consistent differences in base composition, carbohydrate utilization and NaCl tolerance

[6]growth at pH 7

[7]emended description from Vedder 1934

Table 2.4: Overall characteristics of *Bacillus* and closely related genera

Character	*Bacillus*	*Virgibacillus*	*Paenibacillus*	*Aneurinibacillus*	*Brevibacillus*	*Halobacillus*	*Alicyclobacillus*	*Amphibacillus*
No. of species	>60	1	26	3	10	3	3	1
Murein	V	DAP	DAP	DAP	DAP	Orn-D-Asp	ND	DAP
Cell shape	Rods	rods	rods	rods	rods	rods/cocci	rods	rods
spores	+	+	+	+	+	+	+	+
Spore shape	E,C,S,B	E-S	E	E	E	E,S	E	E
Anaerobic growth	V	+	V	-	+/-	-	V	+
Temperature optimum°C	15-55	37	28-40	37	30-48	35	48-65	37
pH optimum	7-9.5	7	7 – 10	7	7	7.5	3 – 4	9 – 10
Growth in 10% NaCl	V	+	-	-	-	+	-	-
Major isoprenoid quinine	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	None
Major cellular fatty acids	V	iso / anteiso$C_{15:0}$	anteiso-$C_{15:0}$	iso$C_{15:0}$, $C_{16:0}$, iso-$C_{16:0}$	anteiso-$C_{15:0}$+iso-$C_{15:0}$ or just iso-$C_{15:0}$	ND	ω-alicyclic acids	anteiso-$C_{15:0}$, $C_{16:0}$, iso $C_{16:0}$
G+C mol%	32-69	36.9# -38.3†	40-54	41.1-43.4	42.8-57.4	40-43	51.6-60.3	36-38
Reference	Claus+ Berkeley 1986	Heyndrickx *et al.* 1998	Heyndrickx *et al.* 1996	Heyndrickx *et al.* 1997; Shida *et al.* 1996	Shida *et al.* 1996	Spring *et al.* 1996	Deinhard *et al.* 1988; Wisotzkey *et al.* 1992	Niimura *et al.* 1990

Abbreviations: DAP (direct-linked meso-diaminopimelic acid), E, ellipsodal, S, spherical, E-S, ellipsoidal to spherical, C cylindrical, B bent, V character varies depending on species. # from Fahmy *et al.* (1985), † mean of two measurements by the DNA melting temperature method.

Table 2.5a: Comparative growth characteristics of *Bacillus* and closely related genera

Bacillus species	Temp.opt. Temp.range (or maximum) °C	pHopt.[1]	mol % (G + C) DNA	O_2 or ANO_2	Growth at 65°C	Oxidase / Catalase Activity
B. thermocloaceae sp. DSM 5250	55 – 60 37 – 70	8 – 9	42 – 44	O_2	+	+ / +
B. agaradhaerens sp. DSM 8721	- 10 – 45	>10	39.3-39.5	O_2	-	ND
B. vedderi DSM 9768	40 (50)	10	38.3	Facultat ANO_2	-	+ /+
B. alkaliphilus S-8	30 (50)	10 – 11.5	-	Facultat O_2	-	ND / +
Aneurinibacillus thermoaerophilus DSM 10154	- 40 – 60	7 – 8	46.7	O_2	-	ND /variable
Bacillus sp. strain TAR-1	50 -	10.5	-	O_2	ND	ND
Bacillus sp. strain C-125 (*B. lentus* Group3)	- 20 – 57 at pH 10	6.8 –10.8 at 37°C	42.2	O_2	-	+ /+
B. caldotenax	80 (85)	7.5 – 8.5	64.8[2]	O_2	ND	ND
B.alkaliphilus subsp. *halodurans* ATCC 27557	48 (54)	8.9[3]	42.5[4]	Facultat ANO_2	-	ND
B. flavothermus DSM 2641	60 – 65 30 – 72	6 – 9	61	O_2 / ANO_2	+	+ / +
Bacillus thermoantarcticus DSM 9572	63 at pH 6.0 (65)	6.0	53.7	O_2	+	+ / -
Bacillus thermoglucosidasius	61 -- 63 (69)	6.5	45-46	O_2	+	+ / +
Bacillus alcalophilus DSM 485	- (40)	around 9.7	36.5	O_2	-	+ / +
Bacillus alcalophilus DSM 485 (Nielsen *et al.* 1995)	- 10 - 40	10	36.2-38.4	O_2	-	ND
B. clarkii sp. nov. DSM 8720	- 15 – 45	More than 10	42.4-43.0	O_2	-	ND
B. thermoalcaliphilus	60 -	8.5 – 9.0	48.6	Facultat ANO_2	ND	ND
B. clausii DSM 8716	- 15 – 50	8	42.8-45.5	O_2	-	ND
B. gibsonii DSM 8722	- 10 – 37	8	40.6-41.7	O_2	-	ND
B. halmapalus DSM 8723	- 10 – 40	8	38.6	O_2	-	ND
B. halodurans DSM 497	- 15 – 55	9-10	42.1-43.9	O_2	-	ND

Table 2.5a continued: Comparative growth characteristics of *Bacillus* and closely related genera

Bacillus species	Temp.opt. Temp.range (or maximum) °C	pHopt.[1] / range	mol % (G + C) DNA	O_2 or ANO_2	Growth at 65°C	Oxidase / Catalase Activity
B. horikoshii DSM 8719	- 10 – 40	8	41.1-42.0	O_2	-	ND
B. pseudalcalophilus DSM 8725	- 10 – 40	10	38.2-39.0	O_2	-	ND
B. pseudofirmus DSM 8715	- 10 – 45	9	39.0-40.8	O_2	-	ND
B. horti JCM 9943	- 15 – 40	8-10	40.2-40.9	O_2	-	+ / +
B. haloalkaliphilus DSM 5271	<50 -	9.7	37-38	O_2	-	+ / +
B. licheniformis Strain IC	55 37 – 58	- 7 – 10	44.4	Fac ANO_2 /O_2	-	ND / +
Bacillus species TTUR 2-2	25 – 37 (43)	10 7 – 11	-	O_2	-	+ / +
Bacillus species NCIM 59	50 27 – 55	9 – 10 8 – 11	46.7	O_2	-	+ / +
Paenibacillus campinasensis KCTC 0364BP	40 10 – 45	10 7.5 – 10.5	50.9	Fac ANO_2	-	- / +
Bacillus stearothermophilus ATCC 12980	50 – 60[5] 40 – 75	6.8	51.9[6]	O_2	+	ND
Bacillus smithii NRS-173	- 25 – 65	5.7 - 6.8	40.2	Fac ANO_2	+	+ / +

[1] in many instances the temperature at which the pH optimum has been measured was not stated. It should be noted that pH is medium and temperature dependant

[2] Sharp *et al.* 1980

[3] Horikoshi and Akiba 1982

[4] Fritze *et al.* 1990

[5] Gibson and Gordon 1974

[6] Nakamura *et al.* 1988

Terms : ( ) means weakly, O_2 = aerobic growth, ANO_2 = anaerobic growth, Fac or Facultat = facultative, ND = not done

Table 2.5b: Comparative growth characteristics of *Bacillus* and closely related genera

Bacillus species	Growth in NaCl	Hydrolysis of Casein / Starch	Sucrose Utilisation	Trehalose Utilisation	Glucose Utilisation	Fructose Utilisation	Reference
B. thermocloaceae DSM 5250	3%, weak at 5%	- / -	-	-	-	-	Demharter + Hensel 1989
B. agaradhaerens DSM 8721	16%	+/+	+	+	+	+	Nielsen *et al.* 1995
B. vedderi DSM 9768	7.5% not at 10%	- / -	+	+	+	ND	Agnew *et al* 1995
B. alkaliphilus S-8	ND	ND / +	+	ND	+	+	Takahara and Tanabe 1962 Horikoshi 1991b
Aneurinibacillus thermoaerophilus DSM 10154	3% not at 5% or higher	+ / -	-	-	+	-	Meier-Stauffer *et al.* 1996; Heyndrickx *et al.* 1997
Bacillus sp. strain TAR-1	ND	ND	ND	ND	ND	ND	Nakamura *et al.* 1994
Bacillus sp. strain C-125 (*B. lentus* Group3)	≤ 10%	+ / +	+	+	+	+	Aono 1995
B. caldotenax	ND	ND	ND	ND	ND	ND	Heinen and Heinen 1972
B. alkaliphilus subsp. *halodurans* ATCC 27557	7% (slow in 15%)[1]	+ / +[1]	+	ND	+	ND	Boyer *et al.* 1973
B. flavothermus DSM 2641	up to 2.5%	ND / ND	+	ND	+	ND	Heinen *et al.* 1982
Bacillus thermoantarcticus DSM 9572	No	- /+	(+)	+	+	ND	Nicolaus *et al* 1996
Bacillus thermoglucosidasius	No	+/+	+	+	+	ND	Suzuki *et al* 1983
Bacillus alcalophilus DSM 485	-	+ / ND	ND	ND	+	ND	Vedder 1934
Bacillus alcalophilus DSM 485	below 5%	+ / +	+	+	+	+	(reclassified Nielsen *et al.* 1995)
B. clarkii DSM 8720	16%	+/-	+	+	+	+	Nielsen *et al.* 1995
B. thermoalcaliphilus	-	-	-	-	-	-	Sarkar 1991
B. clausii DSM 8716	Up to 10%	+/+	+	+	+	+	Nielsen *et al.* 1995
B. gibsonii DSM 8722	9%	+/-	+	+	+	+	Nielsen *et al.* 1995
B. halmapalus DSM 8723	Below 5%	+/+	+	+	+	+	Nielsen *et al.* 1995
B. halodurans DSM 497	Up to 12%	+/+	+	+	+	+	Nielsen *et al.* 1995

Table 2.5b continued: Comparative growth characteristics of *Bacillus* and closely related genera

Bacillus species	Growth in NaCl	Hydrolysis of Casein / Starch	Sucrose Utilisation	Trehalose Utilisation	Glucose Utilisation	Fructose Utilisation	Reference
B. horikoshii DSM 8719	Max 8-9%	+/+	+	+	+	+	Nielsen *et al.* 1995
B. pseudalcalophilus DSM 8725	Up to 10%	+/+	+	+	+	+	Nielsen *et al.* 1995
B. pseudofirmus DSM 8715	Up to 16%	+/+	+	+	+	+	Nielsen *et al.* 1995
B. horti JCM 9943	3 – 11 %	+/+	-	ND	+	+	Yumoto *et al.* 1998
B. haloalkaliphilus DSM 5271	0.5 – 25 %	(+) / (+)	ND	ND	ND	ND	Fritze 1996
B. licheniformis Strain IC	7 %	+ / +	ND	ND	+	ND	Kitada *et al.* 1987
Bacillus species TTUR 2-2	10%	ND / +	(+)	+	(+)	+	Kimura *et al.* 1994
Bacillus species NCIM 59	ND	+ / +	+	ND	+	+	Dey *et al.* 1992
Paenibacillus campinasensis KCTC 0364BP	7 %	+ / +	-	-	(+)	+	Yoon *et al.* 1998
Bacillus stearothermophilus ATCC 12980	ND	(+) / +	+[2]	ND	+	(+)[3]	Donk 1920
Bacillus smithii NRS-173	less than 3%	- / (+)	-	+	+	+	Nakamura *et al.* 1988

Terms : ( ) means weakly, ND = not determined

[1] Horikoshi 1991b

[2] Ljungdahl 1979 [3] growth in fortified but not in minimal media

2.4 Thermophilic alkaliphilic bacteria

Research on extremophiles has primarily focused on isolating organisms from conditions of one extreme, for example high temperature (thermophiles) or high pH (alkaliphiles). For a long time it was assumed that bacteria could not grow both at elevated temperatures (above 60°C) and alkaline conditions (above pH 8), since this would place too great a strain on the stability and integrity of cell walls and membranes. Subsequent research has proven that this is not the case.

Engle *et al.* (1995) stated that only a few of the known alkaliphiles were also (moderate) thermophiles. Despite the fact that they are relatively rare, alkali-thermophiles and alkali-tolerant thermophiles have been isolated from a variety of environments, including garden soil (Horikoshi 1996), mesobiotic sewage sludge, river and lake sediments, microbially heated compost and geothermally heated springs with a wide variety of pH values (Wiegel 1998). Many of these isolates are found in places where the conditions are less suitable for their optimum growth, for example, where the macro-environment is neither very alkaline nor necessarily very hot. For example, *Clostridium paradoxum* (Li *et al.* 1993) an alkali-tolerant anaerobic thermophile with a maximum pH of 11.1 at 25°C was isolated from a mesobiotic sewage facility where the macro-environmental temperature and pH were never above 35°C and pH 7.8. This facultative alkaliphile has a short doubling time (10 minutes) which presumably enables it to respond quickly if micro-environmental conditions occur that are suitable for growth.

As stated by Wiegel (1998) there is an inherent problem when comparing published growth pH data for thermophiles since many investigators do not specify how the given pH values were obtained. Published pH values could have been determined at room temperature or at the elevated (growth) temperature. Measurements could have been taken without using a temperature probe and using a pH meter calibrated at room temperature, or as it should be done, correctly measuring the pH at the elevated temperature using an electrode and pH meter calibrated at the same temperature.

One of the challenges in comparing bacteria that tolerate two growth condition extremes, is that whichever of the extreme characteristics one focuses on, the comparative picture with other extreme condition is different. In other words, alkali-thermophilic organisms are not the most thermophilic nor the most alkaliphilic organisms known. To gain a true picture of their growth characteristics an overview of their combined temperature and pH traits must be taken into account. In order to make comparison between the different bacterial species easier, for the basis of this discussion, they are divided into aerobes or anaerobes.

2.4.1 Anaerobic thermo-alkaliphilic bacteria

An overview of the key features of described anaerobic thermo-alkaliphilic bacteria are presented in Table 2.6. The first thermophilic and anaerobic bacterium isolated able to grow above pH 9 was *Clostridium paradoxum* which was isolated from sewage plants in the USA and was described as being alkaliphilic and thermophilic (Li *et al.* 1993). However, since its conditions for growth range from pH 7.0-11.1 and 30-63°C it should probably be defined as thermo- and alkali-tolerant.

Engle *et al.* (1996) isolated *Thermobrachium celere,* a Gram positive alkali- and thermo-tolerant obligate anaerobe, from geothermally and anthropogenically heated locations as well as mesobiotic environments, located on three continents. The alkali- and thermo-tolerant *Anaerobranca horikoshii* was isolated from springs with an alkaliphilic as well as an acid pH (Engle *et al.* 1995). Few alkalitolerant (or alkaliphilic) thermophilic anaerobes have been isolated from exclusively alkaline thermobiotic environments. Svetlitshnyi *et al.* (1996) described an obligately anaerobic, thermophilic lipolytic alkalitolerant bacterium, *Thermosyntropha lipolytica* from Kenyan alkaline hot springs. *T. lipolytica* differed from other anaerobic biopolymer-degrading thermophiles, and glycolytic alkali-thermophiles (Engle *et al.* 1995, 1996; Li *et al.* 1993, 1994) in that it could not utilize cellulose, starch, pectin and xylan, but in the presence of hydrogen utilizers syntrophically uses the fatty acids from lipids.

Meijer *et al.* (1996) reported the isolation of *Thermopallium natronophilum* a Gram negative bacterium that grew fermentatively at 70°C and pH 10 (25°C) and according to 16S rDNA sequence analysis is a member of the *Thermotogales*. Since further details of this strain are as yet unpublished it is not included in Table 2.6. Kitada *et al.* (1987) stated that a thermophilic bacterium which could grow at over 90°C under alkaline conditions had been reported, however, no reference or further details were given for this statement, meaning that it can not be substantiated.

Table 2.6: Comparison of key features of described anaerobic alkaliphilic (alkali-tolerant) thermophilic (thermotolerant) bacteria

	Temp.opt. Temp.range.	pHopt.[1] pH range	mol % (G + C) DNA	
Clostridium paradoxum	56°C 30- 63°C	10.1 7.0 - 11.1 at 25°C	30.0	Li *et al.* 1993
Clostridium thermoalkaliphilum DSM 7309	48 – 51°C at pH 10.1 27 – 57.5°C	9.6 – 10.1 7.0 – 11.0 at 50°C	32	Li *et al.* 1994
Thermobrachium celere (JW/YL-NZ35)	62 - 66°C at pH 8 (66°C) >37 < 75°C	8.0 – 8.5 at 66°C 5.0 – 9.7 at 66°C	30 – 31	Engle *et al.* 1996
Anaerobranca horikoshii DSM 9786	57°C 34 - 66°C at pH 8.5	8.5 6.9 – 10.3 at 60°C	34	Engle *et al.* 1995
Thermosyntropha lipolytica	- 52-70°C pH 8.5 (25°C)	- 7.15-9.5 at 25°C	-	Svetlitshnyi *et al.* 1996

[1] in many instances the temperature at which the pH optimum or range was measured was not stated. pH is media and temperature dependant

2.4.2 Aerobic thermo-alkaliphilic bacteria

The first isolation of a thermo-tolerant alkaliphilic bacteria was *Bacillus alkaliphilus* S-8 (Takahara and Tanabe) in 1962. The aerobic *Bacillus* species and strains for which temperature range data is available are all thermo-tolerant rather than thermophilic. (Table 2.7). Horikoshi and Akiba (1982) commented that almost all alkaliphilic bacteria showed optimum temperatures for growth in the range of 25–45°C. While *Thermomicrobium roseum* had a higher temperature optimum than a number of the *Bacillus* strains (Table 2.7), it had an optimal pH that was only moderately alkaline.

The highest growth temperature for an aerobic alkali-tolerant bacteria growing above pH 10, up to 1982, was 57°C for *Bacillus* sp. No. 221 (ATCC 21591) an alkaline proteinase producer (Horikoshi 1990). The second highest growth temperature was 55°C for *Bacillus* sp. No. P-4-N which produced an alkaline pectinase most active at pH 10.5. More recently (Table 2.7) *Bacillus* NCIM 59 and *Bacillus* species strain TAR-1 have been isolated (Dey *et al.* 1992; Nakamura *et al.* 1994) with optimal growth conditions of 50°C and pH 9-10 or near pH 10.5 respectively.

Table 2.7: Comparison of key features of described aerobic[1] alkaliphilic (alkali-tolerant) thermophilic (thermotolerant) bacteria

	Temp.opt. Temp.range.	pHopt.[2] pH range	mol % (G + C) DNA	
B. alkaliphilus S-8	30°C to 50°C	10 - 11.5 -	-	Takahara + Tanabe 1962
B. caldotenax	80°C to 85°C	7.5 - 8.5 -	64.8 [3]	Heinen + Heinen 1972
B. alkaliphilus subsp. *halodurans* ATCC 27557	48°C to 54°C	8.9 [4] -	42.5 [5]	Boyer *et al.* 1973
B. flavothermus DSM 2641	60 - 65°C 30 - 72°C	6 – 9 -	61	Heinen *et al.* 1982
B. thermocloaceae DSM 5250	55 - 60°C 37 - 70°C	8 – 9 (not at 7)	42 - 44	Demharter+ Hensel 1989
B. thermoalcaliphilus	60°C -	8.5 - 9.0 -	48.6	Sarkar 1991
Bacillus NCIM 59	50°C 27 – 55°C	9.0 – 10.0 8.0 – 11.0	46.7	Dey *et al.* 1992
Bacillus sp. strain TAR-1	50°C -	around 10.5 -	-	Nakamura *et al.* 1994
Bacillus sp. strain C-125 (*B. lentus* Group3)	- 20 - 57°C at pH 10	- 6.8 -10.8 at 37°C	42.2	Aono 1995
B. haloalkaliphilus DSM 5271	<50°C -	9.7 -	37 - 38	Fritze 1996
Thermomicrobium roseum ATCC 27502	70 - 75°C to 85°C	8.2 - 8.5 -	64.3	Jackson *et al.* 1973
Bacillus TX-3	55°C	8.5 - 10	-	Kitada *et al* 1989b
Bacillus sp. NCIM 59	50°C 27 – 55°C	9 – 10 8 – 11	-	Dey *et al.* 1992
Bacillus strain IC	58°C 37 – 60°C	- 8 – 10	-	Kitada *et al.* 1987
Bacillus sp. No. 221	- ≤ 57°C[6]	- 7 – 11	-	Horikoshi 1990c

[1] or facultatively (an)aerobic

[2] in many instances the temperature at which the pH optimum or range was measured was not stated. pH is media and temperature dependent

[3] Sharp *et al.* 1980

[4] Horikoshi and Akiba 1982

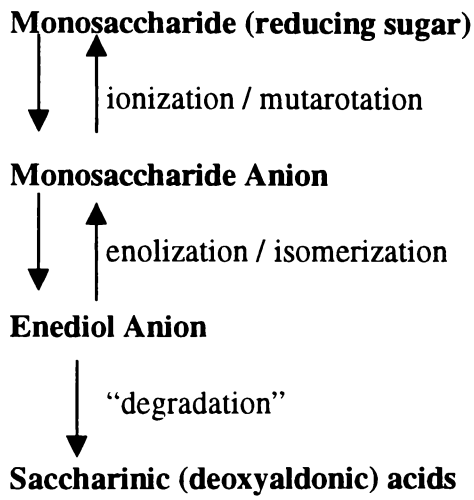
[5] Fritze *et al.* 1990

[6] described as ≤ 55°C in Horikoshi 1971a

2.5 Effects of alkaline conditions on monosaccharide reducing sugars and sucrose

2.5.1 Monosaccharides in alkaline medium

Monosaccharides are often found in nature in the form of various polymers, from which they can be liberated by hydrolysis with aqueous mineral acids or with enzymes. Monosaccharides are stable at neutral pH, however reducing sugar monosaccharides are unstable at alkaline pH. A simplified overall reaction scheme of monosaccharides (reducing sugars) in alkaline medium (Clarke *et al.* 1997) contains both reversible and irreversible reactions.



2.5.2 The alkaline decomposition of sucrose

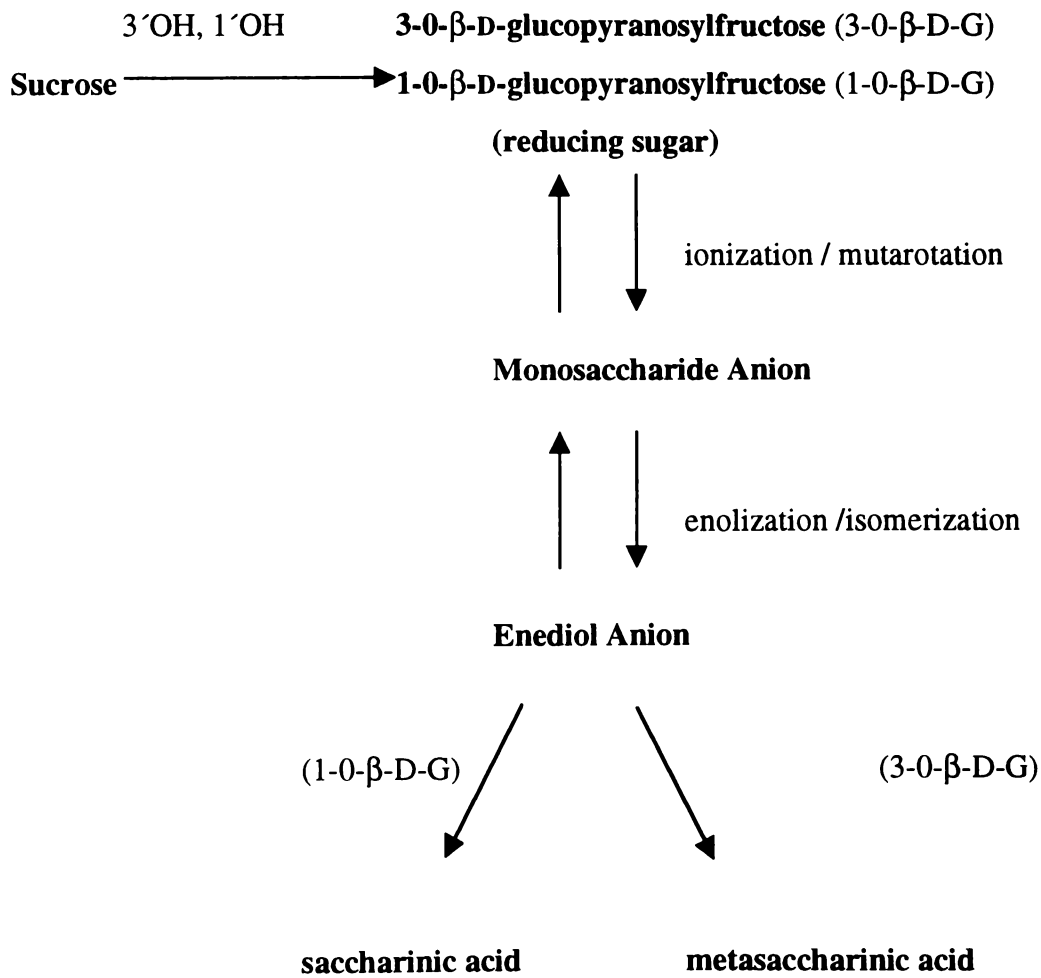
Under acidic conditions (pH 2-6) the products of sucrose hydrolysis, glucose and fructose, are sufficiently stable to permit the progress of the reaction to be followed accurately by the determination of the change in their concentrations over a period of time (Parker 1971). In contrast, under alkaline conditions sucrose undergoes a generalized decomposition with the ultimate formation of acidic products, several of which have not been characterized (Parker 1971). It is possible to show that neither glucose nor fructose are formed even transiently during the hydroxyl ion-catalyzed

decomposition of sucrose. Under alkaline conditions the rate of sucrose decomposition must be followed by the determination of residual sucrose. In addition, constant pH must be maintained by the continuous addition of alkali. Uncertainty as to the true value of the pH in sucrose solution at elevated temperature is considered to be the source of greatest error in such a determination, since the rate of decomposition is directly proportional to hydroxyl ion activity.

It is a common but erroneous belief that the alkaline decomposition of sucrose proceeds initially via cleavage of the glycosidic linkage, resulting in either D-glucose and D-fructose or ionic forms of these monosaccharides as products (Clarke *et al.* 1997). This issue is further confused by the fact (Parker 1970) that sucrose can hydrolyze to D-glucose and D-fructose by an acid-catalyzed mechanism in slightly alkaline conditions (up to pH 8.3). Parker (1970) reported that neither D-glucose or D-fructose are even transiently formed, during the hydroxyl-ion catalyzed decomposition of sucrose. Parker favored a mechanism whereby an internal nucleophilic displacement at the glycosidic linkage of a sucrose anion, resulted initially in ionic forms of the monosaccharides, which rapidly formed acids of lower molecular weight. In contrast Kelly and Brown (1978/79) favored an inversion or hydrolysis mechanism.

In contrast to reducing sugars, sucrose and related alkyl glycosides are considerably more stable in alkali. Several investigators (Overend 1972; Ballou 1954; Lai and Ontto 1979) have investigated the alkaline degradation of glycosides. In glycosides, a *trans* relationship between the aglycon and the 2-hydroxyl group of an aldoside or the 3-hydroxyl group of a ketoside allows a much more facile alkaline degradation than is the case for the *cis* analog. Because of this α-D-fructofuranosides degrade faster than β-D-fructofuranosides (like the fructose moiety in the sucrose molecule) and β-D-glucopyranosides degrade faster than α-D-glucopyranosides (like the glucose moiety in the sucrose molecule). In the case of sucrose, the ring C-OH groups adjacent to the anomeric carbon atoms on the glucose and fructose moieties are both *cis* to the glycosidic bonds (Clarke *et al.* 1997). Thus sucrose should be a relatively alkali-stable glycoside. However, sucrose is relatively labile to alkaline degradation. Manley-Harris and Richards (1981) proposed that the alkaline degradation reaction proceeds by a slow

mechanism where the substitution at the C-1 of the D-glucose moiety by oxyanions derived from 1'-OH or 3'-OH resulted in 1- or 3-0-β-D-glucopyranosyl-D-fructose. An important point of this mechanism is that the alkali-catalyzed degradation of sucrose does not initially proceed via hydrolysis of the glycosidic linkage and the formation of D-glucose and D-fructose, or their ionic forms. Since the methods used by Manley-Harris and Richards (1981) were more sensitive than those used by Parker (1970) the mechanism proposed by Manley-Harris and Richards is more likely to represent actual events. The proposed mechanism of alkaline decomposition of sucrose is outlined below:



Sucrose can hydrolyse to D-glucose and D-fructose in slightly alkaline solutions (at pH up to 8.3) but this hydrolysis proceeds by the normal acid-hydrolysis mechanism. In

alkali, degradation of sucrose proceeds more slowly than the rate of degradation of D-glucose or D-fructose. Thus if these monosaccharides were intermediates in the degradation process they would be detected.

Vukov (1965) developed equations based on experimental data that predict the effect of temperature, pH, ionic strength on rate constants of sucrose decomposition in acid and alkaline conditions. When pure crystalline sucrose is heated it remains relatively stable until melting, after which the disappearance of sucrose occurs rapidly. Richards (1986) noted that the thermolysis of pure sucrose can be observed at much lower temperatures if it is not crystalline.

2.5.3 Colour formation from glucose, fructose and sucrose in solution

D-glucose and D-fructose are present in both cane and beet juice and decompose on the addition of lime at high temperatures during purification of the juice (Clarke *et al.* 1997). Dark coloured products in sugar juice are formed via the Maillard reaction of amino acids and reducing sugars (non-enzymatic browning). Sucrose is relatively stable under alkaline conditions (Kelly and Brown 1978/79) because it lacks the unsubstituted hemiacetyl group present in reducing disaccharides. Eggleston *et al.* (1995) noticed a pale yellow colour in a sucrose solution in water, after 2 hours at 100°C, due to Maillard type browning reactions between reducing sugars and ammonia. Imming *et al.* (1996) described a kinetic model of colour formation, especially by the Maillard reaction, in heated concentrated carbohydrate solutions. They included the main parameters of colour formation such as reaction time, temperature, pH, amounts of sucrose, glucose, fructose, amino-nitrogen and dry matter. They stated, however, that due to the complex mechanisms of the colour formation reactions an exact calculation was not achieved.

2.5.4 Detection of sugars

High performance liquid chromotography (HPLC) with either refractive index, constant or pulsed amperometric detectors, is the most commonly used technique for sugar determination in food matrices (Mannino *et al.* 1996). Pulsed amperometric detection is

sensitive for disaccharides but care must be taken that the electrode does not become fouled by additives in the mobile phase. Refractive index detection has been used for determination of saccharides but allows neither determination of sugars at trace levels nor the use of gradient elution. Since sugars do not exhibit absorbance at wavelength above 200nm, direct UV detection is not applicable due to low sensitivity and high background interference. Lu *et al.* (1995) developed an indirect UV-detection method in the separation of saccharides by reversed-phase LC using an alkaline sodium hydroxide-methanol mobile phase.

2.6 Substrate uptake in bacteria

Energy sources are often limiting for bacterial growth in natural environments. The level of available nutrients in any environment usually fluctuates so any organism must be able to regulate its metabolism to cope with external changes. Transport systems are important sites of metabolic regulation (Saier 1985).

Bacteria have a variety of mechanisms by which solutes are transported in and out of cells. Solutes may be either concentrated on one side of a biological membrane or be in equilibrium across the membrane. Five substrate transport mechanisms for micoorganisms have been described (Dills *et al.* 1980; Cook 1992). It is important to realize than one organism may use more than one method for different substrates. Even within the same genus the same substrate may be transported in different ways.

Each of these substrate transport mechanisms will be described briefly in turn, with more emphasis on active transport, because of the relevance of this uptake mechanism to work later in this thesis.

2.6.1 Passive Diffusion

This involves substrates not interacting with the protein components of the cell membrane. The substrate crosses the lipid phase of the membrane until there is an equilibrium between the internal and external concentrations. Usually passive diffusion involves hydrophobic compounds because these are soluble in the membrane lipid bilayer. The first enzyme of the metabolic pathway normally controls the affinity of uptake and rate of transport (Russell *et al.* 1990).

2.6.2 Facilitated Diffusion

This uptake method is also a reversible and energy independent process. Facilitated diffusion aids the quick equilibration, along already existing gradients, of substrate across a membrane. Unlike passive diffusion, facilitated diffusion is mediated by an

integral membrane protein. Because this protein has stereospecificity towards substrates there is competition between the solute and any similar compounds. Because this is not an energy driven process, solute uptake via this method can not happen against a concentration gradient. Rates of uptake are much more rapid than for passive diffusion. Since there is a carrier protein involved, this method can exhibit saturation kinetics, and so the affinity of the transporter for the substrate can be measured (Russell *et al.* 1990). The driving force for continued uptake is intracellular solute transformation, which maintains a concentration gradient across the membrane, which allows more solute to be transported. The most well characterized bacterial facilitated system is that for glycerol (Lin 1976).

2.6.3 Osmotic shock sensitive active transport systems

These transport systems are involved in the transport of amino acids, peptides, organic acids and inorganic cations and anions (Gottschalk 1986) particularly in Gram negative bacteria. Transport using this system is binding-protein dependent. Substrate molecules are bound by binding proteins in the periplasmic space and then interact with membrane-embedded transport-system components and the substrate is taken up at the expense of ATP or another high energy phosphate compound (Ames and Higgins 1983).

2.6.4 Enzyme-mediated group transfer reactions

This method of substrate uptake is different from facilitated diffusion or carrier mediated active transport systems in that the solute is chemically altered during transport and arrives intracellularly in a chemically modified form, usually as a phosphate ester. The most well characterized group translocation systems are the phosphoenolpyruvate-dependent sugar phosphotransferases (Saier and Chin 1990; Meadow *et al.* 1990). This system is important in oral microbiology since oral streptococci transport sugars by this system (St Martin and Wittenburger 1979) and also amongst marine bacteria, for example members of the genus Vibrio (Meadow *et al.* 1987). Generally phosphotransferase systems (PTS) are favoured by facultatively anaerobic bacteria (Dills *et al.* 1980), however, there are exceptions, for example phosphoenolpyruvate (PEP)-

dependent glucose PTS have been found in *Bacillus* species (Romano *et al.* 1979) and in the transport of fructose in *Bacillus subtilis* (Delobbe *et al.* 1976).

2.6.5 Active Transport

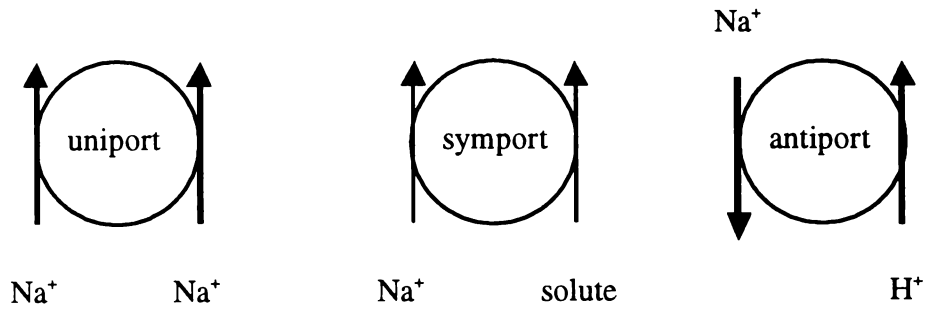
This is an energy-dependent process which allows substrate saturation of metabolic enzymes at much lower external substrate concentrations than those that are dependent on diffusion. This is important since most natural environments have low substrate concentrations.

The involvement of the membrane potential in energizing active transport is widely accepted. Mitchell (1961) proposed a theory of the establishment of a proton motive force and ATP production. The basic concept of Mitchell's energy conservation theory is that hydrogen and electron carriers of the respiratory chain are arranged in loops across the cytoplasmic membrane, so that oxidation of substrate brings about the transport of protons outward across the membrane, which generates an electrochemical gradient. The hydrolysis of ATP in the membrane-bound F_1F_0-ATPase is also able to translocate protons outward, also generating an H^+ gradient. This means that substrate oxidation via the respiratory chain and ATP hydrolysis both result in the same form of conserved energy, an electrochemical potential designated the proton motive force (Mitchell 1963). Gradient formation can also occur by ion pumps linked to product excretion or metabolic reactions (Gätje *et al.* 1991; Michels *et al.* 1979). A gradient like this is made up of a proton gradient (ΔpH) and an electrical potential ($\Delta\psi$). Under "normal" (non –alkaliphilic) bacterial growth the movement of ions / molecules proceeds in a direction which reduces a concentration or electrical gradient. In other words molecules move from a high concentration to a low concentration. For a discussion on energy generation in alkaliphiles and how this differs from energy generation in other bacteria refer to Section 2.2.3.

Most systems utilize the $\Delta\mu H^+$ by using carriers that have binding sites for both protons, and a particular substrate and both are transported into the cell. The $\Delta\mu H^+$ drives transport by different mechanisms depending on the substrate (Kaback 1986). The terms antiport(er), symport(er) or uniport are used to define how metabolites are linked to ion

transport. Uniports are processes where a solute moves across a membrane but is not tightly coupled to the movement of something else (Ambudkar and Rosen 1990). Symports are processes involving two or more substrates that are translocated obligately, by the same carrier and in the same direction (Ambudkar and Rosen 1990). Antiports are exchanges (counter-transports) where the two substrates are translocated in opposite directions (Ambudkar and Rosen 1990). Examples to illustrate these different processes are given below:

Figure 2.3 Three types of active transport processes



2.7 Objectives

The aims of this thesis were two-fold:

(1)To characterise *Bacillus* isolate TA2.A1, a novel alkaliphilic thermophilic aerobic strain, in terms of its growth characteristics and phylogeny.

(2) To characterise the bioenergetic processes utilized by *Bacillus* isolate TA2.A1 to overcome the energetic difficulties resulting from growth in conditions of extreme pH and temperature. In particular the mechanisms used for the uptake of a growth supporting amino acid (glutamate) and carbohydrate (sucrose) were investigated.

The description of glutamate uptake by *Bacillus* isolate TA2.A1 was published in the Journal of Bacteriology (1999) 181(10): 3172-3177. Because of this the format of Chapter 5 differs from that in the rest of the thesis.

Chapter 3 - Media Development and Growth

3.1 General features of media for alkaliphilic aerobic cultures

The development of a medium in which to grow *Bacillus* isolate TA2.A1 was initially based on an analysis of the spring water from which the organism was isolated. This analysis was displayed at the baths' office at the Te Aroha (New Zealand) domain. The isolate was obtained from a percolator fed with spring water from a thermal bore on Mount Te Aroha (Figure 3.1). The source temperature and pH were 77°C and pH 8.28 respectively. The ability of the organism to grow aerobically helped define what needed to be added to the basal medium. In naturally occurring alkaliphilic environments sodium carbonate is the major source of alkalinity. In media designed for alkaliphilic organisms sodium carbonate is predominantly used; although potassium carbonate, sodium bicarbonate, sodium borate, sodium orthophosphate and even sodium hydroxide have been used in some media. Many alkaliphiles can survive in media with low concentrations of divalent cations (Mg^{2+} and Ca^{2+}) which are normally scarce in alkaline environments, however, high levels in media can be toxic. No universal growth factors required for alkaliphilic growth have been reported. Wiegel (1998) observed that all isolated alkali-thermophiles required yeast extract, which could be replaced with tryptone (and sometimes peptone) but not with vitamins or trace elements, as a supplement of the medium. This requirement for yeast extract has been widely reported, for example, *Thermobrachium celere* an alkali-tolerant thermophilic obligate anaerobe required yeast extract for growth and in the presence of yeast extract could utilize a range of carbohydrates including glucose, sucrose, fructose, galactose and maltose (Engle *et al.* 1996). *Bacillus thermoalcaliphilus* (Sarkar 1991) a heterotrophic, thermophilic, alkaliphilic, facultative anaerobe required peptone or yeast extract, which could not be replaced with amino acids or vitamins, for growth. Yeast extract is also required for the growth of the anaerobic alkali-tolerant thermophile *Anaerobranca horikoshii* (Engle *et al.* 1995). *Bacillus* isolate TA2.A1 was able to utilize a wider range of carbon sources in minimal medium containing $0.1 g.l^{-1}$ peptone (Tables 4.9 and 4.10) than in the same medium without peptone (Table 4.11). Takahara *et al.* (1961) isolated a 'growth factor' for *Bacillus alkaliphilus* No. S-8, without the peptide Ala-Ile-

Figure 3.1: Thermal bore at Te Aroha, New Zealand

Leu-Val-Lys-Glu-Gly the bacterium was unable to reduce indigo blue. Most metabolic and physiological studies on thermophilic bacteria have been carried out using complex media with high levels of undefined compounds such as yeast extract or peptone (Heyne *et al.* 1991; Cook 1988; De Vrij *et al.* 1989).

No medium has been expressly developed for isolation of thermophiles per se. This is probably not surprising given the broad range of habitats and substrates utilized by organisms growing at high temperatures. Usually a medium for mesophilic bacterium is trialled for a physiologically similar thermophile. Two key modifications need to be made to mesophilic media before it is used for thermophiles; the effect of temperature on any bicarbonate in the media needs to be taken into account and for solid media the solidifying agent needs to be able to set at high temperatures. Agar becomes less solid or fails to set at all above 70°C; for this reason Phytagel™ (Sigma) or Gelrite™ (Kelco) were used to solidify media for *Bacillus* isolate TA2.A1. Gelrite™ is a low-acetyl clarified gellan gum that is solidified with aid of divalent cations, for example, magnesium and calcium. Contributing to this requirement for a firmer setting agent was the fact that at alkaline pH (above pH 9) agar is hydrolyzed at elevated temperatures. Bicarbonate is not stable above 50°C and releases CO_2 causing a shift to alkaline pH, obviously a move towards high pH is less of a problem when the organisms for whom the medium is being designed are both alkaliphic and thermophilic. Liquid media incubated at high temperature will show increased evaporation. This is especially true with increased aeration. Hence the need for tinfoil covers on top of non-absorbent cotton wool flask bungs. The increased humidity in sealed containers can cause internal condensation, leaving smears on surfaces of solid medium. For this reason media plates were incubated "upside down" i.e. with the agar surface at the top. To prevent solid media from drying out during incubation the circumference of the petri dishes was covered with masking tape. The caramelisation of sugars, which occurs readily under the pH and temperature growth conditions of isolate TA2.A1, is further enhanced under aerobic and high phosphate concentration conditions. At elevated temperatures the solubility of gases is lower. Because of this, aerobic culture vessels need to have a high ratio of surface to liquid volume i.e. a shallow layer of culture fluid per flask volume. Flask cultures of *Bacillus* isolate TA2.A1 were grown in an orbital-shaking incubator (Gallenkamp) to assist with this.

In most cases the pH of alkaline media and enrichments is achieved and maintained by adding an appropriate quantity of Na_2CO_3. This is the additive of choice since high concentrations of this compound are found in most naturally occurring alkaline environments, and there is some evidence that growth is enhanced in its presence (Nakamura and Horikoshi 1976c; Grant *et al.* 1979).

This chapter outlines (a) the development of both a minimal and rich medium capable of supporting the growth of *Bacillus* isolate TA2.A1 and (b) growth under flask or fermenter conditions.

3.2 Materials and methods

3.2.1 Chemicals

Unless otherwise stated, chemicals were of analytical grade and obtained from well-known chemical distributors. The suppliers of special chemicals are indicated in the appropriate section describing their use.

3.2.2 Incubation

The incubation of liquid culture was routinely carried out in an orbital shaking incubator (Gallenkamp) at 120 revolutions per minute and 65°C or 70°C. The incubation of solid media was carried out at the same temperatures in a stationary incubator (Clayson).

3.2.3 Assessment of growth

Growth was assessed by a combination of the following methods: optical density by spectrophotometer (650 or 450nm), examination under a phase contrast microscope (Olympus BH-2), an increase in total protein (by one of the methods outlined below), or detection of enzymatic activity. Growth on soluble substrates was evaluated by optical

density and/ or an increase in the total protein of the culture. Growth on less soluble substrates, for example, casein and ovalbumin, was assessed by phase contrast microscopy. This was particularly important for those substrates on which *Bacillus* isolate TA2.A1 grew in clumps rather than with an even distribution throughout the medium. Where samples were examined microscopically a number of fields of view (minimum of eight) were counted for each sample.

Perkin Elmer (lambda 3B), Pharmacia Biotech (Ultrospec 3000) or Pye Unicam (SP6 450 UV/VIS) spectrophotometers were used to monitor growth and unless stated were zeroed against RO water. Samples were placed in 3.5ml (1cm path) disposable cuvettes with a useful range 340 - 800nm (Biorad). All measurements in the same experiment were performed on the same instrument. This was important because different spectrophotometer brands gave slight variations in optical density on the same sample at the same wavelength. An increase in optical density of less than 0.02 was designated as no growth. Growth on each carbon source was tested in minimal medium and minimal medium without $0.1 g.l^{-1}$ of peptone.

3.2.3.1 Protein Assays

(1) Modified Lowry Assay (Peterson 1979)

Reagents:
Reagent A: 0.5% (w/v) $CuSO_4.5H_2O$ and 1% (w/v) Na_3citrate
Reagent B: 2.0% (w/v) Na_2CO_3 and 0.4% (w/v) NaOH
Working solution (use within 1.5hours): 1ml of reagent A and 50ml of reagent B
BSA (Bovine Albumin Fraction V –Sigma) standards: 0 – 1000µg/ml in Milli-Q water

Method:
Add 200µl of the protein samples (diluted appropriately in RO water) and standards to 1ml of the working solution. Vortex, then incubate at room temperature for 10 minutes. Add 100µl of 2N Folins-Ciocalteu reagent (Sigma) (diluted 1:1 with RO water) and mix immediately. Allow to stand for at least 1 hour at room temperature. Read the

absorbance by spectrophotometer at 750nm. This assay was linear over a range of protein concentrations to 100µg/ml.

(2) Modified Lowry for samples containing interfering substances (Peterson 1983)

Reagents:

	%	Molarity
$CuSO_4.5H_2O$	0.1	
Sodium and/or potassium tartrate	0.2	
Na_2CO_3	10	
Sodium dodecyl sulphate (SDS)	5.0	
NaOH		0.8
Folin-Ciocalteu phenol reagent		2.0N
Sodium deoxycholate (DOC)	0.15	
Trichloroacetic acid (TCA)	72, 6	

BSA (Bovine Albumin Fraction V) standards: 0–1000µg.ml^{-1} in Milli-Q water

CTC Reagent: Dissolve the Na_2CO_3 into 40ml of RO water. Dissolve the $CuSO_4.5H_2O$ and tartrate into a second 40ml of RO water. Add the first solution slowly, with stirring, to the second solution and make the final volume up to 100mls. This mixture will keep indefinitely at 10°C.

Reagent A: Mix one part of the CTC reagent with two parts of the SDS solution and one part of the NaOH solution. With time the SDS will precipitate out, but can be redissolved by warming. Reagent A will keep for 2-3 weeks at room temperature (20°C) but should be discarded if a dark precipitate appears.

Reagent B: Mix one part 2 N Folin-Ciocalteu phenol reagent with five parts of RO water. This is stable for months at room temperature (20°C) when protected from the light.

Method:

(1) Sample Pretreatment:

To a 400µl aliquot of sample or standard in a 1.5 ml reaction vial, add 40 µl of DOC, mix and allow to stand at room temperature for 10 minutes. Add 40µl of 72% TCA mix and centrifuge for 5 minutes in a Beckman microfuge E. Carefully remove the supernatant from the reaction vial. If the original protein solution contained an interfering substance that the Lowry method is extremely sensitive to, e.g. Hepes buffer or histidine, wash the pellet by adding 1ml of 6% TCA, mix and centrifuge as before and remove the supernatant. Repeat this washing process. Make a pin-hole through the parafilm covering the top of the reaction vials, then dry the pellet *in vacuo* for about an hour. Resuspend the pellet in 400µl of Milli-Q water. Any remaining precipitate will redissolve in the first step of the standard assay procedure (see below).

(2) Standard assay procedure:

To a 400µl aliquot of sample or standard, add 400µl of reagent A. Mix throughly and allow to stand for 10 minutes at room temperature. Add 200µl of reagent B and mix immediately. Allow to stand for 30 minutes (but no more than 60 minutes) at room temperature, then read the absorbance at 750nm. This assay was linear over a range of protein concentrations to 100µg/ml.

(3) Lowry Method of Waterborg and Matthews (1994)

This method was used in preference to those outlined in (1) and (2) above in two situations: (a) to solubilise or lyse cells to assess total protein and (b) to directly assess higher protein concentrations, since this assay can determine protein concentrations to 2000µg/ml. The assay is linear to a concentration of 500µg/ml of protein when read at 750nm and linear to a concentration of 2000µg/ml when read at 550nm.

Reagents:

(1) Complex- forming reagent (prepare immediately before use) Mix stock solutions, a, b and c in the proportion 100 : 1 : 1 (v:v:v).

solution a : 2% (w/v) Na_2CO_3. in distilled water

solution b : 1% (w/v) $CuSO_4.5H_2O$ in distilled water

solution c : 2% (w/v) sodium potassium tartrate in distilled water
(2) 2N NaOH
(3) Folin-Ciocalteu phenol reagent use at 1N concentration

Standards : Use a stock solution of standard protein (bovine serum albumin fraction V) containing 4mg / ml protein in Milli-Q water stored frozen at -20°C. Prepare standards by diluting the stock solution with Milli-Q water to give these concentrations (µg/ml) 0, 10, 20, 50, 100, 200, 500, 1000, and 2000.

Method:
(1) To 0.1ml of sample or standard add 0.1ml of 2N NaOH and hydrolyze at 100°C for 10 minutes in a boiling water bath
Note: The precipitation step of Peterson (1983) which allows the separation of the protein sample from interfering substances (and concentrates the sample allowing the determination of proteins in dilute solution) can be carried out as the first step with the pellet then being treated as in step (1) above.
(2) Cool the hydrolyzate to room temperature and add 1 ml of freshly mixed complex-forming reagent. Let the solution stand at room temperature for 10 minutes.
(Note: The reaction is very pH dependent and it is important to maintain the pH between 10 and 10.5. The incubation period is not critical (as long as it is consistent) and can vary from 10 minutes to several hours without affecting the final absorbance.)
(3) Add 0.1ml of Folin-Ciocalteu phenol reagent, using a vortex mixer (mix immediately and well), and let the mixture stand at room temperature for 30 - 60 minutes (do not exceed 60 minutes)
(4) Read the absorbance at 750nm if the protein concentration is below 500 µg/ml or at 550nm if the protein concentration is between 100 and 2000µg/ml
(5) Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations

Note: A range of substances (buffers, sugars, nucleic acids etc) interfere with the Lowry assay. In many cases the effect of these can be minimized by dilution assuming that the protein concentration is high enough to be detected after dilution. Interference caused by detergents, sucrose and EDTA can be eliminated by SDS (Markwell *et al.* 1981).

(4) Bradford Protein Assay (Reade and Northcote 1981)

This method of protein determination was used in preference to those outlined above for a fast assessment of protein concentrations. The only reagent in this assay has a long shelf-life and once prepared can be used to ascertain protein concentrations after 5 minutes.

Reagents:
Dye reagent: Coomassie blue G (Serva) in 1.6 M phosphoric acid / 0.8 M ethanol
BSA standards : 0–200 µg/ml in Milli-Q water

Method:
Add 40µl of sample/standard to 1.2 ml of the dye reagent. Mix briefly and allow to stand for 5 minutes at room temperature. Read the absorbance by spectrophotometer at 595 nm The assay is linear at protein concentrations from 0 to 100µg/ml.

3.2.3.2 SDS-PAGE sample preparation and electrophoresis

The PhastGel method (Pharmacia) using homologous 20%, 10-15% gradient or high density SDS polyacrylamide Phastgels (Pharmacia) was used for electrophoresis. Sample buffer was prepared as a five times concentrated stock solution and contained (final concentration) 10mM Tris/HCl pH 8.0, 1mM EDTA, 2.5% SDS (may require heating to dissolve), 5% β-mercaptoethanol and 0.01% bromphenol blue.

2µl of the five times concentrated SDS PAGE sample buffer was added to samples or standards and mixed well. If the solution was a yellow colour, concentrated NaOH was added 2µl at a time until the solution turned blue. The minimum volume of NaOH was added since any more would cause protein precipitation. The samples were boiled for 10 minutes then centrifuged in a microfuge on full speed for 5 minutes. The supernatants were loaded onto the PhastGel and then run according to the Pharmacia protocol.

3.2.3.2.1 Silver staining of polyacrylamide gels

The SDS polyacrylamide Phastgels were stained using the method outlined in Table 3.2; all reagents were freshly prepared immediately prior to use. Molecular weight standards (Table 3.1) were loaded onto the Phastgels and run according to the Pharmacia protocol.

3.2.4 Maintenance of stock cultures

Stock cultures were maintained in rich medium (refer to section 3.3.1.3) by sub-culturing on a weekly basis. These cultures were grown for 18 hours at 65°C in an orbital shaking incubator and then stored at room temperature (18–22°C). Ampoules of *Bacillus* isolate TA2.A1 were also stored as freeze-dried preparations of rich medium at room temperature and in 15% (v/v) sterile glycerol in nutrient broth at –70°C.

Table 3.1: SDS-PAGE standards from Pharmacia

standards	$mg.ml^{-1}$	molecular weight (kDa)
Phosphorylase b	6.4	94
BSA	8.3	67
Ovalbumin	14.7	43
Carbonic anhydrase	8.3	67
Soybean trypsin inhibitor	8.8	20.1
α –lactalbumin	12.1	14.4

The standard mixture was diluted as appropriate (1/20 to 1/50) for silver staining.

Table 3.2: Silver staining of electrophoresis gels (Biorad Method)

Step	Time	Solution	Comment
1	30 minutes	40% (v/v) methanol, 10% (v/v) acetic acid	Fixative
2	15 minutes	20% (v/v) ethanol, 5% (v/v) acetic acid	Fixative
3	15 minutes	20% (v/v) ethanol, 5% (v/v) acetic acid	Fixative
4	5 minutes	8.3% (v/v) gluteraldehyde	Sensitizing
5	-	Milli-Q water	Wash
6	20 minutes	0.25% (w/v) $AgNO_3$ (prepare immediately before use)	Stain
7	-	Milli-Q water	Wash
8	3 – 10 minutes	0.04% (v/v) formaldehyde, 2.5% (w/v) Na_2CO_3	Developer
9	10 minutes	5% (v/v) acetic acid	Stop
10	5 minutes	10% (v/v) acetic acid, 5% (v/v) glycerol	Preservative

3.2.5 Preparation of liquid media

3.2.5.1 Influence of peptone brand

When added as a trace ($0.1 g.l^{-1}$) to minimal medium, peptone was added either directly, prior to autoclaving, or from a filter sterilized stock after the medium was autoclaved. In rich medium, peptone ($10 g.l^{-1}$) was added prior to autoclaving with the other medium components. A comparison of growth using peptone from different suppliers was carried out.

3.2.5.2 Trace element solutions and vitamins

Dictyoglomus Trace Elements (Saiki *et al.* 1985) contained final concentrations of ($mg.l^{-1}$): $CoCl_2.6H_2O$ 2.9, $Na_2MoO_4.2H_2O$ 2.4, Na_2SeO_3 0.17, $MnCl_2.4H_2O$ 2.0 and $ZnSO_4$ 2.8.
This was made as a 1000 X concentrated stock in Milli-Q water and stored at 4°C. A working stock, was then prepared using 1ml of concentrated stock to a litre of Milli-Q water, stored at 4°C.

The vitamin solution of Wolin (1963) contained (mg per litre) biotin 2.0, folic acid 2.0, pyridoxine-HCl 10.0, riboflavin 5.0, thiamine-HCl 5.0, nicotinic acid 5.0, pantothenic acid 5.0, vitamin B_{12} 0.1, p-aminobenzoic acid 5.0 and thioctic acid 5.0. This solution was filter sterilized through a 0.45µm disposable filter and stored in an amber bottle (protected from light) at 4°C until required. Wolins vitamins were added aspectically to the medium after it had been autoclaved and cooled to 65°C.

3.2.5.3 Carbon sources

All soluble carbon sources were added to medium separately after autoclaving from 10% (w/v) filter sterilized stock solutions. Insoluble carbon sources were added to the medium prior to autoclaving.

3.2.5.4 Flask preparation and inoculation

Where possible (due to incubator space and flask availability) 100ml aliquots of medium were dispensed in 500ml flasks. The surface area to volume ratio under these conditions proved optimal for growth. Where it was necessary to grow smaller culture volumes the volume of medium added to the different flask size was calculated to give a surface area to volume ratio as close to optimal as possible. The flasks were stoppered with non-absorbent cotton wool bungs, covered in cheesecloth and the bungs were capped with aluminium foil, to reduce volume loss due to evaporation. Cultures, unless otherwise stated, were inoculated using a 2% (v/v) inoculum from an 18 hour culture grown at 65°C, in the same medium.

3.2.5.5 Sterilization

All autoclaving was at 121°C and one atmosphere overpressure for 15 minutes. Filtration, unless otherwise stated, was through 0.45μm pore size disposable filters (Minisart).

3.2.5.6 pH measurement and adjustment

3.2.5.6.1 pH standards

Measurement and adjustment of pH was conducted using a Philips (PW 9421) pH-meter. For standardizing pH electrodes (Orion 9156SC) at pH 7 at room temperature Colourkey buffer solutions (BDH) were used. For pH adjustment at pH 10 (70°C) and for any pH adjustment at temperatures above room temperature, Philips pH standards were used.

3.2.5.6.2 pH adjustment / effect of temperature and media type on pH

The pH of media, unless otherwise stated, was adjusted to pH 10.7 at 20°C before autoclaving. This gave a post-autoclaving pH of 10.6 (20°C) which was pH 9.8 at incubation temperature (65 / 70°C). During the preparation of media the pH was adjusted with the addition of 10 N NaOH or 10 N HCl as required.

To follow how pH varied under different temperature conditions, media containing different buffering agents was adjusted to a range of pH values at 70°C, shown in the first column of Tables 3.4 and 3.5 (Section 3.3.1.5). The range of pH values for each buffering system was selected to ensure an overlap occurred. Each pH or buffer combination had ten replicates. After pH adjustment at 70°C each replicate was cooled to 20°C, the pH meter re-equilibrated at 20°C and the pH recorded at 20°C. After autoclaving (15 minutes at 15 psi) the pH readings were repeated at both 20°C and 70°C.

3.2.6 Original liquid medium composition

The original liquid medium composition, based on spring water analysis, was as follows (per litre of distilled RO water):
Na_2SO_4 0.5g, $(NH_4)_2SO_4$ 0.1g, $MgSO_4.7H_2O$ 0.1g, $CaCl_2.2H_2O$ 0.05g (added after autoclaving), K_2HPO_4 0.2g, $NaHCO_3$ 9.0g, Dictyoglomus Trace Elements 5.0ml, Peptone (Oxoid Tryptone) 10g and Wolins Vitamins 1.0ml (added after autoclaving). The pH of the medium was adjusted to pH 10 (at 70°C) by the addition of 10 N NaOH. After autoclaving at 15psi for 15 minutes the medium pH was 9.8 (70°C). Wolin's vitamins and calcium chloride were added from filter sterilized stock solutions stored at 4°C when the medium had cooled to room temperature, after autoclaving. Addition of calcium chloride after autoclaving assisted in reducing the level of precipitation in the medium.

Further development of this medium is outlined in the results section (3.3.1.1).

3.2.7 Other alkaline liquid media

Two alternative liquid medium preparations were trialled for their ability to support the growth of *Bacillus* isolate TA2.A1.
(a) Modified A1 growth medium (B. Patel, Griffith University, Australia, personal communication) was composed of (% w/v) glucose 1.0*, peptone 0.5, yeast extract 0.5, K_2HPO_4 0.1*, $MgSO_4.7H_2O$ 0.02, $CaCl_2.2H_2O$ 0.03*, Na_2CO_3 1.0, casamino acids 0.4. The chemicals designated with an asterisk were autoclaved separately.

(b) Synthetic medium (B. Patel, Griffith University, Australia, personal communication) (% w/v) glucose 1.0*, K_2HPO_4 0.1*, $MgSO_4.7H_2O$ 0.02, $CaCl_2.2H_2O$ 0.03*, Na_2CO_3 1.0, casamino acids 0.4. The chemicals designated with an asterisk were autoclaved separately.

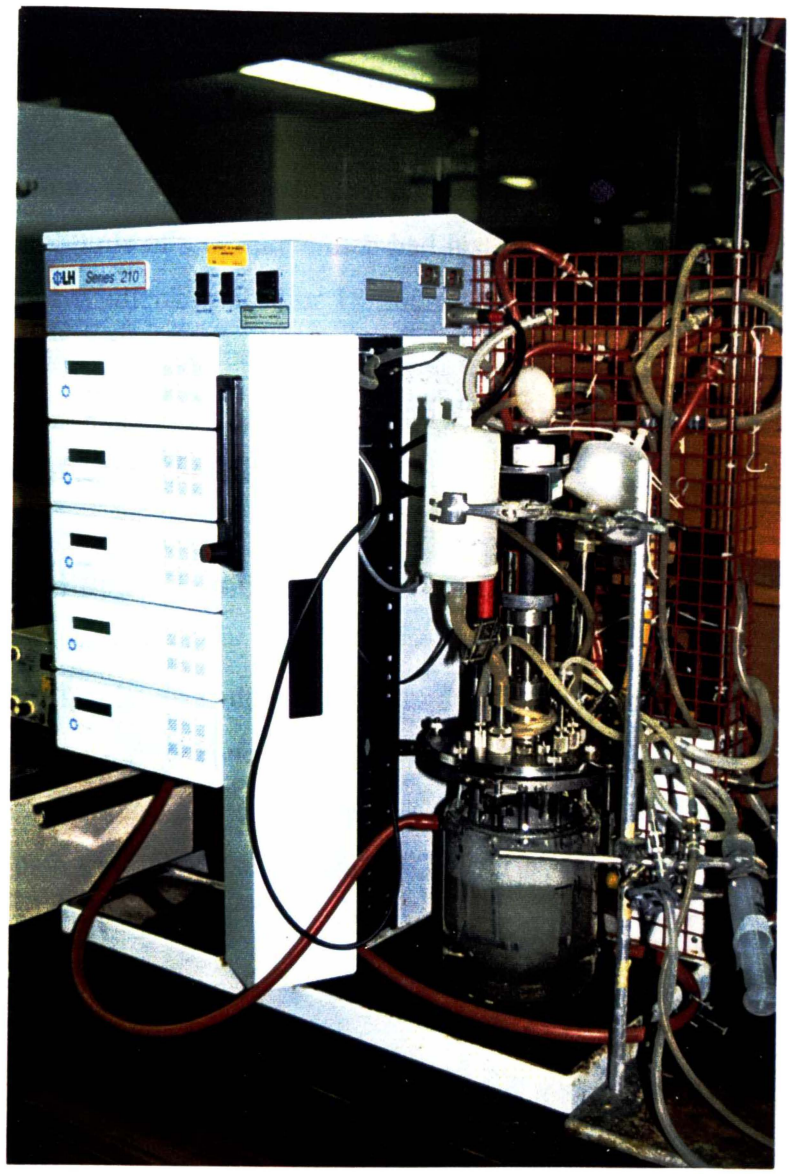
3.2.8 Preparation for growth in a fermenter vessel

Two fermenter types (LH Fermentation / LH series 210) were used for scaling up the culture volume of *Bacillus* isolate TA2.A1 (Figure 3.2). The first used a water jacketed fermenter with a 1.5 litre working volume which was heated by circulating water from a 66°C water bath. A stable temperature was maintained, with the temperature inside the fermenter being 1°C lower than the waterbath. The other fermenter had a 2 litre working volume and was heated via heating elements (Watlow). In either case the temperature was maintained at 65°C. The preparation of medium for use in these fermenters followed the same procedure, the only adjustments being for the different working volumes.

The fermenter vessel containing 500ml RO water was autoclaved for 30 minutes at 15psi. If the fermenter was not used immediately after autoclaving it was left sealed in the autoclave until immediately prior to use. This precaution was to prevent the change in air pressure, as the autoclave was opened, forcing non-sterile air into the vessel because filters or tubing were occasionally dislodged during autoclaving. Minimal medium components for 2 litres of medium (as per section 3.3.1.2) were made up in a quarter of the total volume of RO water. After pH adjustment the medium was filtered (Sartorius, Sartobran PH Capsule Filters 0.2µm) into a sterile fermenter vessel. The remaining RO water was then filtered into the vessel, enabling the filter to be washed of any remaining medium components. If a double batch of medium was run through the fermenter i.e. one vessel full harvested and then another lot of medium added immediately and growth continued, any medium additions to an already hot vessel were warmed to prevent any cold shock and drop in growth rate.

Before connection to the fermenter the pH electrode (Ingold) was connected to a conventional pH meter (Philips PW 9421) to check that it was responding accurately. The pH electrode was then washed with ethanol, placed into the fermenter vessel and the pH parameters for the fermenter set. Regulation of pH, to pH 10 at 70°C, was made by dosing with 2M H_2SO_4 and 2M NaOH which had been autoclaved at 15 psi for 15 minutes. The dosing parameters for the fermenter pH control were: dead band 5%, derivative time 0 seconds, integral time 0 seconds and proportional band 15%.

Figure 3.2: Fermenter vessel LH Series 210



These settings were important in preventing the pH dosing pump from overdosing which would cause the volume in the fermenter vessel to increase to such an extent that it would overflow.

An oxygen probe was used to measure the percentage saturation of oxygen under fermenter conditions. It should be noted that this is not equivalent to partial pressure.

Prior to inoculation but once the medium was at growth temperature the medium in the fermenter was flushed with nitrogen for five minutes. This level was used to calibrate the oxygen probe at the 0% oxygen level, after a further five minutes of flushing with oxygen the 100% oxygen level was set. The agitation level in the fermenter (unless stated otherwise) was set to 500rpm. Airflow into the fermenter was at 1 litre per minute.

Once the medium in the fermenter vessel had reached temperature the vessel was inoculated with 30mls of a culture which had just been removed from the 65°C shaking orbital incubator (Gallenkamp) after 18 hours of growth. The inoculum was grown on the same medium present in the fermenter.

3.2.9 Preparation of Solid media

3.2.9.1 Agar addition to liquid medium

2 % (w/v) agar (Davis) was added to rich liquid medium (section 3.3.1.3) and dissolved by heating prior to autoclaving. To prevent drying of the plates at the incubation temperature of 65°C the plates were poured thicker than normal and the circumference of each petri-dish and lid was sealed with masking tape.

3.2.9.2 Gelrite™ Plates

Medium for Gelrite™ plates contained (per litre of distilled RO water): Na_2SO_4 0.5g, $(NH_4)_2SO_4$ 0.1g, $MgSO_4.7H_2O$ 1.8g, K_2HPO_4 0.2g, $NaHCO_3$ 9.0g, Dictyoglomus Trace Elements 5.0ml and peptone. The concentration of peptone was either at 0.1 or $10g.l^{-1}$. The results of these trials are discussed in section 3.3.2.2.

The concentration of Mg^{2+} in this medium was increased over that used in broth medium in order for the Gelrite™ to set. After adjusting the pH of the medium to pH 8.5 (at 20°C) $8g.l^{-1}$ of Gelrite™ was added, mixed and brought to the boil in a microwave. The medium was then autoclaved without delay at 15 psi for 15 minutes. After autoclaving the medium was immediately dispensed into petri dishes, because it sets irreversibly on cooling.

3.2.9.3. Phytagel™ Plates

Medium for Phytagel™ (Sigma) plates contained (per litre of distilled RO water): Na_2SO_4 0.5g, $(NH_4)_2SO_4$ 0.1g, $MgSO_4.7H_2O$ 0.1g, K_2HPO_4 0.2g, $NaHCO_3$ 9.0g, Dictyoglomus Trace Elements 5.0ml and peptone. The concentration of peptone was either at 0.1 or 10g.l^{-1}. The results of these trials are discussed in section 3.3.2.3.

After the medium had been adjusted to the correct pH Phytagel™ was added (8g.l^{-1}) and dissolved by heating in a microwave. Sterilization was then conducted by autoclaving for 15 minutes at 15 psi. On removal from the autoclave the medium was poured directly into petri-dishes.

3.2.9.4 Other alkaline solid media

There are two standard basal alkaliphilic media used by Horikoshi and co-workers. These contain (g.l^{-1}):
Horikoshi-I: glucose 10.0, polypeptone 5.0, yeast extract 5.0, KH_2PO_4 1.0, $Mg_2SO_4.7H_2O$ 0.2, Na_2CO_3 10.0 and agar 20.0.
Horikoshi-II: soluble starch 10.0, polypeptone 5.0, yeast extract 5.0, KH_2PO_4 1.0, $Mg_2SO_4.7H_2O$ 0.2, Na_2CO_3 10.0 and agar 20.0.

These media are identical with the exception that Horikoshi-I contains glucose and Horikoshi-II soluble starch both at 10 g.l^{-1}. This medium was prepared with sucrose (0.5 g.l^{-1}) added instead of either the glucose or the starch. Peptone (Tryptone Oxoid) was used instead of polypeptone.

3.3 Results and Discussion

3.3.1 Liquid media

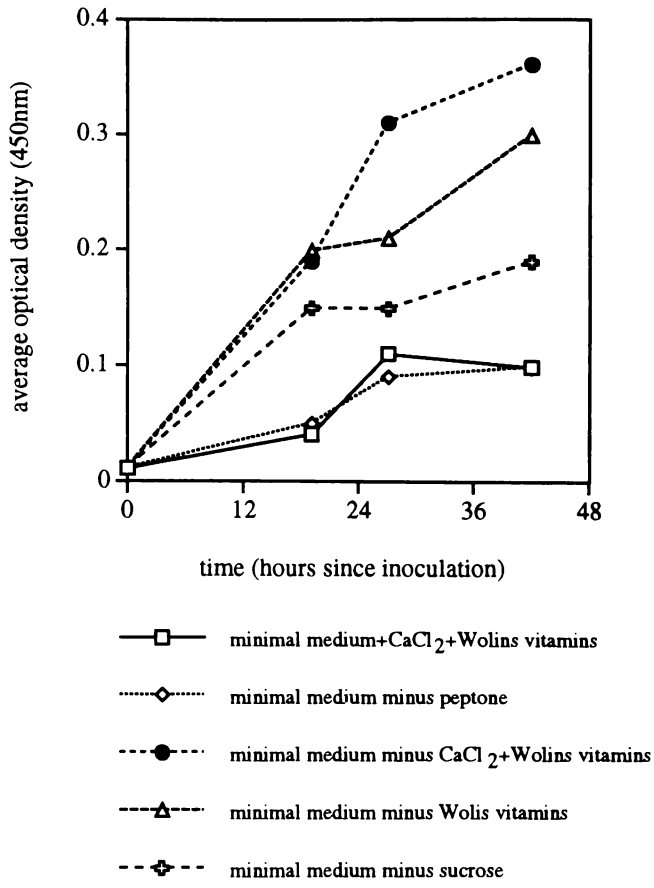
3.3.1.1 Optimization of growth on liquid media

Beginning with the "Original Liquid Medium", as outlined in section 3.2.6 experiments were carried out to optimize a liquid medium for growth. Growth of *Bacillus* isolate TA2.A1 in media with and without calcium chloride was evaluated by microscopic examination (Phase Contrast Olympus BH-2) and measurement of optical density (Perkin Elmer Spectrophotometer). Growth was found to be better in the absence of calcium. Similarly the addition of Wolin's vitamins appeared to hold no growth advantages for the organism, indeed in the case of minimal medium it appeared to be inhibitory (Figure 3.3).

The addition of urea to minimal medium was not growth enhancing. This alternative nitrogen source was tested because it was possible that ammonium ions added in the form of ammonium sulphate, the main source of nitrogen in the medium, could have been volatized under the incubation conditions of high pH and temperature.

In order to reduce the cloudiness which was sometimes present in minimal medium the concentrations of K_2HPO_4 and Na_2SO_4 were adjusted to 20% (w/v) of their original concentration. The refined medium contained (per litre) K_2HPO_4 0.04g and Na_2SO_4 0.1g. The concentration of other medium components remained unaltered.

Figure 3.3: Growth of *Bacillus* isolate TA2.A1 in sucrose ($0.5g.l^{-1}$) supplemented minimal medium



3.3.1.2 Minimal liquid medium

After development (as outlined in section 3.3.1.1) minimal medium contained the following (per litre of distilled RO water):

Na_2SO_4 0.1g, $(NH_4)_2SO_4$ 0.1g, $MgSO_4.7H_2O$ 0.1g, K_2HPO_4 0.04g, $NaHCO_3$ 9.0g, Dictyoglomus Trace Elements 5.0ml, and Peptone (Oxoid) 0.1g.

The $0.1g.l^{-1}$ peptone in minimal medium enabled growth on other carbon sources, sucrose for example, to generate a higher final yield than on either $0.1g.l^{-1}$ peptone or sucrose alone (Figure 4.23a). The low concentration of peptone in minimal medium was to keep the medium as defined as possible so that growth on added carbon sources could clearly be ascribed to that carbon source. For evaluation of growth on different carbon

sources using this medium refer to Chapter four, Section 4.4.1 for an overview, and Section 4.4.2 for details on specific carbon sources.

3.3.1.3 Rich liquid medium

The composition of rich liquid medium was as for the original liquid medium (Section 3.2.6) without the Wolins vitamins or calcium chloride.

3.3.1.4 Influence of peptone brand on growth

The growth of isolate TA2.A1 on a range of comparable peptones; Tryptone (Oxoid code L42), Bacto™ Tryptone (Difco 0123-17-3), Trypticase® Peptone (BBL) was compared. The analysis was conducted at three concentrations; the concentration added to minimal medium ($0.1 g.l^{-1}$), $1 g.l^{-1}$ and $10 g.l^{-1}$ (rich medium). Each test was conducted in Universal bottles with six replicates, the average optical density readings are given below.

Table 3.3: Growth of *Bacillus* isolate TA2.A1 in media containing different brands of peptone

	Average optical density[1] (450nm) after 20 hours incubation at 65°C			
	Peptone $g.l^{-1}$	Oxoid (tryptone)	Difco (bactotryptone)	BBL (trypticase)
Minimal medium with no sucrose	0.1	0.200	0.14	0.10
	1.0	0.304	0.269	0.338
	10.0	0.650	0.588	0.668
minimal medium with sucrose	0.1	0.394	0.229	0.17
	1.0	0.507	0.449	0.498
	10.0	ND	ND	ND

[1] in Universal bottles ND = Not Done

Addition of peptone at $0.1 g.l^{-1}$ final concentration, either directly into minimal medium prior to autoclaving or from a filter sterilized stock solution produced best growth if the

peptone was from Oxoid (Tryptone) (Table 3.3). The product Trypticase® Peptone from BBL at the same concentration was not as effective. At 1 and 10g.l^{-1} all brands supported similar levels of growth. The addition of 10g.l^{-1} peptone to minimal medium with sucrose was not tested (ND in Table 3.3) because rich medium (i.e. peptone at this concentration) never contained sucrose. In the rich medium (10g.l^{-1}) the source of peptone did not influence growth. However, in the minimal medium (0.1g.l^{-1} peptone) the source of peptone influenced growth. There are two possible explanations for this phenomenon: (1) there is something inhibitory in brands of peptone other than Oxoid (Tryptone) or (2) there is some component lacking in the other peptone brands that is required for growth. Unless competitive inhibition were taking place, where the ratio of inhibitory compounds and the 'real' substrate stayed the same at increasing peptone concentrations, the first explanation can be discounted, since something inhibitory in the peptone would have greater effect at higher concentrations. Since the source of the peptone only affected growth in minimal medium this was clearly not the case. It is possible however, that there is something present in Oxoid peptone (Tryptone) at low concentrations that is required for *Bacillus* isolate TA2.A1 to grow. At higher peptone concentrations this requirement could be met by other media components. Because Oxoid peptone supported better growth at minimal medium concentration (0.1g.l^{-1}) and growth at rich medium concentration (10g.l^{-1}) was similar irrespective of the peptone brand; unless otherwise stated Oxoid peptone was used in all media.

3.3.1.5 pH variation under different media and temperature conditions

The fact that temperature affects pH is not surprising. Often however when pH optima or pH ranges for growth are reported in the literature the temperature at which this characteristic was tested is not recorded. For organisms living near neutral pH under mesophilic conditions this omission may not be significant. Often this omission is made when referring to thermophilic organisms where it is important to know at what temperature the readings were taken. Whether a pH was recorded at 20°C or 70°C, for example, can drastically change the significance of that statistic. Hence in this thesis the temperature that a pH was measured at is recorded along with the pH reading itself. When working with an isolate that is living under two extreme conditions, that of both high pH and temperature, small alterations in pH or temperature can become significant.

The average pH data from six replicates for rich and minimal media at 20 and 70°C is represented in Table 3.4 and 3.5 respectively. It is worth noting that not only does temperature influence pH, so too does autoclaving and medium type. Conventionally, media was buffered with $9g.l^{-1}$ $NaHCO_3$ unless otherwise stated and was pH adjusted to pH 10 at 70°C (pH 10.7 at 20°C) prior to autoclaving. This meant that after autoclaving the pH at growth temperature (65/70°C) was 9.8 (10.6 at 20°C).

Table 3.4: Average pH in rich medium at 20°C and 70°C pre# and post* autoclaving for 15 minutes at 15 psi

Buffer	pH at 70°C #	pH at 20°C #	pH at 20°C*	pH at 70°C*
50mM MOPS	6.5	6.9	7.1	6.6
	7	7.45	7.74	7.17
	7.5	8.06	8.5	7.75
	8	8.76	9.34	8.36
50mM TAPS	7.5	8.42	8.66	7.73
	8	8.95	9.22	8.20
	8.5	9.47	9.74	8.69
	9	10.32	10.33	9.35
50mM CAPS	8.5	9.67	9.99	8.73
	9	10.2	10.36	9.06
	9.5	10.76	10.8	9.47
$9g.l^{-1}$ $NaHCO_3$	9.5	10.08	10.19	9.45
	10	10.7	10.6	9.8
	10.5	11.55	11.05	10.15
	11	12.52	11.86	10.62

Table 3.5: Average pH in minimal medium at 20°C and 70°C pre# and post* autoclaving for 15 minutes at 15 psi

Buffer	pH at 70°C #	pH at 20°C #	pH at 20°C*	pH at 70°C*
50mM MOPS	6.5	6.9	7.1	6.7
	7	7.46	7.84	7.39
	7.5	7.99	8.83	8.23
	8	8.57	9.52	8.9
50mM TAPS	7.5	8.31	8.52	7.72
	8	8.82	9.01	8.15
	8.5	9.31	9.67	8.7
	9	10.21	10.25	9.28
50mM CAPS	8.5	9.43	10.01	8.98
	9	10.05	10.38	9.3
	9.5	10.61	10.77	9.69
$9g.l^{-1}$ $NaHCO_3$	9.5	10.08	10.10	9.41
	10	10.7	10.6	9.8
	10.5	11.41	11.02	10.1
	11	12.32	11.71	10.5

3.3.1.6. Growth of *Bacillus* isolate TA2.A1 on other alkaliphilic media

Both modified A1 and the synthetic growth media (section 3.2.7) were prepared and inoculated as outlined in section 3.2.5.4. Growth was monitored as outlined earlier (section 3.2.3). *Bacillus* isolate TA2.A1 grew in both media, however, the final cell yield was 50% of that in a minimal medium with $0.5g.l^{-1}$ sucrose culture.

3.3.2 Growth of *Bacillus* isolate TA2.A1 on solid media

3.3.2.1 Agar addition to liquid media

Rich liquid medium, as per section 3.3.1.3, had 2% (w/v) agar (Davis) added and was poured into petri-dishes. An 18 hour rich medium culture was then spread over the surface of the plates, however, no growth was observed after 72 hours incubation in a stationary incubator (Clayson) at 70°C. It was difficult to evenly spread the inoculum on these plates because of their soft nature. A lack of growth on agar containing media has been observed previously for thermophilic organisms. The growth of thermophilic

Alicyclobacillus-like isolate COMP.A2 (Huang 1998) was inhibited by the presence of agar. For this reason and because of the soft nature of plates set with agar, under incubation conditions of high temperature and pH, alternative setting agents were investigated.

3.3.2.2 Gelrite ™Plates

Plates with rich medium and 8g.l^{-1} Gelrite™ (Kelco) were inoculated with a culture grown in a broth of the same composition and streak plated for single colonies. Plates were incubated at 65°C and 70°C in stationary incubators. After nineteen hours colonies were visible on plates incubated at 65°C but not at 70°C. No colonies were evident at 70°C even after extended (96 hours) incubation. Colonies of *Bacillus* isolate TA2.A1 on solid media were small and translucent in appearance. Three colonies from each of the Gelrite™ plates were inoculated back into liquid culture and their morphology was checked. In liquid culture cells of isolate TA2.A1 were often long tangled filaments with an average length of 6µm and a constant width of 0.3µm. For scanning electron microscope photographs of *Bacillus* isolate TA2.A1 refer to Figures 4.1 to 4.3. Growth of colonies from the streak plate was assessed by optical density and carbon source utilization by HPLC when inoculated back into liquid culture. The colonies were capable of growing on the range of carbon sources utilized by *Bacillus* isolate TA2.A1.

In rich medium Gelrite™ plates over a range of incubation temperatures (45, 55, 65, 70°C) and medium pH values (8, 8.5, 9.0, 9.5, 10.0) best growth, assessed by spread plate colony counts, was at pH 8 (65°C) and 65°C. This was equivalent to a pH of 8.5 (20°C) prior to autoclaving. Minimal medium with sucrose (0.5g.l^{-1}) added as a carbon source was also solidified into plates using Gelrite™. Despite the fact that this medium in liquid form was able to support good growth of *Bacillus* isolate TA2.A1 no growth was observed using an inocula grown on either minimal medium supplemented with sucrose or rich medium. A range of incubation temperatures (45, 55, 65, 70°C) and medium pH's (8, 8.5, 9.0, 9.5, 10.0) were assessed with the same lack of growth resulting.

3.3.2.3 Phytagel™ Plates

Plates of rich and minimal media were set with 8g.l^{-1} Phytagel™ (Sigma). No growth was achieved in the minimal medium plates which had sucrose (0.5g.l^{-1}) as their carbon source at any of the pH (8.0, 8.5, 9.0, 9.5, 10.0) or temperature (45, 55, 65, 70°C) variations tested. Growth was achieved at all of the pH (8.0, 8.5, 9.0, 9.5, 10.0) and temperature variations (45, 55, 65, 70°C) tested on rich medium plates set with Phytagel™. Best growth, by spread plate colony counts, was at pH 8 (65°C) and 65°C. This is equivalent to pH 8.5 (20°C) prior to autoclaving. At lower temperatures the plating efficiency was lower. Three representative colonies from each of the Phytagel™ plates were inoculated back into liquid culture and their morphology was checked. The expected long filamentous cells were cultured and these were capable of utilizing a range of carbon sources typical of isolate TA2.A1. Colony numbers were ten fold higher on rich medium Phytagel™ plates than on identical plates set with Gelrite™.

3.3.2.4 Other alkaline solid media

Horikoshi (1991b) noted that the sodium carbonate in Horikoshi I and Horikoshi II media (refer to section 3.2.9.4 for media components) needed to be sterilized separately, otherwise the microorganisms exhibited poor growth. Unaltered, the basal medium used by Horikoshi and co-workers was unsuitable for growth of isolate TA2.A1 due to the fact that it contained either glucose (Horikoshi I) or soluble starch (Horikoshi II) which were inhibitory or non-utilizable by isolate TA2.A1 as carbon sources. However, *Bacillus* isolate TA2.A1 did not grow on these media when sucrose (0.5g.l^{-1}) replaced the glucose or starch.

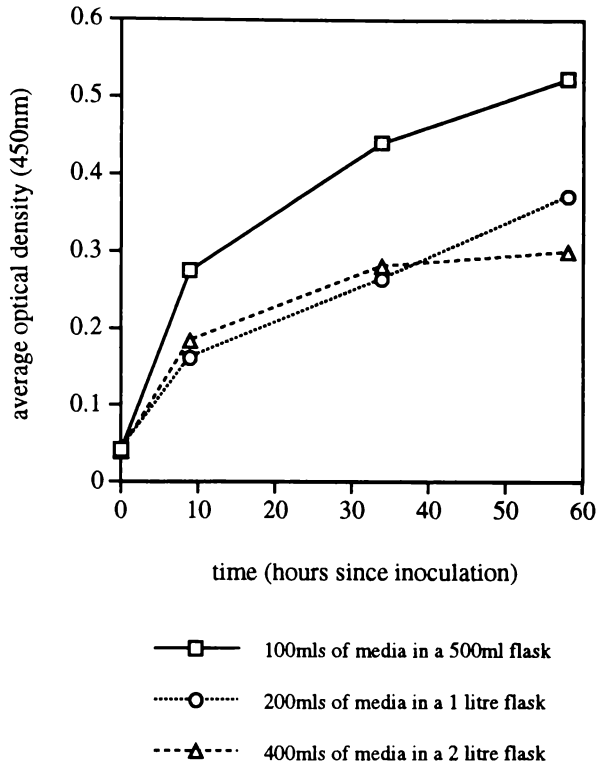
3.3.3 Scale up of media volume

3.3.3.1 Flask growth

Flask cultures in a rotary shaking incubator need a high surface area to volume ratio in order to get good growth; this is particularly important as the size of the culture volume is increased. For example, much better growth was achieved with 100mls of medium in

a 500ml flask, than with 200mls of the same medium, using the same (2% v/v) inoculum, in a one litre flask (Figure 3.4). The volume of inoculum used for each replicate was the same in all instances.

Figure 3.4: Growth of *Bacillus* isolate TA2.A1 in minimal medium plus 0.5g.l^{-1} sucrose with different media volumes and flask sizes



When scaling up culture size from 500ml (100mls of medium) to two litre flasks (400mls of minimal medium) there was no corresponding proportional increase in growth. Baffled flasks, despite giving more aeration, failed to produce a higher growth yield. Identical non-baffled flasks were used for comparison in case too much aeration was growth limiting. Both baffled and non-baffled flasks were incubated in orbital shaking and stationery incubators for comparison. Numerous attempts were made to scale up culture volume, using different medium volumes and flask sizes, in an attempt to achieve a proportional increase in yield, but this was not achieved on any of the carbon sources tested. The disadvantage in best growth being achieved with a small volume of medium is that it limits the number and size of samples that can be removed without significantly altering the culture volume, this is particularly important when

growth is being analyzed over a long time period.

Dissolved oxygen in a flask culture was measured with an oxygen electrode (Ingold) to ascertain whether the limited growth in larger volumes was due to a lack of dissolved oxygen. It was thought that this might explain the differences in growth (optical density and cell yield) between flask and fermenter grown cultures. However, flask cultures in an orbital incubator at 65°C had a percentage of dissolved oxygen of 100% which was the same level as in the fermenter. Thus the percentage of dissolved oxygen did not explain why culture volumes of 1 or 2 litres grew well in a fermenter but not in a flask. The degree of culture agitation was investigated as a possible reason for the difference. The fermenter and flask cultures were normally agitated at 500 and 120 rpm respectively. When the agitation in the fermenter was reduced to 120 rpm the final cell yield did not decrease to the cell yield from the same volume of identical medium incubated in an orbital incubator. This suggests that pH adjustment during growth may be critical to growth of *Bacillus* isolate TA2.A1 in culture volumes of 1 or 2 litres.

3.3.3.2 Fermenter growth

Initially 0.5ml of anti-foam (Rhône-Poulenc, Bevaloid 5901, BN 2625) was added per 1.5 litres of medium in a fermenter vessel. Though the final concentration was quite low this proved to be inhibitory to growth. Consequently no control of foaming within the fermenter vessel was used. As long as the agitation within the vessel was slowly increased to the level used during growth (500 rpm) most of the foaming could be avoided.

Growth yield was better when the inoculation was carried out by pouring the inoculating culture aseptically in through a sterile port in the top plate of the fermenter. This achieved better growth than inoculation with the same volume of identical culture by sterile syringe. Two identical fermenter vessels, run for the same time, using the same medium and inoculated by syringe and by pouring achieved maximum optical densitites (450nm) of 0.35 and 0.60 respectively. The reason for this phenomenon, which was replicated on three separate occasions, was unclear. It seems unlikely that, despite having long filamentous cells, *Bacillus* isolate TA2.A1 would be harmed by being

forced through a needle, since there would be little pressure involved. However, it is possible that the syringe inoculated fermenter grew slower due to some of the inoculated cells becoming damaged in some way, resulting in a lower 'initial inoculum' than anticipated.

Chapter 4 – *Bacillus* isolate TA2.A1

4.1 General characteristics of *Bacillus* isolate TA2.A1

4.1.1 Materials and Methods

4.1.1.1 Cell morphology of *Bacillus* isolate TA2.A1

Gram reaction:

Growth from a young Phytagel™ plate culture (less than 18 hours of incubation at 65°C) was suspended in sterile saline and Gram stained following the method of Lillie (1928) as modified by Preston and Morrell (1962). A KOH lysis test (Buck 1982) was carried out to ascertain a 'Gram-type' result, using the *E. coli* culture as a positive control. Isolate TA2.A1 was grown on rich medium and Phytagel™ plates (pH 8.5) at 65°C. A positive control of *E. coli* was grown on Luria agar at 25°C.

Pigmentation:

Inoculated media samples were centrifuged (Beckman Microfuge E) to investigate whether the tan/light brown colour seen in rich culture medium was due to pigmentation of isolate TA2.A1 cells.

API Testing:

Bacillus isolate TA2.A1 was analyzed using the biochemical test API strips. Rich and minimal media along with freeze dried ampoules of cells grown both in rich and minimal media (on sucrose) were sent for analysis (Grayson Laboratories, Auckland) and the tests were conducted at 65°C.

Electron Microscopy:

The use of rich liquid medium to grow isolate TA2.A1 for scanning electron microscope photographs gave a background cluttered with medium components. Cells were grown in rich medium and washed gently in minimal salts (rich medium without peptone) which had been filtered (Millipore 0.45μm) to remove any medium precipitation. To prevent cell lysis medium at the same pH was used as the washing solution.

4.1.1.2 Antibiotic / sodium azide sensitivity

The effect of antibiotics on liquid culture of isolate TA2.A1 was assessed. Antibiotic stock solutions in ethanol (100% v/v) were prepared at a concentration of 50mg/ml for penicillin-g, chloramphenicol and erythromycin and 5mg/ml for ampicillin and tetracycline. Stock solutions of neomycin sulphate, nalidixic acid, bacitracin and kanamycin sulphate were prepared at a concentration of 50mg/ml in Milli-Q water. Antibiotics were added from stock solutions to final concentrations in media of 25, 50, 100, 250 and 500µg/ml. Since some of the antibiotic stock solutions were prepared in ethanol, ethanol controls were evaluated, to confirm that any inhibitory effect was due to the antibiotic and not ethanol. Growth was monitored by optical density (650nm) using a Varian DMS 80 spectrophotometer, zeroed on uninoculated medium. Every antibiotic was tested in quadruplicate and compared to non-antibiotic containing growth controls. The effectiveness of sodium azide in preventing growth in liquid media over a range of concentrations (500 µg/ml to 0.08 µg/ml) was also assessed. Filter sterilized sodium azide stock in Milli-Q water was added to the same volume of double strength rich and minimal media to give a final concentration of 500 µg / ml. The sodium azide in medium was serially diluted to give a range of concentrations to 0.08 µg/ml in both rich and minimal medium. Growth controls in rich and minimal medium (without sodium azide) were also prepared. Media containing each sodium azide concentration was replicated six times.

The effect of antibiotic impregnated discs (Difco / Alpha Biological) on the growth of isolate TA2.A1 on rich medium with Phytagel™ plates (pH 8.5) was assessed. The inoculum was grown on rich medium at 65°C for 18 hours and 0.1ml was spread by sterile glass spreader over the surface of the agar plates and left to soak in for 15 seconds. Any excess culture was removed by sterile pipette. Both commercially prepared antibiotic disks and sterile blank filter discs impregnated with freshly prepared antibiotic solutions were placed aseptically onto the agar surface. Lack of growth, seen as a zone of inbibition around the antibiotic disc, was recorded after incubation of the plates for 18 hours at 65°C.

4.1.1.3 Sodium dependence

Minimal medium was prepared as per section 3.3.1.2 (without 0.1g.l^{-1} peptone) with the modification that potassium-containing salts were substituted for the sodium salts and the medium pH was adjusted with KOH instead of sodium hydroxide. Sodium free medium was dispensed into flasks that had been acid washed to remove any sodium attached to the glass. Prior to use these flasks were then given multiple washes in Milli-Q water. Defined concentrations of sodium, in the form of NaCl, were added to sodium free medium to achieve sodium concentrations of (mM) 0.5, 1, 2, 5, 10, 20, 50, 100, 200 and 500. Sucrose was added as a carbon substrate to these media which were inoculated from an 18 hour sucrose supplemented minimal medium culture. After incubation in a shaking orbital incubator (Gellankamp) at 65°C, growth was measured by optical density at 650nm (Varian DMS 80 spectrophotometer) and phase contrast microscopic examination.

4.1.1.4 Phospholipid and cell residue fatty acid composition

Bacillus isolate TA2.A1 cells were grown in rich medium for 18 hours at 65°C and harvested by centrifugation at 15200 g for 20 minutes. The cell pellet was freeze dried and sent to CSIRO Marine Laboratories (Australia) for phospoholipid and cell residue fatty acid analysis. Total lipid was extracted from the sample by the modified one-phase chloroform-methanol-water Bligh and Dyer method (Bligh and Dyer 1959; White *et al.* 1979). After phase separation, the lipids were recovered from the lower chloroform layer, concentrated using a rotary evaporator, sealed under nitrogen and stored at –20°C. The upper aqueous layer was collected for cell residue analysis.

The total lipid was fractionated into neutral-glyco and phospho-lipid using 1 gram of activated 100-200 mesh Unisil silicic acid (chloroform 10ml, acetone 20ml, methanol 10ml). The phospholipid fatty acid fraction was treated with 3 mls of methanol:concentrated hydrochloric acid:chloroform (10:1:1 v/v/v) at 100°C for 60 minutes (Edlund *et al.* 1985) to produce the corresponding fatty acid methyl esters which were then extracted with hexane:chloroform (4:1 v/v). The normal and hydroxy fatty acids from the aqueous layer were recovered following acidification of the lipid

extracted cell residue with 50 ml of 1M hydrochloric acid which was then heated under reflux for three hours. After cooling, the sample was extracted with chloroform and the fatty acids were methylated using the same procedure as for the phospholipids (CSIRO Marine Laboratories, Australia).

Fatty acid analysis was performed using a Hewlett Packard 5890 gas chromatograph (GC) fitted with a 50m x 0.32mm i.d. HP1 cross-linked methyl silicone fused-silica capillary column, with hydrogen as the carrier gas. The GC was fitted with a flame ionisation detector set at 310°C and a purged split/splitless injector set at 290°C. Samples were injected using a Hewlett Packard 4673A auto-sampler. The oven temperature was programmed from 50 to 150°C at 30°C/min, then at 2°C/min to 250°C and 5°C/min to 300°C. Indentifications were confirmed by gas chromatography-mass spectrometry (GC-MS, Fisons MD800) and by comparison of retention time and mass spectral data from laboratory standards (CSIRO Marine Laboratories, Australia).

4.1.1.5 Sporulation

Sporulating (incubated at 55°C) and non-sporulating *Bacillus* isolate TA2.A1 cultures (incubated at 65°C) grown in rich medium were heated to 80°C in a waterbath (Julabo). There was a significant difference in the number of sporulating cells seen after heating, with 2% of the number of cells in the sporulating culture present in the non-sporulating culture after 2 hours at 80°C. The experiment was repeated with the sporulating and non-sporulating cultures being heated at 100°C, in a saucepan of boiling water, since that temperature was further from the growth range of isolate TA2.A1. Prior to each sampling the temperature of the culture was checked by thermometer and a 1ml sample was removed at 0, 10, 30, 40, 60 and 120 minutes. The samples were diluted, using uninoculated medium as diluent, to 10^{-8} and then 100 µl samples of each dilution were spread onto rich medium Phytagel plates. After incubation at 65°C the number of colonies were counted.

Photographs were taken of sporulating isolate TA2.A1 cells using Delta 100 (Ilford) black and white film and an Olympus SC35 camera. To immobilise the organism to enable photographs to be taken, 1% (w/v) Noble agar (Difco) was heated at 60°C in a

waterbath and then added to the same volume of bacterial culture, mixed gently and placed on a glass slide.

4.1.1.6 The search for other alkaliphilic and thermophilic isolates

Alkaline pool and bore samples of both water and sediment gathered from various geothermal sites around the North Island of New Zealand were inoculated into rich liquid medium and incubated in a gently shaking orbital and a stationary incubator at 65 and 75°C. Growth was evaluated by phase contrast microscopic examination and transfers into identical medium following inoculation and incubation.

4.1.2 Results and Discussion

4.1.2.1 Cell morphology of *Bacillus* isolate TA2.A1

Bacillus isolate TA2.A1 stained as a Gram positive rod. Isolate TA2.A1 gave a negative KOH lysis result in contrast to *E. coli* which gave a positive 'stringy' result. General characteristics of the *Bacillus* genus and comparable results for *Bacillus* isolate TA2.A1 are summarized in Table 4.1. Following centrifugation the pellet of TA2.A1 cells was an off-white colour suggesting that majority of the colour in the growth medium was due to the high level of peptone in the medium rather than pigmentation of cells. *Bacillus* isolate TA2.A1 cells were long irregular rods with an average length of 6μm and a constant width of 0.3μm (Figure 4.1).

The results from biochemical test analysis (API) (Grayson Laboratories) characterized *Bacillus* isolate TA2.A1 as a *Bacillus stearothermophilus* strain, with 99.9% probability. Considering that *Bacillus stearothermophilus* is the only option on the API kit for an isolate that grows at elevated temperature this result is not surprising. API test systems have been used successfully for the classification and identification of *Bacillus* strains (Logan and Berkeley 1984) but Nielsen *et al.* (1995) found that some alkaliphilic *Bacillus* strains were unable to produce a detectable reaction, even though the system had been modified for use at high pH. Some alkaliphilic *Bacillus* strains in the study by Nielsen *et al.* (1995) failed to grow or produce reliable indication of carbon utilization in

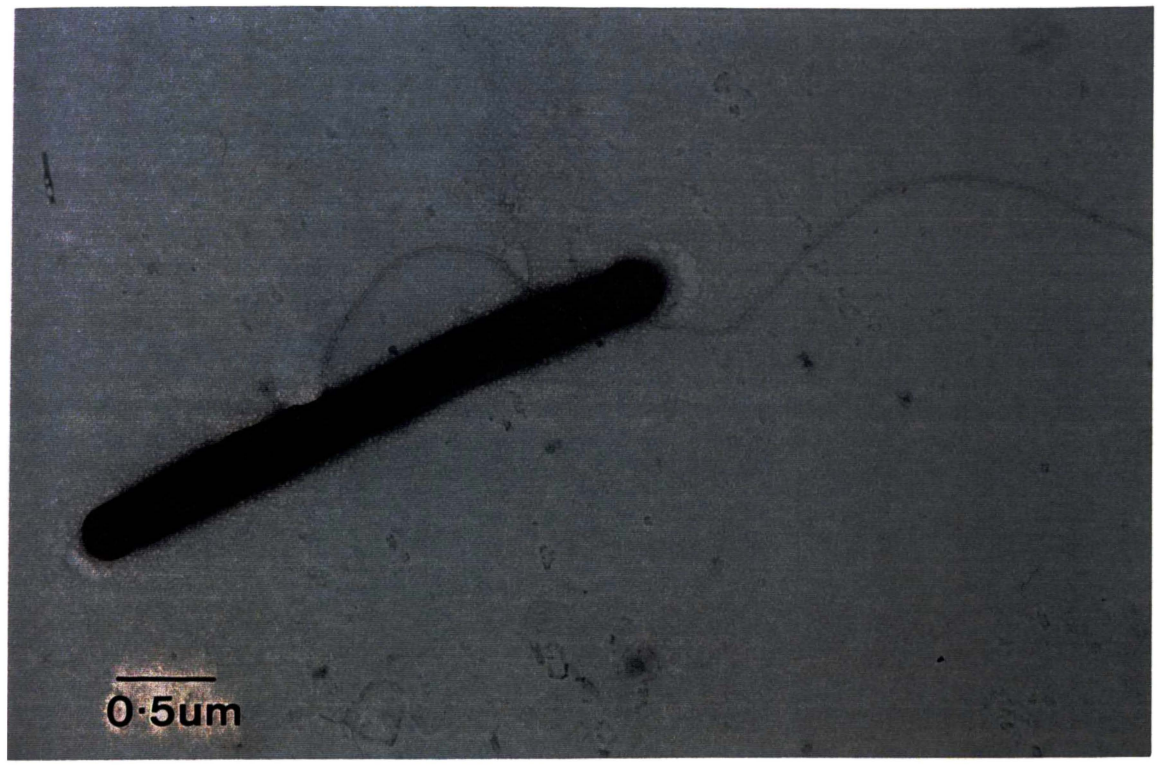
a modified API 50CH. For this reason the API tests were not used independently for the characterization of *Bacillus* isolate TA2.A1.

Table 4.1: General characteristics of the *Bacillus* genus and isolate TA2.A1

Characteristics	*Bacillus* genus	*Bacillus* isolate TA2.A1
Rod shaped	+	+
Diameter over 2.5µm	-	-
Filaments	-	+
Rods or filaments curved	-	+
Endospores produced	+	+
Motile	+	+
Stain Gram-positive (young cultures)	+	+
Strict aerobes	D	+
Facultative anaerobe / microaerophile	D	-
Strict anaerobe	(-)[1]	-
Homolactic fermentation	D	-
Sulphate actively reduced to sulphide	-	-
Catalase	+	+
Oxidase	D	+
Marked acidity from glucose	+	-
Nitrate reduced to nitrite	D	ND
Mol % G + C	32-69	47.3

D = results vary depending on the species
ND = not determined
( )[1] = except *B. infernus* (Boone *et al.* 1995)

Figure 4.1: Electron micrograph of *Bacillus* isolate TA2.A1 with polar flagella.



The motility of *Bacillus* isolate TA2.A1 was not restricted to the exponential growth phase and was not dependent on the presence of any particular utilisable carbon source. This was in contrast to the thermotolerant facultative alkaliphile *Clostridium thermoalkaliphilum* (Li *et al.* 1994) which was only motile through the exponential growth phase and exhibited little motility in the absence of glucose and in contrast to the anaerobic alkalithermophile *C. paradoxum* which changed motility drastically after sporulation had occurred (Li *et al.* 1993).

Figure 4.2: Electron micrograph of a cross-section showing internal organelles.

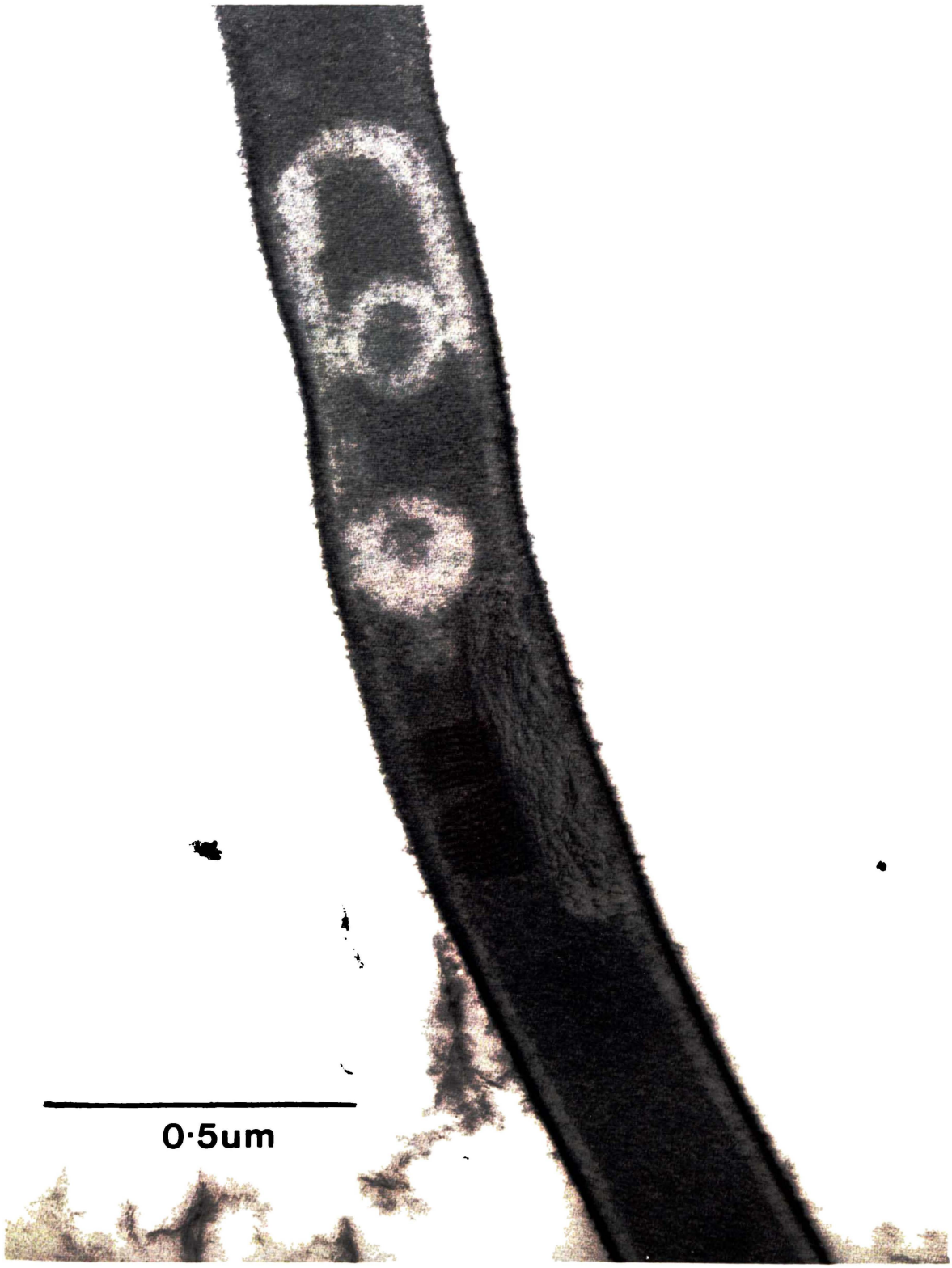
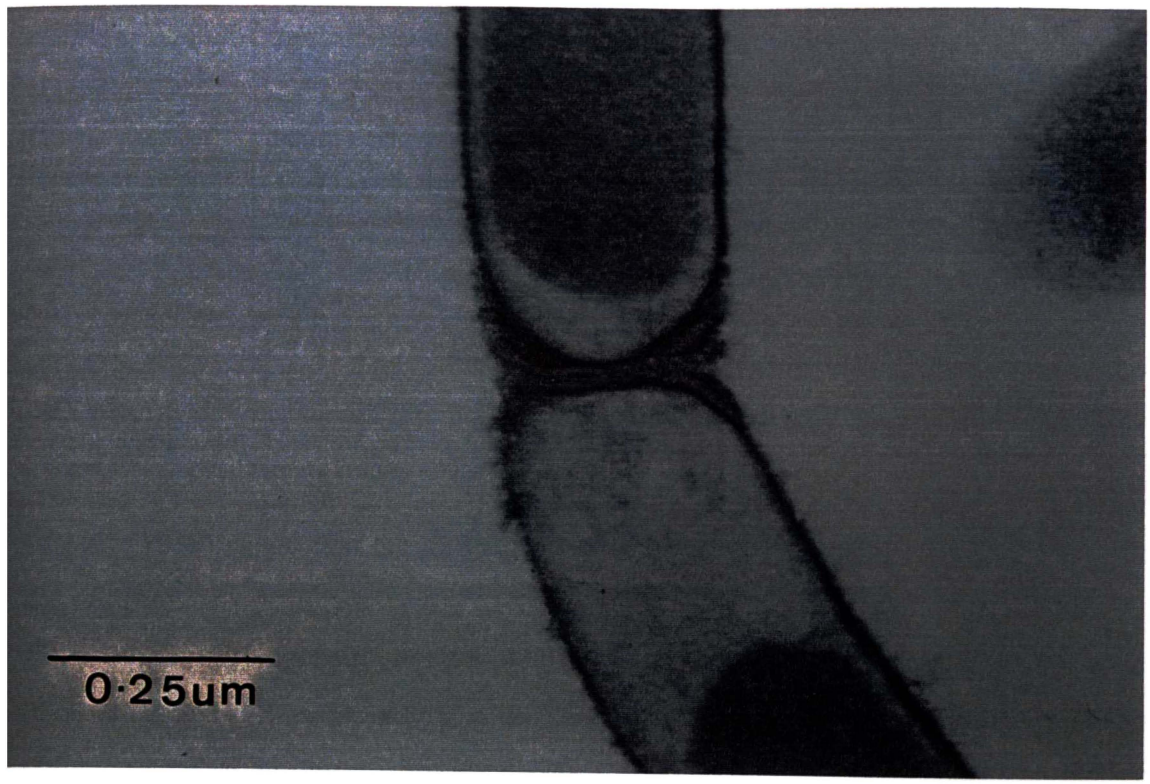


Figure 4.3: Electron micrograph of a cross-section showing the ends of divided cells



4.1.2.2 Antibiotic / sodium azide sensitivity

The same overall antibiotic sensitivity results were obtained in broth and on agar. Results are summarized in Table 4.2. Isolate TA2.A1 was resistant to all antibiotics tested at a concentration of 25µg/ml. The organism was sensitive to neomycin sulphate, tetracycline and erythromycin, which interfere with protein synthesis. Isolate TA2.A1 was resistant to kanamycin sulphate and nalidixic acid to a concentration of least 500 µg/ml. The ineffectiveness of the quinolone nalidixic acid against *Bacillus* isolate TA2.A1 was not surprising since it is active against Gram negative bacteria by inhibiting the replication of bacterial DNA. Kanamycin sulphate is an aminoglycoside which is more active under alkaline conditions but does not interfere with protein synthesis of isolate TA2.A1. In contrast, erythromycin which also has increased activity at high pH does interfere with protein synthesis of isolate TA2.A1. Antibiotics which inhibit protein synthesis are more effective against isolate TA2.A1 than those that affect cell wall synthesis.

Table 4.2: Antibiotic sensitivity of *Bacillus* isolate TA2.A1

Antibiotic	Antibiotic target	25µg/ml	50µg/ml	100µg/ml	250µg/ml	500µg/ml
Ampicillin	cell wall synthesis	+	+	+	+	-
Penicillin G	cell wall synthesis	+	+	+	-	-
Chloramphenicol	protein synthesis	+	+	-	-	-
Neomycin Sulphate	protein synthesis	+	-	-	-	-
Tetracycline	protein synthesis	+	-	-	-	-
Erythromycin	protein synthesis	+	-	-	-	-
Nalidixic Acid	DNA replication	+	+	+	+	+
Bacitracin	cell wall synthesis	+	+	+	+	-
Kanamycin Sulphate	protein synthesis	+	+	+	+	+

+ = growth eqivalent to controls (optical density 650nm 0.2-0.4
- = growth inhibited (optical density 450nm <0.2)

Wiegel (1998) stated that the antibiotic sensitivity spectrum of the alkali-thermophiles did not show any peculiarities and was similar to that of other bacteria from the *Bacillus-Clostridium* phylogenetic branch. For example, the anaerobic thermophilic alkali-tolerant species *C. paradoxum*, at pH (25°C) 7.1 and 10.1, was sensitive towards chloramphenicol, erythromycin, tetracycline, monensin, gramicidin S and lasalocid, all at 25 µg/ml and against 50 µg/ml of gentamicin. *C. thermoalcaliphilum* was sensitive to 50 µg/ml penicillin G, ampicillin, metroimidazole, gentamicin and rifampin. *Thermosyntropha lipolytica* was sensitive to 50 µg/ml ampicillin, chloramphenicol, kanamycin, neomycin, rifampin and vancomycin. However, as stated by Peteranderl *et al.* (1990) the testing of an antibiotic resistance pattern is equivocal because of the stability of the antibiotics under the growth conditions of combined high temperature and pH is not known. This has been stated to be under current investigation (Wiegel 1998) but the results are as yet unpublished. Thus results in which isolate TA2.A1 appeared to be resistant to a particular antibiotic may be the result of the inactivation of that antibiotic under one or both of the extreme conditions required for isolate TA2.A1 growth.

The effect of sodium azide on the growth of *Bacillus* isolate TA2.A1 was assessed; the data in Table 4.3 represents the average from six replicates at each sodium azide concentration. Growth occurred in rich medium at concentrations of sodium azide up to and including 500µg/ml (Table 4.3). In rich medium with a sodium azide concentration of 0.16 µg/ml or above, growth exceeded that of controls. In minimal medium

supplemented with sucrose growth was affected by the presence of sodium azide from 10 μg/ml down to 0.08 μg/ml. The different effect of sodium azide depending on the medium type used could have been due to the lower cell density in the minimal medium or perhaps a different mechanism for utilization of the carbon source in the rich medium (peptone) compared with that in the minimal medium (sucrose). Sodium azide inhibits oxidative phosphorylation suggesting that this may have a role in isolate TA2.A1's utilization of sucrose.

Table 4.3: Sodium azide sensitivity of *Bacillus* isolate TA2.A1

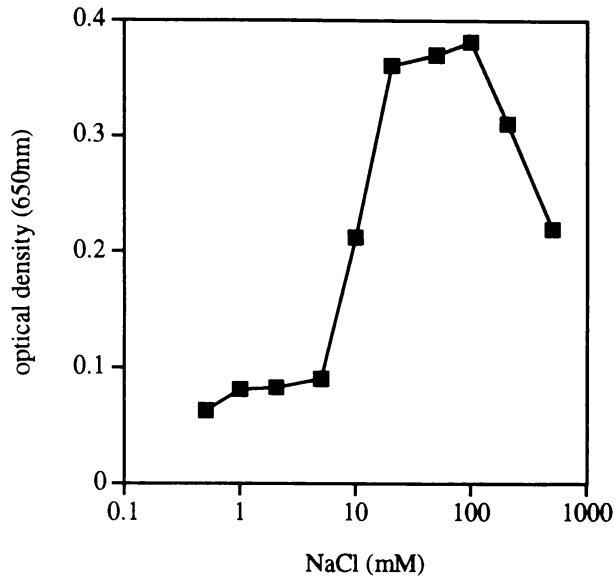
	sodium azide (μg/ml)													
	0	0.08	0.16	0.3	0.6	1.3	2.5	5	10	25	50	100	250	500
Rich medium	+	+	++	++	++	++	++	++	++	++	++	++	++	++
Minimal medium	+	-	-	-	-	-	-	-	-	+	+	+	+	+

+ = growth eqivalent to controls (optical density 650nm 0.2-0.4), ++ = growth increased compared to controls (optical density 650nm >0.4), - = growth inhibited (optical density 650nm <0.2)

4.1.2.3 Sodium ion dependence

Like all true alkaliphiles growth of *Bacillus* isolate TA2.A1 was sodium ion-dependent. Below a concentration of 5mM sodium ions, growth did not occur in minimal medium supplemented with 0.5g.l^{-1} of either sucrose, trehalose or glutamate. Growth was assessed by optical density after 24 hours incubation at 65°C. The data for the growth of isolate TA2.A1 in minimal medium supplemented with glutamate is shown in Figure 4.4.

Figure 4.4: The dependence of *Bacillus* isolate TA2.A1 on sodium ions for growth in minimal media



The dependence on the presence of sodium ions for growth has been shown for other alkaliphilic *Bacillus* species, for example, *B. agaradhaerens* and *B. clarkii* (Nielsen *et al.* 1995). These species were obligately alkaliphilic (unable to grow at pH 7) and grew optimally at pH 10 or above, and at up to 45°C. Growth of *Bacillus* isolate TA2.A1 was inhibited when the sodium ion concentration was above 100mM (Figure 4.4).

4.1.2.4 Phospholipid and cell residue fatty acid composition

Isolate TA2.A1 contains relatively high levels of saturated branched and unbranched fatty acids (Table 4.4). The phospholipid fraction of isolate TA2.A1 contained the highest levels of saturated branched chain fatty acids (91%). The cell residue fatty acids contained 98-100% branched or straight chain saturated fatty acids.

Table 4.4: Phospholipid and cell residue fatty acid composition of *Bacillus* isolate TA2.A1

Fatty acids	Percentage composition (%)	
	Phospholipid fatty acids	Cell residue fatty acids
branched chain		
i14:0	0.2	0.4
i15:0	12.6	7.5
a15:0	2.0	1.7
i16:0	20.7	35.7
i17:0	45.9	24.5
a17:0	7.9	6.6
i18:0	1.8	1.7
i19:0	0.1	-
sum	91.3	78.1
straight chain		
14:0	0.1	0.6
15:0	1.7	2.2
16:0	4.6	15.0
17:0	1.8	1.9
18:0	0.5	1.9
sum	8.7	21.6
other		
18:1	-	0.3
16:0 ß-OH	-	-
total sum	100	100

i = iso, a = anteiso, ß-OH = beta hydroxy fatty acid.

The dominant phospholipid fatty acid in isolate TA2.A1 was il7:0 (Table 4.4, Figure 4.5). The relative level of il7:0 was lower in the cell residue fatty acid sample (Figure 4.6) compared with the phospholipid fatty acid (Figure 4.5). Hydroxy fatty acids were not observed in the phospholipid or cell residue fraction of the strain. Similar levels of saturated fatty acids have been reported for other thermophilic bacteria with il7:0 and il5:0 commonly reported as the dominant fatty acids (Hensel *et al.* 1986; Prado *et al.* 1988; Patel *et al.* 1991). Only one monounsaturated fatty acid was noted at low levels in the TA2.A1 cell residue fatty acid fraction (18:1). Cyclic fatty acids have been reported in thermophilic *Bacillus* species including *Bacillus cycloheptanicus* (Deinhard *et al.* 1987b; Wisotzkey *et al.* 1992) and *Alicyclobacillus acidocaldarius* (Wisotzkey *et al.* 1992).

In bacterial cells fatty acids occur mainly in the cell membranes as the acyl constituents of phospholipids. Membrane fatty acids can be divided in two major groups on the basis of their biosynthetic relationships: (a) straight-chain fatty acids, which are most common in bacteria, and (b) branched-chain fatty acids, which do not occur as commonly in bacteria in general but predominate in *Bacillus* species (Kaneda 1977). The difference between these two groups of cell membranes is in the mechanism that controls their fluidity. The fluidity of membranes composed of straight chained fatty acids is adjusted by the inclusion of monounsaturated fatty acids, whereas that of membranes with branched-chain fatty acids is controlled mainly by 12- and 13-methyltetradecanoic acids. For this reason bacteria with straight-chain membrane systems usually require unsaturated fatty acids for growth, but these fatty acids are non-essential for bacteria with the branched chain membrane system. For *Bacillus* species, four factors which contribute to fatty acid patterns have been identified on the basis of biochemical mechanisms. These factors are the ratio of three classes of branched-chain fatty acids, the proportion of straight-chain fatty acids, the occurrence of unsaturated fatty acids, and the relatively high proportion of unique fatty acids (Kaneda 1977). The occurrence of hydroxy fatty acids must be considered in examining fatty acid patterns of whole bacteria having branched-chain fatty acids as major cellular fatty acids. Many species of *Bacillus* have branched-chain fatty acids as major cellular fatty acids. Most thermophiles, which grow at neutral pH, do not possess unusual fatty acids - although the proportions of iso- and anteiso- fatty acids are adjusted for their growth temperatures (Oshima and Miyagawa 1973). Kaneda (1991) did not comment on the presence of unusual fatty acids in thermophiles growing under alkaline conditions. Many bacteria capable of growing at high pH (up to pH 10.5) (for example, *Bacillus fermus* and *Bacillus alcalophilus*) like other *Bacillus* species possess branched-chain fatty acids as their major fatty acids. However, unlike many other species, 20% of their total fatty acids are unsaturated. *Bacillus thermoantarcticus* (Nicolaus *et al.* 1996) a thermophile isolated from the antarctic had lipids with ester linkages, however, no glycolipids which are normally found in *Bacillus* species were found. The fatty acid composition showed that the isolate possessed a high amount of iC17:0 and aiC17:0 (Nicolaus *et al.* 1995). The high concentration of aiC17:0 was a distinct and unusual feature.

Figure 4.5: Phospholipid profile of *Bacillus* isolate TA2.A1

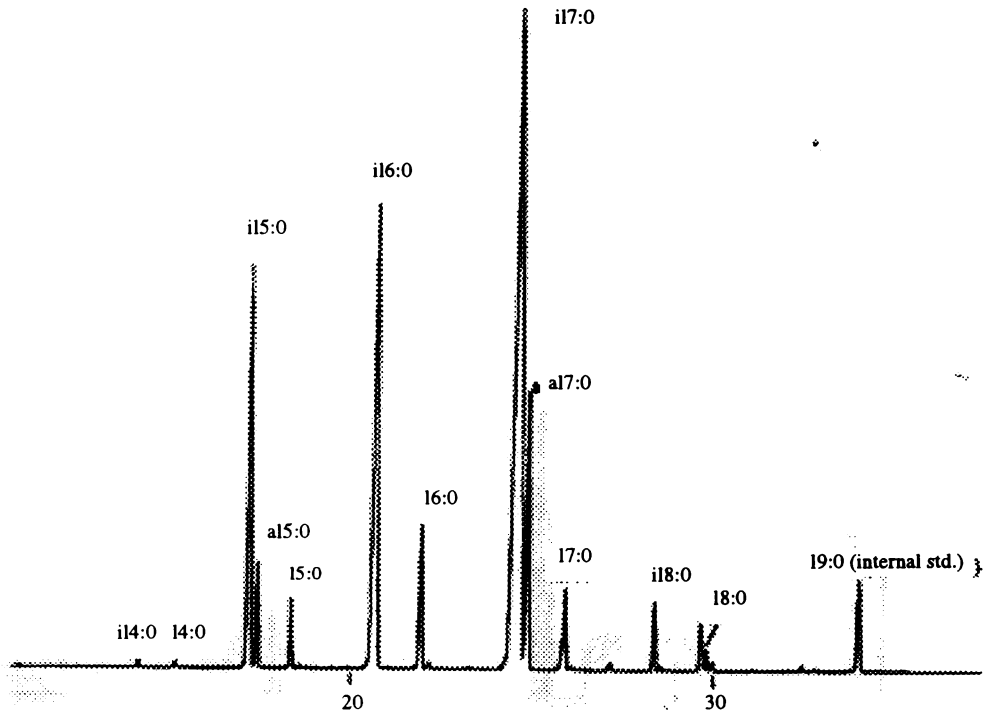
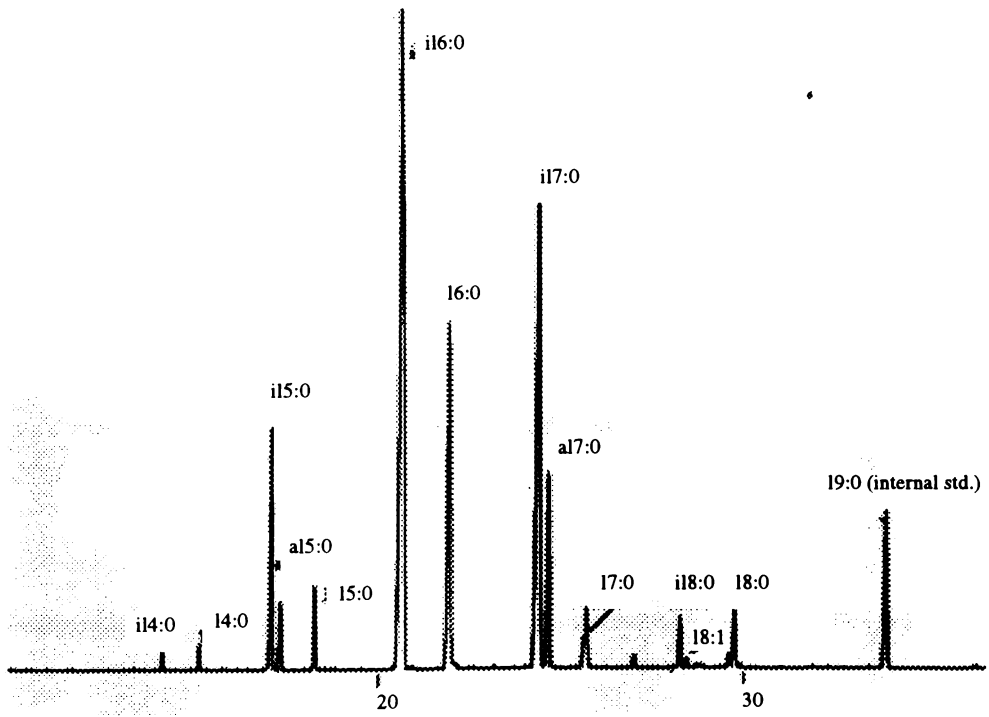


Figure 4.6: Cell residue fatty acid profile of *Bacillus* isolate TA2.A1



4.1.2.5 Sporulation

When *Bacillus* isolate cells were grown at 55°C and lower temperatures, in liquid and solid media, terminal spores were formed. When incubated at 55°C the ratio of sporulated to non-sporulated cells was 50:1. Cells grown in any of the media tested did not produce spores when incubated at 65°C to 70°C. When cells grown at 55°C were sub-cultured into rich or minimal media and incubated at 65°C there was a return to vegetative cells with only a few spores present.

Horikoshi and co-workers (Horikoshi and Akiba 1982; Horikoshi 1991b) stated that while the environmental factors affecting the sporulation of *Bacillus* species have been reported by many investigators, only one report of sporulation of alkaliphilic *Bacillus* species had been presented. Kudo and Horikoshi (1979) isolated an obligately alkaliphilic *Bacillus* sp. (No. 2b-2) from soil and used it throughout their experiments because of its excellent spore yield.

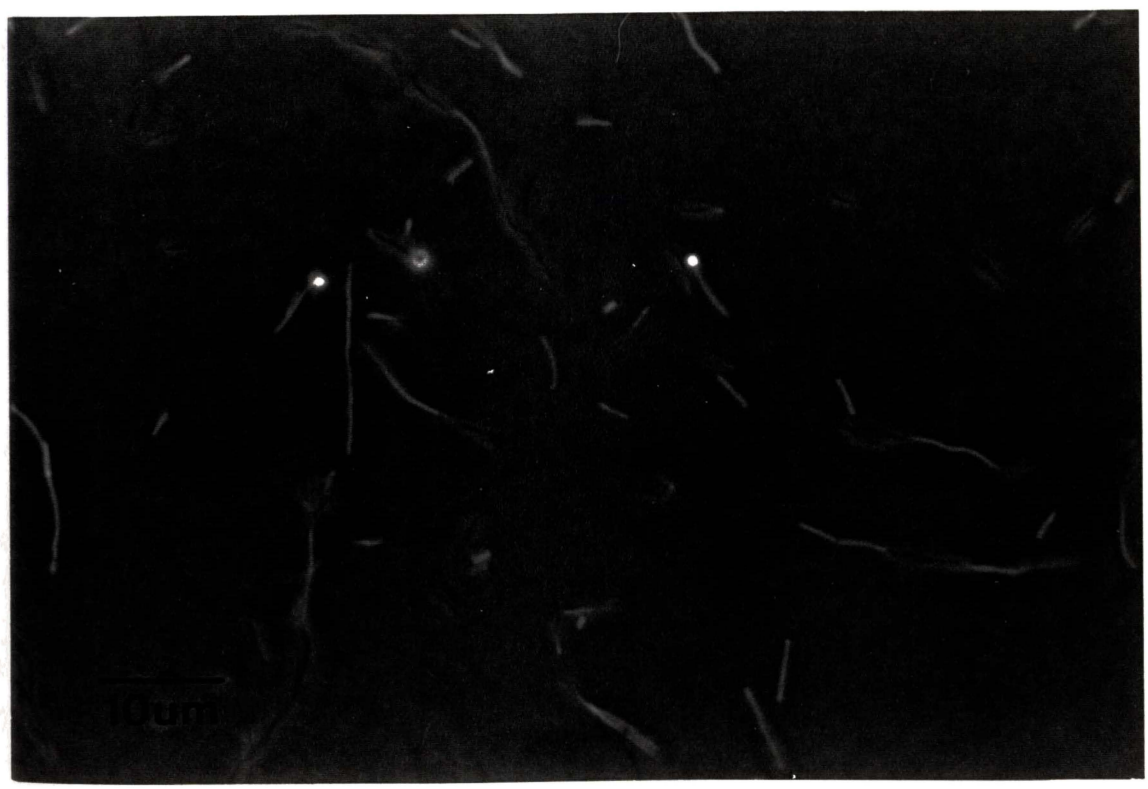
Sporulation was observed at pH 9.0–9.5 in the presence of 0.2M NaCl. There was general agreement that the optimum pH for sporulation was close to that for growth, but the range was narrow. The optimum temperature for sporulation was almost the same as that for growth (34–37°C). Sodium ions stimulated germination of *Bacillus* sp. No. 2b-2 spores, but other cations (K^+, NH_4^+, Rb^+, Cs^+ and Ca^{2+}) did not show this stimulating effect. Only Li^+ showed weak stimulation (Kudo and Horikoshi 1979). In the case of *Bacillus* isolate TA2.A1 sporulation occurred at temperatures at least 10°C lower than that which was optimal for growth. Sporulation also occurred at the lower end of the pH range for growth at pH 8.5.

Table 4.5: Heat resistance of sporulating and non-sporulating[1] cultures of *Bacillus* isolate TA2.A1. Bacteria / ml of original culture heated at 100°C for the times indicated.

Time (minutes) at 100°C	Non-sporulating[1]	Sporulating
0	6.02×10^6	1.9×10^7
10	4.14×10^7	1.0×10^8
30	1.72×10^7	1.7×10^7
40	2.49×10^5	2.9×10^7
60	0	7.1×10^6
120	0	1.3×10^3

[1] no spores observed over twenty microscope fields of view

Figure 4.7: Phase contrast photograph of *Bacillus* isolate TA2.A1 showing terminal spores.



4.1.2.6 The search for other alkaliphilic and thermophilic isolates

Water and sediment samples (source temperature and pH in brackets) from the following alkaline thermal sites: Tokaanu, Rotorua (Rachel's Spring Rt 10) (95°C pH 8) and (Whakarewarewa Rt 400) (85°C pH 8) and Tikitere (TK 43 bore) (99°C pH 8) failed to exhibit growth in rich medium following incubation at either 65 or 75°C. The source temperature and pH of *Bacillus* isolate TA2.A1 were 77°C and pH 8.28, suggesting that sampling sites with a higher temperature and lower pH than the optimal conditions for isolate TA2.A1 should not in itself be a barrier to isolation of similar organisms. Cells were seen in the Whakarewarewa and Rachel's spring samples, after 56 hours of incubation at 65°C; however, cell numbers failed to increase on further incubation and transfer into the same medium.

4.2 Effect of pH on growth of *Bacillus* isolate TA2.A1

4.2.1 Methods

Unless otherwise stated rich or minimal media was buffered with $9g.l^{-1}$ sodium hydrogen carbonate. When commercial buffers (Sigma) were used, usually at 50mM, the concentration of $NaHCO_3$ in the medium was reduced to $1g.l^{-1}$. pH measurement was completed using a pH meter (Philips PW 9421) with an Orion 9156SC electrode. Where the use of a pH electrode was not possible, the surface of agar plates for example, Alkalit indicator paper (Merck) was used. The pH electrode was calibrated with Philips pH standards (pH 6.88 and pH 9). These standards are stable at temperatures higher than 18-20°C and have accompanying manufacturer's buffer pH values to calibrate a pH meter from 0 to 95°C. The d(pKa) / dt values of these buffers was quite low and between 20 and 70°C were (pH 6.88) –0.0007 and (pH 9) –0.006 per °C.

The useful pH range of the commercial buffers used was, according to the manufactures instructions; Bis-tris propane (6.3-9.5), MOPS (3-[N-Morpholino]propane-sulfonic acid) (6.5–7.9), TAPS (N-tris[Hydroxymethyl]methy-3aminopropanesulfonic acid) (7.7-9.1), CAPS (3-[Cyclohexylamino]1-propanesulfonic acid) (9.7-11.1). However, it must be remembered that these values are calculated for use at room temperature (20°C) rather than the higher temperatures utilized by thermophilic microorganisms. The range of pH values over which these buffers were useful at high temperature (65–70°C) was uncertain; in order to ascertain this both minimal and rich media were prepared and the pH adjusted (at 70°C) to a range of values. The pH values of a variety of buffers at 20 and 70°C in different media pre- and post-autoclaving are shown in Table 3.4 (rich medium) and Table 3.5 (minimal medium) in Chapter 3.

Rich or minimal media was heated in a water bath (Julabo) to 70°C and the temperature was checked by thermometer before the medium had its pH adjusted. The pH meter was calibrated at 70°C using temperature stable pH standards (Phillips) of pH 9.0 and 6.88. Rich or minimal media (supplemented with $0.5g.l^{-1}$ sucrose) was initially buffered and pH adjusted to the following pH values at 70°C before autoclaving; MOPS (pH 6.5, 7, 7.5, 8), TAPS (pH 7.5, 8, 8.5, 9), CAPS (pH 8.5, 9, 9.5) and $NaHCO_3$ (pH 9.5, 10, 10.5,

11). The pH of these media after autoclaving are shown in the first columns of Tables 4.6 and 4.7. In a separate experiment, rich medium was buffered with either 50mM CAPS or with $9g.l^{-1}$ sodium hydrogen carbonate to cover the pH range 9 to 9.6 (70°C) at intervals of 0.2 pH units. In each experiment five replicates were evaluated for each set of pH/buffer conditions. Medium was dispensed in 100ml aliquots into 500ml flasks as per section 3.2.5.4. After inoculation growth was monitored by optical density at 650 or 450nm and phase contrast microscopic examination. Uninoculated medium controls were included to ensure that the pH was maintained at the desired value and to correct for any colour development on inoculation.

Table 4.6 Buffer conditions at different pH values in rich medium

PH at 70°C (after autoclaving)	Buffer conditions	d(pKa)/ dt
6.6, 7.17, 7.75, 8.36	50mM MOPS (1g/l $NaHCO_3$)	-0.015
7.73, 8.2, 8.69, 9.35	50mM TAPS (1g/l $NaHCO_3$)	-0.018
8.73, 9.06, 9.47	50mM CAPS(1g/l $NaHCO_3$)	-0.032
9.45, 9.8, 10.15, 10.62	9 g/l $NaHCO_3$	-0.009

Table 4.7 Buffer conditions at different pH values in minimal medium

PH at 70°C (after autoclaving)	Buffer conditions	d(pKa)/ dt
6.7, 7.39, 8.23, 8.9	50mM MOPS (1g/l $NaHCO_3$)	-0.015
7.72, 8.15, 8.7, 9.28	50mM TAPS (1g/l $NaHCO_3$)	-0.018
8.98, 9.3, 9.69	50mM CAPS(1g/l $NaHCO_3$)	-0.032
9.41, 9.8,10.1, 10.5	9 g/l $NaHCO_3$	-0.009

Solid medium was prepared as per section 3.3.2. The adjustment of pH was carried out by addition of 1N NaOH or 1N HCl. The pH of solid medium was checked after the plates had set by measuring the pH on the agar surface (at 20°C).

pH gradient plates were prepared using rich medium without Na_2CO_3. After setting, a trough was removed from one end and agar containing 20% Na_2CO_3 and 0.2M NaOH was poured into the trough. This agar was prepared by mixing together equal volumes of sterile 0.4M NaOH / 40% (w/v) Na_2CO_3 and 4% (w/v) Phytagel™ at 60°C. After setting and incubation, the plates contained a pH gradient from pH 12 to pH 7 (20°C). *Bacillus* isolate TA2.A1 was streaked across the plate at right angles to the trough.

4.2.2 Results and Discussion

Rich medium with different buffers covering a range from pH 6.6 to 10.62 at 70°C (Table 4.6) was tested for its ability to support growth of isolate TA2.A1. The average optical density results at 45 hours since inoculation are shown in Figure 4.8. In rich medium the peptone ($10g.l^{-1}$) will have a buffering effect making this medium more highly buffered than the minimal medium. In some cases the optical density appears to decrease with one buffer but support growth at a similar pH with another buffer. For example CAPS buffered medium which was at pH 9.47 and TAPS buffered medium at pH 9.35 (after autoclaving) supported less growth than $NaHCO_3$ (pH 9.45) (Figure 4.8). The fact that this was caused by a lack of buffering capacity by TAPS and CAPS at these pH values at 70°C was confirmed by a shift in the medium pH during growth. The differing abilities of buffers to work under high temperature conditions explains why the optimal pH for growth varies depending on the buffering system in use. Minimal medium with a range of pH values from pH 6.7 to 10.5 at 70°C (Table 4.7) using a range of buffering systems, was tested for its ability to support growth of isolate TA2.A1. The average optical density results at 48 hours since inoculation are shown in Figure 4.9.

Figure 4.8: Growth of *Bacillus* isolate TA2.A1 in rich medium at different pH values (45 hours since inoculation)

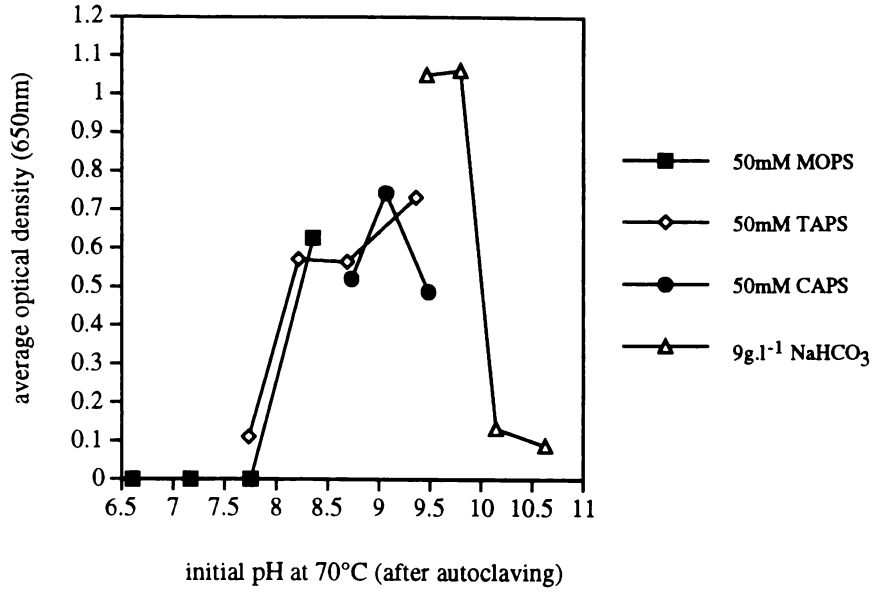
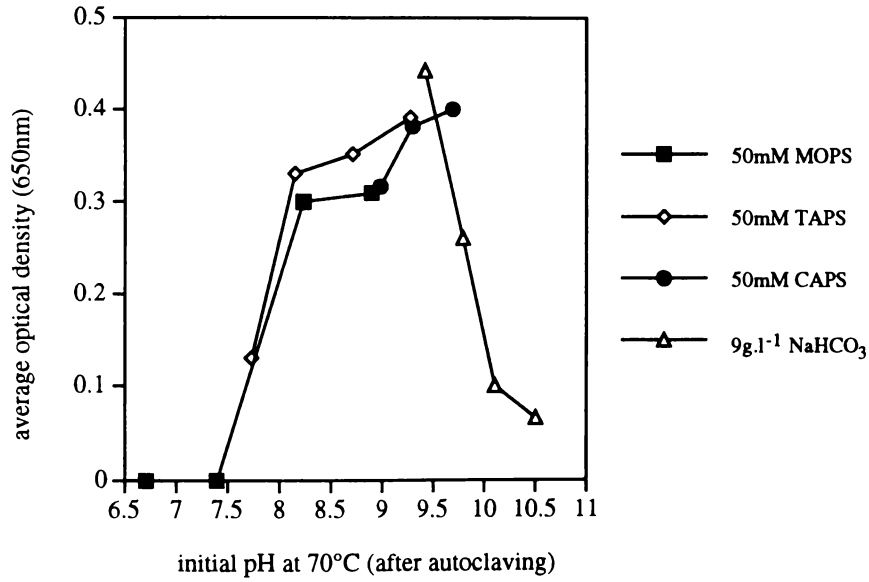


Figure 4.9: Growth of *Bacillus* isolate TA2.A1 in minimal medium at different pH values (48 hours since inoculation)



In rich medium buffered with $NaHCO_3$ the pH optimum was between pH 9.2 and 9.45 at 70°C (Figures 4.8 and 4.10), however, in medium buffered with CAPS the pH optimum was 9.2 at 70°C (Figure 4.11). In minimal medium buffered with $NaHCO_3$ the optimal pH was 9.4–9.7 (Figure 4.9). No growth occured even after extended incubation (52 hours) at pH 7.5 or below in rich or minimal medium. This confirmed that *Bacillus* isolate TA2.A1 was an obligate alkaliphile. To confirm that this lack of growth was not

simply an effect of buffer, medium was buffered at pH 7 with either MOPS or Bis-Tris; however, the same lack of growth resulted. Reduced growth was seen in rich or minimal medium at pH 10.5 (Figures 4.8 and 4.9), with an average optical density (650nm) of 0.1 or less. After extended incubation (52 hours) the growth at pH 10.5 in either media did not increase above the level following 45 hours of incubation (Figures 4.8 and 4.9).

Figure 4.10: Growth of *Bacillus* isolate TA2.A1 in bicarbonate buffered rich medium

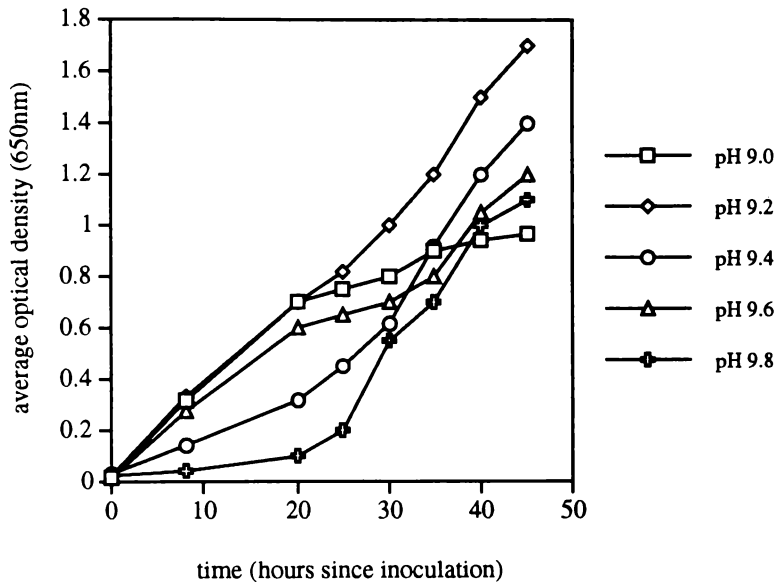
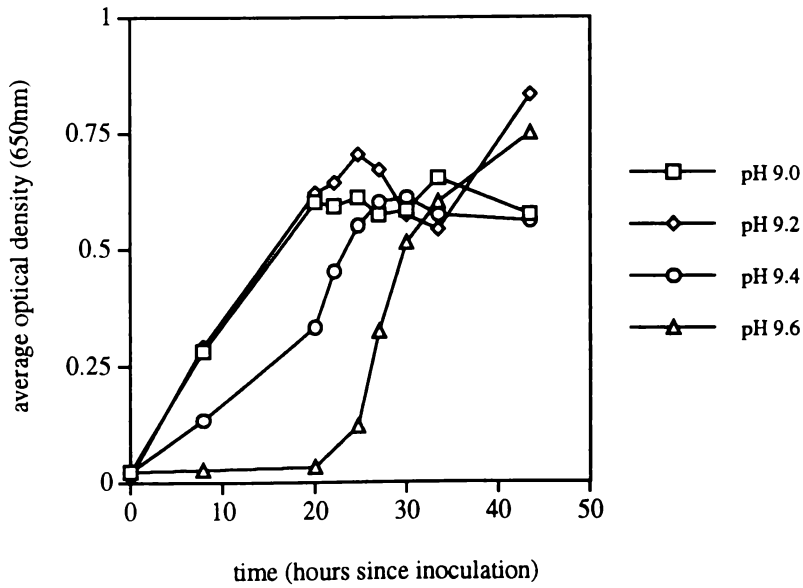


Figure 4.11: Growth of *Bacillus* isolate TA2.A1 in CAPS buffered rich medium



Growth of *Bacillus* isolate TA2.A1 on solid medium was assessed over a range of pH values (pH 8, 8.5, 9.0, 9.5, 10.0) by spread plate colony counts. In rich medium solidified with either Gelrite™ or Phytagel™ best growth was at pH 8 (65°C). Colony numbers were ten-fold higher on rich medium Phytagel™ plates than on identical plates set with Gelrite™. Minimal medium with sucrose ($0.5g.l^{-1}$) added as a carbon substrate with either solidifying agent failed to support growth. This lack of growth in the minimal medium plates was consistent over a range of pH values from 8 to 10 even after extended (96 hours) incubation. The fact that the pH optima on solid medium was lower than that achieved in liquid medium could be due to the reduced water activity of the solid plates. The use of pH gradient plates to ascertain the pH range of growth for alkaliphilic bacteria is well documented (Grant and Tindall 1980). *Bacillus* isolate TA2.A1 grew on the pH gradient plates from pH 8 to 9 confirming the inability of the isolate to grow at neutral pH.

It is widely recognized that most aerobic alkaliphilic bacteria change the pH of their growth medium in the acid and / or alkaline direction (Kelly and Fogarty 1978; Horikoshi and Akiba 1982; Kelly *et al.* 1983; Kitada and Horikoshi 1976; Paavilainen *et al.* 1994; Horikoshi 1991b). In the case of *Bacillus* isolate TA2.A1 the pH of inoculated liquid medium declined slightly during growth and the pH of uninoculated medium rose slightly during the same time period. Growth measured by optical density and corresponding pH (20°C) is shown for rich (Figure 4.12) and minimal medium supplemented with $0.5g.l^{-1}$ sucrose (Figure 4.13). The fact that this effect is less in minimal (Figure 4.13) compared to rich (Figure 4.12) medium suggests that this effect may be due to the acid production from the oxidation of peptone in the medium.

Horikoshi and Akiba (1982) reported that alkaliphilic bacteria inoculated on agar plates at pH 12 and incubated for several days, slowly changed the plate pH to around pH 9. Under more acidic conditions, pH 7.5, some alkaliphiles can increase the pH value to around 9 and finally show similar growth to that at pH 10.2 (Horikoshi and Akiba 1982). This phenomenon may explain why some alkali-tolerant bacteria can live in acidic soil. Ueyama and Horikoshi (1981) isolated an alkaliphilic *Arthrobacter* species capable of changing the broth pH to an optimum. Paavilainen and co-workers (1994) examined the

Figure 4.12: Growth of *Bacillus* isolate TA2.A1 in rich medium

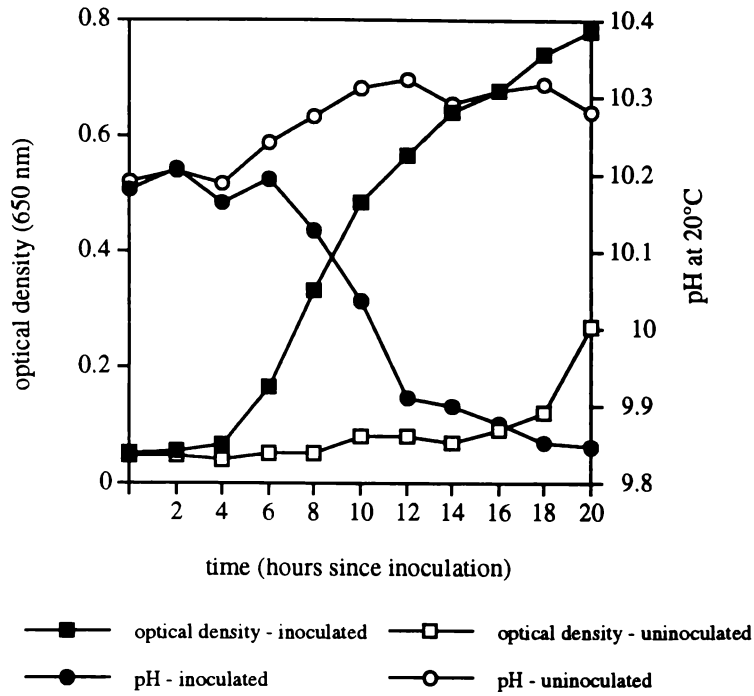
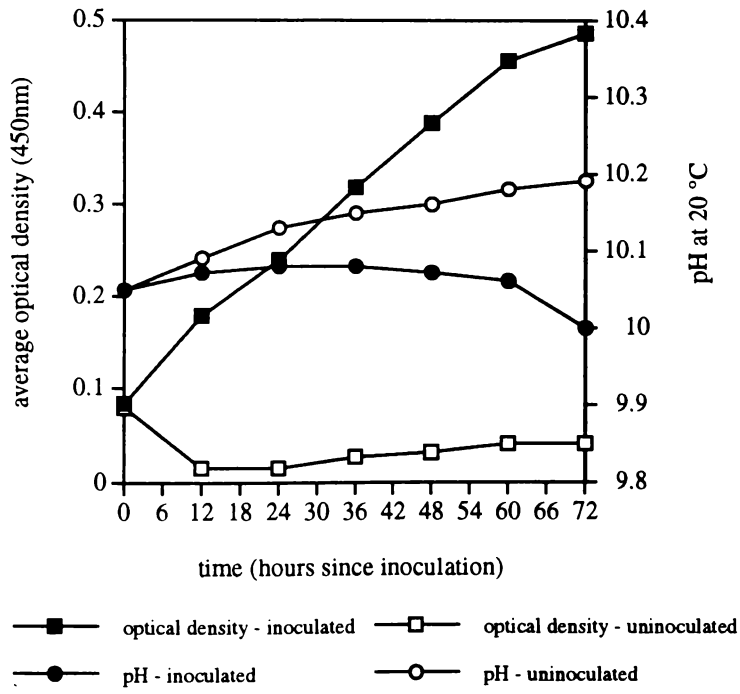


Figure 4.13: Growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with 0.5g.l^{-1} sucrose



acids produced via sugar catabolism by four obligately or facultatively alkaliphilic bacilli and showed a difference in timing between the medium pH and the appearance of acid anions. This suggested that the cation / proton / electron translocation took place through the cell membrane more or less independently of the anions, which are the

origin of the typical pH changes in the growth medium. Paavilainen and co-workers (1995) described the sugar catabolism and pH-dynamics of the culture medium of alkaliphilic *Bacillus circulans* var. *akalophilus*. The pH of the medium increased rapidly from the minimum pH during the cessation of bacterial growth. In this case the increase in pH was not due to the utilization of the organic acids because only a small quantity of them was used. Also the rise in pH continued steadily although there was little metabolic activity, except later by sporulating cells. The moles of acids needed to achieve the minimum pH of the medium during bacterial growth approximately corresponded to the moles of produced acids. However, the timing of the pH minimum did not match with the appearance of the acids, but the required amounts of acids were detected several hours later in the medium. They proposed that the eventual rise of the pH arose from the re-equilibration of the redox-state, and not from consumption of acids or generation of basic compounds, which had previously been suggested. Obviously the pH decrease in the growth medium is due either to the formation of acids or the removal of bases. The pH changes can be aggravated by either changes of buffering capacity of nutrients in the pH region in question, or by the equilibria between carbonate, bicarbonate and carbon dioxide. The fixation of carbonate to a carboxylic acid to form a new acid stronger than carbonic acid could change the pH or the buffering capacity (Paavilainen *et al.* 1995). Paavilainen *et al.* (1995) excluded the simple explanation for pH changes in the medium, of organic acid production, followed by their consumption. They suggested that the bacterium extruded protons (equal to electron uptake) without the acid anions. In other words that the essence of the growth of alkaliphilic bacilli in sugar medium was the redox-potential obtained from sugar catabolism. When sugar was exhausted from the medium, the consumption of oxygen and growth finished, and the pH started to increase for re-equilibration of the redox reactions. In the case of alkaliphilic thermophilic *Bacillus* isolate TA2.A1 the pH declined during growth possibly due to acid production from substrate utilization.

4.3 Effect of temperature on growth of *Bacillus* isolate TA2.A1

4.3.1 Methods

(a) Growth response in a temperature gradient incubator

A temperature gradient incubator (University of Waikato workshop) was set to cover a temperature range from 45°C to 88°C and with a rocking speed of 15 oscillations/min. Temperatures at each tube position were checked by thermometer and culture tubes were held at each temperature in triplicate. Seven ml volumes of rich medium (as per section 3.3.1.3) were dispensed into 25ml Bellco tubes. Once the medium had equilibrated at each temperature, inoculation (3% v/v) was carried out via a sterile syringe from an 18 hour culture in the same medium. Growth across the temperature gradient was measured by placing the tubes directly into a spectrophotometer (Pye Unicam SP6–450 UV/VIS) set to 650nm. Readings were taken as quickly as possible to prevent cooling and the tubes were aligned in the same position for each reading, to prevent differences due to irregularities in the wall thickness of the tubes. The effect of additions of air or oxygen (BOC Gases N.Z. Limited), on growth response in tubes incubated at temperatures between 57 and 70°C was compared with tubes with no additional terminal electron acceptor. Air and oxygen were sterilized through a 0.45µm filter, and 18ml was injected into the headspace of each tube.

(b) Growth response in orbital shaking incubators

A range of temperatures (35°C, 45°C, 55°C, 65°C) was set in a series of shaking incubators (Thermolyne Rosi 1000) at 130rpm. Four flasks of rich medium and of minimal medium with 0.5g.l^{-1} sucrose buffered with either $NaHCO_3$ or 50mM CAPS pH 9.2 were incubated at each temperature. The medium was pre-warmed prior to inoculation (3% v/v) with an 18 hour culture of the same medium type. Growth was monitored by following increases in optical density (650nm) on samples withdrawn at time intervals. In addition, growth was followed microscopically, by plate counts and by protein assay (Section 3.2.3.1). For the plate counts, dilutions of inoculated medium were prepared in minimal salts (minimal medium without peptone) and plated out onto

rich medium Phytagel™ plates. In subsequent trials the range of temperatures that were investigated changed (55°C, 60°C, 65°C, 70°C) but the methods involved remained the same.

4.3.2 Results and Discussion

(a) Growth response in a temperature gradient incubator

Growth was recorded over a temperature range from 45-72°C with an optimum between 61-63°C. This is illustrated in Figure 4.14 which represents the mean growth from triplicate tubes at each temperature after 24 hours of incubation. The maximum temperature that supported growth was 72°C, the temperature minimum was harder to ascertain in this experiment since isolate TA2.A1 grew to the lowest temperature (45°C) in the gradient. Growth around the 70°C region was slower than might have been expected from growth with the same medium in shake flasks (Figure 4.16). To ascertain if oxygen was limiting, the experiment was repeated and oxygen and air were injected into tubes incubated between the temperatures of 57 and 70°C. No significant difference in growth was recorded compared to tubes which received no additional oxygen or air (Figure 4.15).

Figure 4.14: Growth of *Bacillus* isolate TA2.A1 in a temperature gradient incubator

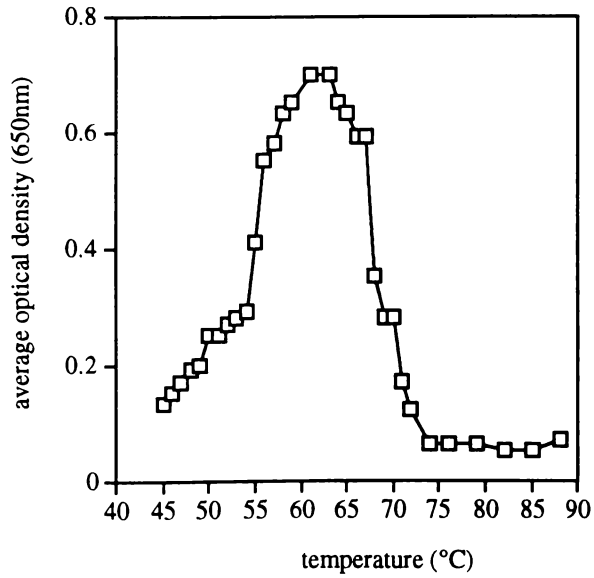
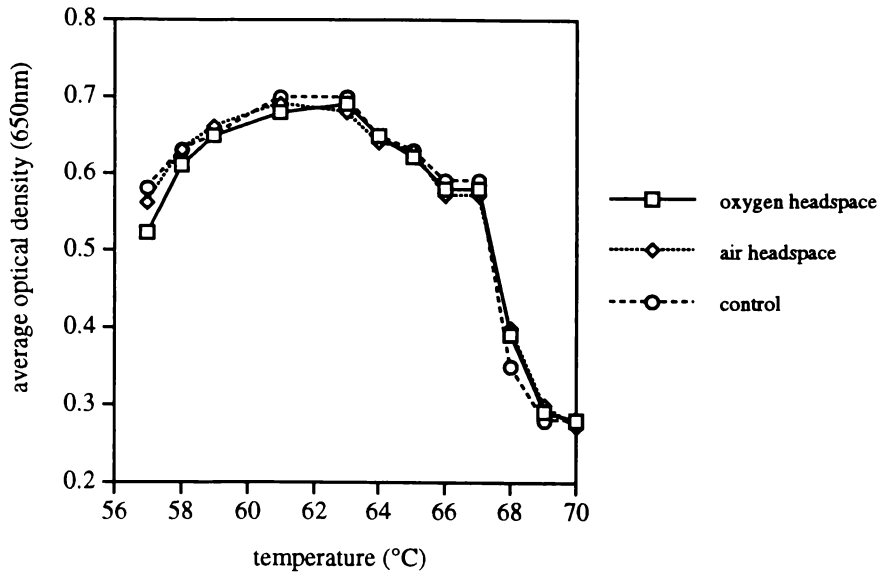


Figure 4.15: Growth of *Bacillus* isolate TA2.A1 in tubes with different headspace gases



(b) Growth response in orbital shaking incubators

No growth, as measured by optical density, and confirmed by examination under the phase contrast microscope was observed after incubation at 35°C in either rich or minimal medium. Limited growth at 45°C was observed after extended incubation (84 hours) with 0.1% of the colony numbers at 65°C after the same incubation period. Growth at 55°C was observed after 24 hours of incubation but there were fewer cells than at 60 or 70°C after the same length of incubation. Temperatures above 70°C were unable to be tested in orbital shaking incubators since this was the incubator's temperature maximum.

The highest final cell yield when isolate TA2.A1 was grown in rich medium was obtained after incubation at 65°C. The average results of five replicate experiments are shown in Figure 4.16; the temperature profile (shown over the first eight hours since inoculation) showed the same range of growth trends at each incubation temperature over 48 hours of inoculation. When the carbonate in the medium was replaced with 50mM CAPS at pH 9.2 essentially the same response to temperature was shown but with an overall reduction in the optical density obtained. Growth in minimal medium supplemented with 0.5g.l^{-1} sucrose under the same incubation conditions gave similar

results (Figure 4.17). Because growth in minimal medium was slower, than in rich medium, the temperature profile in minimal medium was conducted over 48 hours.

Figure 4.16: Growth of *Bacillus* isolate TA2.A1 in rich medium in flasks incubated at a range of temperatures

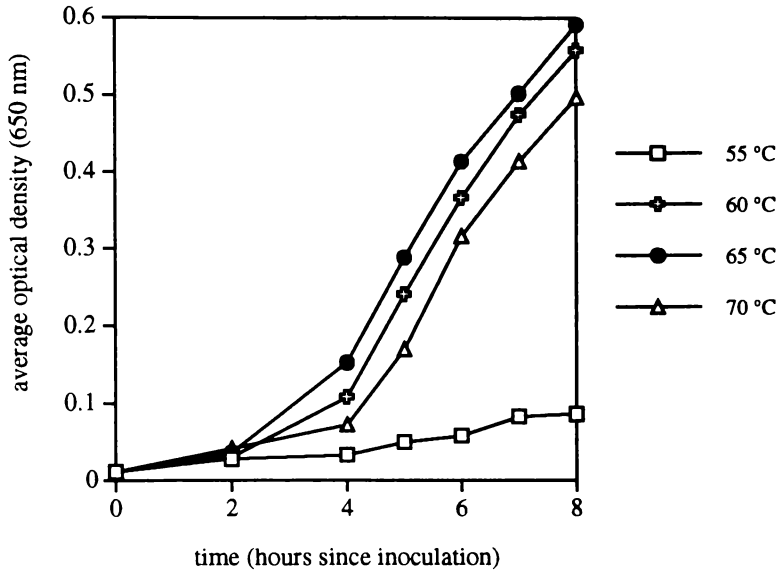
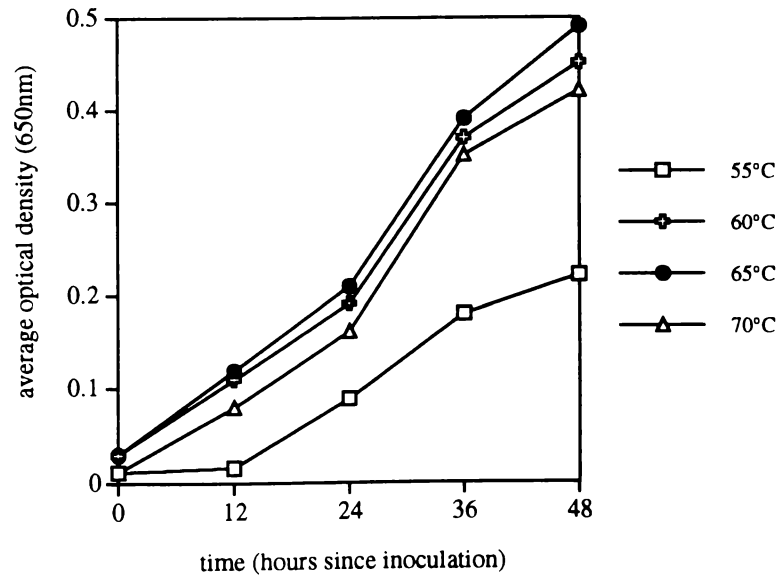


Figure 4.17: Growth of *Bacillus* isolate TA2.A1 in minimal medium with sucrose ($0.5g.l^{-1}$) in flasks incubated at a range of temperatures



In both rich and minimal medium the optimum temperature, in terms of final optical density, was 65°C. The generation times in both media are summarized in Table 4.8. The generation times in rich medium at incubation temperatures from 60 to 70°C were similar ranging from 48 to 66 minutes. In minimal medium the generation times were slower, for example 360 minutes at 65°C. For growth rate data on different carbon sources both in flask and fermenter cultures refer to section 4.4.1.2. After 48 hours of incubation the total protein produced at each incubation temperature was assessed by Lowry assay (Section 3.2.3.1). The final cell yield was greatest for the cultures incubated at 65°C in either rich or minimal media.

Table 4.8: Generation times (minutes) of *Bacillus* isolate TA2.A1 at different incubation temperatures

Temperature (°C)	55	60	65	70
Rich medium	110	52	66	48
Minimal medium	279	384	360	240

Ascertaining the maximum temperature for growth, under optimal conditions, was difficult because the orbital shaking incubators (Gallenkamp / Thermolyne Rosi 1000) had a maximum temperature of 70°C. Under the growth conditions in the temperature gradient incubator the maximal temperature for growth was 72°C. Growth at 75°C in rich or minimal media incubated in a stationery incubator (Clayson) required longer incubation, probably due not only to the increased temperature but also the decrease in oxygen availability. The optical densities obtained after incubation at 75°C were 5% of those in the same medium incubated at 65°C. *Bacillus* isolate TA2.A1 grew over a temperature range from 45 to at least 75°C.

4.4 Growth of *Bacillus* isolate TA2.A1 on different carbon substrates in minimal medium

4.4.1. Growth of *Bacillus* isolate TA2.A1 on a range of carbon substrates

4.4.1.1 Methods

Minimal medium (section 3.3.1.2) was prepared and inoculated in both flasks and fermenter vessels as outlined in sections 3.2.5.4 and 3.2.8 respectively. Stock solutions (10% w/v) of water-soluble carbon substrates were prepared in Milli-Q water and then filtered using a 0.45µm filter (Minisart). Less soluble compounds such as xylan, starch, inulin and indulin were added directly to the medium ($0.5g.l^{-1}$) and sterilized by autoclaving for 15 minutes at 15 psi. Lipids and fatty acids were added, unless otherwise stated, at a concentration of 0.04% (v/v) to minimal medium supplemented with 5mM$CaCl_2.2H_20$ from a filter sterilized stock solution. Palmitic and lauric acid were melted in a 70°C waterbath and then added by pre-warmed pipette tips, directly into minimal medium. Casein ($5g.l^{-1}$) was added to minimal medium and evenly dispersed throughout the media in a blender (Waring) before dispensing and autoclaving as normal.

Carbon substrates were added to a final concentration, unless otherwise stated, of 0.5 $g.l^{-1}$ and growth was assessed as described in section 3.2.3. Growth rates of *Bacillus* isolate TA2.A1 grown in flasks and fermenter vessels were compared on key carbon substrates.

Pre-warmed minimal medium supplemented with the carbon substrate under test was inoculated with an 18 hour culture grown in rich media at 65°C in an orbital shaking incubator. The viability of the inoculum was confirmed by inoculation simultaneously into a flask of rich medium. Additionally minimal medium without supplemented carbon source was inoculated to compensate for background increases in optical density; and uninoculated controls were used to compensate for increases in optical density from the incubation of the carbon source under conditions of high pH and temperature. Once growth on a particular substrate was established, a minimum of three serial transfers were carried out from an 18 hour old culture into the same medium, to ensure the growth

response was not due to carry over of substrate from the initial inoculum. Using data from the exponential section of a growth curve of *Bacillus* isolate TA2.A1 the doubling time on specific substrates was calculated. The average growth rate constant (k) is defined as:

$(\log_{10} \text{growth time}_2 - \log_{10} \text{growth time}_1) / 0.3010 \times t_D$

4.4.1.2 Results

Table 4.9: Growth of *Bacillus* isolate TA2.A1 on carbohydrate substrates in minimal medium

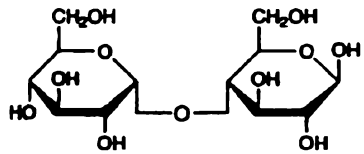
Substrates supporting growth	**Substrates failing to support growth**
Monosaccharides	**Monosaccharides**
6 deoxy-L-mannose	arabinose
rhamnose	β–D(-)fructose
	galactose
	D(+) glucose
	L-glucose
	mannose
	methyl α–D-glucopyranoside
	D(-) ribose
	L(+) ribose
	sorbose
	xylose
Disaccharides	**Disaccharides**
αD(+) melibiose	cellobiose
sucrose	lactose
D(+)trehalose (αα-trehalose)	
D(+) turanose	
Trisaccharides	**Trisaccharides**
D(+)raffinose	

Table 4.9 continued: Growth of *Bacillus* isolate TA2.A1 on carbohydrate substrates in minimal medium

Substrates supporting growth	**Substrates failing to support growth**
Polysaccharides	**Polysaccharides**
arabinogalactan	amorphous cellulose
fructooligosaccharides (Nutraflora™)	amygdalin
mannan	carboxymethylcellulose (CMC)
	dextran
	glycogen
	indulin
	inulin
	pectin
	pullulan
	starch (soluble / potato)
	xylan (oatspelts / birchwood / larchwood)

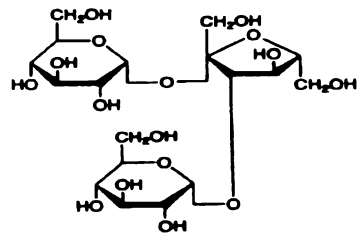
Maltose and D(+) melezitose were able to support growth of *Bacillus* isolate TA2.A1 on some occasions but not all. The variable growth results may be caused by the instability of maltose and melezitose under conditions of high pH and temperature. The structures of the two compounds are given below:

Figure 4.18: Di- and trisaccharides that do not consistently support the growth of *Bacillus* isolate TA2.A1



maltose

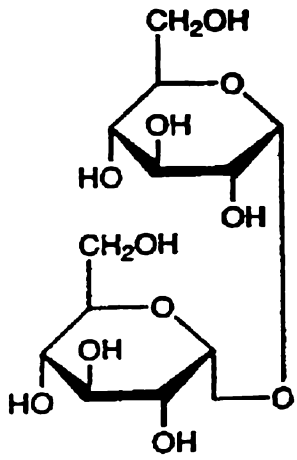
4,0α-D-glucopyranosyl-D-glucopyranose



melezitose

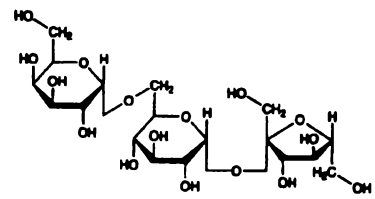
0-αD-glucopyranosyl-(1-3)β–D-fructofuranosyl-α–D-glucopyranoside

Figure 4.19: Di- and trisaccharides that support the growth of *Bacillus* isolate TA2.A1



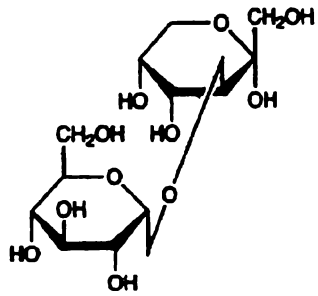
trehalose

1–0α–D-glucopyranosyl α–D-glucopyranoside



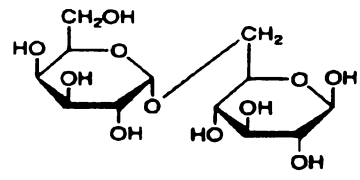
raffinose

β–D-fructofuranosyl-0α–Dgalactopyranosyl-(1-6)-α–D-glucopyranoside



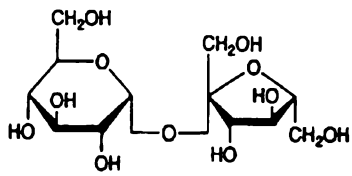
turanose

3-0α–D-glucopyranosyl-D-fructose



melibiose

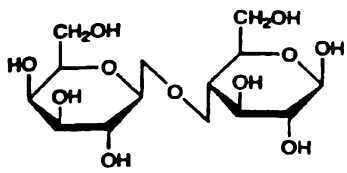
6,0 α–D-galactopyranosyl-D-glucose



sucrose

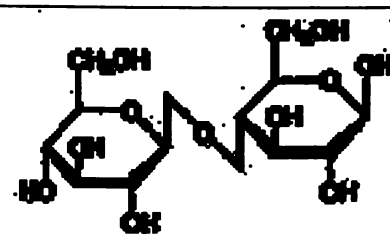
1-0α–D-glucopyranosyl-β–D-fructofuranoside

Figure 4.20: Di- and trisaccharides that do not support the growth of *Bacillus* isolate TA2.A1



lactose

4,0β–D-galactopyranosyl-D-glucopyranose



cellobiose

4,0β–D-glucopyranosyl -D-glucose

Table 4.10: Growth of *Bacillus* isolate TA2.A1 on non-carbohydrate substrates in minimal medium

Substrates supporting growth	**Substrates failing to support growth**
Lipids / Fatty acids	**Lipids / Fatty acids**
tricaprylin / Caprylic acid	acetic acid
triacetin	butyric acid
trilaurin / lauric acid	tripalmitin / palmitic acid
tributyrin	triolein / oleic acid
Krebs' Cycle Intermediates	**Krebs' Cycle Intermediates**
sodium pyruvate	DL-gylceric acid
sodium acetate	α-ketoglutarate
sodium succinate	sodium citrate
sodium fumarate	sodium lactate
L-malate	
Amino Acids	**Amino Acids**
L-asparagine	ß-alanine
L-aspartate	L-alanine
L-glutamate	L-arginine
D-glutamine	D-aspartate
L-glutamine	L-cysteate
L-lysine	cysteine
L-proline	D-glutamate
D-serine	glycine
L-serine	L-histidine
L-threonine	isoleucine
	DL-leucine
	L-methionine
	L-ornithine
	D-proline
	D-valine

Table 4.10 continued: Growth of *Bacillus* isolate TA2.A1 on non-carbohydrate substrates in minimal media

Substrates supporting growth	**Substrates failing to support growth**
Sugar Alcohols / Polyols	**Sugar Alcohols / Polyols**
glycerol	dithiothreitol
	erythritol
	inositol
	maltitol
	mannitol
	meso-inositol
	ribitol (adonitol)
	sorbitol
Proteins	**Proteins**
casein	bovine serum albumin (BSA)
ovalbumin	elastin
	gelatin
	hemoglobin
	keratin
Others	**Others**
acetamide	acetoin
casamino acids	dihydroxyacetone
glutaric acid	ethanediol/ethylene glycol monoether
peptone	D-glucuronic acid
	DL glyceraldehyde
	α–methyl-glutamate
	polyethylene glycol 300, 4000
	salicin
	thioglycollate
	Tween 80

Table 4.11 : Carbon substrates that support the growth of *Bacillus* isolate TA2.A1 in minimal medium without peptone (Oxoid) :

Complex	Simple
casamino acids	arabinogalactan
casein	L-glutamate
digested inulin [1]	glycerol
ovalbumin	Nutraflora ™
	sodium pyruvate
	trehalose

[1] refer to section 4.4.2.5 for description

The 0.1g.l^{-1} peptone in minimal media led to the utilization of a much broader range of carbon substrates (Table 4.9 and 4.10) than in its absence (Table 4.11).

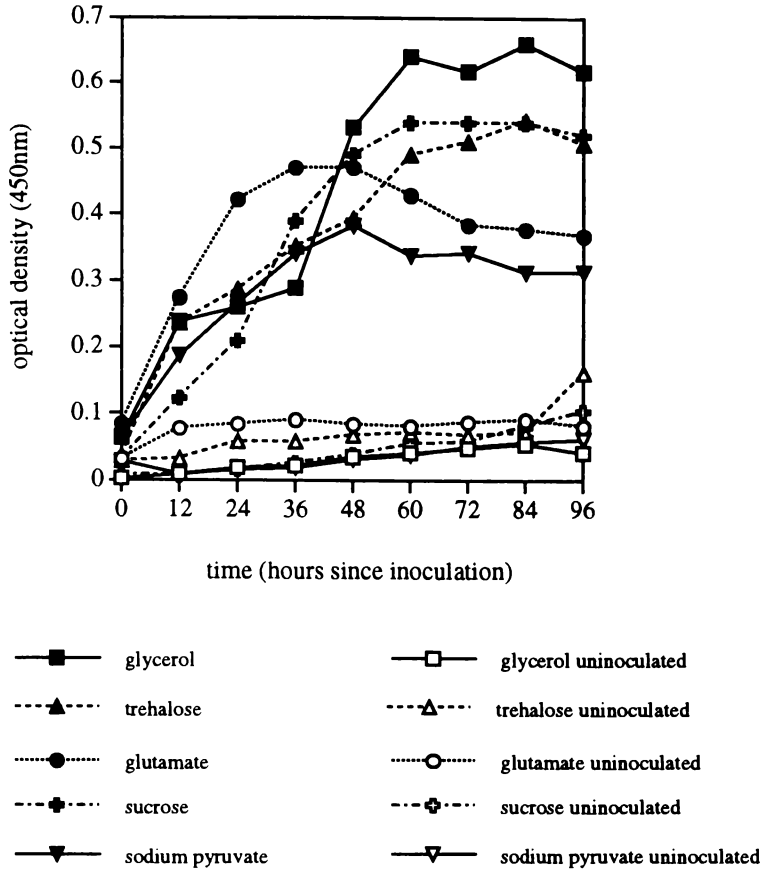
Table 4.12: Generation time of *Bacillus* isolate TA2.A1 on different carbon substrates at 0.5g.l^{-1}.

Carbon substrate	Fermenter growth (minutes)	Flask growth (minutes)	Section reference
Nutraflora™ (fructooligosaccharide)	69	849	4.4.2.5
trehalose	78	259	4.4.2.2
sodium pyruvate	95	461	4.4.2.4
glycerol	97	380	4.4.2.3
sucrose	103	360	4.4.2.1
10 minute inulin digest (fructooligosaccharide)	158	953	4.4.2.5
sucrose + trehalose [1]	170	-	4.4.2.1
glutamate	-	420	Chapter 5

[1] 0.25 g.l^{-1}final concentration of each

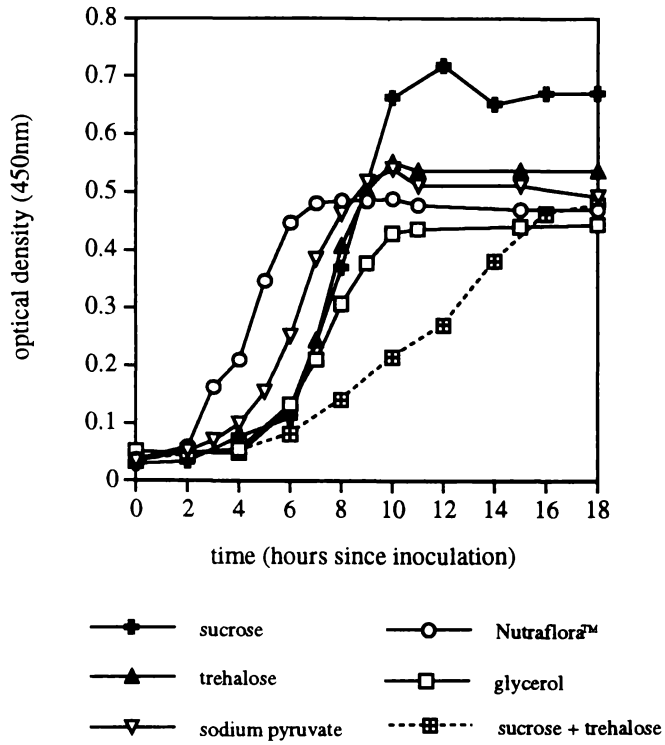
When the culture was grown in flasks the best carbon substrate in terms of overall yield was glycerol (Figure 4.21). The fastest generation time in a flask culture was obtained with trehalose as a substrate. Growth on sodium pyruvate was complete by 48 hours after inoculation. Growth on glycerol, trehalose and sucrose continued for at least 60 hours after incubation. Under fermenter conditions with the same media, the best growth in terms of cell yield was on sucrose (Figure 4.22), however, fastest growth occurred on fructooligosaccharides (Nutraflora™).

Figure 4.21: Growth of *Bacillus* isolate TA2.A1 in minimal medium with different carbon substrates



Many thermophilic organisms have been shown to utilize more carbon substrates in the presence of yeast extract or peptone. For example, *Thermobrachium celere* in the presence of 0.1% yeast extract (Engle 1996; Wiegel 1998) could also utilize several sugars including, glucose, fructose, galactose and maltose. The thermophilic alkali-tolerant species *Clostridium paradoxum* (Li *et al.* 1993) utilized glucose, fructose, sucrose, maltose and pyruvate but required yeast extract or tryptone, which could not be replaced by peptone, for growth.

Figure 4.22: Growth of *Bacillus* isolate TA2.A1 on supplemented minimal medium under fermenter conditions



In order to further characterize *Bacillus* isolate TA2.A1 growth on specific carbon substrates in minimal media was analyzed in more detail. The results of these investigations are outlined in section 4.4.2.

4.4.2 Growth of *Bacillus* isolate TA2.A1 on specific carbon sources

4.4.2.1 Sucrose

4.4.2.1.1 Methods

Culture preparation

Minimal medium (Section 3.3.1.2) was prepared and inoculated as outlined in section 3.2.5.4. Sucrose was added from a filter sterilised (0.45μm) stock solution (10% w/v) immediately prior to inoculation to give a final concentration, unless otherwise stated, of 0.5 $g.l^{-1}$. Growth was assessed (Section 3.2.3) and fermenter cultures were set up (section 3.2.8) as described earlier. All growth experiments were performed in triplicate. In the replicates where growth was supported, maximum variability between replicates was ± 0.03 optical density units (nm). Where growth was not supported the lack of growth was consistent over all replicates.

Sample preparation for HPLC

Culture samples were centrifuged in a microfuge on full speed for 5 minutes. Culture supernatant samples were stored at –20°C until analyzed. 50 μl samples of the culture supernatant was injected by Hamilton syringe onto the HPLC column. Standards were loaded onto the HPLC columns to confirm a linear response of peak area to concentration and to prepare a standard curve. Carbohydrate standards (50 mM to 1.5 mM) diluted in HPLC solvent were loaded onto the HPX.87H or HPX.42A columns. A mixture of carbohydrate standards (sucrose, trehalose, glucose and fructose) was made up in Milli-Q water at a concentration of $1g.l^{-1}$. This mixture was diluted over a range of concentrations down to a 50-fold dilution in Milli-Q water and injected into the Carbopac PA1 column. Substrate concentrations at or above $0.5g.l^{-1}$ gave a non-linear response to peak area on the Carbopac PA1 column.

HPLC column/detector systems:

(a) HPX.87H Aminex™ column (Bio-Rad)

This column separates low molecular weight carbohydrates and organic acids using a refractive index detector and a 0.01N H_2SO_4 solvent. The solvent was prepared in Milli-Q water from a 1.0N H_2SO_4 stock solution and filtered through a 0.45µm filter (Whatman) prior to use. The flow rate was set to 0.5 ml/minute (Waters model 510 pump) and the temperature to 50 ± 1°C in a column oven (Waters).

(b) HPX.42A Aminex™ column (Bio-Rad)

This column is designed for oligosaccharide analysis using a refractive index detector and Milli-Q water as the solvent. The Milli-Q water was filtered (0.45µm filter), boiled and then degassed under vacuum. The solvent was stirred on a hot plate during use to prevent oxygen redissolving. The column oven (Waters) was set between 85°C and 86°C and the flow rate to 0.6 ml / minute (Waters model 510 pump).

(c) Carbopac PA1 column (Dionex)

This HPLC uses a pulsed amperometric system of detection. The method for sucrose analysis involved an isocratic elution with 150mM noncarbonated NaOH (Fisher Scientific / Fisher Chemical 50% (w/w) Sodium Hydroxide Solution) constantly maintained under helium. The solvent flow rate was at 1 ml/minute. Unless otherwise stated the column was in a column oven (Waters) at 30°C.

Method development using the carbopac PA1 column

The stability of glucose, fructose and sucrose at high pH and temperature was analyzed using the Carbopac PA1 column. The retention time of the standards changed over a series of injections, the average minimum and (maximum) times in minutes were as follows; sucrose 7.43 (9.99), fructose 3.92 (4.95) and glucose 3.72 (4.13). The pump speed and solvent flow rate were consistent throughout these shifts in retention time. To avoid the shifts in atmospheric temperature which were responsible for these changes the column was placed in a column oven (Waters) at 30°C.

Regardless of whether the medium was inoculated or not, there was an increase in the sucrose peak area detected by the HPLC (Dionex) for the first sample taken after incubation commenced (for example: Figures 4.23b to 4.27). Four flasks of minimal medium and $0.5g.l^{-1}$ sucrose incubated at 65°C or 22°C (room temperature) had multiple samples removed from each flask. Multiple injections of supernatant from these samples onto the Carbopac PA1 column showed that this initial increase in peak area from the time of incubation until the first sample timepoint was reproducible. The effect was the same whether the first sample timepoint was after 12 hours of incubation (as shown in Figure 4.23b and 4.24) or 1 hour after incubation. The variation in peak area between injections of the same sample was ± 3%. The average increase in sucrose peak area from the start of incubation at either temperature until the first sample was removed was 120% of the initial peak area; after this initial increase the sucrose peak area increased no further. This was an effect of high pH or medium components, since it was observed at either temperature in medium, but not in water.

Search for evidence of enzymatic activity

1 ml samples of inoculated rich medium or sucrose supplemented minimal medium were centrifuged in a microfuge at full speed for 15 minutes. Cell pellets were resuspended in 100µl of a $0.5g.l^{-1}$ sucrose solution. Supernatants were concentrated 100-fold in a 10kDa Centricon (Amicon) tube. The removal of sucrose from the sucrose solution after incubation at 65°C, in the presence of concentrated sucrose grown cell pellet or supernatant, was monitored by HPLC. Concentrated supernatant or cell pellet samples from growth in minimal medium with sucrose were also screened with pNP (para-nitrophenyl) substrates; α-D glucopyranoside, β-D glucopyranoside, α-L fucopyranoside, β-D lactopyranoside, α-L arabinopyranoside, β-D galactopyranoside for indirect evidence of sucrase activity. The only substrate that gave a positive assay result, shown by para-nitrophenyl release, was p-Nitrophenyl-α–D glucopyranoside. The optimal conditions for the p-Nitrophenyl-α–D glucopyranoside assay were; 200µl of 5mM substrate in 50mM MOPS (pH 7) incubated with 50µl of enzyme sample (concentrated supernatant, cell pellet, cell fragments or supernatant after sonication) for 15 minutes at 65°C. After incubation the assay was stopped by addition of 500µl of 1M Na_2CO_3. Following centrifugation of the assay mixture, the optical density of the

supernatant was determined spectrophotometrically at 400nm and compared to a pNP standard curve in order to calculate the concentration of pNP released during each assay.

The glucose oxidase (GOD) assay (Boehringer Mannheim) was used as a direct measure of sucrase activity by assessing glucose release. Culture samples (1ml) taken at different time intervals during growth on sucrose, were centrifuged and the resulting cell pellet sonicated (Dynatech) on ice for 5 minutes. After sonication the sonicate-cell pellet and sonicate-supernatant were incubated in a 5mM sucrose solution at 65°C and glucose accumulation was measured by GOD assay. The GOD assay was scaled down from the manufacturer's instructions so that it could be read in a 1.5 ml cuvette. The manufacturer's Solution 2, containing peroxidase (POD) (≥0.35U/ml), GOD (≥8U/ml) and ABTS® (1.0mg/ml) (di-ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) was made up at a concentration of 1.54 g/100ml and was stored protected from the light at 4°C. A 50µl sample of culture supernatant or cells (resuspended in their original volume of uninoculated minimal medium) was mixed with 1.25 ml of solution 2 and incubated in a water bath at 25°C until a colour change occurred (for a maximum of 50 minutes). The samples were then centrifuged in a microfuge on full speed and the optical density of the supernatant was read at 610nm, using a reagent blank to zero the spectrophotometer. If the sample on which the GOD assay was being conducted had initially been incubated at 65°C, for example, the samples were cooled on ice prior to being assayed. The optical density (610nm) of the samples was compared to the GOD assay results from both standard concentrations of glucose prepared in Milli-Q water and the glucose standard solution (Solution 1, 0.505 $mmol.l^{-1}$ glucose) provided by the manufacturer.

Intracellular enzymatic activity was measured by assaying sucrose grown cells that had either been treated with toluene:acetone (1:1 (v/v)) or had been sonicated for 5 minutes on ice. After sonication the sucrose grown cells were centrifuged in a microfuge on full speed for 15 minutes. The sonicate-supernatant and the sonicate-cell fragments were then assayed separately for p- Nitrophenyl-α–D glucopyranoside activity. Cell pellets from 1ml aliquots of inoculated sucrose supplemented minimal medium cultures were resuspended in their original volume of minimal medium (sucrose free) and had 100µl of toluene:acetone (1:1 (v/v)) added. The cell pellet and toluene:acetone mixture was left

standing at room temperature for 10 minutes; aliquots were then removed and assayed for p-Nitrophenyl-α–D glucopyranoside activity as outlined above.

In addition to utilizing sucrose as a carbon substrate, *Bacillus* isolate TA2.A1 could also grow in minimal medium supplemented with trehalose; details on trehalose utilization are given in section 4.4.2.2. To assess whether the sucrose degrading enzyme acted specifically on sucrose, or could act on trehalose, for example, and whether sucrose was utilized preferentially before trehalose when both carbohydrates were present in inoculated medium, the following assays were performed. Cell pellets from 1ml samples of culture, with both sucrose and trehalose ($0.25g^{-1}$ of each) as carbon sources, were resuspended in 100µl of 20mM MOPS (pH 7) buffer. The samples were sonicated on ice for 10 minutes and centrifuged in a bench top microfuge on full speed for 15 minutes. The sonicate supernatant was mixed in a 1:1 (v/v) ratio with either 5mM sucrose or 5mM trehalose in 50mM MOPS (pH 7) buffer. The samples were placed in a water bath (65°C) for 60 minutes, after which time duplicate 50µl aliquots were removed on which GOD assays were performed.

4.4.2.1.2 Results and Discussion

Inoculated minimal medium with no carbohydrate addition supported some growth of *Bacillus* isolate TA2.A1 over the first twenty-four hours of incubation, presumably using the $0.1g.l^{-1}$ peptone as the carbon and energy source. After twenty-four hours no further growth was evident (Figure 4.23a). Increased growth was recorded in minimal medium supplemented with sucrose (Figure 4.23a). Uninoculated medium produced a slight increase in optical density (450nm). All significant increases in optical density were confirmed by microscopic examination, as being due to growth of *Bacillus* isolate TA2.A1, rather than colour development in the medium. There was a slight increase in optical density in the inoculated medium containing sucrose alone (no peptone), however, no decrease in sucrose concentration above the level observed for uninoculated medium was detected (Figure 4.23b).

Figure 4.23a: Growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with 0.5g.l^{-1} sucrose

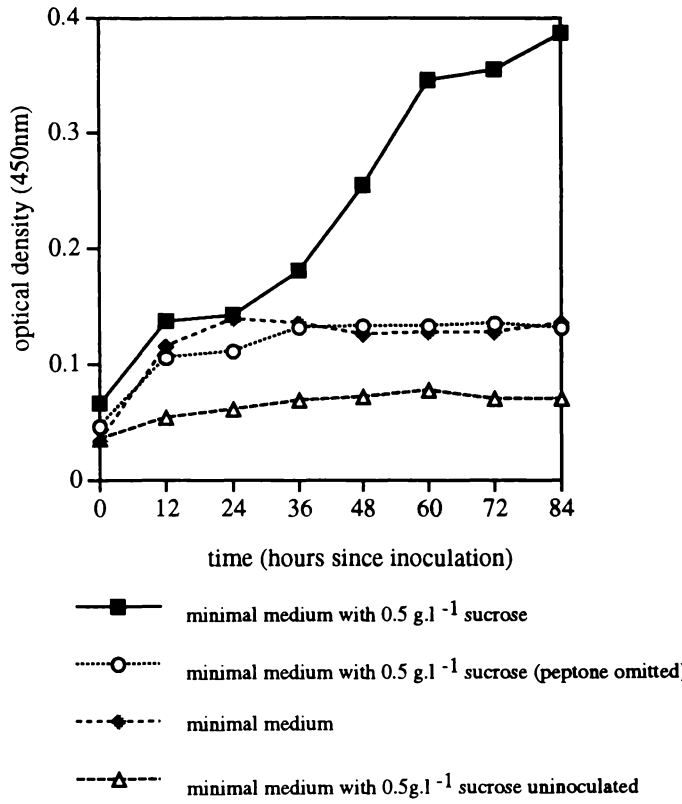
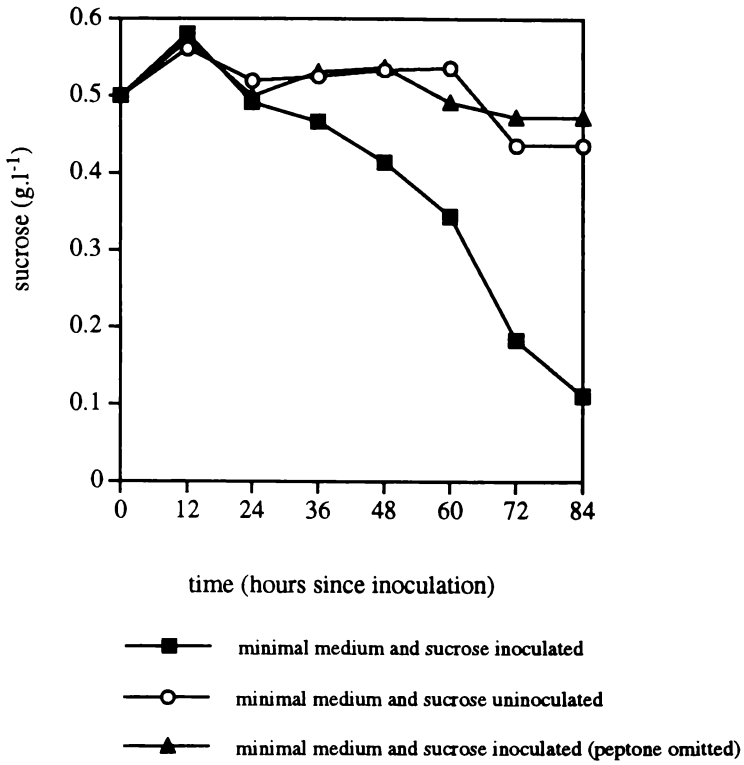
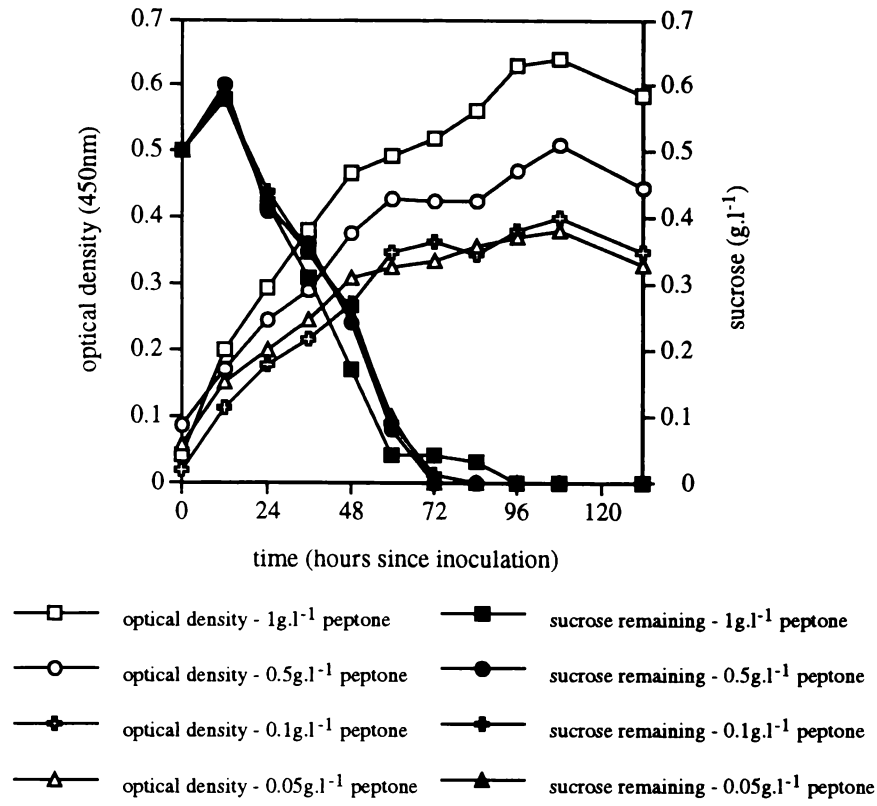


Figure 4.23b: Sucrose disappearance from minimal medium in the presence or absence of 0.1g.l^{-1} peptone



Clearly *Bacillus* isolate TA2.A1 is able to utilize sucrose as a substrate more efficiently in the presence of a small concentration of peptone (Figures 4.23a and 4.23b). Tests were carried out to ascertain the optimal concentration of peptone in the sucrose supplemented minimal medium. Over a range of peptone concentrations (0.05, 0.1, 0.5 and 1.0g.l^{-1}) the final cell yield of *Bacillus* isolate TA2.A1 in minimal medium supplemented with 0.5g.l^{-1} sucrose was highest in medium containing 1g.l^{-1} peptone (Figure 4.24). The final yield was not, however, proportional to the concentration of peptone in the medium. Also the rate of sucrose removal from the medium was not significantly influenced by the peptone concentration (Figure 4.24). Sucrose utilization by *Bacillus* isolate TA2.A1 was not influenced by the concentration of peptone supplied, thus there is no evidence of diauxic growth on these substrates.

Figure 4.24: Growth of *Bacillus* isolate TA2.A1 in minimal medium with 0.5g.l^{-1} sucrose and different concentrations of peptone



After a small initial increase in sucrose peak area (as described in the Dionex HPLC method development, section 4.4.2.1.1) following incubation in minimal medium at 65°C the sucrose concentration decreased as growth occurred (Figures 4.25 and 4.26).

The response recorded in Figure 4.25 was typical of growth of *Bacillus* isolate TA2.A1 in sucrose supplemented minimal medium, however, on occasion a prolonged lag phase was evident followed by a slower growth response, as illustrated in Figure 4.26. This occurred despite standardizing the age of the inoculum, pre-warming the medium prior to inoculation and ensuring that the inoculum was taken from a log-phase culture.

Figure 4.25: Growth of *Bacillus* isolate TA2.A1 in minimal medium with 0.5g.l^{-1} sucrose

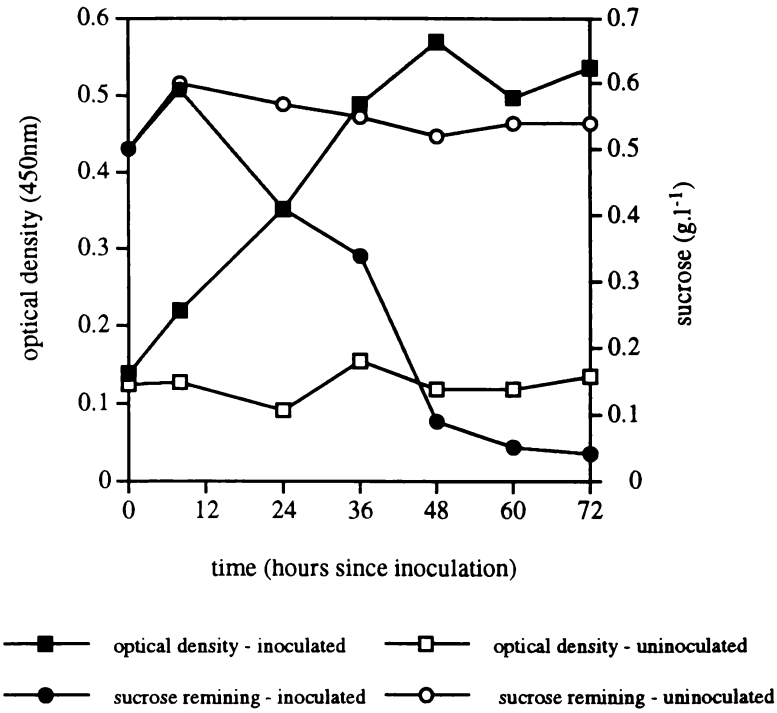
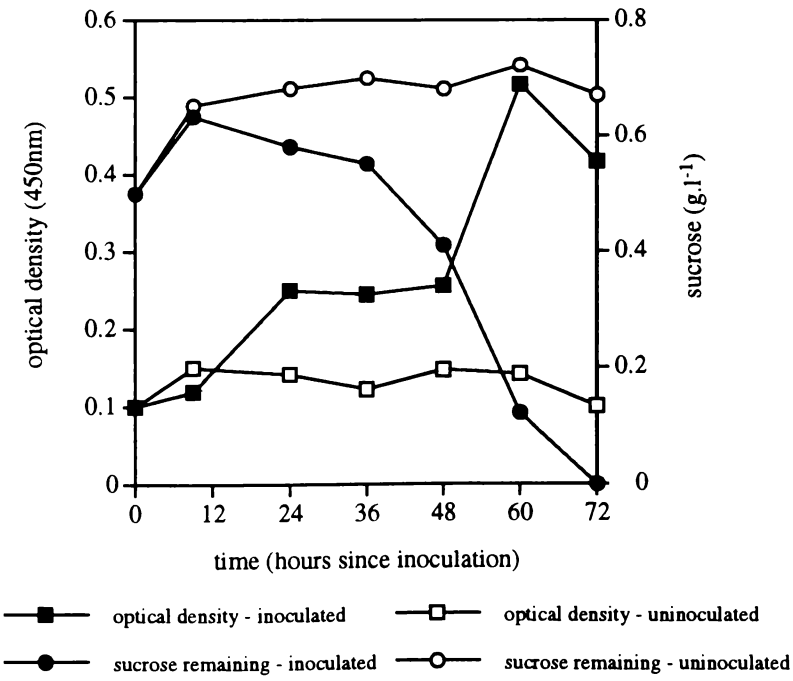
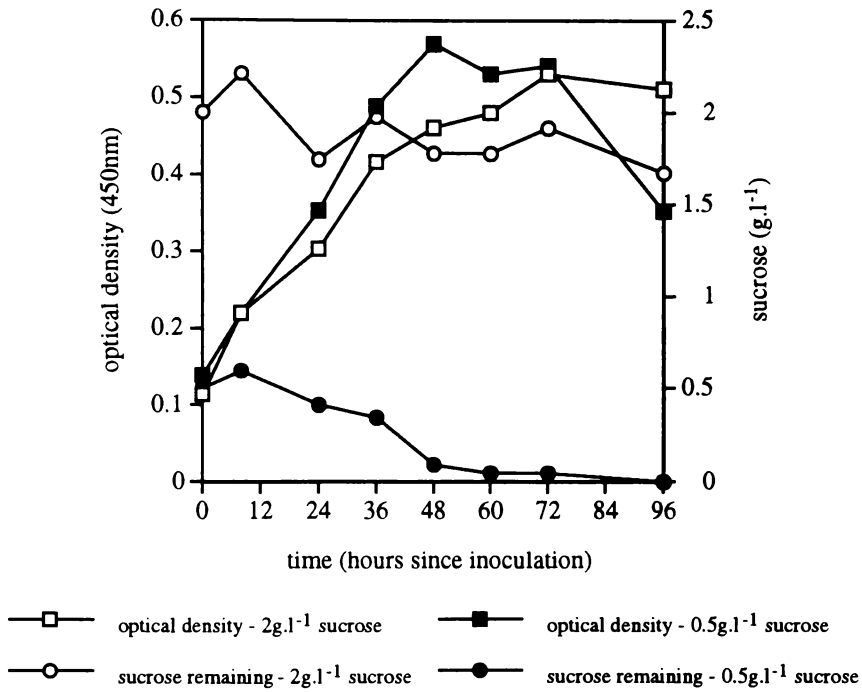


Figure 4.26: Growth of *Bacillus* isolate TA2.A1 in minimal medium with 0.5g.l^{-1} sucrose



The maintenance of stable growth over multiple transfers, on minimal medium supplemented with two sucrose concentrations, was observed (Figure 4.27). No difference in growth of isolate TA2.A1 was seen between medium where the sucrose was added from a filter sterilised stock solution after autoclaving and medium where the sucrose was autoclaved with other components.

Figure 4.27: Growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with either 0.5 or 2g.l^{-1} sucrose

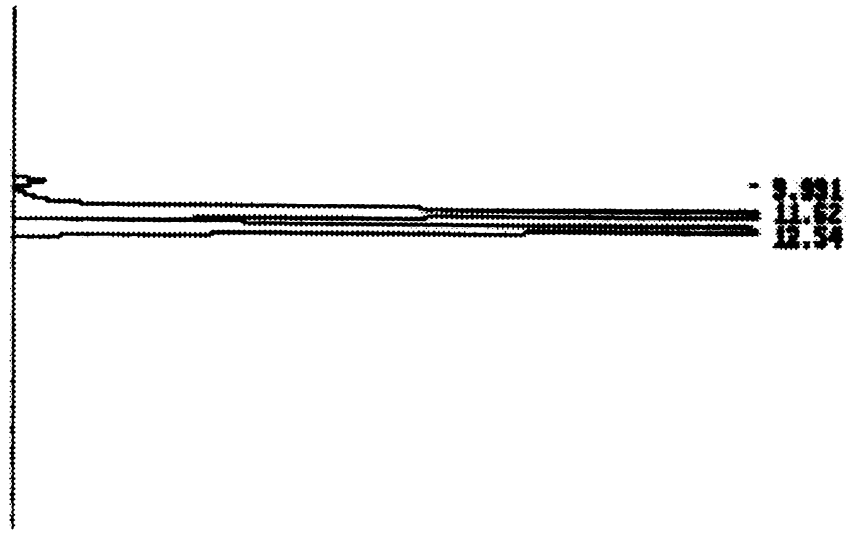


The growth yield achieved by *Bacillus* isolate TA2.A1 grown in minimal medium with 0.5g.l^{-1} sucrose was not significantly increased when the sucrose concentration was increased four-fold (Figure 4.27). The proportion of the initial sucrose concentration removed from the medium was greater in the medium supplemented with 0.5g.l^{-1} sucrose compared to 2g.l^{-1} sucrose (Figure 4.27). No growth of *Bacillus* isolate TA2.A1 was observed (data not shown) at sucrose concentrations of 10 and 15g.l^{-1}.

Initially the evidence for growth on sucrose was an increase in optical density when compared to all necessary controls (uninoculated and inoculated, with and without sucrose and/or peptone). Finding a suitable method for analyzing sucrose concentrations in media proved problematic. Despite repeated attempts and different HPLC columns and solvents, sucrose was shown to invert on the Aminex™ (Bio-Rad) HPLC columns

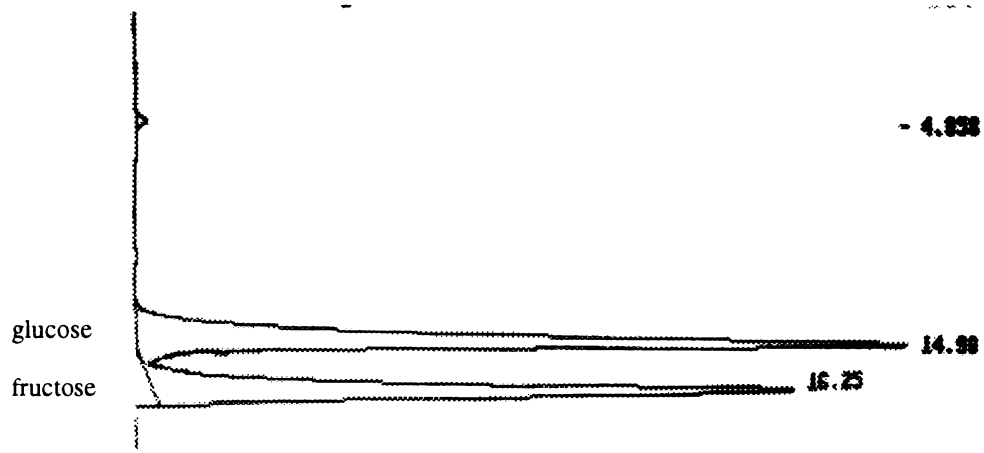
tested. This is illustrated in the chromatogram showing two peaks eluting close together (Figure 4.28a), but not corresponding to either glucose or fructose in retention time (Figure 4.28b). These two peaks failed to decrease or increase consistently in proportion to the sucrose concentration being analyzed. For this reason sucrose concentration results by this method of detection were inconclusive.

Figure 4.28a: Aminex™ HPLC profile of sucrose inversion products



The difficulty in resolving sucrose as a single peak was not due to molecular weight since cellobiose has the same molecular weight and gave a single peak. Fructose or glucose standards from 50mM to 3.125mM, prepared in Milli-Q water, gave a perfectly linear response of concentration to peak area and were resolved as sharp single peaks.

Figure 4.28b: Aminex™ HPLC profile of glucose and fructose standards

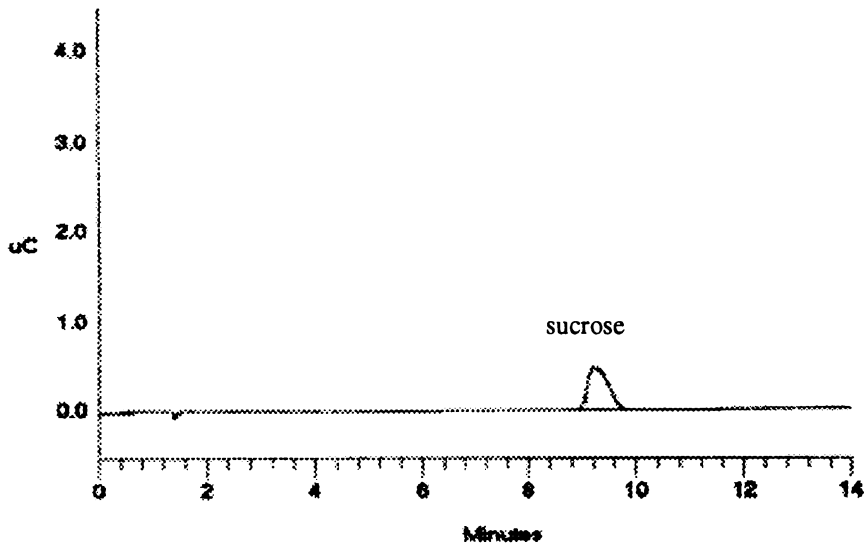


A mixed solution of fructose and glucose could be resolved as two distinct adjacent peaks with similar retention times on the HPLC column (Figure 4.28b). The pH of the solvent system with the HPX.87H Aminex™ column was pH 2, which may have increased the problems of sucrose inversion. However, changing the solvent system to CH_3CN for example, does not alleviate the problem of sucrose inversion (Bio-Rad - HPLC columns for carbohydrate analysis). Despite using Milli-Q water as a solvent, which should be a less harsh option than the low pH solvent used for the HPX.87H column, the problem of sucrose inversion was not rectified by the use of the HPX.42A column (Aminex™).

Analyzing culture supernatants on the Carbopac PA1 column using pulsed amperometric detection and isocratic elution with 150mM NaOH solvent provided conclusive data. This enabled the growth of isolate TA2.A1 in minimal medium with sucrose to be measured not only by an increase in optical density and protein concentration over time, but also by sucrose disappearance. The identification of the sucrose peak was checked both by analysis of sucrose standards in Milli-Q water and the fact that the sucrose peak disappeared immediately on addition of mesophilic invertase (baker's yeast $0.4g.l^{-1}$) in 10mM MOPS pH 7 buffer, with the appearance of corresponding amounts of glucose and fructose. Despite the sucrose concentrations decreasing considerably during growth of isolate TA2.A1 in sucrose supplemented minimal medium, no glucose or fructose accumulation was detected (Figure 4.29) even though all three carbohydrates could be

resolved using the Carbopac PA1 column (Figure 4.30). The fact that glucose and fructose were not detected may be explained by the degradation of sucrose to saccharinic and metasaccharinic acids (Section 2.5.2) in conditions of high pH (above pH 8.3). No commercial source of saccharinic or metasaccharinic acids was found, so it was not possible to determine the HPLC detector parameters necessary to ascertain the presence of these compounds.

Figure 4.29: Carbopac PA1 (Dionex) HPLC profile of culture supernatant from growth of *Bacillus* isolate TA2.A1 on sucrose



The stability of sucrose, glucose and fructose (all at 0.5g.l^{-1}) in Milli-Q water (pH 7) or minimal medium (pH 10) was assessed at 65°C and room temperature. The formation of a yellow colour (Y) and fragmentation of the sugar peak (F) (seen by HPLC (Dionex) analysis) under different temperature and solvent conditions is shown in Table 4.13.

Figure 4.30: Carbopac PA1 (Dionex) HPLC profile of glucose, fructose and sucrose (1.25mM) in minimal medium

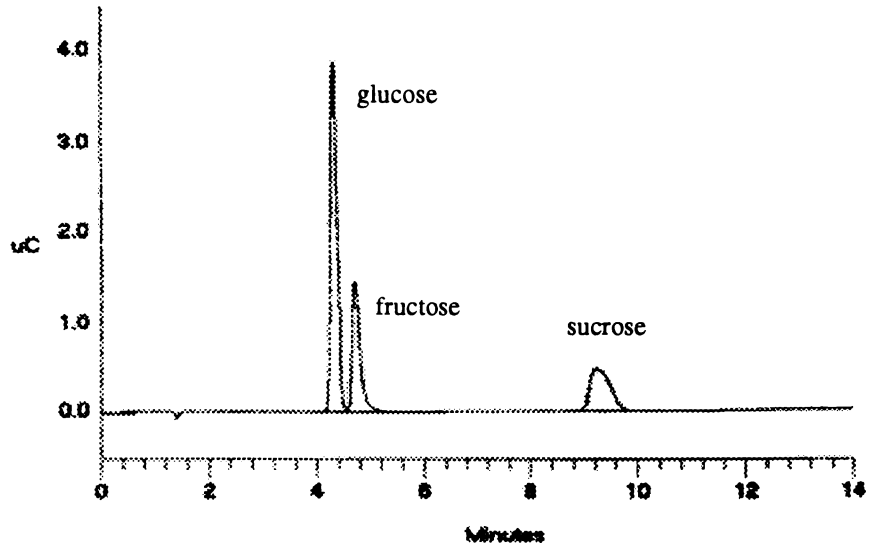


Table 4.13: The stability of glucose, fructose and sucrose under different solvent and temperature conditions

	room temperature (22°C)						65°C					
	glucose		fructose		sucrose		glucose		fructose		sucrose	
	Y	F	Y	F	Y	F	Y	F	Y	F	Y	F
Water (pH 7)	-	-	-	-	-	-	-	-	-	-	-	-
minimal medium (pH 10)	-	-	-	-	-	-	+	+	+	+	-	-

At room temperature glucose, fructose and sucrose were detected as single HPLC peaks and there was no colour development in either solvent; these results confirmed their stability at either pH. At 65°C glucose, fructose and sucrose were stable at pH 7. Even after extended incubation (16 hours), increased concentration (1.6M) and temperature (70°C) these substrates remained stable. Sucrose was stable at pH 10 at 65°C when

analyzed by Dionex HPLC. Glucose and fructose showed the development of yellow colour and a loss of HPLC peak definition at pH 10 and 65°C. The development of yellow colour under these conditions occurred more quickly for fructose (four hours) compared to glucose (six hours) at the same concentration. The fragmentation (shoulder-peak formation) of the glucose and fructose peaks began after two hours at pH 10 and 65°C (Figures 4.31 and 4.32).

Since the fragmentation of the glucose and fructose peaks preceded medium colour development, it is possible that medium colouration was caused by an alteration in glucose or fructose structure or the formation of other compounds detected as shoulder peaks. The stability of both glucose and fructose at high pH is dependent on the temperature of incubation. This is shown by the decrease in the concentration of glucose and fructose at pH 10 when incubated at 70°C compared to the relative consistency of concentration at 22°C (Figure 4.33). The fructose concentration decreased more rapidly than that of glucose at 70°C, which confirms that fructose is the less stable of these monosaccharides under conditions of high pH and temperature.

Figure 4.31: Carbopac PA1 (Dionex) HPLC profile of glucose in minimal medium (pH 10) after 0 to 8 hours of incubation at 65°C

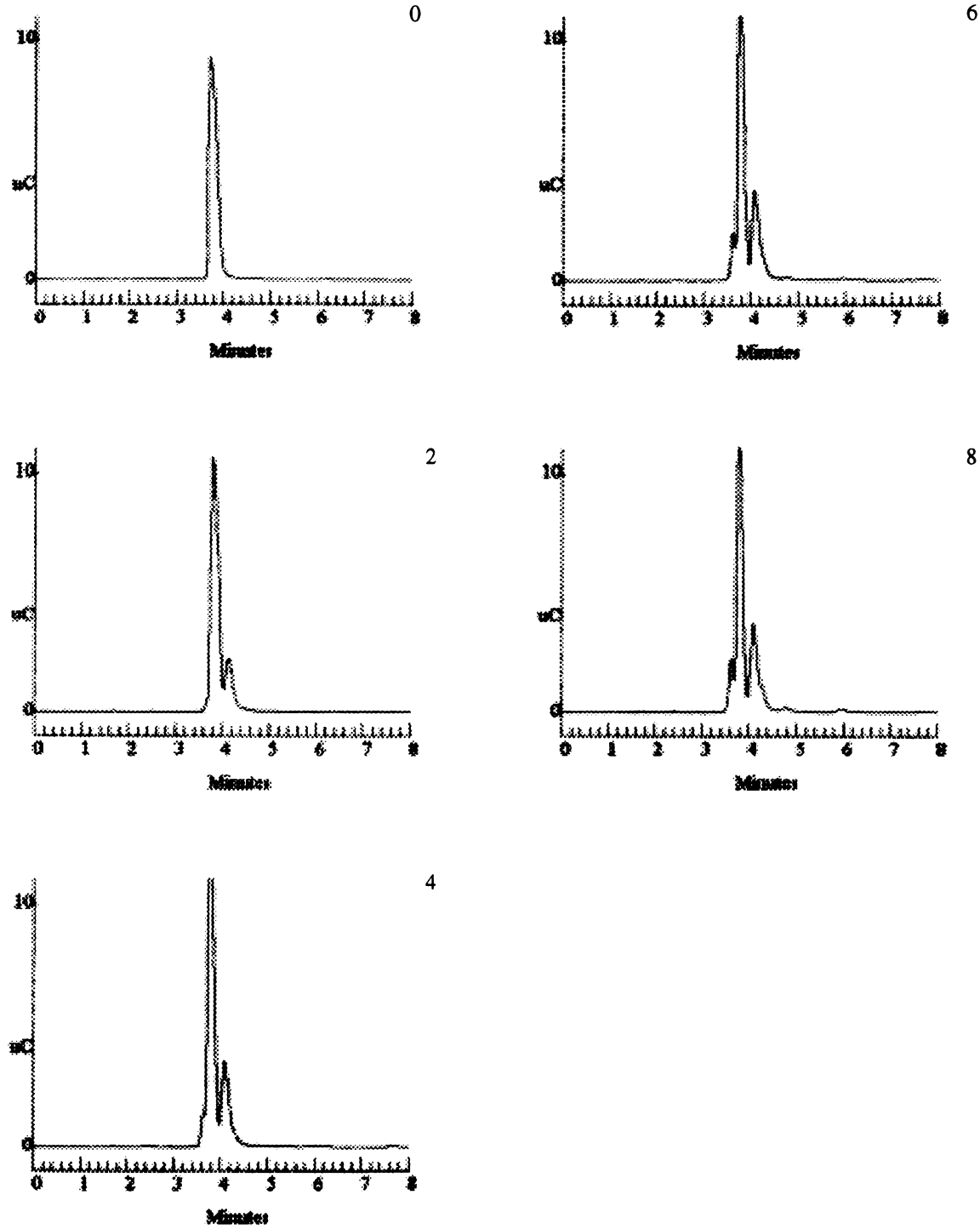


Figure 4.32: Carbopac PA1 (Dionex) HPLC profile of fructose in minimal medium (pH 10) after 0 to 8 hours of incubation at 65°C

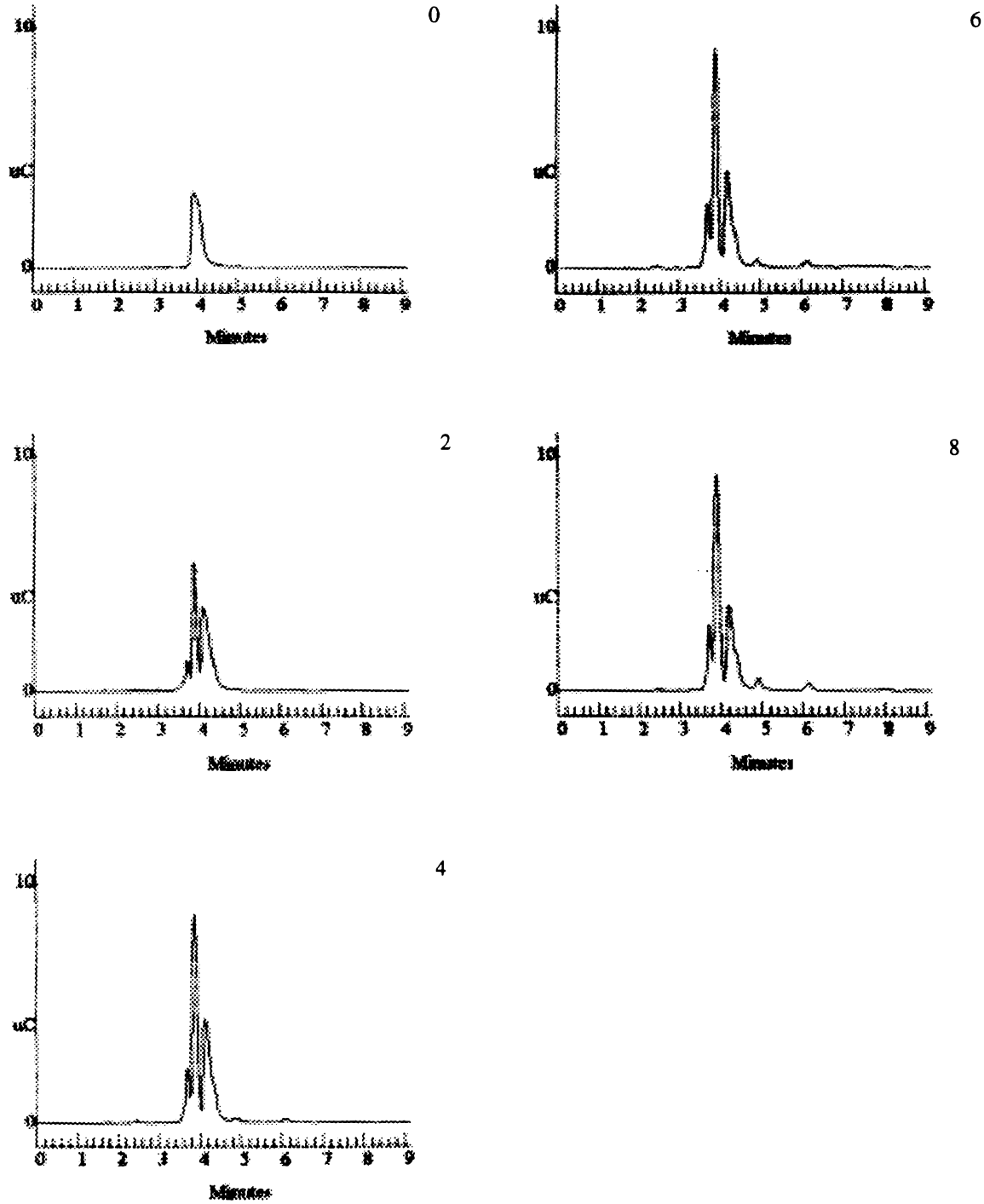
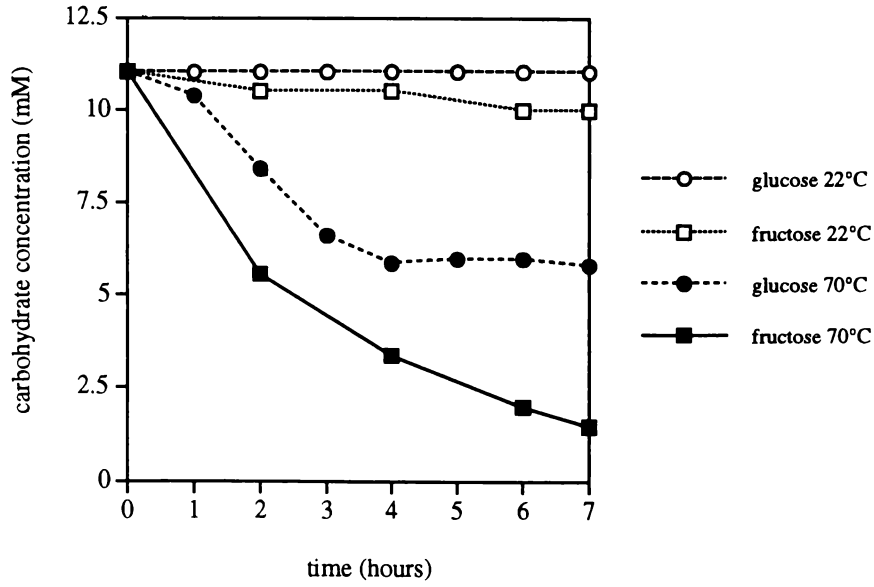


Figure 4.33: The temperature dependent stability of glucose and fructose incubated at pH 10



The further addition of sucrose, from a filter sterilized stock solution, to a *Bacillus* isolate TA2.A1 culture grown for 48 hours on sucrose supplemented minimal medium, resulted in an extension of the log phase of growth and an increased cell yield (Figure 4.34a). This shows that growth in minimal medium supplemented with sucrose is not nitrogen or phosphorus limited, or limited by some growth factor supplied by the peptone in the medium. The removal of sucrose from inoculated sucrose supplemented minimal medium, with and without additional sucrose after 48 hours of growth is shown in Figure 4.34b.

In most organisms the utilization of disaccharides is by an initial hydrolysis into constituent monosaccharides, followed by their entry into conventional metabolic pathways. This hydrolysis can occur either extracellularly or intracellularly depending on the species and the transport system. Sucrose utilization was further investigated in *Bacillus* isolate TA2.A1 to try and establish the enzyme involved and its location. In a search for evidence of sucrase enzymatic activity isolate TA2.A1 cells or supernatant from growth in sucrose supplemented minimal medium were incubated in the presence of a sucrose solution ($0.5g.l^{-1}$) at 65°C; however no decrease in sucrose concentration was detected by HPLC analysis, which suggested the presence of an intracellular enzyme. To characterize the enzyme which *Bacillus* isolate TA2.A1 was using to metabolize sucrose, pNP (para-nitrophenyl) assays were conducted using a range of pNP substrates (Section 4.4.2.1.1).

Figure 4.34a: Effect of sucrose addition to a mid-log phase culture of *Bacillus* isolate TA2.A1 growing in sucrose supplemented minimal medium

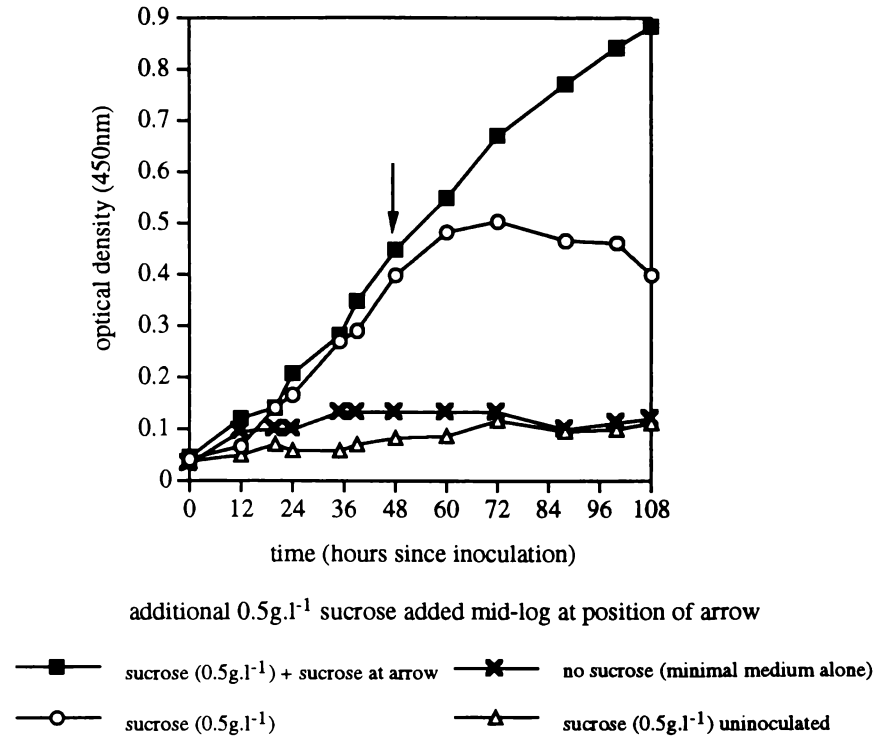
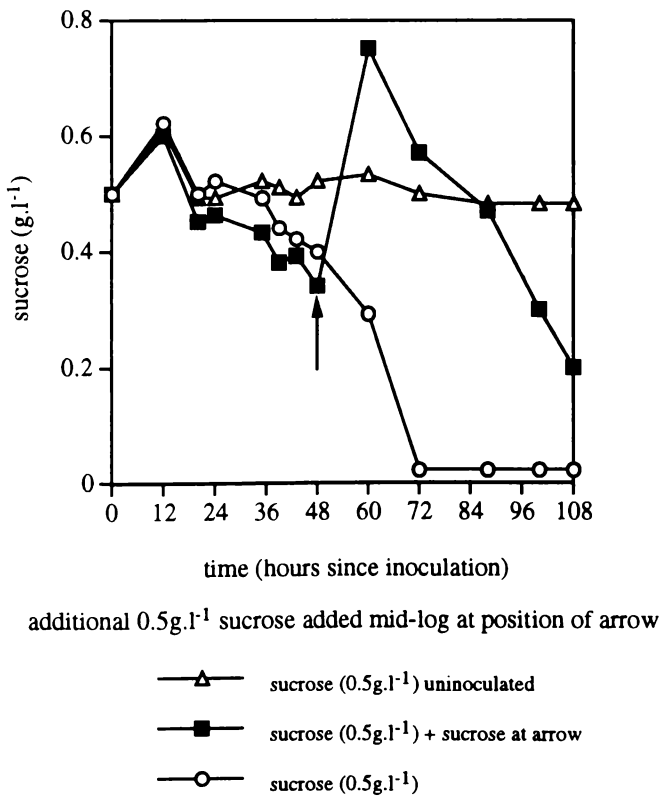


Figure 4.34b: Sucrose remaining during growth of *Bacillus* isolate TA2.A1 in sucrose supplemented minimal medium



Whole culture, culture supernatant and cells from growth in minimal medium supplemented with sucrose, were all assayed for pNP activity. No activity was detected in the culture supernatant. The cells were sonicated and the cell fragments and supernatant separated by centrifugation. pNP assays on the cell-sonicate fragments and cell-sonicate supernatant showed that there was enzymatic activity in both fractions detected by p-Nitrophenyl-α-D glucopyranoside. The fact that activity was detected in both the sonicate supernatant and cell fragments suggested an enzyme associated with the cell wall. The enzymatic activity was specific to the alpha form of the p-Nitrophenyl glucopyranoside substrate and was also specific in its ability to degrade sucrose. Sonicate supernatant from isolate TA2.A1 cells grown in sucrose containing medium would not degrade trehalose, another disaccharide which supported the growth of isolate TA2.A1. The specificity of the enzymatic action was confirmed by measuring glucose release (GOD assay) during incubation of cell sonicate supernatant with sucrose or trehalose and by HPLC analysis of sucrose or trehalose concentrations during the same incubation.

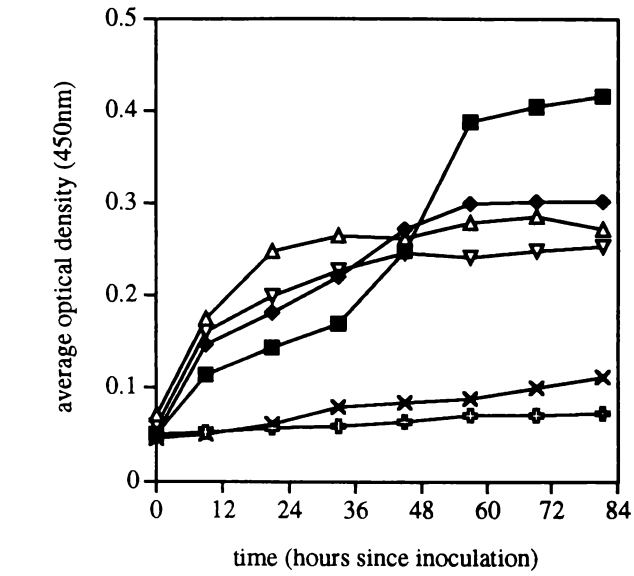
Since *Bacillus* isolate TA2.A1 grows on sucrose, if this substrate was utilized by conventional metabolic routes then the products of its hydrolysis, glucose and fructose, might also be expected to support the growth of the isolate. Glucose and fructose had been shown not to support the growth of isolate TA2.A1 as carbon substrates in minimal medium inoculated with a rich medium inoculum (Table 4.9). Further investigations were carried out on carbohydrate supplemented minimal medium where the inoculum was grown on sucrose supplemented minimal medium. Figures 4.35a, 4.35b, 4.36a and 4.36b illustrate the results obtained; the data presented are averages from triplicate flasks of each medium and carbohydrate combination.

Sucrose addition resulted in the highest cell yield, and in contrast to previous results glucose and fructose either in combination or alone, produced a higher culture optical density than unsupplemented medium (Figure 4.35a). Despite the higher optical density in the fructose and/or glucose supplemented media no increase in the numbers of isolate TA2.A1 cells was observed by microscopic examination. The decrease in the carbohydrate concentrations during the incubation of the carbohydrate supplemented

media are shown in Figure 4.35b. The variance in peak area between medium replicates was never more than 3%, irrespective of whether the glucose and/or fructose was in inoculated or uninoculated medium; hence to increase graph clarity the glucose and/or fructose results in inoculated and uninoculated medium appear identically on Figure 4.35b. Glucose and fructose as a mixture decreased in concentration slower than when added as individual media supplements under the same conditions (Figure 4.35b). Interestingly rhamnose (Figure 4.36b) reduced in concentration slower than any other carbohydrate tested, except sucrose, whether in the inoculated or uninoculated medium. Perhaps the better stability of rhamnose under the conditions necessary for the growth of *Bacillus* isolate TA2A1 is one of the reasons why it can be utilized while other carbohydrates cannot. Previous results (Table 4.9) showed that cellobiose and lactose did not support growth of isolate TA2.A1 in minimal medium when a rich medium inocula was used. However, when a sucrose supplemented minimal medium inocula was used there was an increase in optical density above that for uninoculated medium supplemented with the same carbohydrate (Figure 4.36a). Despite the increase in optical density no increase in the number of isolate TA2.A1 cells was seen after microscopic examination. Higher increases in optical density were observed for medium supplemented with sucrose, rhamnose and maltose, carbohydrates that had been shown earlier to support the growth isolate TA2.A1, although only variably in the case of maltose. An increase in isolate TA2.A1 cell numbers was seen after microscopic examination of the inoculated sucrose, maltose and rhamnose supplemented media confirming that the increases in optical density in these cases correlated with growth. The concentration of substrate remaining throughout the period of incubation was evalulated by HPLC and clearly the decrease in lactose, cellobiose and maltose concentration occurred at the same rate in inoculated and uninoculated media (Figure 4.36b). The increase in optical density in the inoculated medium continued after the maltose, lactose, and cellobiose concentration had decreased to less than 10% of the initial concentration (Figure 4.36b) this initially suggested that *Bacillus* isolate TA2.A1 was growing on the degradation products rather than the actual carbohydrate. These degradation products which were not detected with the HPLC detector settings being used, would presumably accumulate in the uninoculated medium and be consumed by the cells in the inoculated medium. However, against the hypothesis that isolate TA2.A1 was growing on carbohydrate breakdown products was the fact that no increase in cell

numbers was seen in microscopic examination of the inoculated medium containing lactose or cellobiose which showed a similar increase in optical density of the inoculated compared to uninoculated medium. Similarly no increase in cell numbers was observed in microsopic examination of the fructose and/or glucose containing inoculated medium (Figure 4.35a) despite the fact that the optical density in these media was above that in minimal medium alone. It is possible that these carbohydrates are so unstable under the growth conditions of *Bacillus* isolate TA2.A1 that they degrade or modify so as to be unusable by the isolate. Driskill *et al.* (1999) stated that the periodic addition of carbohydrates in fed-batch cultures of the hyperthermophilic archaeon *Pyrococcus furiosis* led to significant increases in cell yield. *Pyrococcus furiosis* was unable to grow on glucose, unless it was added during growth, due to its thermochemical liability in the growth environment. As stated by Driskill *et al.* (1999) it is not completely clear what specific effects the intermediates generated by the thermochemical modifications of carbohydrates (Maillard reactions, caramelization) have on the nutritional value of sugars and how these effects are influenced by pH, temperature and salt concentrations. Certainly in the case of *Bacillus* isolate TA2.A1 which grows under conditions of high pH and temperature the modifications of carbohydrates under the organism's growth conditions could not only effect their nutritional value but also whether they are available in a form that is utilizable by the organism.

Figure 4.35a: Growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with carbohydrate ($0.5g.l^{-1}$)



inoculated from a 48 hour minimal medium supplemented with sucrose culture

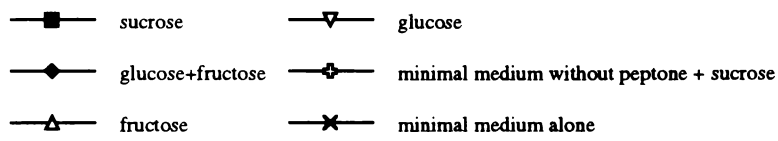
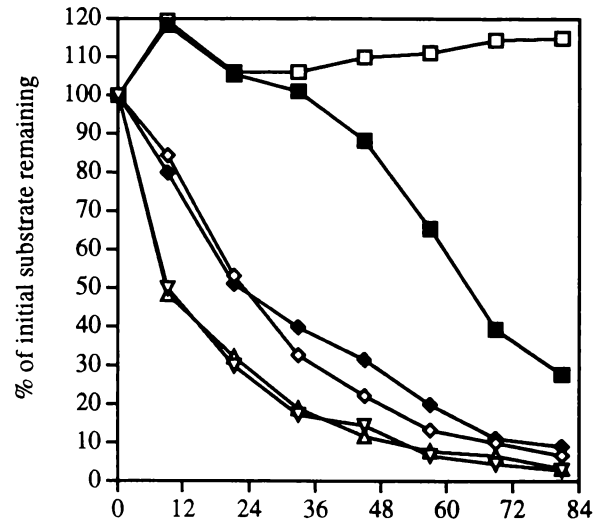


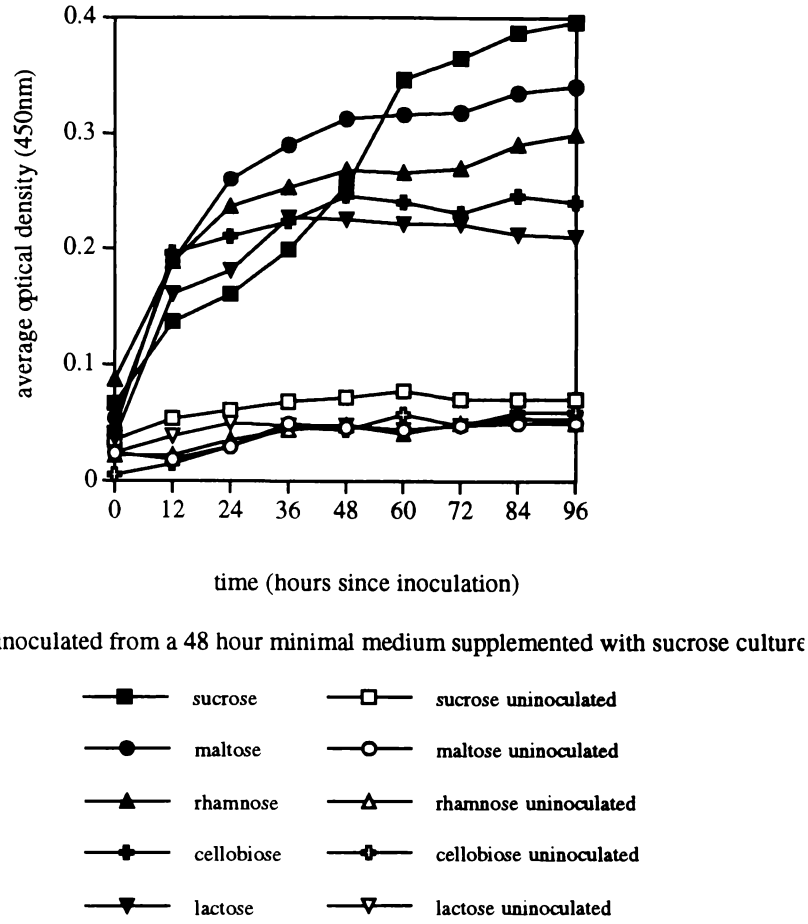
Figure 4.35b: Substrate remaining during growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with carbohydrate ($0.5g.l^{-1}$)



inoculated from a 48 hour minimal medium supplemented with sucrose culture

- sucrose - uninoculated
- sucrose - inoculated
- glucose from glucose and fructose mix - inoculated/uninoculated
- fructose from glucose and fructose mix - inoculated/uninoculated
- glucose - inoculated/uninoculated
- fructose - inoculated/uninoculated

Figure 4.36a: Growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with carbohydrate ($0.5g.l^{-1}$)



To ascertain whether monosaccharides, unable to support the growth of isolate TA2.A1, would affect sucrose utilization, glucose or fructose at $2g.l^{-1}$ final concentration were added either at inoculation (from a sucrose grown culture) or at mid-log to a minimal medium and $0.5g.l^{-1}$ sucrose culture. Fructose was inhibitory to growth of *Bacillus* isolate TA2.A1 on sucrose four hours after addition at inoculation (Figure 4.37) or immediately after addition at mid-log phase. Glucose added at inoculation was not inhibitory for the first eight hours, after which growth was inhibited. Glucose added to isolate TA2.A1 cells grown on sucrose in mid-log phase had no effect on growth, with growth resembling that achieved by the control sucrose culture, even twenty hours after glucose addition (Figure 4.37).

Figure 4.36b: Substrate remaining during growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with carbohydrate ($0.5g.l^{-1}$)

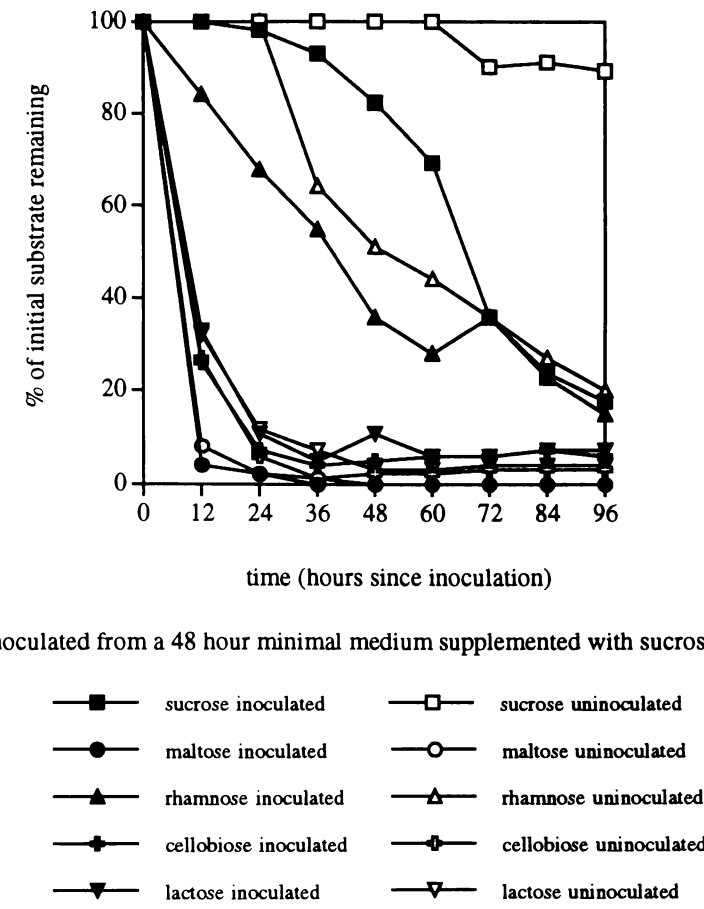
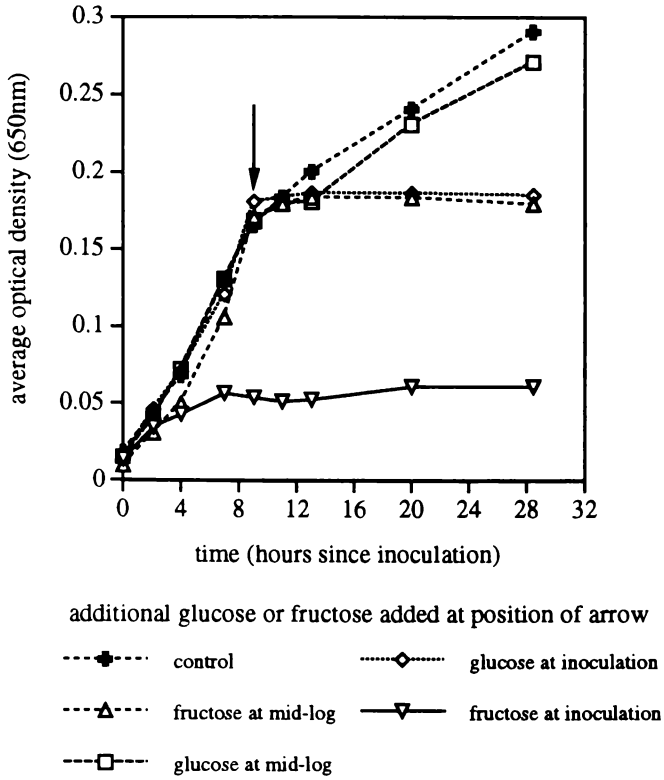
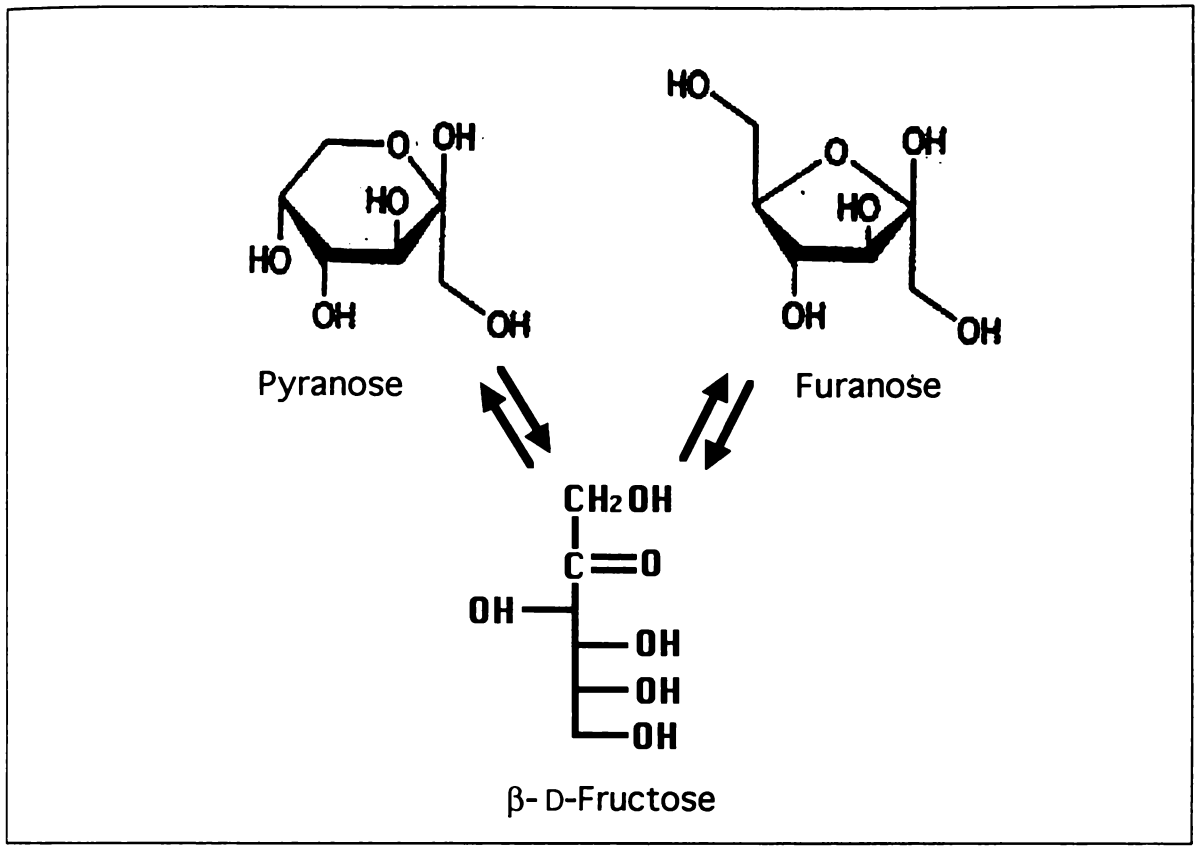


Figure 4.37: Growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with sucrose ($0.5g.l^{-1}$)



The inability of *Bacillus* isolate TA2.A1 to utilize fructose, while being able to utilize fructose-containing compounds such as fructooligosaccharides, might be explained by the fact that in each case the fructose has a different structure (Figure 4.38). In aqueous solution fructose exists as a 6-membered pyranose ring structure, whereas fructose in compounds of biological origin has a 5-membered furanose ring structure; these different forms of fructose are outlined in Figure 4.38. It is perhaps the 6-membered ring structure of fructose, or the instability of fructose at high pH and temperature, that makes this monosaccharide non-metabolizable by isolate TA2.A1.

Figure 4.38: Different structures of fructose



Growth under fermenter conditions of *Bacillus* isolate TA2.A1 in minimal medium with $0.5g.l^{-1}$ sucrose yielded a higher density of cells (Figure 4.39) than when grown in the same medium in a flask. The strict pH control and better aeration of the fermenter may explain the increase in cell yield. Growth on $0.5g.l^{-1}$ sucrose reached a higher optical density than growth on an equal concentration of sucrose and trehalose together ($0.25g.l^{-1}$ of each) (Figure 4.39). The utilization of sucrose was faster when sucrose was present as the sole carbon source than when the medium contained both sucrose and trehalose (Figure 4.39). When the medium contained both sucrose and trehalose, the trehalose was used preferentially over the sucrose (Figure 4.39).

The effect of ionophores on the growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with sucrose was assessed. Monensin, a carboxylic ionophore that disrupts sodium or potassium gradients across bacteral membranes, was added after commencement of growth on sucrose. Even at a low concentration (0.1μM) this had an inhibitory effect on growth (Figure 4.40). Amiloride, which inhibits Na^+/H^+ antiporters,

had no effect on growth at 100 µM and 500 µM final concentration (Figure 4.40). 2,4 di-nitrophenol, a proton gradient uncoupler, also had no effect on growth on sucrose (Figure 4.41). The optical density of cultures to which 2,4 di-nitrophenol was added, were read by spectrophotometer at 500nm to remove interference from colour absorbance. Further discussion on the implications of these ionophore results can be found in Chapter 6. Growth of *Bacillus* isolate TA2.A1 on sucrose was dependent on the presence of sodium ions (Figure 4.40). No growth on sucrose was possible in sodium-free medium. The sodium requirement for sucrose utilization is discussed in more detail in Chapter 6.

Discussion on the utilization of sucrose by other thermophilic or alkaliphilic organisms can be found in section 4.4.2.7.

Figure 4.39: Growth of *Bacillus* isolate TA2.A1 in minimal medium under fermenter conditions

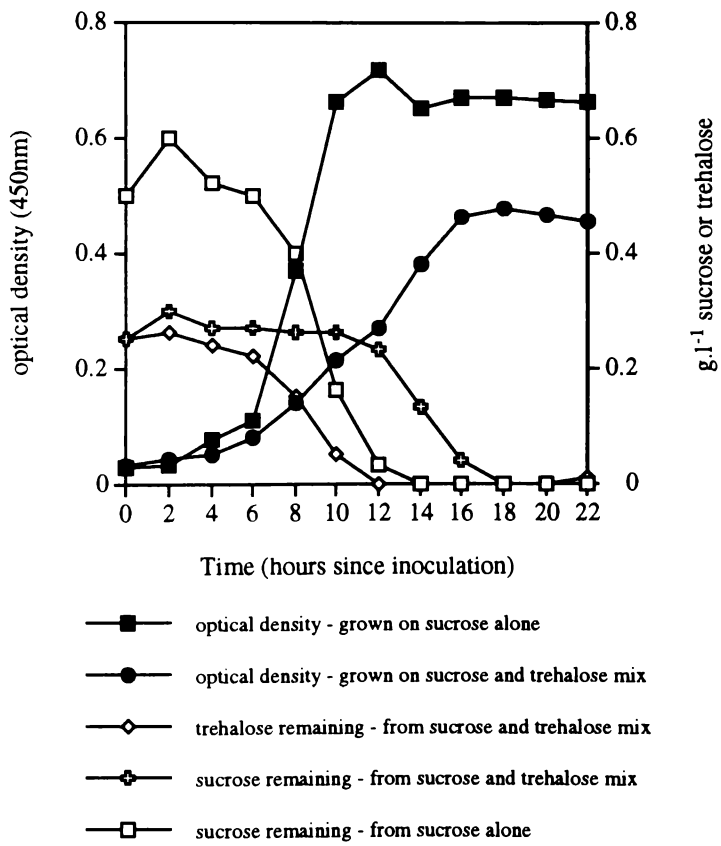


Figure 4.40: The effect of amiloride, monensin and sodium on the growth of *Bacillus* isolate TA2.A1 in minimal medium with $0.5g.l^{-1}$ sucrose

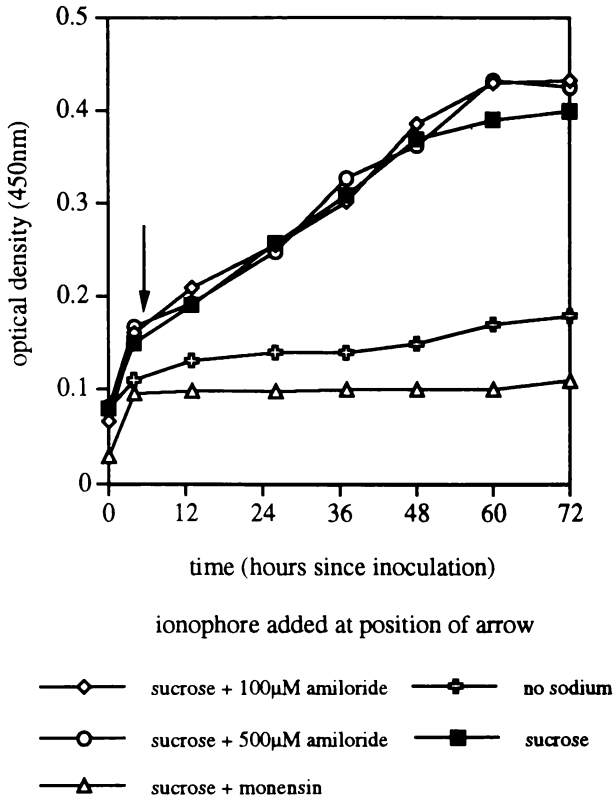
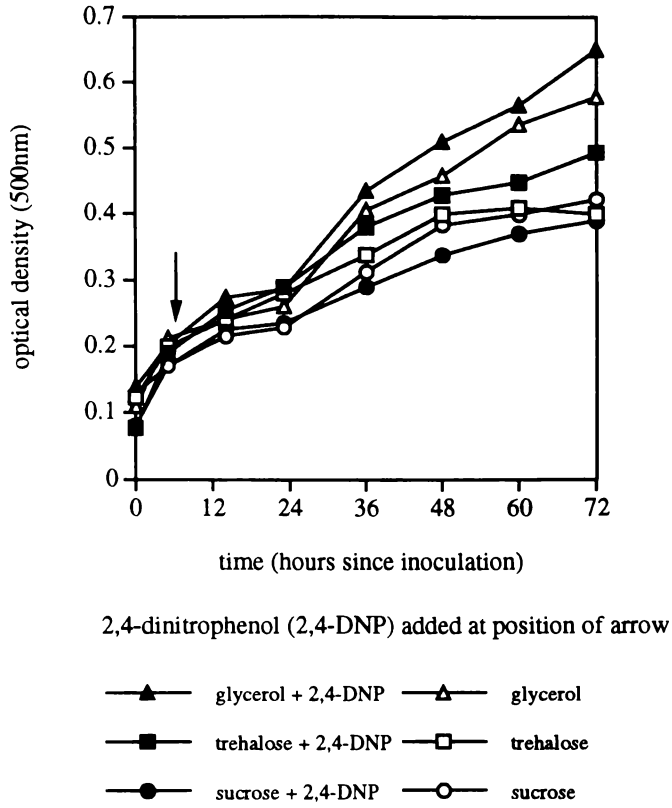


Figure 4.41: The effect of 2,4 –dinitrophenol on the growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with trehalose, glycerol or sucrose ($0.5g.l^{-1}$)



4.4.2.2. Trehalose

4.4.2.2.1 Methods

Culture preparation

Minimal medium (section 3.3.1.2) was prepared and inoculated as outlined in section 3.2.5.4. Trehalose was added from a filter sterilised (0.45µm) stock solution (10% w/v) immediately prior to inoculation to give a final concentration, unless otherwise stated, of $0.5g.l^{-1}$. Growth was assessed (Section 3.2.3) and fermenter cultures were set up (section 3.2.8) as described earlier. All growth experiments were performed in triplicate. In the replicates where growth was supported, maximum variability between replicates was ± 0.03 optical density units (nm). Where growth was not supported the lack of growth was consistent over all replicates.

Culture samples were prepared and analyzed on a Dionex HPLC system as outlined previously (Section 4.4.2.1.1). Supernatant and cell pellet samples from growth in minimal medium plus $0.5g.l^{-1}$ trehalose were screened for trehalase activity by direct (glucose oxidase assays - Section 4.4.2.1.1.) and indirect (pNP assays - Section 4.4.2.1.1) measurement of glucose released.

4.4.2.2.2 Results and Discussion

Bacillus isolate TA2A1 grew on $0.5g.l^{-1}$ trehalose in minimal medium with no added peptone. However, more growth was achieved in the complete minimal medium (including $0.1g.l^{-1}$ peptone) (Figure 4.42). As the concentration of peptone in the medium increased the final cell yield increased but not proportionally (Figure 4.42). The rate of trehalose removal from the medium was not significantly influenced by the peptone concentration (Figure 4.42). There was no proportional increase in growth yield when *Bacillus* isolate TA2.A1 was grown in minimal medium with trehalose concentrations ranging from 0.5 to $2g.l^{-1}$ (Figure 4.43a). Over all the concentrations of trehalose tested, the final cell yield was in fact very similar. The concentration of trehalose in the medium decreased over the same time interval, during growth of

Bacillus isolate TA2.A1, regardless of the initial concentration of trehalose (Figure 4.43b). At all trehalose concentrations tested 0.5g.l^{-1} had been utilized by *Bacillus* isolate TA2.A1 after 72 hours of incubation.

Figure 4.42: Effect of peptone concentration on growth of *Bacillus* isolate TA2.A1 in minimal medium with 0.5g.l^{-1} trehalose

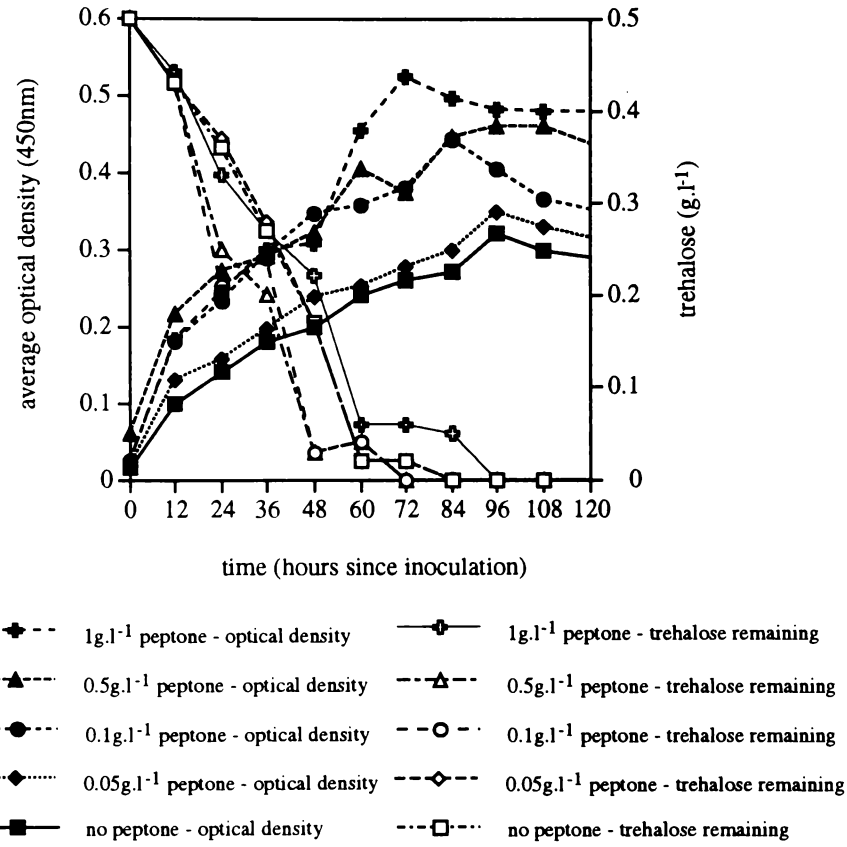
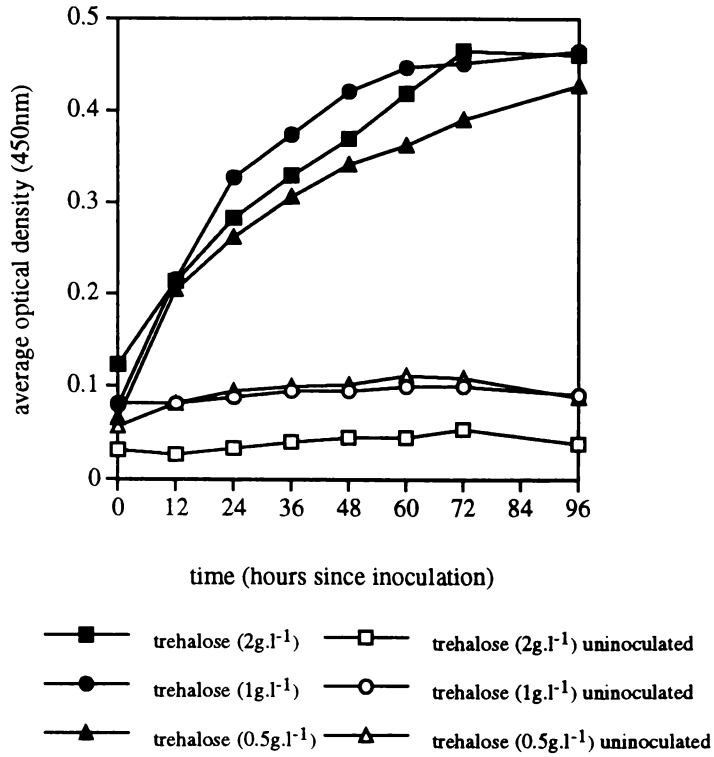
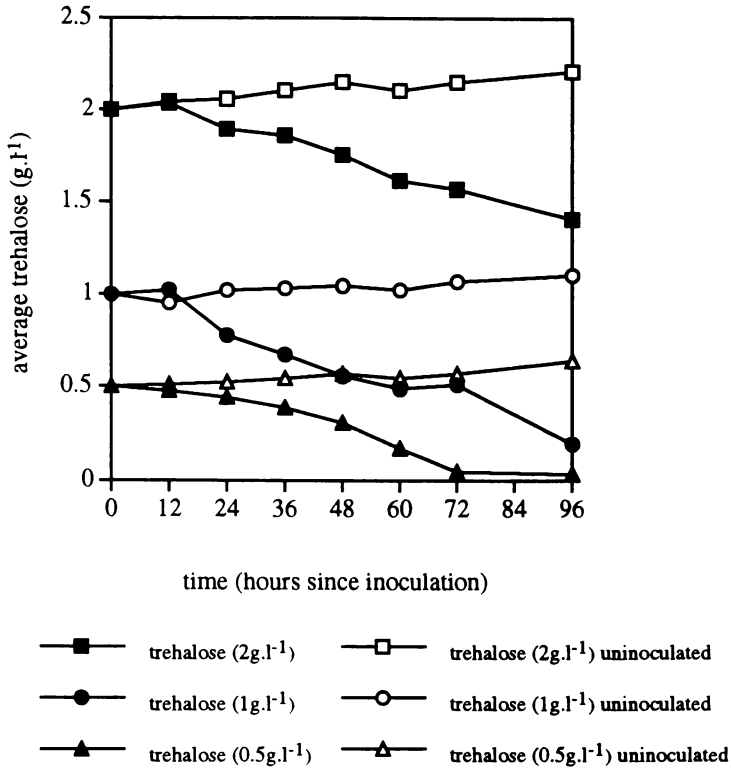


Figure 4.43a: Growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with different concentrations of trehalose



Uninoculated minimal medium supplemented with trehalose showed a slight increase in optical density (450nm) over the time course of the experiment (Figure 4.43a). No decrease in trehalose was seen in uninoculated minimal medium supplemented with any concentration of trehalose (Figure 4.43b). In all instances increases in optical density were confirmed as being due to growth, rather than colour development in the medium, by cell counts using phase contrast microscopy.

Figure 4.43b: Removal of trehalose from minimal medium supplemented with different trehalose concentrations during growth of *Bacillus* isolate TA2.A1



In a search for trehalase activity, pNP assays (to indirectly measure glucose release) were conducted on minimal medium plus trehalose ($0.5g.l^{-1}$) culture that had grown for 18 hours at 65°C. Culture supernatant, whole cells, sonicated cell fragments and sonicate supernatant were all assayed for activity. The same range of pNP substrates trialled to detect sucrase activity (section 4.4.2.1.1) was evaluated for the detection of trehalase activity. pNP-release was only detected using p-nitrophenyl α-D-glucopyranoside and was specific to the alpha form of this substrate. No trehalase activity was detected in the culture supernatant of trehalose grown cells which suggested that the trehalase enzyme was intracellular. The fact that all pNP activity was in the sonicate-supernatant and none was detected in the sonicate-cell fragments suggested that the enzyme was non-cell wall associated. The enzyme was specific in its activity since the cell sonicate from trehalose grown cells would not release glucose after incubation with sucrose, for example, and vice versa. This was confirmed by measuring glucose released by both GOD assay and HPLC.

To ascertain whether monosaccharides, unable to support the growth of isolate TA2.A1 would affect trehalose utilization, glucose or fructose at $2g.l^{-1}$ final concentration was added either at the time of inoculation or at mid-log to a culture growing on trehalose in minimal medium. Fructose was inhibitory to growth on trehalose eight hours after addition at inoculation (Figure 4.44); however, eight hours after fructose addition at mid-log phase, growth was not inhibited. Glucose added at inoculation or at mid-log phase had no effect on the growth of *Bacillus* isolate TA2.A1 on trehalose (Figure 4.44).

Growth of *Bacillus* isolate TA2.A1 in minimal medium with trehalose (Figure 4.45) under fermenter conditions gave an increased yield, compared to flask cultures, and maximum cell density was achieved over a shorter time period. Most of the trehalose in the fermenter was utilized in the ten hours after inoculation. The increased cell yield in fermenter may have been due to the better aeration and pH control in the fermenter vessel. To ascertain whether isolate TA2.A1 grew better on trehalose alone or could grow diauxically on trehalose and sucrose together, growth on these substrates individually, and in combination, was compared under fermenter conditions (Figure 4.39 and Figure 4.45). Growth on $0.5g.l^{-1}$ trehalose reached a higher optical density than growth on an equal concentration ($0.25g.l^{-1}$) of sucrose and trehalose together (Figure 4.39). The utilization of trehalose was faster when trehalose was present as the sole carbon source (Figure 4.45) than when the medium contained both trehalose and sucrose (Figure 4.39). When the medium contained both trehalose and sucrose, the trehalose was used preferentially over the sucrose (Figure 4.39).

The effect of ionophores on the growth of *Bacillus* isolate TA2.A1 on trehalose in minimal medium was assessed. Monensin and amiloride were added to cultures of *Bacillus* isolate TA2.A1 once growth was established. Monensin at a concentration of 0.1μM completely inhibited growth on trehalose, however, amiloride at 100μM or 1.5mM final concentration had no effect on growth (Figure 4.46). Growth of *Bacillus* isolate TA2.A1 on trehalose at $0.5g.l^{-1}$ final concentration in minimal medium was unaffected by 2,4-dinitrophenol (Figure 4.41). The optical density of the cultures to which 2,4-dinitrophenol was added, was read by spectrophotometer at 500nm to remove interference from colour absorbance by the 2,4-dinitrophenol. Further discussion on the

implications of these ionophore results can be found in Chapter 6. When *Bacillus* isolate TA2.A1 was inoculated into sodium-free medium, no growth was evident either by optical density or microscopic examination (Figure 4.46). The sodium requirement for trehalose utilization is discussed in more detail in Chapter 6. Discussion on the utilization of trehalose by other thermophilic or alkaliphilic organisms can be found in section 4.4.2.7.

Figure 4.44: Effect of glucose or fructose additions on growth of *Bacillus* isolate TA2.A1 in minimal medium with trehalose

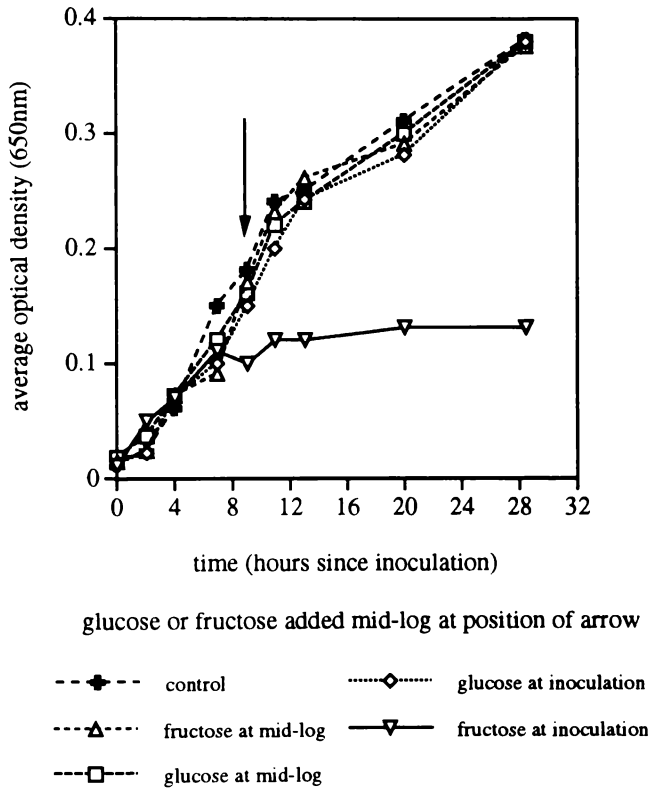


Figure 4.45: Growth of *Bacillus* isolate TA2.A1 under fermenter conditions in minimal medium with 0.5g.l^{-1} trehalose or glycerol

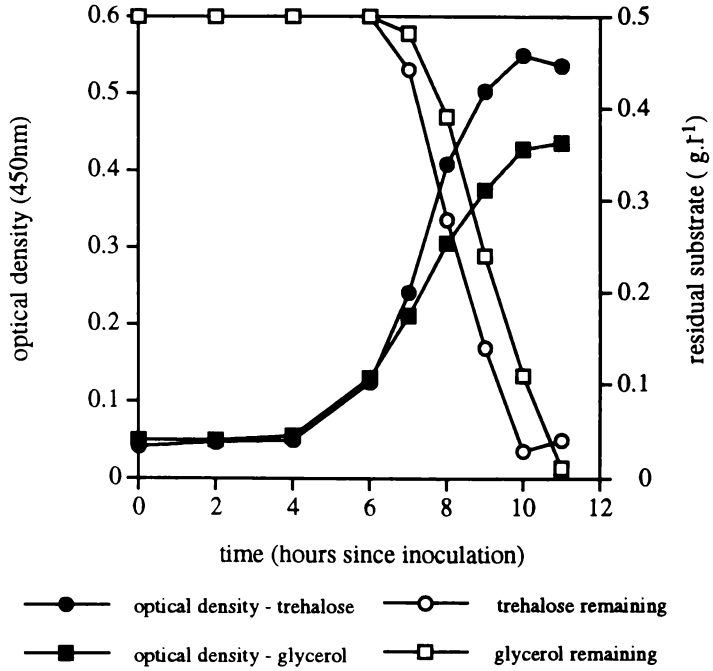
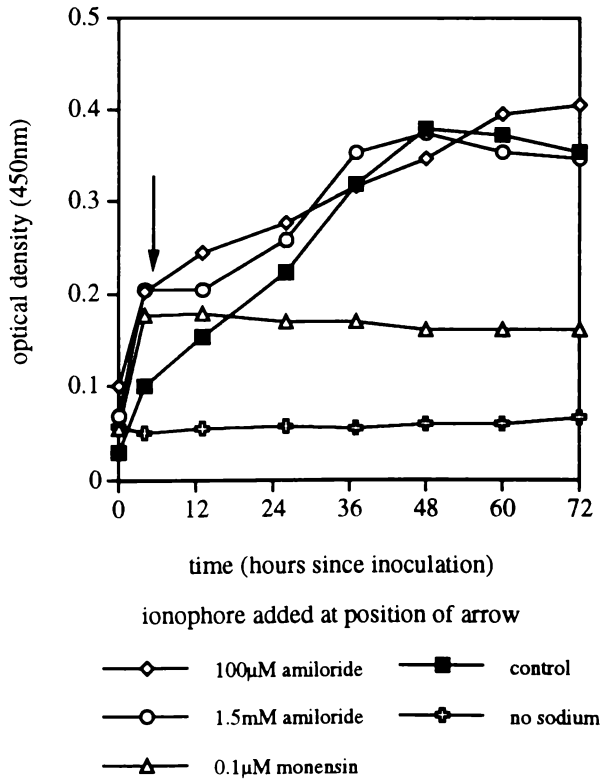


Figure 4.46: The effect of monensin, amiloride and sodium ions on the growth of *Bacillus* isolate TA2.A1 in minimal medium with 0.5g.l^{-1} trehalose



4.4.2.3. Glycerol

4.4.2.3.1 Methods

Culture preparation

Minimal medium (Section 3.3.1.2) was prepared and inoculated as outlined in section 3.2.5.4. Glycerol was added from a filter sterilised (0.45μm) stock solution (10% w/v) immediately prior to inoculation to give a final concentration, unless otherwise stated, of $0.5g.l^{-1}$. Growth was assessed (Section 3.2.3) and fermenter cultures were set up (Section 3.2.8) as described earlier. All growth experiments were performed in triplicate. In the replicates where growth was supported, maximum variability between replicates was ± 0.03 optical density units (nm). Where growth was not supported the lack of growth was consistent over all replicates.

Sample preparation for HPLC

Samples were prepared and analyzed on a Dionex HPLC system as outlined previously (Section 4.4.2.1.1). Glycerol concentrations above $0.25g.l^{-1}$ gave a non-linear response to peak area on the Carbopac PA1 column.

4.4.2.3.2 Results and Discussion

Bacillus isolate TA2.A1 grown in minimal medium with 0.5, 1 and $2g.l^{-1}$ glycerol produced a similar final yield which was not proportional to the glycerol concentration (Figure 4.47a). Likewise the rate of glycerol removal from the medium was similar at all three glycerol concentrations (Figure 4.47b). No significant increase in optical density was seen in the uninoculated medium at any of the glycerol concentrations tested (Figure 4.47a). Interestingly the concentration of glycerol in the inoculated medium decreased after 36 hours of growth (Figure 4.47b) even though the culture had already reached an optical density of 0.4 (450nm) by this time (Figure 4.47a). The possibility that isolate TA2.A1 was utilizing the peptone in the medium in preference to glycerol can be discounted because a peptone concentration of $0.1g.l^{-1}$ only produces an optical density

of approximately 0.1 (450nm) in the absence of any other carbon source (Figure 4.23a). No decrease in glycerol concentration was detected in the uninoculated medium at any of the glycerol concentrations tested (Figure 4.47b). *Bacillus* isolate TA2.A1 did not grow as efficiently on glycerol under fermenter conditions (Figure 4.45) compared to flask conditions (Figure 4.47a). Growth of isolate TA2.A1 on glycerol under fermenter conditions occurred quickly over a three hour period, from seven to ten hours after inoculation, by which stage all the glycerol in the medium had been utilized (Figure 4.45). Growth of isolate TA2.A1 on glycerol under flask conditions was slower, reaching a maximal optical density 72 hours after inoculation. Glycerol was the only carbon source, of those tested, which did not give an improved final optical density under fermenter, compared to flask, conditions.

Figure 4.47a: Growth of *Bacillus* isolate TA2.A1 in minimal medium with different concentrations of glycerol

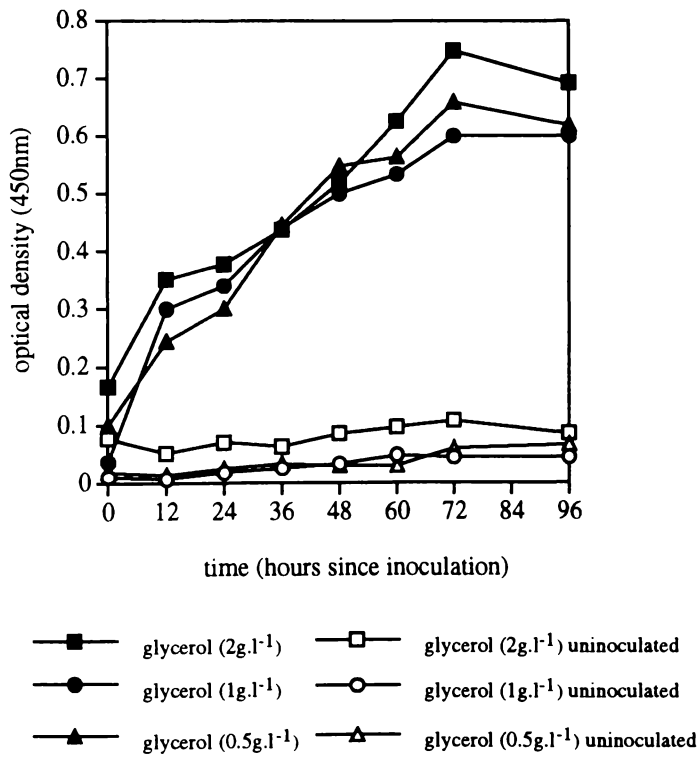
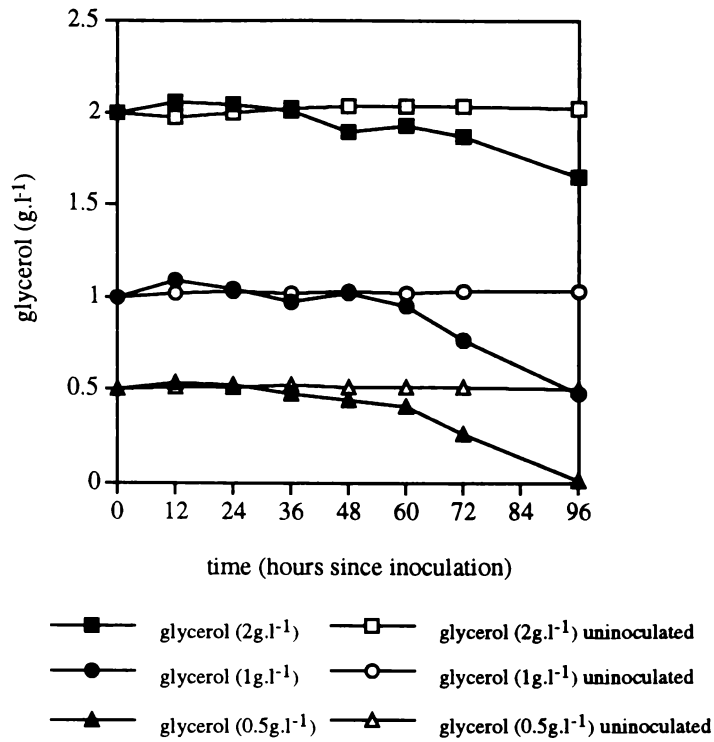


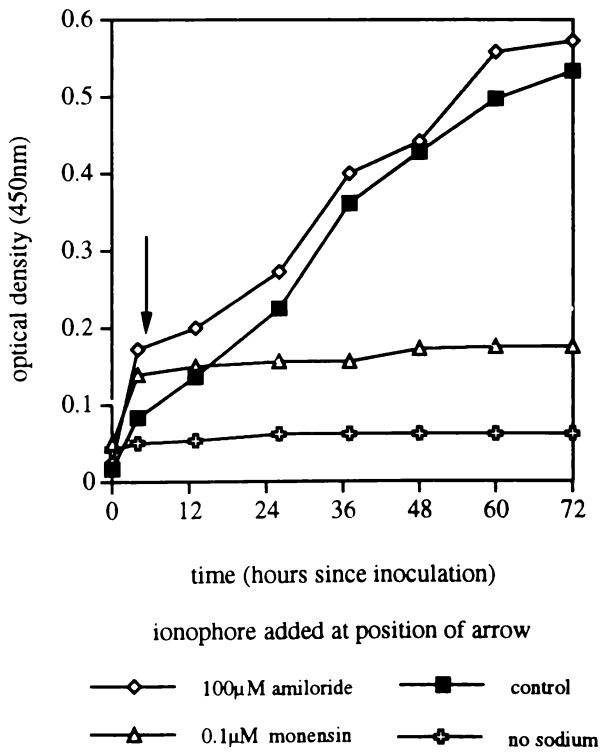
Figure 4.47b: Removal of glycerol from minimal medium during *Bacillus* isolate TA2.A1 growth on glycerol at different concentrations



In an investigation into a possible mechanism of glycerol utilization by *Bacillus* isolate TA2.A1, cells grown in glycerol supplemented minimal medium were centrifuged and resuspended twice in either sodium free (potassium substituted) minimal medium or conventional minimal medium. After the final centrifugation both cell pellets were resuspended in different flasks containing minimal medium and glycerol ($0.5g.l^{-1}$) and incubated at 65°C. The cells that had been washed in sodium containing (conventional) minimal medium grew, however, the cells washed in potassium supplemented (sodium free) minimal medium failed to grow even after extended incubation (72 hours). This suggests that the uptake of glycerol by isolate TA2.A1 is not by diffusion alone and is dependent on the presence of sodium ions. In bacteria glycerol is often taken-up by facilitated diffusion (Lin 1976; Saheb 1972). Based on the results above, it is possible that *Bacillus* isolate TA2.A1 uses sodium ions to move glycerol intracellularly. Certainly growth of isolate TA2.A1 on glycerol was sodium dependent, with no growth evident, either by optical density or microscopic examination, in sodium-free medium (Figure 4.48).

The effect of ionophores on the growth of *Bacillus* isolate TA2.A1 on glycerol in minimal medium was assessed. Monensin and amiloride were added to cultures of *Bacillus* isolate TA2.A1 once growth was established. Monensin at a concentration of 0.1μM inhibited growth on glycerol (Figure 4.48), but amiloride (100μM) and 2,4-dinitrophenol (2,4-DNP) (Figure 4.41) had no effect on growth. The optical density of the cultures to which 2,4-DNP was added, was read by spectrophotometer at 500nm to remove interference from colour absorbance by the 2,4-DNP. The inhibition of growth by monensin, which disrupts sodium gradients across bacterial membranes suggests that sodium is a requirement for the utilization of glycerol by isolate TA2.A1. The fact that 2,4-DNP, a protonophore which abolishes the $\Delta\mu H^+$, did not affect growth suggests that the isolate is not dependent on a Δp for energising glycerol transport. Amiloride, an inhibitor of Na^+/H^+ antiporters, had no effect on the growth of *Bacillus* isolate TA2.A1 on glycerol. Discussion on the utilization of glycerol by other thermophilic or alkaliphilic organisms can be found in section 4.4.2.7.

Figure 4.48: The effect of monensin, amiloride and sodium on the growth of *Bacillus* isolate TA2.A1 in minimal medium with 0.5g.l^{-1} glycerol



4.4.2.4. Sodium pyruvate

4.4.2.4.1 Methods

Culture preparation

Minimal medium (Section 3.3.1.2) was prepared and inoculated as outlined in section 3.2.5.4. Sodium pyruvate was added from a filter sterilised (0.45µm) stock solution (10% w/v) immediately prior to inoculation to give a final concentration, unless otherwise stated, of $2g.l^{-1}$. Growth was assessed (Section 3.2.3) and fermenter cultures were set up (Section 3.2.8) as described earlier. All growth experiments were performed in triplicate. In the replicates where growth was supported, maximum variability between replicates was ± 0.03 optical density units (nm). Where growth was not supported the lack of growth was consistent over all replicates.

Samples were prepared and analyzed on the HPX.87H (Aminex™) HPLC column as outlined previously (Section 4.4.2.1.1).

4.4.2.4.2 Results and Discussion

The stability of sodium pyruvate was assessed under different conditions. Sodium pyruvate polymerizes and decomposes on standing, even as a powder, unless it is pure and kept in an airtight container. The decomposition of sodium pyruvate can be seen by the development of an increasingly deep yellow colour, and confirmed by the detection of additional peaks under HPLC analysis.

The effect of oxygen on the stability of a freshly filter sterilized sodium pyruvate solution was assessed. Decomposition was reduced when the headspace of the solution container was kept free from oxygen. In the absence of oxygen (solution flushed with nitrogen), sodium pyruvate in HPLC solvent (0.01N H_2SO_4), and also in Milli-Q water at the same concentration, had no colour and gave a single HPLC peak (retention time approximately 13.4 minutes). When oxygen was bubbled through the two sodium pyruvate solutions, the sodium pyruvate peak split into several shoulder peaks and there

was a distinct and immediate increase in colour confirmed by spectrophotometric scanning from 500 to 350nm.

Differences were observed between freshly filter sterilized, 2 year old filter sterilized, and autoclaved (15 minutes /15psi) sodium pyruvate solutions in terms of their colour. A freshly filter sterilized sodium pyruvate solution was colourless, while an autoclaved sodium pyruvate solution had a distinct yellow colour, which was duplicated in filter sterilized sodium pyruvate solutions that were more than three weeks old. Over time, both the freshly filter sterilized, or autoclaved, sodium pyruvate solutions deepened in colour to resemble the more intense yellow colour of the 2 year old filter sterilized solution.

pH was also observed to have an effect on sodium pyruvate degradation. Samples of freshly filtered sodium pyruvate were left at room temperature for 24 hours in minimal medium (pH 10), Milli-Q water (pH 7), and HPLC solvent (0.01 N H_2SO_4) (pH 2). The sample left in minimal medium (pH 10) had the deepest yellow colouration, with the least yellow colour being seen the HPLC solvent sample.

Temperature (4, 20 and 65°C) did not have a significant effect on the breakdown of sodium pyruvate in Milli-Q water and minimal medium. The HPLC profiles of sodium pyruvate in minimal medium after 24 hours at room temperature (22°C) and 65°C are shown in Figure 4.49.

Growth of *Bacillus* isolate TA2.A1 on autoclaved, freshly filter sterilized and 2 year old filter sterilized sodium pyruvate at $2g.l^{-1}$ final concentration was tested. *Bacillus* isolate TA2.A1 produced the best cell yield in minimal medium containing old filter sterilized sodium pyruvate (Figure 4.50). Sodium pyruvate that had been autoclaved supported better growth than freshly filter sterilized sodium pyruvate (Figure 4.50). The fact that the old filter sterilized sodium pyruvate supported better growth than the freshly filtered sodium pyruvate suggests that isolate TA2.A1 grows better on breakdown products of sodium pyruvate. The higher yield obtained with old sodium pyruvate compared to autoclaved sodium pyruvate (Figure 4.50) suggests that autoclaving either does not cause degradation to the same extent, or inactivates some breakdown product that is

utilized by *Bacillus* isolate TA2.A1. When *Bacillus* isolate TA2.A1 is growing in minimal medium, with sodium pyruvate from any source, growth is of short duration with maximal yield achieved close to twelve hours after inoculation (Figure 4.50 and Figure 4.52).

Figure 4.49: HPLC profiles of sodium pyruvate in uninoculated minimal medium after 24 hours at room temperature and at 65°C

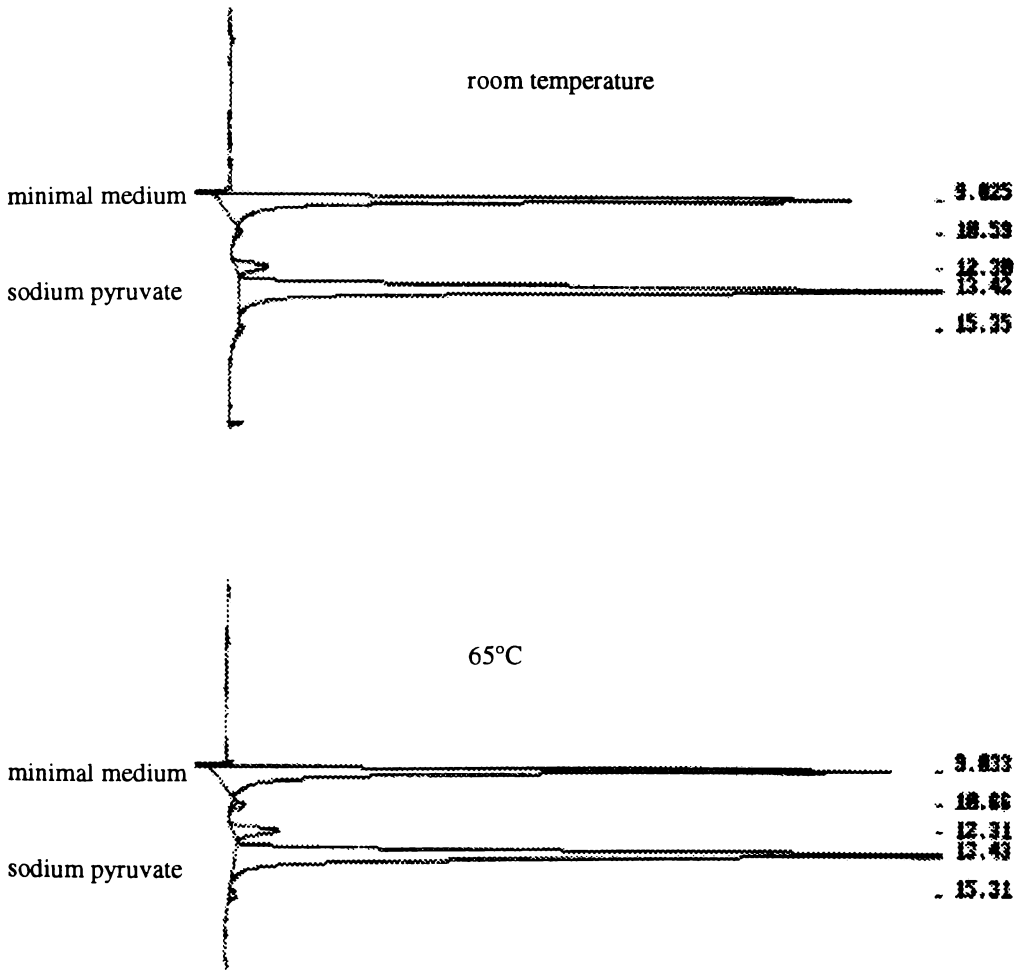
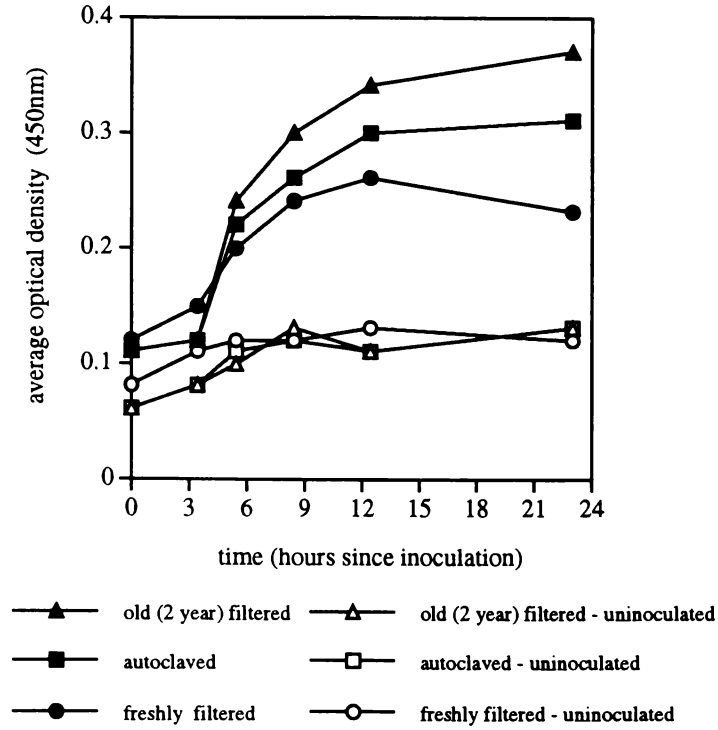


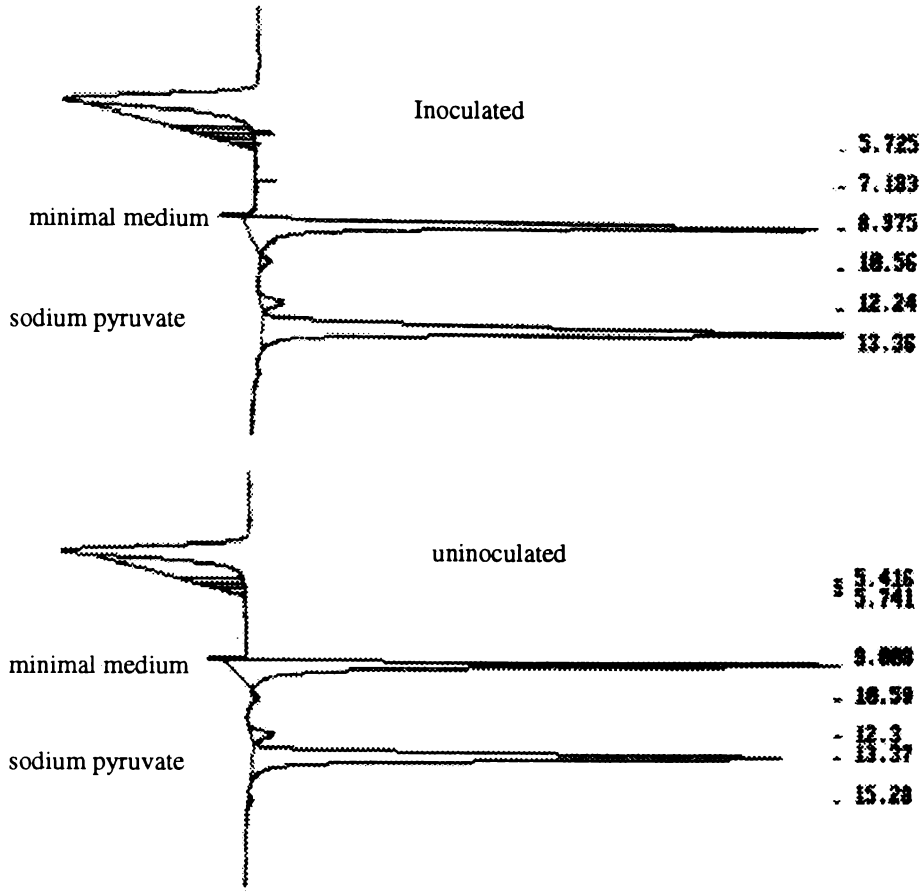
Figure 4.50: Growth of *Bacillus* isolate TA2.A1 in minimal medium with sodium pyruvate



The concentration of sodium pyruvate in inoculated minimal medium did not differ significantly from the concentration in uninoculated minimal medium after the same period of incubation at 65°C (Figure 4.51), despite the fact that the inoculated medium supported the growth of *Bacillus* isolate TA2.A1. This suggests that growth may largely be supported by the breakdown products of the pyruvate which were not detected by HPLC analysis.

While isolate TA2.A1 was growing, the pH in the growth medium decreased slightly and began to rise again when growth was complete. By contrast, in identical but uninoculated medium, the pH rose over the same period of incubation at 65°C (Figure 4.52). *Bacillus* isolate TA2.A1 was able to grow on sodium pyruvate ($2g.l^{-1}$) in minimal medium without $0.1g.l^{-1}$ peptone (Figure 4.53) but a much higher total cell yield was reached with peptone in the minimal medium. However, the growth achieved on sodium pyruvate in minimal medium without peptone was better than that in minimal medium alone or minimal medium without both peptone and sodium pyruvate (Figure 4.53). No significant increase in optical density was seen in any of the uninoculated minimal media over the same period of incubation at 65°C (Figure 4.53).

Figure 4.51: HPLC profiles of sodium pyruvate in inoculated and uninoculated minimal medium after 24 hours at 65°C



Growth of *Bacillus* isolate TA2.A1 in minimal medium with 0.5g.l^{-1} sodium pyruvate under fermenter conditions achieved an optical density above 0.5 (450nm) after nine hours of incubation at 65°C (Figure 4.54). The cell yield and the rate of growth under fermenter conditions were higher than in a flask, despite having only 25% of the substrate (Figure 4.54). The reason for the decreased yield under flask conditions is uncertain. There is only a pH shift during growth on sodium pyruvate in a flask of 0.1 pH units (Figure 4.52) discounting pH control, which is better in the fermenter, as a possible explanation. It is possible that the higher concentration of sodium pyruvate (and sodium pyruvate breakdown products) in the flask cultures had a growth limiting effect on *Bacillus* isolate TA2.A1. Discussion on the utilization of carbon substrates by other thermophilic or alkaliphilic organisms can be found in section 4.4.2.7.

Figure 4.52: Growth of *Bacillus* isolate TA2.A1 in minimal medium with $2g.l^{-1}$ sodium pyruvate

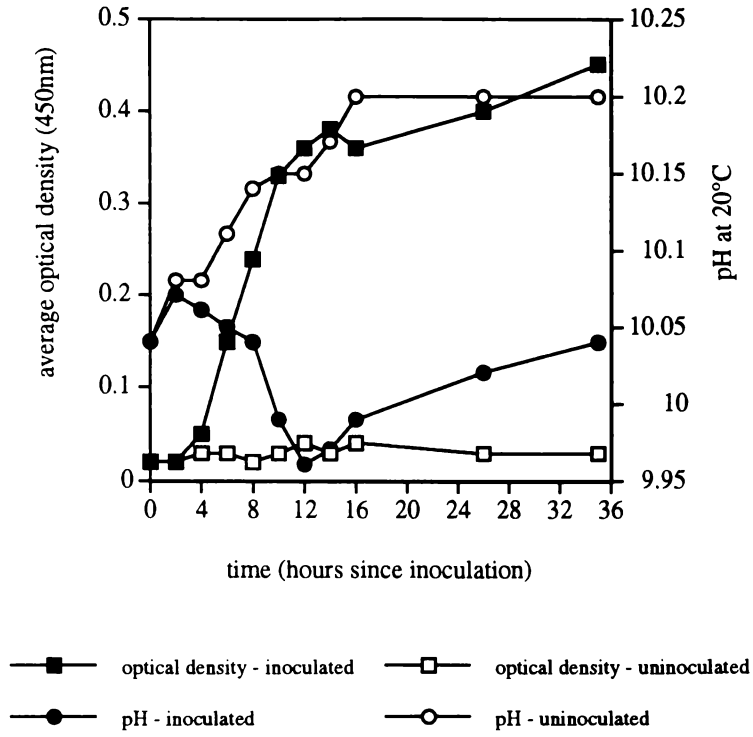


Figure 4.53: Growth of *Bacillus* isolate TA2.A1 in minimal medium (with and without $0.1g.l^{-1}$ peptone) and $2g.l^{-1}$ sodium puruvate

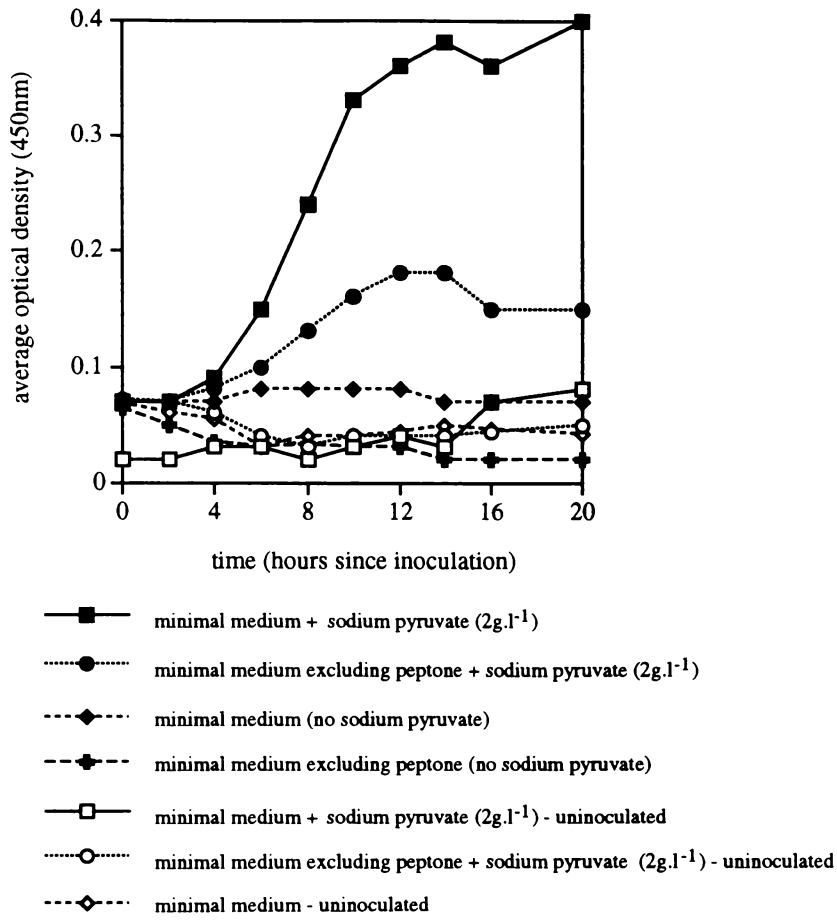
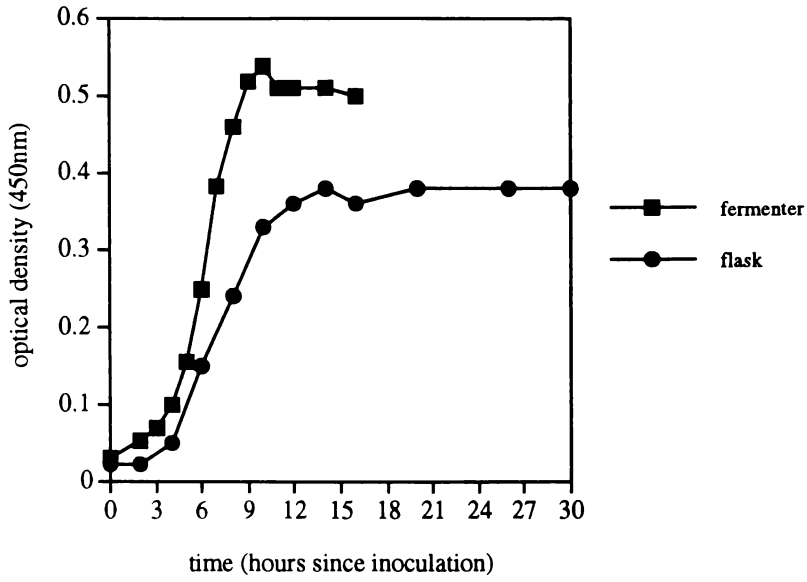


Figure 4.54: Growth of *Bacillus* isolate TA2.A1 in minimal medium in a fermenter (0.5g.l^{-1} sodium pyruvate) and in a flask (2g.l^{-1} sodium pyruvate)



4.4.2.5. Fructooligosaccharides

4.4.2.5.1 Methods

Culture preparation

Minimal medium (Section 3.3.1.2) was prepared and inoculated as outlined in section 3.2.5.4. Nutraflora™(Life's Finest®, Marion, IL; Golden Technologies) was added from a filter sterilised (0.45µm) stock solution (10% w/v) immediately prior to inoculation to give a final concentration, unless otherwise stated, of $2g.l^{-1}$. Fructooligosaccharides prepared from inulin, as outlined below, were added directly into minimal medium immediately prior to inoculation. Growth was assessed (Section 3.2.3) and fermenter cultures were set up (Section 3.2.8) as described earlier. All growth experiments were performed in triplicate. In the replicates where growth was supported, maximum variability between replicates was ± 0.03 optical density units (nm). Where growth was not supported the lack of growth was consistent over all replicates.

Laboratory preparation of fructooligosaccharides

Nutraflora™ is a commercially available fructooligosaccharide. Fructooligosaccharides were also prepared from inulin (BDH) a 2,1-β linked polymer of D-fructofuranose (M. Manley-Harris, University of Waikato, personal communication). Ten grams of inulin in 100mls of 1M acetic acid were heated in a water bath at 90°C for 60 minutes. The acetic acid was removed under vacuum by rotary-evaporater (Büchi R110) at 50°C, and as the volume decreased water was added to wash the digested inulin. This process was repeated until testing with pH indicator paper (Alkalit Merck) showed that the pH of the product was close to neutral. The length of time that the inulin was digested was varied from 60 to 10 minutes to vary the size of the inulin fragments.

Sample preparation for HPLC

Samples were prepared for HPLC analysis as outlined previously (Section 4.4.2.1.1). Methods for using the HPX.42A Aminex column (Bio-Rad) were as outlined in section

4.4.2.1.1. The initial method for fructooligosaccharide/ Nutraflora™ analysis using the Carbopac PA1 column (Dionex) was as outlined in Section 4.4.2.1.1 but with a 45 minute elution time per sample.

Using the Carbopac PA1 column, more sensitive analysis of both standards and supernatants from culture growth on fructooligosacchaides was possible using modifications of the method of Slaughter and Livingston (1994). These methods involved the use of a sodium acetate gradient prepared in sodium hydroxide. Analyses of inulin (30 mg/ml) and high molecular weight inulin digest products were resolved using a gradient from 100mM NaOH and 200mM sodium acetate to 50mM NaOH and 700mM sodium acetate. Low molecular weight fructooligosaccharides, including Nutraflora™, had poor resolution using this gradient, but were separated using a 0 to 500mM sodium acetate gradient in 150mM NaOH.

Thin layer chromatography of inulin digest samples

Culture supernatant samples from the growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with products of the acetic acid digest of inulin or Nutraflora™, were concentrated ten-fold by freeze drying and re-dissolving in a smaller volume of sterile Milli-Q water. Samples of glucose, sucrose, inulin and Nutraflora™ (10% w/v) were run as standards. A total of 10µl of each sample was dispensed, in 2 µl aliquots, onto thin layer chromatography (TLC) solid phase plates (Merck). The TLC plates were placed in a tank whose atmosphere had been saturated with solvent (n-butanol:ethanol:Milli-Qwater (4:1:2). After the samples had migrated the plates were removed from the solvent and dried. Development involved flooding with silver nitrate in acetone and then 0.5N NaOH in ethanol (Dawson *et al.* 1986).

4.4.2.5.2 Results and Discussion

Nutraflora™ is a commercially available mixture of fructooligosaccharides, marketed as a dietary supplement, which is predominantly composed of nystose and 1-kestose (M. Manley-Harris, University of Waikato, personal communication). *Bacillus* isolate TA2.A1 was able to grow on Nutraflora™ as a sole carbon and energy source. In

addition to growth on commercially available fructooligosaccharides, such as Nutraflora™, isolate TA2.A1 could also grow on fructooligosaccharides produced from acetic acid hydrolysis of inulin (Figure 4.55a). Although *Bacillus* isolate TA2.A1 was able to use the products of inulin hydrolysis as a sole carbon source, it was unable to grow on inulin (2,1-β linked polymer of D-fructofuranose) itself (Figure 4.55a). The structures of 1-kestose, nystose and inulin (Figure 4.56) show that these may be derived from sucrose by successive additions of (2→1)-linked β-fructofuranose residues. As detailed earlier (Section 4.4.2.1) *Bacillus* isolate TA2A1 is able to utilize sucrose. Investigations were carried out to see if an actively growing isolate TA2.A1 culture grown in minimal medium supplemented with sucrose could grow on inulin, however, no growth was evident even after extended incubation (96 hours) at 65°C (Figure 4.55a).

Figure 4.55a: Growth of *Bacillus* isolate TA2.A1 on fructooligosaccharides ($2g.l^{-1}$) in minimal medium

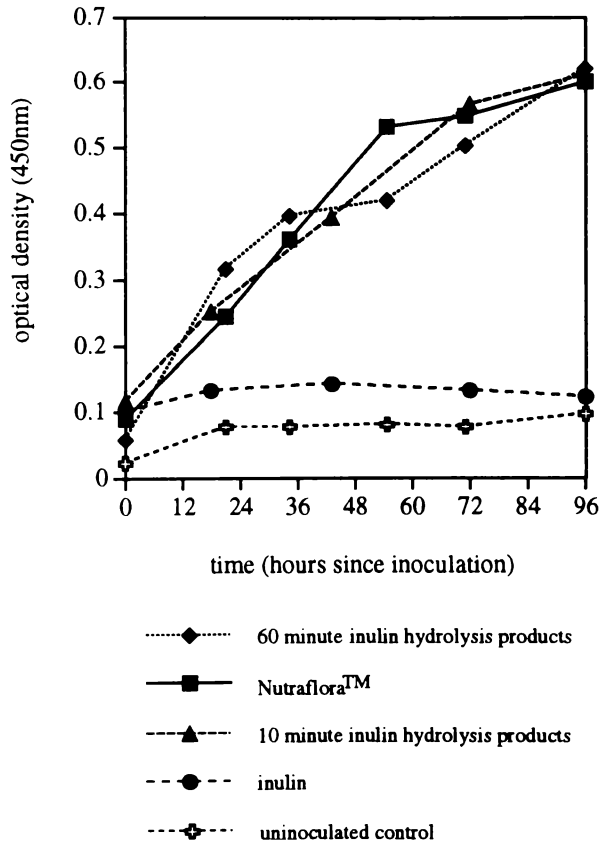


Figure 4.55b: Utilisation of fructooligosaccharides ($2g.l^{-1}$) as inulin hydrolysis products or Nutraflora™ in minimal medium by *Bacillus* isolate TA2.A1

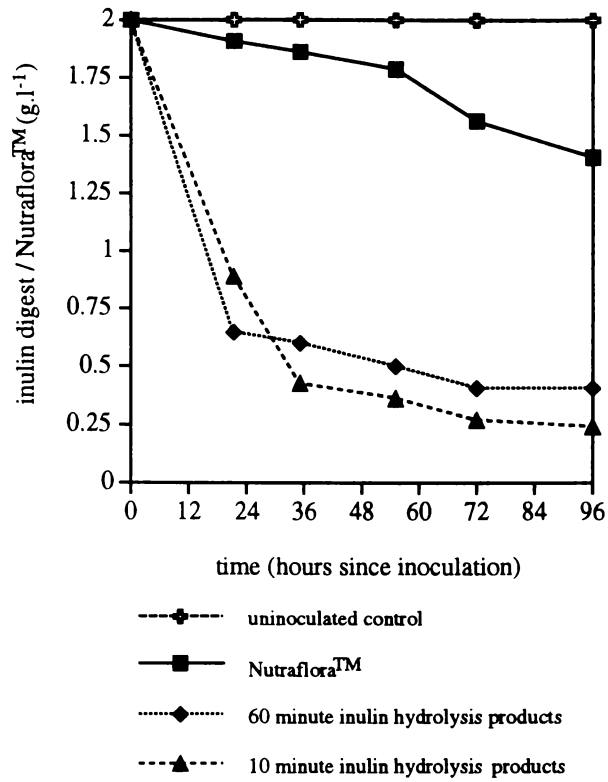
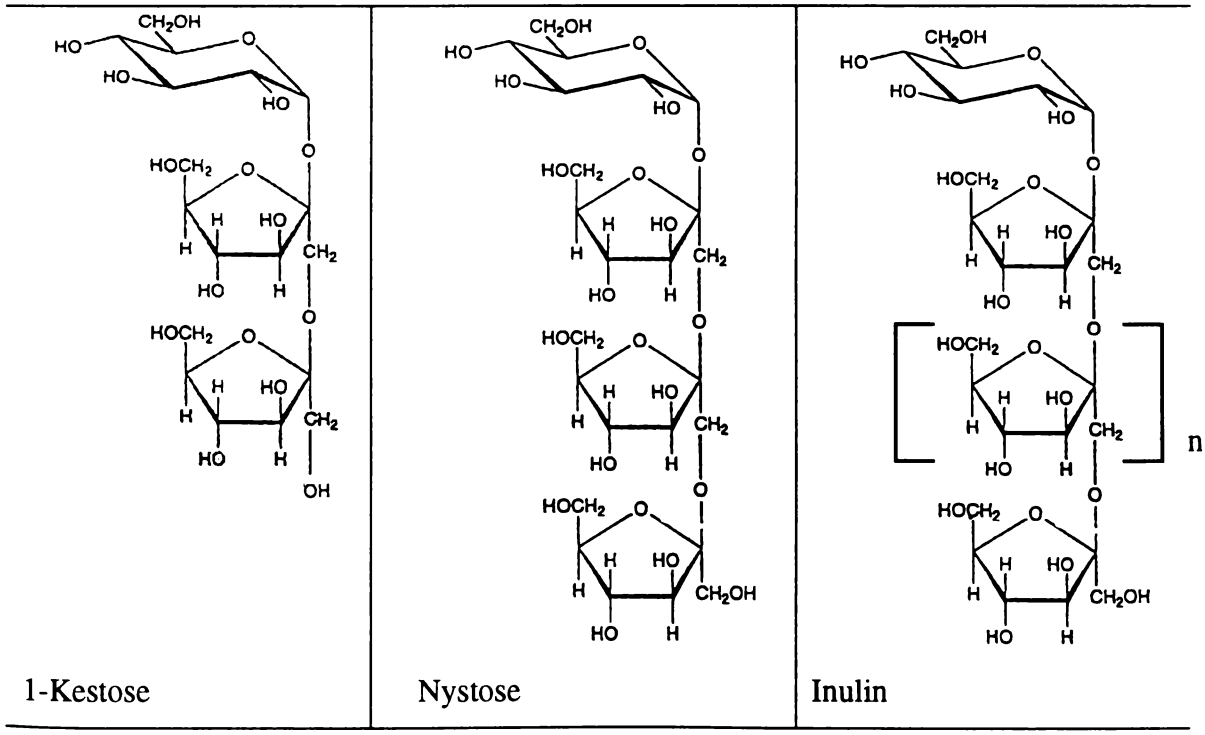


Figure 4.56: The structure of 1-kestose, nystose and inulin



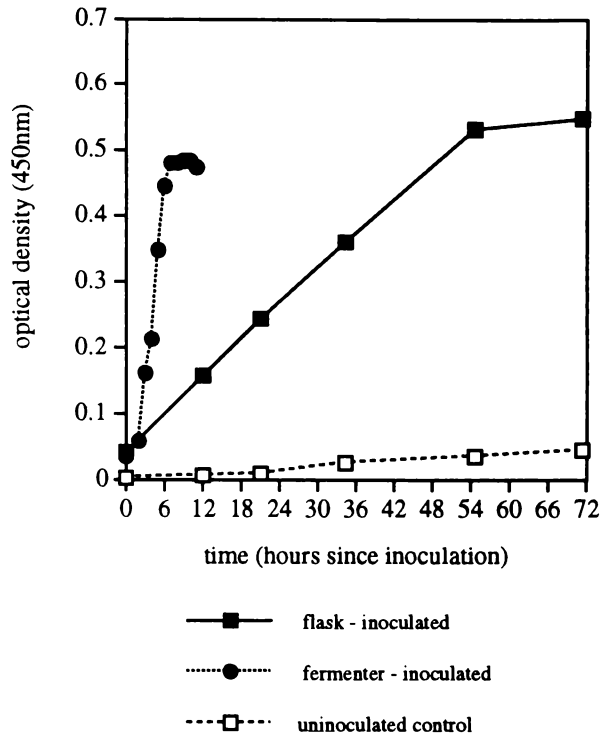
Growth of *Bacillus* isolate TA2.A1 in minimal medium with commercial fructooligosaccharides (Nutraflora™) was compared with growth on fructooligosaccharides made from inulin at the same concentration ($2g.l^{-1}$) (Figure 4.55a). The final cell yield on fructooligosaccharides prepared in the laboratory with both a 10 and 60 minute inulin digest was similar to that on commercial fructooligosaccharides (Figure 4.55a). The only visible difference was that after three hours of incubation at 65°C the long and short inulin digests in minimal medium both showed a yellow colouration, in contrast to the commercial fructooligosaccharides, which under the same conditions of temperature and pH were colourless. Since the hydrolysis of inulin exposed reducing ends, there were two possible reasons for the yellow colour formation. The most likely reason was non-specific alkaline degradation forming unsaturated compounds. However, Maillard reactions from the reaction of reducing sugars with amino acids, could also cause colour formation. The relatively low levels of amino acids in minimal medium make this second possibility less likely.

The growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with Nutraflora™ under fermenter conditions was assessed. *Bacillus* isolate TA2.A1 grew more slowly in a flask with minimal medium with $0.5g.l^{-1}$ Nutraflora™ than in identical medium in a fermenter (Figure 4.57). In a fermenter a maximum cell yield was achieved in six hours (Figure 4.57); however, with the same fructooligosaccharide in a flask, a similar cell yield was achieved after 54 hours of incubation. The increased availablity of oxygen under fermenter conditions may account for the faster rate of growth.

The profiles of the inulin digest and Nutraflora™ culture media supernatants analyzed by HPLC (Bio-Rad HPX.42A column) were compared. Despite the similar total cell yield achieved by the growth of *Bacillus* isolate TA2.A1 on the different inulin-digest and Nutraflora™-containing minimal media (Figure 4.55a), there were variations in the disappearance of fructooligosaccharides as shown by HPLC analysis (Figure 4.55b). For example, after 36 hours of growth, the concentration of fructooligosaccharides remaining was approximately $0.5g.l^{-1}$ for the 60 and 10 minute inulin digest containing media and $1.9g.l^{-1}$ in the Nutraflora™-containing medium (Figure 4.55b). There was no

change in fructooligosaccharide concentration in uninoculated minimal medium, even after extended incubation (96 hours) at 65°C (Figure 4.55b).

Figure 4.57: Comparative growth of *Bacillus* isolate TA2.A1 in minimal medium with 0.5g.l^{-1} Nutraflora™ in a fermenter and a flask



The exposure of reducing ends on the inulin molecule by the acetic acid and heat hydrolysis may have enabled easier utilization of the 60 and 10 minute inulin digest components by *Bacillus* isolate TA2.A1. The initial rate of growth, over the first eighteen hours, of *Bacillus* isolate TA2.A1 was fastest in minimal medium containing the 60 minute inulin digest products (Figure 4.55a). However, despite only 0.5g of the Nutraflora™ being utilized by *Bacillus* isolate TA2.A1 after 96 hours of incubation (Figure 4.55b), the cell yield achieved was similar to that in medium where more than 1.5g of the fructooligosaccharides (from 10 and 60 minute inulin digests) had been utilized after the same period of incubation.

Analysis of fructooligosaccharide growth medium supernatants was also carried out using an isocratic elution with 150mM NaOH (Carbopac PA1 column). However, at the concentrations used in media (0.5 and 2g.l^{-1}) no peaks were detected using this method. At a fructooligosaccharide concentration of 10% (w/v) multiple peaks were observed over 45 minutes of elution. The modification of the HPLC solvent system to a sodium acetate gradient prepared in sodium hydroxide allowed for a more compact elution of peaks and for the profile of an inulin solution to be obtained (Figure 4.58). As shown on the HPLC profile inulin is composed of a range of different oligosaccharides (Figure 4.58). The inability of *Bacillus* isolate TA2.A1 to grow on these oligosaccharides is probably because of the lack of reducing ends on the molecule. The digest of inulin in acetic acid at high temperature (Section 4.4.2.5.1) exposes reducing ends so that they are accessible to *Bacillus* isolate TA2.A1. When *Bacillus* isolate TA2.A1 is inoculated into minimal medium supplemented with 10minute inulin hydrolysis products the concentration of all the hydolysis products decreases over 72 hours of growth (Figure 4.60).

Using a sodium acetate gradient prepared in sodium hydroxide, Nutraflora™ samples in Milli-Q water were resolved by HPLC as two peaks corresponding to 1-kestose and nystose (Figure 4.59). Immediately on the addition of Nutraflora™ to minimal medium the high pH caused it to break down into a number of different compounds, detected by the same HPLC method, instead of its two principal components of nystose and 1-kestose (Figure 4.59). This formation of multiple peaks under conditions of high pH made analysis of supernatants from growth on fructooligosaccharide supplemented minimal medium by HPLC difficult.

Figure 4.58: HPLC profile of an inulin solution by Dionex HPLC (30mg/ml)

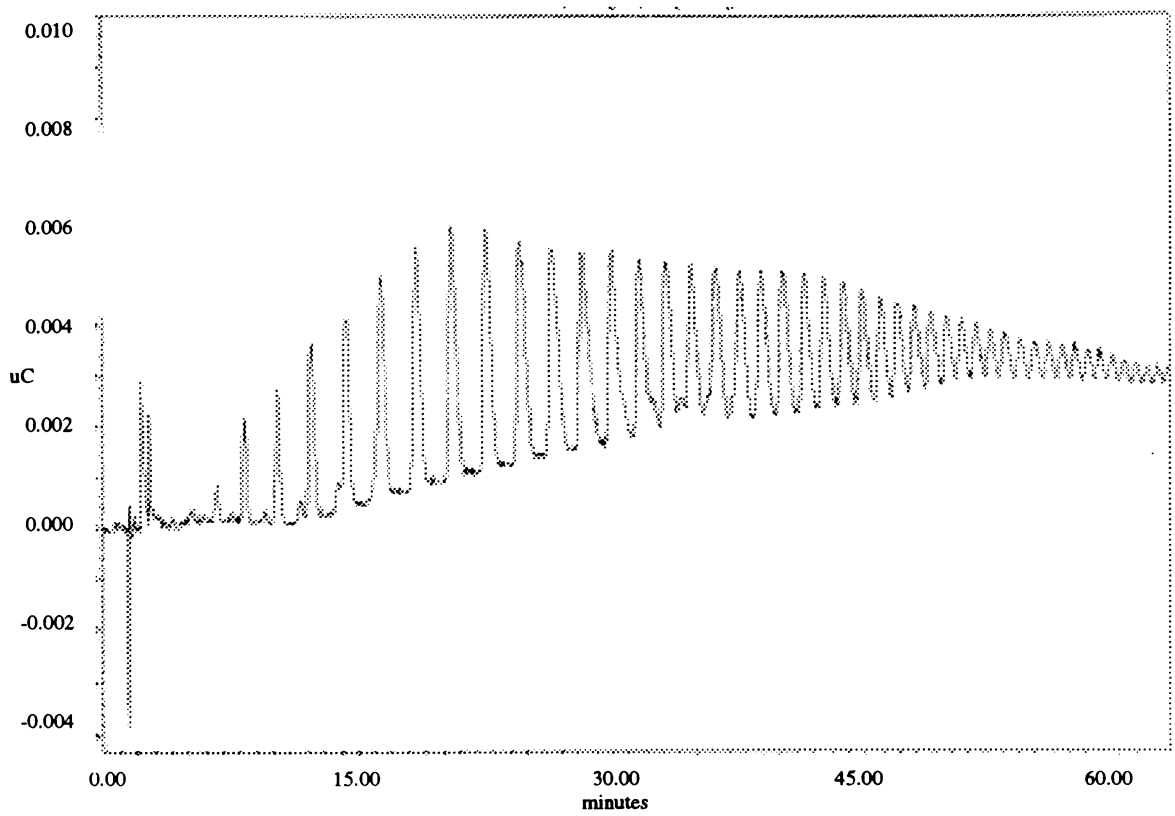


Figure 4.59: 1-kestose stock (0.417mg/ml) and nystose stock (0.355mg/ml) in Milli-Q water (50-fold dilution)

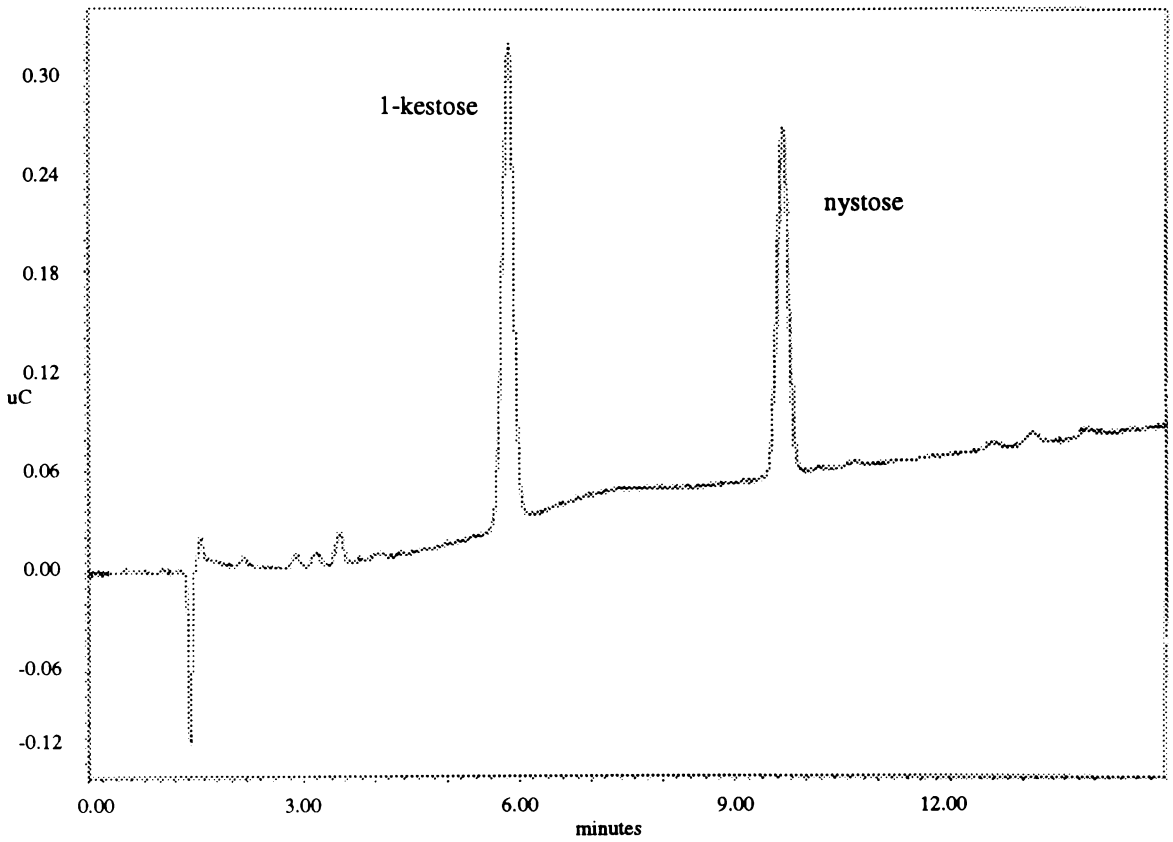
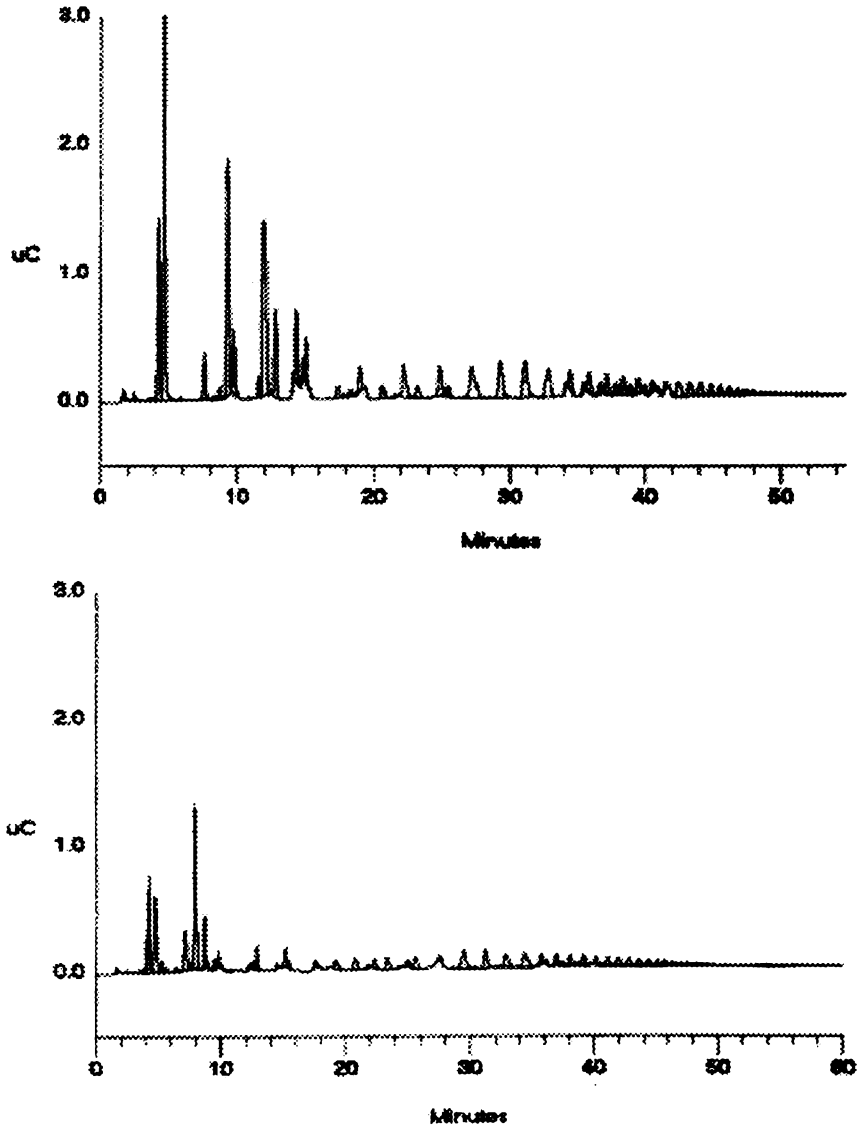
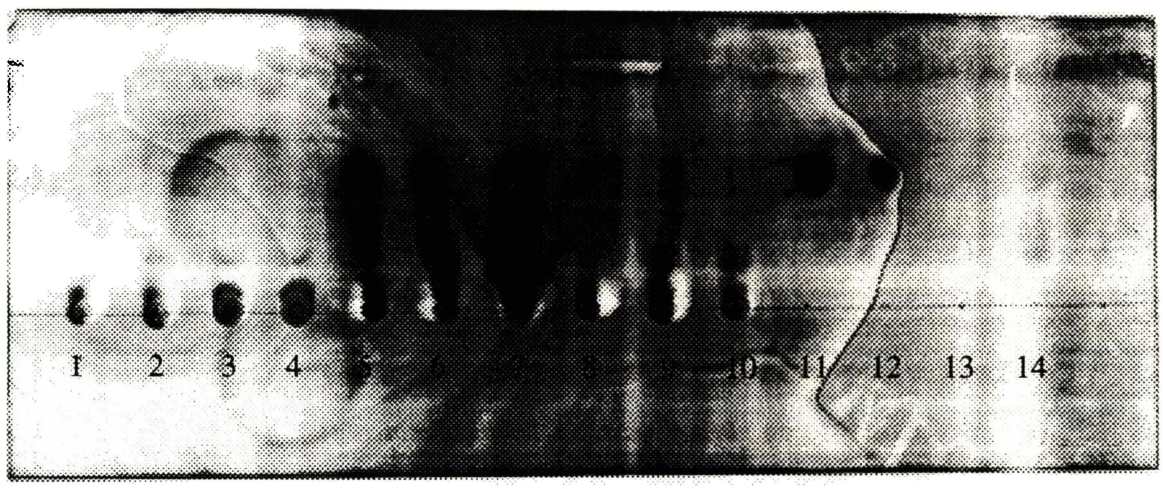


Figure 4.60: Dionex HPLC profile of minimal medium supplemented with 10 minute inulin hydrolysis products; at inoculation (top) and after 72 hours of *Bacillus* isolate TA2.A1 growth (bottom)



The analysis of concentrated media samples from the growth of *Bacillus* isolate TA2.A1 on fructooligosaccharides from inulin acetic acid digests and on Nutraflora™ by thin layer chromatography was inconclusive (Figure 4.61).

Figure 4.61: TLC analysis of concentrated media from growth on fructooligosaccharides from inulin digests and on Nutraflora™



Lanes 1 to 4 contained samples from growth on $1g.l^{-1}$ Nutraflora™ at 0, 24, 48 and 72 hours after inoculation. These samples only migrated slightly from their loading position and there was no difference between the samples despite their having come from media after growth periods of differing duration. Lanes 5 to 7 contained samples from the growth of *Bacillus* isolate TA2.A1 on long inulin digest products for 0, 35 and 72 hours respectively; these samples migrated over the thin layer chromatography plate and more breakdown products were detected as the duration of growth increased. Lanes 8 to 10 contained samples from growth on short inulin digest products. Here the range of fragments separated by the TLC plate decreased as the length of time of growth increased from 0, 43 to 72 hours respectively. The concentration of breakdown products was less for the 10 minute inulin digest sample than for the 60 minute digest sample.

Due to the mixed chain lengths of the inulin digest discrete product spots were not formed. The glucose and fructose standards (10% w/v) (lanes 11 and 12) migrated with the solvent font, however, the inulin and Nutraflora™ standards (10% w/v) (lanes 13 and 14) were not detected.

The utilization of fructooligosaccharides suggests that *Bacillus* isolate TA2.A1 may be producing a β–fructosidase. An alternative possibility is that isolate TA2.A1 is growing on saccharinic acids from acidic degradation of inulin, which would commence presumably with the degradation of the sucrose moiety exposing a reducing end. Further evidence to support this was the fact that isolate TA2.A1 was able to grow well on arabinogalactan as a sole carbon source. In the high pH growth medium required for isolate TA2.A1, this compound would degrade extremely rapidly into oligosaccharides containing β-1-6 D-galactopyranose and / or α-1,3 L-arabinofuranose, and terminating in metasaccharinic acids (Manley-Harris 1997). Because of its polymeric nature, inulin does not degrade as quickly in the alkaline growth medium of isolate TA2.A1, compared to the rate of degradation under acid hydrolysis when producing fructooligosaccharides. Thus *Bacillus* isolate TA2.A1 can utilize acid hydrolysis products of inulin (fructooligosaccharides) but not inulin itself. Discussion on the utilization of carbon substrates by other thermophilic or alkaliphilic organisms can be found in Section 4.4.2.7.

4.4.2.6. Lipids / Fatty acids / Casein / Ovalbumin

4.4.2.6.1 Methods

Culture preparation

Minimal medium (Section 3.3.1.2) was prepared and inoculated as outlined in Section 3.2.5.4. Lipids and fatty acids were added, unless otherwise stated, at a concentration of 0.04% (v/v) to minimal medium supplemented with 5mM $CaCl_2.2H_20$ from a filter sterilized stock solution. Palmitic and lauric acid were melted in a 70°C waterbath and then added by pre-warmed pipette tips, directly into minimal medium. Attempts to get casein to dissolve in minimal medium by pH adjustment failed because when the pH was adjusted back to 10 the casein precipitated out of solution. Casein ($5g.l^{-1}$) was added to minimal medium and evenly dispersed throughout the medium in a blender (Waring) before dispensing and autoclaving as normal.

Growth was monitored as outlined in Section 3.2.3. Due to the fact that growth of *Bacillus* isolate TA2.A1 on lipids, fatty acids, casein and ovalbumin occurred mainly in large clumps, growth assessment was made by microscopic examination of a minimum of twenty fields of view. The large number of microscopic fields of view counted was important because examination of fewer fields could lead to an incorrect growth assessment by the non-observation of a large clump where most of the isolate TA2.A1 cells were aggregating.

Lipase / Esterase Assays

Lipase / Esterase assays were based on the p-Nitrophenyl / Copper Phosphate Triton X100 method (Janssen *et al.* 1994)

Reagents and Reagent Preparation
100mM EPPS buffer, 7.5mM $CaCl_2$ pH 8.2 at 20°C (= pH 7.5 at 70°C)
substrate p-NP butyrate, p-NP caprylate, p-NP acetate, p-NP laurate or p-NP palmitate in ethanol (1mg/ml)

copper phosphate suspension: (a) 6.85 % (w/v) $Na_3PO_4.12H_2O$ in Milli-Q water
(b) 2.80% (w/v) $CuCl_2.2H_2O$ in Milli-Q water
(c) 19.1g.l^{-1}(pH 9.1) $Na_2B_4O_7.10H_2O$

While stirring 40ml of the sodium phosphate solution, 20ml of the copper chloride solution was added. The resultant precipitate was centrifuged, and the supernatant discarded. The copper phosphate was resuspended in 100 ml of borax buffer, centrifuged, and the supernatant discarded. The resuspension/centrifugation steps were repeated, the supernatant discarded, and the pellet resuspended in 90 ml of borax buffer and 10 ml of Triton X-100. 6 grams of NaCl were then added to the mixture.

Assay Method

Substrate solution (100 µl) and buffer (800 µl) were dispensed into microfuge tubes, mixed and preincubated for 10 minutes at 70°C. Enzyme (whole cell culture) (100 µl) was added and the mixture incubated at 70°C for 60 minutes. The samples were removed from the waterbath and 500µl of a continually stirred copper phosphate suspension (as above) was added. After centrifugation in a microfuge at full speed for 5 minutes and cooling on ice, the absorbance of the supernatant was read by spectrophotometer at 400nm.

In addition to the method outlined above, which used 100mM EPPS buffer and 7.5mM $CaCl_2$ pH 7.5 at 70°C, assays (30 minutes at 70°C) were repeated at a pH closer to the growth pH of *Bacillus* isolate TA2.A1 by using 100mM CAPS and 10mM $CaCl_2$ (pH 9 at 70°C). Due to the instability of pNP substrates at pH 9, but the desire to assay at a pH as close to the growth pH of *Bacillus* isolate TA2.A1 as possible, assays were also attempted at pH 8.6 and pH 8 (at 70°C) in 100mM EPPS and 7.5mM $CaCl_2$ with incubation for 60 minutes at 70°C.

Thin layer chromatography

Culture supernatant from the growth of isolate TA2.A1 on trilaurin, tricaprylin, lauric acid and caprylic acid had 100mM CAPS buffer (pH 9 at 65°C) and 1% final

concentration of the corresponding lipid or fatty acid added. After mixing, 10µl samples were spotted onto thin layer chromatography plates (Nano-SIL C_{18}-100UV_{254} plates for nano-TLC layer 0.20mm Silica gel C_{18}-100UV_{254} with fluorescent indicator from Macherey-Nagel Duren) before incubation. After incubation at 70°C in a water-bath for 15, 30 and 45 minutes samples were removed and loaded onto thin layer chromatography plates The samples were run in a chloroform solvent and the plates were developed with Ninhydrin spray, which was able to resolve lipids (Alltech catalog).

Proteinase assays

Peptide analysis

Duplicate 1ml culture aliquots of isolate TA2.A1 inoculated and uninoculated minimal medium with casein were removed at 12 hour intervals throughout the growth of *Bacillus* isolate TA2.A1. Each sample had 500 µl of 15% (w/v) TCA (trichloroacetic acid) added and was then centrifuged at full speed in a microfuge for 5 minutes. The absorbance of the supernatant was read, in a quartz curvette, at 280nm. The average absorbances at 280nm, from both uninoculated and inoculated media gave an indication of the level of released peptides.

Azocasein or azoalbumin substrates (0.2% w/v) were prepared in 50mM CAPS and 5mM $CaCl_2$ (pH 9 at 65°C). 1ml of substrate was dispensed into 1.5ml microfuge tubes and prewarmed for 15 minutes at 65°C. Crude enzyme (50µl) (whole culture or culture supernatant) was added sequentially and the samples were incubated for an hour at 65°C. The reaction was stopped by adding 0.5ml of 15% TCA to each in the same sequence. The samples were cooled on ice for 5 minutes and then 50µl of crude enzyme sample was added to each of the blank tubes. After a further 10 minutes on ice the samples were spun in a microfuge, before absorbances were read by spectrophotometer at 420nm.

The substrate concentration was increased to 0.4% azocasein or azoalbumin in 100mM CAPS and 10mM $CaCl_2$ (pH 9 at 65°C) and assays were performed on 0.5ml of substrate and 0.5ml of crude enzyme (diluted 50% in Milli-Q water). The assays were

also performed at pH 6 with substrate prepared at the same concentration in 100mM MES and 10mM $CaCl_2.2H_2O$. All samples were assayed in triplicate.

Kunitz assays

Substrates of 0.4% (w/v) Hammarsten Casein (BDH) or Ovalbumin (Albumin-Egg) (Sigma) were prepared in 100mM CAPS and 10mM 10mM $CaCl_2.2H_2O$ (pH 9). 500µl of substrate and 500 µl of whole culture or culture supernatant was incubated for 30 or 60 minutes at 65°C, then placed on ice and 500 µl of 15% TCA added. After 15 minutes the assays were centrifuged in a microfuge for 5 minutes, then the absorbance was read in a quartz curvette at 280nm. All assays were completed in triplicate. The definition of a unit of activity was a Δ Absorbance$_{420}$ or a Δ Absorbance$_{280}$ of 1.0 per hour.

In the search for evidence of a proteinase enzyme, cell-free casein culture supernatant was prepared by filtration (0.2 µm) of minimal medium and casein cultures. To prevent further growth of the isolate TA2.A1 cells grown on casein during the assay, nonactin was added to a final concentration of 10µM from a concentrated stock in ethanol, to the casein-culture cell pellets. The same volume of ethanol, as used in the addition of nonactin, was added to the control medium. At each sampling prior to peptide determination microscopic analysis was used to confirm a lack of growth in the cell-free supernatant during the assay period.

4.4.2.6.2 Results and Discussion

Bacillus isolate TA2.A1 grew over three serial transfers into the same medium, on tricaprylin, triacetin, trilaurin and tributyrin. Best growth was on tributyrin and then trilaurin and triacetin. *Bacillus* isolate TA2.A1 was unable to sustain growth on serial transfer in minimal medium supplemented with tripalmitin and triolein. The lipids may have been degrading at the high culture incubation temperature; for example, tributyrin releasing glycerol, which supports the growth of *Bacillus* isolate TA2.A1 (Section 4.4.2.3). For this reason the ability of fatty acids to support the growth of *Bacillus* isolate TA2.A1 was also examined. Caprylic and lauric acid supported growth of isolate TA2.A1 while palmitic, acetic, oleic and butyric acid did not. Minimal medium in which

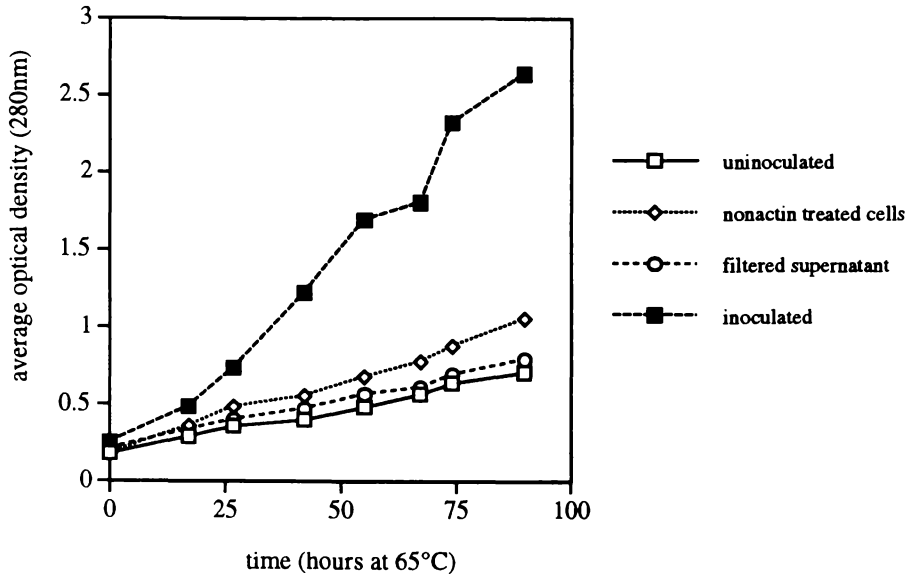
isolate TA2.A1 had grown on tributyrin, triacetin, tricaprylin or lauric acid as carbon substrates was tested for lipase/esterase activity at pH 7.5, 8, 8.5 and 9 (at 70°C). Of the substrates tested, p-NP butyrate was unsuitable for use at 70°C at any of the assay pH values tested because it was hydrolysed during incubation in non-enzyme containing controls. At pH 8 and 8.6 p-NP caprylate also gave high background results. No activity was detected in any of the cultures tested, in whole cells or culture supernatant, using p-NP laurate, p-NP caprylate or p-NP palmitate as substrates. Thin layer chromatography of isolate TA2.A1 culture supernatant from growth on trilaurin, tricaprylin, lauric acid and caprylic acid failed to detect any breakdown products and only the loaded substrate was detected.

Growth of *Bacillus* isolate TA2.A1 was supported by the addition of casein or ovalbumin at 2, 5 and 20g.l^{-1} concentrations to minimal medium and minimal medium without peptone. Growth, assessed by cell counts under the phase contrast microscope, was seen on both substrates as large clumps/mats of cells. On initial exposure to casein supplemented minimal medium, with or without peptone, growth of *Bacillus* isolate TA2.A1 was slow, with an average of only fifteen bacteria per field of view after 16 hours incubation at 65°C. During this initial growth bacterial cells were concentrated around any particulate casein matter in the medium. However, a transfer of growing culture (2% (v/v)) into identical medium gave dense growth after 24 hours of incubation at the same temperature. There were too many bacteria per field of view to count, a large thick mat of growth, with only a few bacteria free (non-clumped) in the medium. The high bacterial cell density on either casein or ovalbumin was stable over multiple serial transfers into the same medium.

The improvement in the levels of growth achieved on casein or ovalbumin after serial transfer may reflect *Bacillus* isolate TA2A1 adapting to growing on these substrates. However, unlike other carbon sources that dissolved readily and were added into minimal medium, from a concentrated filtered stock immediately prior to inoculation, casein and ovalbumin were autoclaved with the basal medium. Not only was casein, for example, exposed to autoclaving, but it was exposed to the high pH of the minimal medium prior to inoculation. Thus growth in casein containing medium not prepared immediately prior to inoculation, for example medium used for a second transfer, may

have been partially on alkaline breakdown products, such as casamino acids. The combination of high pH and high temperature during culture incubation could enhance this breakdown process. It has been established (Table 4.10 and 4.11) that *Bacillus* isolate TA.2A1 grows well on casamino acids, which are produced from the acid hydrolysis of casein. The suggestion that *Bacillus* isolate TA2.A1 was growing on casein breakdown products was supported by peptide determination of casein in minimal medium at 65°C (Figure 4.62). The background levels of released peptides (uninoculated casein in minimal medium) increased over the incubation (65°C) period. There were higher levels of released peptides and a higher rate of peptide production in inoculated minimal medium with casein in which cells were actively growing (Figure 4.62) than in the same medium with non-growing isolate TA2.A1 cells or with cell-free supernatant. No growth was observed in either the casein grown cells treated with nonactin or in the cell-free supernatant, so any increase in free peptides in these solutions, above the level in the uninoculated control, was probably due to enzymatic action. The increase in absorbance at 280nm is presumably due to an accumulation of short peptide chains which have lower molecular weights than are resolvable on the polyacrylamide gels, so that despite ten times more peptide, than in the controls, being released after 90 hours at 65°C, no bands were seen. The increase in free acid-soluble peptides suggests that isolate TA2.A1 uses a peptidase that produces chain lengths that can either be utilized for growth or accumulate in the medium depending on the chain length. It is probable that the cells of *Bacillus* isolate TA2.A1 utilize very short chain peptides or amino acids. Possibly the peptidase specificity limits its action to a minimum size. Amino acid chains which fall between the longest chain usable by the cell, and the shortest chain hydrolysable by the enzyme, will accumulate in the medium. Alternatively, the activity of the putative peptidase could be high enough that peptide formation is significantly faster than the cells peptide utilization, again leading to an accumulation in the growth medium. The presence of a peptidase could have been confirmed, and the peptidase characterized, by assaying against synthetic chromogenic substrates. However, due to the low levels of peptidase activity in the culture supernatant and the high specificity of the chromogenic substrates, this was not considered feasible within the scope of this work.

Figure 4.62: Release of free peptides from casein in minimal medium at 65°C



Samples from the casein solutions described above (after 0, 17, 27, 42 and 90 hours of incubation) were loaded onto homologous 20%, 10-15% gradient and high density SDS polyacrylamide Phastgels (Pharmacia) as described in Section 3.2.3.2. The uninoculated minimal medium had as many bands as the inoculated samples at the start of incubation at 65°C. However, after 90 hours at 65°C the inoculated samples produced no visible bands.

Minimal medium culture and culture supernatant from the growth of *Bacillus* isolate TA2.A1 on ovalbumin, casein, gelatin and elastin were screened for proteinase activity, but there was no activity above the detection threshold (0.2 U/ml). Samples of whole culture and culture supernatant from growth on minimal medium (with and without $0.1 g.l^{-1}$ peptone) and casein were assayed for proteinase activity, but again no activity was detected. The assay method was confirmed to be working correctly by performing assays under the same buffer and pH conditions (pH 9 and 6) using Pre-Taq (Life Technologies) as a positive control. High levels of activity were detected for the Pre-Taq assays (30 Units/ml) under both pH conditions after 30 minutes at 65°C. Kunitz assays completed on minimal medium and casein cultures after a range of incubation periods from 0 to 48 hours detected no proteinase activity above the level in identical, but uninoculated, medium after incubation at 65°C. Pre-Taq was again used as a positive control.

In summary, while growth of *Bacillus* isolate TA2.A1 on casein and ovalbumin was shown by microscopic examination, no proteinase activity was detected. Further investigation showed that it was highly likely that growth was being supported by the breakdown products, such as casamino acids, produced during incubation under conditions of high pH and temperature. Similarly, although growth on a number of lipids or fatty acids was detected by microscopic examination, no lipase or esterase activity was detected in cultures grown on these substrates.

In terms of the possible biotechnological applications of *Bacillus* isolate TA2.A1 the lack of proteinase, lipase or esterase activity detected was disappointing. Alkaliphilic proteases stable under conditions of high pH and temperature are used in detergents not only for stain removal but to ensure that coagulated protein is not redeposited on fabric to produce a grey rather than white appearance (Kalisz 1988a, 1988b). Proteases from alkaliphilic bacteria are also highly effective in the de-hairing of animal hide in the leather-tanning industry. Many alkaline and/or temperature stable proteases have been isolated from *Bacillus* species (Durham *et al.* 1987; Fujiwara and Yamamoto 1987a; Kembhavi *et al.* 1993; Sinha and Satyanarayana 1991; Fujiwara *et al.* 1993). Lipases are used in detergents and washing powders to degrade fats into more hydrophilic fatty acid salts that can be removed by detergent action (Newmark 1988). Lipases with increased stability under conditions of high pH and temperature are more useful for these applications. *Bacillus* strains have been characterized that fulfil these requirements, for example, Emanuilova *et al.* (1993) reported on an alkali-tolerant thermophilic strain of *Bacillus* species that could produce a thermostable lipase. A thermo-tolerant strain of *Bacillus licheniformis* produced a lipase that was stable up to 60°C and optimally active at pH 10 (Khyami-Horani 1996). In contrast to lipases the action of esterases is generally restricted to short chain fatty acid esters (Matsunaga *et al.* 1974), acting on substrates in solution (EC 3.1.1.1) or predominantly undissolved substrates (EC 3.1.1.2). Thermophilic and alkaliphilic esterases have been characterized from *Bacillus* species. For example, Wang and Saha (1993) purified and characterized a tributyrin extracellular esterase (EC 3.1.1.1) from *Bacillus* strain A30-1 with an optimum pH and temperature for esterase activity of 9.0 and 60°C.

4.4.2.7 Carbon source utilization by thermophilic or alkaliphilic bacteria

The range of carbon substrates *Bacillus* isolate TA2.A1 is able to utilize (Tables 4.9, 4.10 and 4.11) is different from those utilized by other thermophilic (including thermo-tolerant) and/or alkaliphilic (including alkali-tolerant) species. The examples given below illustrate this:

(a) Thermophilic or alkaliphilic *Bacillus* or closely related genera

White *et al.* (1993) conducted an analysis of 234 thermophilic *Bacillus* species and strains which were grouped into 18 clusters based on phenotypic characterization. The number of clusters in which 90% or more of the species or strains produced acid from the stated carbon source is given in brackets; glycerol (12), fructose (12), glucose (14), sucrose (5) and trehalose (11). *Bacillus* isolate TA2.A1 was able to utilize glycerol, sucrose and trehalose, but not glucose or fructose. Sodium pyruvate, which also supports the growth of *Bacillus* isolate TA2.A1, was not mentioned as a carbon source in the analysis conducted by White *et al.* (1993). The carbon substrates listed in Table 4.14 were able to support the growth of at least 90% of the strains assigned to that cluster (White *et al.* 1993). The carbon substrates underlined in the table are utilized by *Bacillus* isolate TA2.A1. Eighteen carbon substrates are listed as supporting the growth of 90% or more of the thermophilic *Bacillus* species and strains tested, only six of these support the growth of *Bacillus* isolate TA2.A1. Thus in comparison with the thermophilic *Bacillus* species analyzed by White *et al.* (1993) isolate TA2.A1 utilizes a distinctive range of carbon substrates. A similar conclusion is reached from an overview of other studies on thermophilic (thermo-tolerant) *Bacillus* or *Bacillus*-related strains which are discussed next.

Table 4.14: Substrate utilization by thermophilic *Bacillus* species (White *et al.* 1993)

cluster		substrates able to support growth of >90% of cluster strains
1	*B. pallidus*	fructose, glucose, glycerol, maltose, mannose, sucrose, trehalose
2	*B. kaustophilus*	casein, fructose, glucose, maltose, starch
3	*Bacillus sp.*	casein, maltose, trehalose, tributyrin,
4	*B. smithii*	fructose, glucose, glycerol, maltose, mannose, pullulan, ribose, trehalose, xylose
5	*Bacillus sp.*	fructose, glucose, glycerol, maltose, mannose, pullulan, raffinose, ribose, starch
6	*B. caldotenax*	glucose, glycerol, maltose, starch, trehalose
7	*B. steaothermophilus*	dextrin, fructose, glucose, glycerol, maltose, mannose, pullulan, starch, sucrose, tributyrin
8	*Bacillus sp.*	glucose, glycerol, mannose, starch
9	*B. thermoglucosidasius*	fructose, gelatin, glucose, glycerol, maltose, mannose, pullulan, ribose, trehalose
10	*Bacillus sp.*	fructose, glucose, glycerol, maltose, mannose, melibiose, pullulan, starch, trehalose
11	*Bacillus sp.*	fructose, gelatin, glucose, glycerol, maltose, mannose, melibiose, ribose, sucrose, trehalose
12	*Bacillus sp.*	gelatin, glucose, starch, trehalose
13	*Bacillus sp.*	glucose, glycerol, tributyrin
14	*Bacillus sp.*	fructose, gelatin, maltose, sucrose, trehalose
15	*B. thermodenitrificans*	fructose, glucose, glycerol, maltose, mannose, melibiose, pullulan, starch, trehalose, tributyrin
16	*B. licheniformis*	casein, cellobiose, fructose, glucose, glycerol, maltose, mannose, starch, sucrose, trehalose
17	*Bacillus sp.*	no set carbon source pattern, 40% of the strains utilized glucose; on the majority of the substrates tested only around 1% grew
18	*B. thermocloacae*	no set carbon source pattern, 67% of the strains utilized glucose; on the majority of the substrates tested only around 1% grew

Key: underlined substrates are utilized by *Bacillus* isolate TA2.A1, maltose which inconsistently supports the growth of isolate TA2.A1 is underlined with a broken-line

Bacillus isolate TA2.A1, like most of the thermophilic or alkaliphilic *Bacillus* or *Bacillus*-related strains described in other studies (Tables 2.5a and 2.5b) utilized sucrose. The species that were negative for sucrose utilization were *B. thermocloacae*, *Aneurinibacillus thermoaerophilus*, *B. thermoalcaliphilus*, *B. horti*, *Paenibacillus*

campinasensis, and *B. smithii*. Amongst the *Bacillus* species described (Tables 2.5a and 2.5b), if the species or strain could utilize sucrose, then it was generally also able to utilize trehalose, glucose and fructose as carbon substrates. Similarly most species which were unable to utilize sucrose were also unable to grow on trehalose glucose or fructose, for example, *B. thermocloacae* and *B. thermoalcaliphilus*. This is unlike *Bacillus* isolate TA2.A1 which can utilize sucrose and trehalose, but not the monosaccharides glucose and fructose as carbon substrates.

(b) Thermophilic and alkaliphilic species

Since *Bacillus* isolate TA2.A1 is both thermophilic and alkaliphilic, a comparison of carbon source utilization with species that live or tolerate both high temperature and high pH was carried out. The thermophilic and alkaliphilic bacterial species and strains described in Tables 2.6 and 2.7 grew on a range of carbon sources. A number of the anaerobic species or strains described in Table 2.6 have their carbon substrate utilization described in Table 4.15. A number of the aerobic thermophilic and alkaliphilic bacteria described in Table 2.7 have their carbon substrate utilization described in Table 4.16. These species or strains, which are most similar to *Bacillus* isolate TA2.A1 in terms of growth conditions, differ in terms of their carbon source utilization.

Table 4.15: Substrate utilization by anaerobic thermophilic (thermo-tolerant) and alkaliphilic (alkali-tolerant) bacteria

species/strain	substrates utilized by the strains	reference
Clostridium paradoxum	casamino acids[1], cellobiose[1], fructose[1], glucose[1], maltose[1], sucrose[1]	Li *et al.*. 1993
Clostridium thermoalkaliphilum DSM 7309	casamino acids[1], cellobiose[1], fructose[1], glucose[1], maltose[1], sucrose[1]	Li *et al.* 1994
Thermobrachium celere JW/YL-NZ35	fructose[2], galactose[2], glucose[2], maltose[2], sucrose[2]	Engle *et al.* 1996
Anaerobranca horikoshii DSM 9786	yeast extract, peptone, fumerate	Engle *et al.* 1995

Underlined substrates are utilized by *Bacillus* isolate TA2.A1. Maltose which inconsistently supports the growth of isolate TA2.A1 is underlined with a broken-line

[1] in the presence of 0.1% (w/v) yeast extract or tryptone.

[2] In the presence of 0.5% yeast extract

Table 4.16: Substrate utilization by aerobic thermophilic (thermo-tolerant) and alkaliphilic (alkali-tolerant) bacteria

Species/strain	substrates utilized by the strains	reference
B. caldotenax	cellobiose, dextran, fructose, glucose, melibiose, pullulan, raffinose, salacin, starch, tributyrin	Heinen + Heinen 1972
B. flavothermus DSM 2641	glucose, maltose, mannose, rhamnose,saccharose	Heinen *et al.* 1982
B. thermocloaceae DSM 5250	peptone	Demharter + Hensel 1989
Bacillus NCIM 59	fructose, glucose, mannose, sucrose, xylan	Dey et al. 1992
Bacillus sp. strain TAR-1	glucose, xylan[1]	Nakamura *et al.* 1994
Bacillus sp. strain C-125 (*B. lentus* Group3)	L-arabinose, D-ribose, D-xylose, D-glucose, D-mannose, lactose, maltose, melibiose, raffinose, sucrose, trehalose	Aono 1995, Aono *et al.* 1995
B. haloalkaliphilus DSM 5271	casein, gelatin, starch	Fritze 1996

Underlined substrates are utilized by *Bacillus* isolate TA2.A1. Maltose which inconsistently supports the growth of isolate TA2.A1 is underlined with a broken-line

[1] in a medium containing polypeptone and yeast extract

Like most other groups of bacteria, there is a wide diversity in the ranges of substrates utilized by both thermophilic and/or alkaliphilic species. The range of carbon substrates utilized by *Bacillus* isolate TA2.A1 does not support the growth of any other described thermophilic alkaliphilic species. Further differences in the characteristics of this isolate are described in the molecular biology section which follows.

4.5 Molecular biology

4.5.1 Materials and Methods

4.5.1.1 DNA isolation protocols

4.5.1.1.1 General DNA extraction method

DNA extraction was based on the method of Marmur and Doty (1962). Cells were grown for 18 hours at 65°C in rich medium and harvested by centrifugation (Beckman) at 4°C for 30 minutes at 12,000 x g. The cell pellet was washed twice in 0.1M EDTA/0.15M NaCl pH 8. Cells were resuspended in 25ml 0.1M EDTA/0.15M NaCl containing 10mg lysozyme and incubated for 45 minutes at 37°C. Two ml of 25%(w/v) sodium dodecyl sulphate was added and the mixture was incubated for 3 hours, until lysed. Lysis was confirmed by examination under the phase contrast microscope. 2µl RNAse (Sigma) was added and the sample incubated for 15 minutes at 37°C. Proteinase K (Sigma) was added at 50µl/ml and the sample incubated at 65°C for 20 minutes. The mixture was then heated for 10 minutes at 60°C before adding an equal volume of chloroform : phenol : isoamyl alcohol (24:24:1 v/v). The extraction was repeated until no more protein precipitate was visible. The extraction mixture was mixed/inverted for 30 minutes at room temperature before being centrifuged for 5 minutes at 10,000 x g. The top aqueous layer containing the nucleic acid was removed into a measuring cylinder to which twice the volume of cold ethanol (-20°C) and sodium acetate (3M) (1/10th the volume) were added. The DNA that precipitated was collected with a glass rod and washed in 70% ethanol. The DNA was redissolved in 1ml EDTA/NaCl pH 8 to which 1 gram of CsCl was added per ml of DNA solution. Ethidium bromide (10mg/ml in Milli-Q water) at 0.8ml : 10ml DNA : CsCl solution was added. The sample was mixed and then centrifuged at 4,000 x g for 5 minutes. The supernatant was transferred to an open topped Beckman 3.0ml thick walled polycarbonate tube or a Beckman polyallomer seal-top tube and then centrifuged for at least 20 hours at 70,000 rpm (165,700 x g) in a Beckman benchtop ultracentrifuge at 15°C. The band which contained DNA was removed and transferred to a new tube. CsCl / ethidium bromide solution was added in the same proportions (0.8ml ethidium bromide / 10ml CsCl /10mM Tris and

1mM EDTA pH 8 (TE buffer). The tubes were then recentrifuged at 70,000 rpm for at least 16 hours at 15°C. The DNA band was transferred to a microfuge tube and extracted in darkness (using UV light) with 4M CsCl-saturated isopropylalcohol to remove the ethidium bromide. The extraction was repeated until no colour from the ethidium bromide could be seen, and then the extraction was performed an additional time. The remaining aqueous phase was placed into a microfuge tube and diluted 1:4 in TE buffer and precipitated using 1/10th the volume of 3M sodium acetate and 0.6 the total volume of isopropanol. The resulting pellet was washed twice in 80% ethanol, dried under vacuum and resuspended in 500 µl TE pH 8.0.

4.5.1.1.2 Methods to increase cell lysis / DNA yield

- Proteinase K digestion - After lysozyme treatment 100 µg/ml Proteinase K and 1% SDS were added to the cells which were incubated at 50°C for two hours. As an additional experiment the concentration of Proteinase K was doubled and the incubation temperature was increased to 65°C.

- SDS-lysis at elevated temperature - Cells were incubated for 6 hours at 70°C in SDS.

- Repeated freeze-thawing - Saline EDTA washed cells were frozen at -20°C and thawed repeatedly.

- pH shock (H_2SO_4) - Cells were washed twice in 0.1M EDTA/0.15M NaCl pH 8, resuspended in the same volume of 5M H_2SO_4 and incubated at 70°C for one hour. Cells were also exposed to H_2SO_4 and then immediately the solution was raised to pH 8 by addition of NaOH.

- Sonication - Sonication of cells on ice for was carried out at 30 second intervals over a total of 10 minutes.

- Absence of sodium ions - Lysozyme was added to sodium-free 0.1M EDTA at pH 8. KOH was added to get sodium-free EDTA into solution.

- Triton X-100 lysis - In one attempt to achieve cell lysis 10% (v/v) Triton X-100 and SDS were added simultaneously to cells. In another attempt cells were repeatedly frozen (-20°C) and thawed before adding a third of their net weight in glycerol. Then 1% (volume / wet cell weight) of 10% Triton X-100 and 0.1% (volume / wet cell weight) ß-mercaptoethanol were added and the mixture was stirred for two hours at 4°C. This was then added to either 50mM MOPS / KOH buffer (pH 7 at 20°C) with 7.5mM disodium EDTA or saline EDTA.

- Microwave treatment - Bollet *et al.* (1991) reported a simple method for the isolation of chromosomal DNA from Gram positive or acid-fast bacteria. Cells were washed in TE buffer and resuspended in 100 µl of TE, 50 µl of 10% SDS was added and the solution was incubated for 30 minutes at 65°C. The sample was centrifuged and the supernatant removed. The cell pellet was microwaved in three bursts of 1minute duration. This method was also modified by the use of saline EDTA instead of TE buffer.

- Glass beads /mixer mill - Cells were washed twice in 0.1 M EDTA/0.15M NaCl pH 8, pelleted by centrifugation and the pellet was frozen at –20°C. After thawing the cells were resuspended in 0.1M EDTA/0.15M NaCl and dispensed in 1ml volumes in microfuge tubes. 1/5th of the microfuge tube, containing the cells, was filled with glass beads (100 mesh) (BDH). The microfuge tubes were shaken in a Mixer-Mill (Type MM2000 Retsch iso-9001) for three one minute intervals at an amplitude of 100.

4.5.1.1.3 DNA extraction method for G+C mol% determination

Bacillus isolate TA2.A1 cells were grown in rich medium for 18 hours at 65°C and centrifuged at 5300g for 20 minutes. The section of this procedure that follows was based on the method of Saul *et al.* (1993). The cell pellet was washed in a fifth of its original volume of TES buffer (0.5M Tris,pH 8.0; 0.05M NaCl; 0.005 EDTA) and re-centrifuged as before. The pellet was resuspended, in a tenth of the original culture volume, of 50mM Tris pH 8.0. EDTA (0.5M) was added at 7.5% (v/v) final concentration and the mixture incubated at 37°C for 20 minutes. Freshly prepared

lysozyme from a stock solution of 10mg/ml Milli-Q water was added at 10% (v/v) of the total volume and the mixture incubated at 37°C for 20 minutes. Proteinase K (Sigma) from a 40mg/ml stock solution was added to a concentration of 5% (v/v). SDS from a 10% stock solution was added to a final concentration of 0.5%. The sample was then incubated overnight in a waterbath at 70°C. After overnight incubation a series of phenol/chloroform extractions were performed until all protein had been removed.

At this position in the protocol the following additional steps were completed as modifications for *Bacillus* isolate TA2.A1. RNAse (DNAse free) was added to 50ug/ml final concentration and the sample was incubated at 37°C for 30 minutes. SDS was added to 0.5% final concentration, EDTA to 20mM final concentration and Proteinase K to 100ug/ml final concentration and the mixture was incubated at 65°C for 1 hour. Phenol/chloroform extractions were performed repeatedly until all protein had been removed. DNA was collected by ethanol precipitation in the presence of NaCl to >0.2M final concentration. The DNA was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in TE buffer.

4.5.1.1.4 Extraction method for PCR

Bacillus isolate TA2.A1 cells were grown in rich medium for 18 hours at 65°C. The cells were centrifuged (Beckman) at 15200g for 20 minutes and washed in 50mM Tris and 30mM EDTA (pH 8). After re-centrifugation the cells were suspended in 1ml of 50mM Tris and 30mM EDTA (pH 8). SDS (10µl of a 10% v/v stock solution) and lysozyme (50µl of a 20mg/ml stock in Milli-Q water) were added and the mixture was incubated at 37°C for 2 hours. SDS was added from a 10% (v/v) stock to a final concentration of 0.5% SDS and the sample was mixed. Pronase (Sigma) which had been pre-incubated for an hour prior to use (to remove DNAses) was added to a final concentration of 0.2 mg/ml and the cells were incubated in a 37°C waterbath overnight. Phenol/chloroform extractions were performed using phenol : chloroform : isoamyl alcohol at a ratio of 25:24:1 (v/v). Equal volumes of cells and the phenol:chloroform: isoamyl alcohol mixture were shaken for 10 minutes, and then after centrifugation at 8000 rpm for 5 minutes the upper phase was removed. The pellet had 100µl of Tris/EDTA buffer added, this was shaken and centrifuged as before and the upper phase

removed. These upper phase portions of the extractions were combined and underwent repeated phenol/chloroform extractions until no protein precipitate was present. A final extraction was completed using the same volume of chloroform alone; this mixture was shaken, centrifuged and the top removed into a new sterile tube. RNAse was added at 0.2mg/ml final concentration and the sample was incubated at 37°C for 30 minutes. To remove the RNAse a phenol/chloroform extraction was completed followed by a chloroform extraction. In each case the volume of extractant added was equal to the volume of the aqueous phase. Sodium acetate was added from a 3M (pH 5.2) stock to one tenth of the total volume. Two volumes of ice cold ethanol were added and the mixture was left overnight at –20°C. After centrifugation the pellet was washed with 70% ethanol before being re-centrifuged and dried under vacuum. The pellet was resuspended in a small volume (100µl) of TE buffer and stored at 4°C.

4.5.1.2 G+Cmol % determination method

The G+C mol% value was determined by the thermal denaturation method (Mandel and Marmur 1973). The DNA sample was dialysed (at 4°C for 24 hours) against 0.5x Standard Saline Citrate (SSC) buffer (50% dilution in Milli-Q water of 0.15M NaCl and 0.015M trisodium citrate (pH 7.0)). The DNA was diluted with 0.5x SSC buffer in a 1ml quartz cuvette to give an absorbance value of 0.4 at 260nm. The temperature of a spectrophotometer quartz cuvette was measured directly by sealing a thermocouple probe (Omega, USA) into the cuvette containing 0.5x SSC. This reference cuvette was placed next to the experimental sample cuvette in the heated cuvette holder of a Lambda 3B spectrophotometer (Perkin-Elmer, USA) equipped with a digital temperature controller. The increase in optical density at 260nm was recorded as the temperature increased at 0.1°C per minute. Optical density readings were noted from 30°C and the gradient was begun from 60°C.

The value for G+C mol% was determined according to Mandel and Marmur (1973).

$$\% \text{ G+C} = \text{G+C}_{\text{reference strain}} + 2.24\ (\text{Tm}_{\text{unknown}} - \text{Tm}_{\text{reference strain}})$$

E.coli strain B DNA (Sigma, USA) and RT2 spirochaete DNA, prepared by the same DNA extraction protocol were used as standards, and their G+C mole % was determined under the same conditions as that of *Bacillus* isolate TA2.A1.

4.5.1.3 16S rDNA PCR protocol

DNA was extracted following the method outlined in Section 4.5.1.1.4. The PCR protocol was as follows:

	Temperature (°C)	Time	
Initial Denaturation	94	3 minutes	1 cycle
Denaturation	94	50 seconds	30 cycles
Annealing	50.7	1 minute	
Extension	72	1 minute 50 seconds	
Final extension	72	5 minutes	1 cycle

Universal bacterial 16S rDNA primers were obtained from Life Technologies (Auckland, NZ). Direct sequencing of purified PCR products was achieved using the Prism™ Ready Reaction Dichlororhodamine Terminator Cycle Sequencing Kit (Perkin Elmer) following the manufacturer's protocol. Sequences were analyzed on the Applied Biosystems Model 377 automated DNA sequencer from Perkin Elmer.

Primers :

pA 5'-AGAGTTTGATCCTGGCTCAG-3' (*E.coli* 8-28)

pH* 5'-AAGGAGGTGATCCAGCCGCA-3' (*E. coli* 1542-1522)

Sequencing Primers :

Sef 1* 5'-CTAACTACGTGCCAGCAGCCG-3'

Sef 2 5'-GGAGCATGTGGTTTAATTCG-3'

The purity of the PCR product was ascertained by electrophoresis (50V, 1hour) on a 1% (w/v) agarose gel in 1x TBE (0.09M Tris, 0.09M boric acid and 0.01M EDTA pH 8.3). The PCR product was purified using the Wizard Prep™ (Promega) system.

4.5.1.4 16S rDNA-based phylogenetic tree

Choice of taxa for molecular phylogeny

29 species (including *Bacillus* isolate TA2.A1) were used to generate the phylogeny. The sequence of isolate TA2.A1 showed only moderate similarity to other members of the *Bacillus* group. It was considered important to perform a phylogenic analysis using the best inference methods available. Maximum likelihood is now regarded as the most robust method but it is only suitable for analysing a limited number of taxa (Swofford *et al.* 1996). Early analyses suggested that isolate TA2.A1 branched deeply into the *Bacillus* group and so it was important to include a representative example of each of the major *Bacillus* subgroups. Additional taxa were added to help to break long branches. *Anaerobranca horikoshii* was chosen as outgroup in order that the tree could be rooted.

Phylogenetic methods

Sequence alignment was performed using the program PILEUP (Feng and Doolittle 1987) with a low gap weight. Manual adjustment of the alignment was required to accommodate secondary structure considerations. Where the alignment could not be ascertained with complete confidence, the characters were excluded from the analysis. Phylogenetic analysis was performed using the program PAUP (Swofford 1990), 1430 sites of the aligned sequences were used and gaps were treated as missing data. Bootstrap values were generated from 1000 replicates of heuristic searches using maximum parsimony as the optimality criterion. The evolutionary model used for maximum likelihood searches is described in the results section. Phylogenetic analysis was carried out with the assistance of Dr D. Saul (University of Auckland).

4.5.1.5 Other methods

Proteinase (Pronase E / Sigma P-5147) stock was prepared in Milli-Q water, filtered (0.45µM) and stored in aliquots at -20°C. RNAse A stock was prepared at a concentration of 10mg/ml in 10 mM Tris / HCl (pH 7.5) and 15 mM NaCl. This was

heated to 100°C for 10 minutes and then allowed to cool slowly at room temperature. Aliquots were dispensed and stored at - 20°C.

Dialysis tubing was boiled for 10 minutes in a large volume of 2% (w/v) sodium hydrogen carbonate and 1 mM EDTA. After rinsing thoroughly with Milli-Q water the tubing was boiled for 10 minutes in 0.001 M EDTA. After cooling the tubing was stored submerged in 1mM EDTA at 5°C and washed in Milli-Q water prior to use.

The purity of DNA was assessed by an optical density measurement at 260 and 280 nm. The DNA was pure if the 260:280 ratio was 1:8. A ratio of 2 or above indicated the presence of RNA and a ratio of 1.7 or below indicated the presence of protein. The concentration of the DNA was calculated from the fact that an optical density at 260nm of 1 was equivalent to 50µg/ml of DNA.

4.5.2 Results and Discussion

A small quantity (approximately 25µg/ml) of DNA was obtained by the method of Marmur and Doty (1962). However, the quantity obtained was not enough for G+Cmol % analysis since an individual DNA temperature melting curve required a minimum of 14µg of DNA and the melting curve for each organism was repeated in quadruplicate. The major difficulty in using this method of DNA extraction on *Bacillus* isolate TA2.A1 cells was that poor cell lysis was achieved. The results of attempts to increase cell lysis were as follows:

- Attempts to increase cell lysis by Proteinase K treatment with an increased incubation temperature of 50 or 65°C failed.

- Since isolate TA2.A1 is an alkaliphile and SDS is alkaline, the temperature of incubation in the presence of SDS was increased to 70°C, however, the presence of SDS failed to induce lysis.

- Repeated freeze-thawing of cells failed to cause cell lysis.

- The use of pH shock produced shorter cells, when examined under the phase contrast microscope. No DNA was extracted, however, following a phenol / chloroform extraction. It is probable that any DNA extracted would have been quickly denatured due to the harshness of this procedure. The large protein layer formed, after phenol chloroform extraction, from acid treated cells (which were then adjusted back to pH 8) was treated with proteinase to ensure that DNA was not trapped in the protein layer. No DNA was extracted from the protein layer.

- Since alkaliphiles exhibit sodium-dependence, to keep membranes intact, a lack of sodium ions in the DNA extraction protocol may have been expected to aid lysis; however, this proved to be unsuccessful.

- The use of 10% (v/v) Triton X-100 in conjunction with SDS failed to cause cell lysis as did the addition of Triton X-100 and ß-mercaptoethanol.

- The method of Bollet *et al.* (1991) for the isolation of chromosomal DNA from Gram positive or acid-fast bacteria failed to work for *Bacillus* isolate TA2.A1.

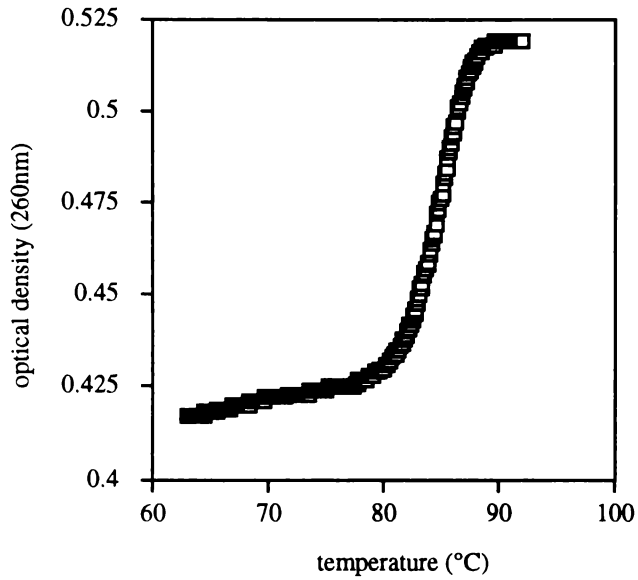
Cell sonication achieved cell lysis but this treatment sheared the DNA so that it was in short pieces which gave inadequate results when used for a G+Cmol% melting curve. Similarily the glass beads and mixer mill were successful in achieving cell lysis but the G+C melting curves conducted using this DNA gave only a small increase in optical density on melting, which was probably due to the extraction of either short strands or single stranded DNA. The method used to extract enough DNA of suitable quality for G+C analysis is outlined in Section 4.5.1.1.3. G+C analysis was carried out in quadruplicate by a DNA melting curve, as described in Section 4.5.1.2; the resulting G+C melting curve is shown in Figure 4.63.

The G+Cmol % of *Bacillus* isolate TA2.A1 DNA was calculated by using both *E.coli* and *Spirochaeta* DNA as reference standards. In the example below the melting point of *Spirochaeta* DNA was used as the reference strain.

$$\% \text{ G+C} = \text{G+C}_{\text{reference strain}} + 2.24\ (\text{Tm}_{\text{unknown}} - \text{Tm}_{\text{reference strain}})$$
$$= 47.3\% + 2.24\ (84-84) \qquad = 47.3\%$$

Not surprisingly for such a diverse genus species of *Bacillus* exhibit a wide range of G+C content in their DNA, from 32 to 69 mol%. In an analysis of the phylogenetic diversity of thermophilic members of the genus *Bacillus* Rainey *et al.* (1994) stated that all thermophilic bacilli had mol%G+C base ratios in the range of 57-63, while mesophilic bacilli had lower G+Cmol% values ranging from 53 to 55.

Figure 4.63: Temperature melting curve of DNA extracted from *Bacillus* isolate TA2.A1



Horikoshi (1991b) analyzed the G+C DNA content of thirty strains of alkaliphilic bacilli from his laboratory and twenty known *Bacillus* reference strains. The G+C contents of the alkaliphilic *Bacillus* strains were distributed over the range 35.5% to 52.4% G+C. The 119 strains of alkaliphilic and alkali-tolerant aerobic spore-forming bacteria examined by Nielsen and co-workers (1995) had G+C contents ranging from 34 to 45.5. The nine new species proposed by Nielsen *et al.* 1995 ranged in G+C content from 38.2 to 45.5. All of these species had lower G+C contents than *Bacillus* isolate TA2.A1; the closest was the alkali- and thermo-tolerant *Bacillus clausii* with a G+Cmol% of 42.8 – 45.5. *Bacillus horti,* a mesophilic alkali-tolerant species (Yumoto *et al.* 1998) which is closely related to *Bacillus* isolate TA2.A1 by 16S rDNA analysis, has a lower G+Cmol% than isolate TA2.A1 of 40.2 – 40.9.

Anaerobic alkaliphilic thermophiles (Table 2.6) range in G+Cmol% content from 30 (*Clostridium paradoxum* (Li *et al.* 1993), *Thermobrachium celere* (Engle *et al.* 1996)) to 34 (*Anaerobranca horikoshi* (Engle *et al.* 1995)). Aerobic alkaliphilic thermophiles (Table 2.7) have G+C contents ranging from 37 for *B. haloalkaliphilus* (Fritze 1996) to 64.8 for *B. caldotenax* (Heinen and Heinen 1972). Species in the genus *Aneurinibacillus* have the ability to tolerate both high pH and high temperature with growth parameters ranging from 20 to 60°C and pH 5.5 to 9.0. These aerobic organisms are alkali- and thermo-tolerant and have G+C contents from 41.1 to 43.4 mol%. The aerobic alkaliphilic thermophile *Bacillus* isolate TA2.A1 with a G+C content of 47.3% is within the range of mol%G+C base ratios for the alkaliphilic thermophiles and has a G+C content similar to the alkali- and thermotolerant *B. clausii* and *Aneurinibacillus* species. In terms of G+Cmol% *Bacillus* isolate TA2.A1 is more closely related to known alkaliphilic or alkali-tolerant rather than thermophilic bacilli.

The 16S rDNA sequence for *Bacillus* isolate TA2.A1 was obtained (Figure 4.64) and has been submitted to GenBank (accession number AF113512).

Figure 4.64: The16S rDNA sequence of *Bacillus* isolate TA2.A1

TGCTGGCGGCACGCCTAATACATGCAAGTCGAGCGCGTGAAGCTTCCAGAAGCCTTCGGGCGGACGGGAGT
GGATCGAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCTGTAAGACCGGGATAACCCCGGGAAACC
GGGGCTAATACCGGATAGGACCTTCGGTCGCATGACCGTTGGTTGAAAGGTGGCCGCAAGGCTACCGCTTA
CAGATGGGCCTGCGGCGCATTAGCTGGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGGCC
TGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCGCAATGGGCGCAAGCCTGACGGAGCGATGCCGCGTGAGCGAGGAAGGCCTTCGGGTCGTAAAGCTCT
GTTGTCAGGGAAGAACAAGTACCGTTCGACAAGGGCGGTACCTTGACGGTACCTGACGAGAAAGCCCCGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATCACTGGGCGTAAAGCGC
GCGCAGGCGGCCTCTTAAGTCTGGTGTGAAAGCCCGCGGCTCAACCGTGGAGACGCACTGGAAACTGGGAG
GCTTGAGTGCAGGAGAGGGAAGCGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAGGAACACC
AGTGGCGAAGGCGGCTTCCTGGCCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGTTTCGACACCCTCAGTGCTGAAG
GCAACCCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCGGT
GCTACCTCCAGAGATGGAGGGTTCTCTTCGGAGACACCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGGCCTTAGTTGCCAGCATTCAGTTGGG
CACTCTAAGGCGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATG
CCCTGGGCTACACACGTGCTACAATGGGCGGTACAAAGGGTTGCGAACCCGCGAGGGGGAGCTAATCCCAA
AAAGCCGCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTCTGTAACA
CCCGAAGTCGGTGAGGTAACCCGCAAGGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCG
TAACAAGGTATCCCTACCGGAAGGTGCGGNTGGATCACCTCC

When the 16S rDNA sequences of *Bacillus* isolate TA2.A1 and other *Bacillus* strains are aligned (refer to Appendix 1 for entire sequence alignment of some of these strains), a small number of strains, including *Bacillus* isolate TA2.A1, contain a five base pair insert near the beginning of the sequence (Figure 4.65), bases 36-40 from the 5′ end of the isolate TA2.A1 sequence. The *Bacillus* strains with an insert in the same position were *B. clarkii*, *B. horti*, *B. agaradhaerens*, and *B. vedderi*. The 16SrRNA gene of an undescribed species isolated from deep-sea mud (HTA454) (Takami *et al.* 1997) and a sequence fragment 'ab009' (complete 16S rDNA sequence not currently available) from a clone library from mesophilic sewage sludge also contained the insertion. Interestingly the inserted fragment "CGTGA" is the same for both *Bacillus* isolate TA2.A1 and the fragment ab009. The insertion "CAGGA" is the same for *B. clarkii* and *B. agaradhaerens* and the insert "AGTGA" is the same for *B. horti* and the undescribed strain HTA454.

Figure 4.65: Comparison of the first bases in the 16S rDNA sequence alignment

Bacillus TA2.A1	TGCTGGCGGCACGCCTAATACATGCAAGTCGAGCG**CGTGA**AGCTTCCAGA
fragment ab009	CGCTGGCGGCACGCCTAATACATGCAAGTCGAGCN**CGTGA**AGCTTCCAGA
B. clarkii	CGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCG**CAGGA**AACAGGCTGA
B. horti	CGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCG**AGTGA**AACTGACGGA
B.agaradhaerens	CGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCG**CAGGA**AGCCGGCGGA
B. vedderi	--CTGGCGGCGTGCCTAATACATGCAAGTCGAGCG**-GATC**AATAAGAGCT
Strain HTA454	----------GGCTAATACATGCAAGTCGAGCG**AGTGA**A-CAAACAGA
*B. t'alkaliphilus**	--CTGGCGGCGTTCCTAAXACATTCAAGTCGAXCG-----GACCGXAGG-
B. alcalophilus	--CTGGCGGCGTGCCTAATACATGCAAGTCGAGCG-----GACAGA-AGG
B. thermophilus	--CTGGCGGCGTGCCTAATACATGCAAGTCGAGCG-----AACCGAT-GA
B. horikoshii	--CTGGCGACGTGCCTAATACATGCAAGTCGAGCG-----GACGTTTTTG
B. subtilis	--CTGGCGGCGTGCCTAATACATGCAAGTCGAGCG-----GACAGAT-GG

**B. t'alkaliphilus* = *B. thermoalkaliphilus*

The characterised *Bacillus* species with a base pair insertion in the same position as *Bacillus* isolate TA2.A1 have the following characteristics:

B. clarkii and *B. agaradhaerens* both have temperature maxima of 45°C, are alkaliphilic and, like *Bacillus* isolate TA2.A1, require sodium ions for growth. The G+C DNA content of these species is 42.4 – 43.0 and 39.3 – 39.5 respectively (Nielsen *et al.* 1994). *B. horti* is a Gram negative, mesophilic, alkalitolerant, areobic, soil isolate with a mol%

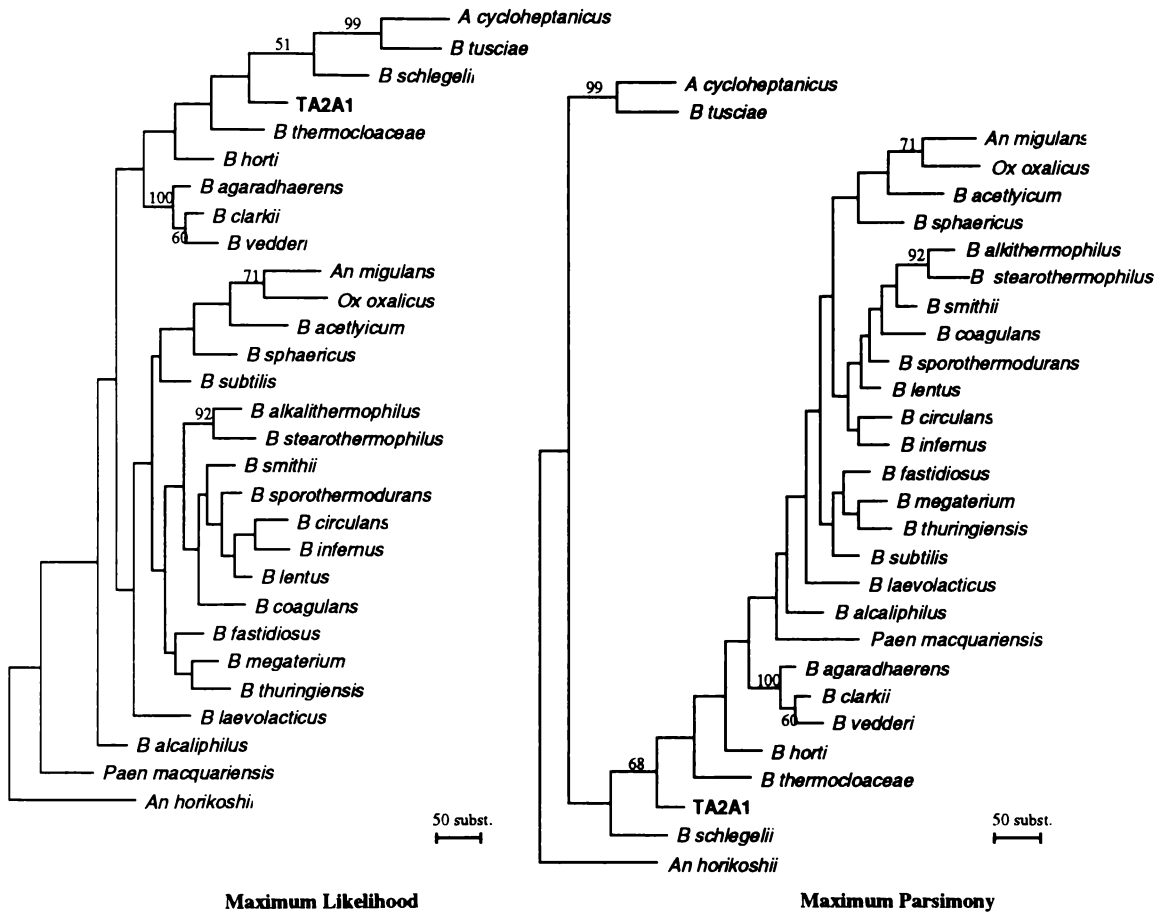
G+C DNA content of 40.2 – 40.9 (Yumoto *et al.* 1998). The facultatively anaerobic *B. vedderi* has a maximum temperature for growth of 50°C, a pH optima of 10 and a mol% G+C DNA content of 38.3 (Agnew *et al.* 1995). As illustrated (Figure 4.65) other alkaliphilic (*B. thermoalcaliphilus*, *B. alcaliphilus*, *B. horikoshii*), thermophilic (*B. thermophilus*) or mesophilic neutralophilic (*B. subtilis*) *Bacillus* species do not have a base pair insertion in the same position.

In order to ascertain the phylogenetic position of *Bacillus* isolate TA2.A1 16SrDNA sequences were aligned and phylogenetic dendrograms were generated (Figure 4.66). A single most parsimonious (MP) tree was generated by an heuristic search with 1000 random sequence additions. It was not possible to generate a maximum likelihood tree *de novo* with this number of taxa without seriously compromising the evolutionary model. Therefore, the MP tree was used as a starting tree and refined using maximum likelihood (ML) and the tree-bisection-reconnection swapping algorithm(TBR). The HKY(85) model of evolution was used (Hasegawa *et al.* 1985) and rates were assumed to follow a gamma distribution. The transition:transversion ratio, base frequencies, proportion of invariant sites and the gamma shape parameter were set to values estimated from the MP tree. A single ML tree was generated. Both the MP and ML trees are shown in Figure 4.66 for comparison.

There is a significant difference between the placing of isolate TA2.A1 when using the alternative methods. However, the reliability of the branching pattern throughout the trees is poor (as is demonstrated by the low bootstrap values). A Kishino-Hasegawa test (Kishino and Hasegawa 1989) on the two alternative trees yielded a probability of 0.894. This figure suggests that neither tree is significantly better at representing the sequence data. If branching patterns with less than 50% bootstrap values are ignored, then few conclusions can be made about the placing of most of the taxa in the trees. At first glance, published Neighbour-Joining trees appear to produce more unequivocal results. However, this data is likely to be misleading. It is now widely accepted that the Neighbour-Joining algorithm is highly susceptable to inconsistencies in evolutionary assumptions (such as a molecular clock and site-site rate heterogeneity) (Swofford *et al.* 1996). What can be deduced from the trees is that isolate TA2A1 is a member of the *Bacillus* group and is deeply branching. There appears to be some affinity with *B.*

thermocloacae, *B. horti*, *B. agaeadhaerens*, *B. clarkii* and *B. vedderi* and these organisms share a common short insertion in their rRNA gene sequences. The apparent affinity with the *Alicyclobaccilli* may be an artefact of 'long branch attraction' (Swofford *et al.* 1996).

Figure 4.66 :Maximum Likelihood and Maximum Parsimony trees



The descriptions: (i) MP tree: Tree length = 2234; Consistency Index (CI) = 0.4167; Retention Index (RI) = 0.3998; -Ln Likelihood = 11714.37757. (ii) ML tree: Tree length (parsimony estimate) = 2256; CI = 0.3565 RI = 0.3897; -Ln Likelihood = 11710.26014. The values on the branches indicate the level of support derived from 1000 bootstrap replicates using maximum parsimony as the optimality criterion. Where no numbers are supplied, bootstrap values are less than 50%.
A – *Alicyclobacillus*; An – *Aneurinicbacillus*; B – *Bacillus*; Ox – *Oxalobacter*; Paen – *Paenibacillus*.

Chapter 5 – Glutamate uptake by *Bacillus* strain TA2.A1

The following has been published in Journal of Bacteriology

(1999) 181(10): 3172-3177.

Sodium-Dependent Glutamate Uptake by an Alkaliphilic, Thermophilic *Bacillus* Strain TA2.A1

Catherine J. Peddie[1], Gregory M. Cook[2], and Hugh W. Morgan[1]*

Thermophile and Microbial Biochemistry and Biotechnology Unit,

University of Waikato, Hamilton[1], and

Department of Microbiology, School of Medical Sciences,

University of Otago, Dunedin[2], New Zealand

*Corresponding author. Mailing address: Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton, New Zealand.

Abstract

A strain of *Bacillus* designated TA2.A1, isolated from a thermal spring in Te Aroha, New Zealand, grew optimally at pH 9.2 and 70°C. *Bacillus* strain TA2.A1 utilized glutamate as a sole carbon and energy source for growth and sodium chloride (> 5 mM) was an obligate requirement for growth. Growth on glutamate was inhibited by monensin and amiloride, both inhibitors that collapse the sodium gradient (ΔpNa) across the cell membrane. N,N- dicyclohexylcarbodiimide inhibited the growth of *Bacillus* strain TA2.A1 suggesting that an F_1F_0-ATPase (H type) was being used to generate cellular ATP needed for anabolic reactions. Vanadate, an inhibitor of V-type ATPases, did not affect the growth of *Bacillus* strain TA2.A1. Glutamate transport by *Bacillus* strain TA2.A1 could be driven by an artificial membrane potential ($\Delta\Psi$), but only when sodium was present. In the absence of sodium, the rate of $\Delta\Psi$-driven glutamate uptake was fourfold lower. No glutamate transport was observed in the presence of ΔpNa alone (i.e., no $\Delta\Psi$). Glutamate uptake was specifically inhibited by monensin, and the K_m for sodium was 5.6 mM. The Hill plot had a slope of approximately 1 suggesting that sodium binding was noncooperative and that the glutamate transporter had a single binding site for sodium. Glutamate transport was not affected by the protonophore carbonyl cyanide m-chlorophenylhydrazone, suggesting that the transmembrane pH gradient was not required for glutamate transport. The rate of glutamate transport increased with increasing glutamate concentration; the K_m for glutamate was 2.90 µM, and the V_{max} was 0.7 nmol.min^{-1} mg of protein. Glutamate transport was specifically inhibited by glutamate analogues.

Introduction

Bacteria display a remarkable capacity to survive and grow in extremely hostile environments. Entire groups of organisms (e.g., thermophiles, halophiles, acidophiles) have adapted their lifestyles to these extreme environments. Even within a given group, a very wide range of environmental limits may be tolerated. In general terms, microorganisms are able to grow over a wide range of pH values from 0 to 11.0 (1). For example, alkaliphiles grow between pH 9.0 and pH 11.5 and maintain their intracellular pH between 8.4 and 9.0 (2, 10, 13, 17). Most aerobic alkaliphiles belong to the genus *Bacillus* (10, 13, 17). *Bacillus* species are gram-positive sporeformers which have been isolated from a diverse range of environments, including those of neutral, acidic and alkaline pH. Few bacteria growing at extremes of both pH and

temperature have been described (18, 19, 22). For example, a novel group of anaerobic alkaliphilic (eu)bacteria capable of optimal growth at 55°C and pH 9.3 (upper limit of pH 10.3) were described by Li *et al.* (18, 19). These bacteria extended the combined range of temperature and pH for bacterial groups, and one of these organisms, *Clostridium paradoxum* was subsequently shown to maintain a pH gradient of 1.3 U at an extracellular pH of 9.0 (2). Bacteria that grow aerobically at alkaline pH and extreme temperature (optimum growth temperature above 65°C) have not previously been described.

Bacteria that grow at alkaline pH are faced with bioenergetic problems in terms of chemiosmotic energy generation and solute transport driven by the proton motive force (Δp) (14). The alkaliphilic bacteria studied maintain an intracellular pH lower than the extracellular pH (inverted pH gradient). It is generally accepted that alkaliphiles use sodium/proton antiporters to acidify the cytosol and generate an inwardly directed sodium motive force (ΔpNa) (16). The use of sodium as a coupling ion circumvents the problem of a low Δp. Growth at extremes of temperature also poses the additional problem of a leaky cytoplasmic membrane to protons (28). Konings and coworkers (3, 4, 8, 25-27) and Holtom *et al.* (9) have demonstrated high proton permeability of membranes at high temperature, and some thermophilic bacteria overcome this problem by using sodium as a coupling ion for solute transport.

In this study, we describe the growth and bioenergetic properties of an aerobic, extremely thermophilic, alkaliphilic *Bacillus* strain TA2.A1. This isolate can grow over a pH range from 7.7 to 10.5, with an optimum of pH 9.2 at 70° C. The growth of this bacterium and glutamate transport were completely dependent on sodium, indicating that this organism uses sodium rather than protons in bioenergetic processes.

Materials and Methods

Chemicals. L-[U-^{14}C]glutamic acid was from Amersham International (Amersham Laboratories, Buckinghamshire, England). Amiloride-HCl, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and monensin were obtained from Sigma Chemical Co. (St Louis, Mo.). N,N-dicyclohexylcarbodiimide (DCCD) and sodium vanadate were from BDH Chemicals Ltd. (Poole, England).

Growth and maintenance. *Bacillus* strain TA2.A1 was isolated from a continuously enriched pool sample. The growth medium contained (per litre) 0.5 g of Na_2SO_4, 0.1 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4.7H_2O$, 0.2 g of K_2HPO_4, 9.0 g of $NaHCO_3$, 5.0ml of dictyglomous trace elements (23), 0.1 g of Trypticase (Oxoid), and 2.0 g of glutamate (BDH). The pH of the medium was adjusted to pH 10 (20°C), which equates to a pH of 9.2 at 70°C after autoclaving, using 2N NaOH. Then 100-ml

aliquots of the medium were dispensed into 500-ml flasks, which were stoppered with a nonabsorbent cotton wool bung and autoclaved at 15 lb/in^2 for 15 min. Cells were cultured in a 65°C orbital shaking incubator and aerated by shaking at 100 rpm. In a typical experiment, flasks were inoculated (1% inoculum) from an overnight grown culture, and cells were grown to mid-exponential phase (0.2 to 0.25 units of optical density at 450 nm).

Glutamate transport assays. Cells were harvested by centrifugation (12,000 x *g*, 5 min, 4°C) during exponential growth (0.22 mg of protein/ml) and washed twice in Tris-HCl buffer (50 mM, pH 9.2 at 70°C). The cell pellet was resuspended in the same buffer to achieve a concentration of 14 to 20 mg of protein per ml. Aliquots (200 µl) of cell suspension were placed into tubes in a shaking (70 rpm) water bath (Julabo, Labortechnik, GmbH, Seelbach, Germany) at 60°C, and transport was initiated by the addition of 100 nCi of L-[U-^{14}C]glutamate (270 mCi/mmol). After 0 to 60 s, transport was terminated by the addition of ice-cold LiCl (2 ml, 100 mM) and rapid filtration (0.45-µm-pore-size cellulose-nitrate filter). Experiments were carried out where the transport rate was first order with respect to protein, and initial rates are reported here. The filters were washed once with 2.0 ml of LiCl, dried for 30 min at 105°C, and counted by liquid scintillation. Cells which were treated with monensin (10 µM) or incubated in the absence of sodium showed essentially no [^{14}C]glutamate uptake, and this result indicated that there was little nonspecific binding of [^{14}C]glutamate to cells.

Artificial membrane potentials. To create an artificial ΔpNa and membrane potential ($\Delta\Psi$), washed cells (100 mM Tris-HCl containing 100 mM KCl, pH 9.2) from exponentially growing cultures were loaded with potassium by valinomycin treatment (10 µM, 0°C, 30 min) and diluted 50-fold (4 µl) into 200 µl of Tris-HCl buffer containing 100 mM NaCl (pH 9.0) plus 1 µM [^{14}C]glutamate. Potassium-loaded cells were diluted into either 100 mM Tris-HCl buffer containing 100 mM NaCl and 100 mM KCl to create a ΔpNa in the absence of $\Delta\Psi$ or 100 mM Tris-HCl buffer alone to create a $\Delta\Psi$. Controls (no driving force) were loaded with K^+ or K^+ and Na^+ and diluted into K^+ or K^+ and Na^+, respectively. Transport was initiated by a 50-fold dilution of concentrated cells (4 µl) into buffer (200 µl) containing the radioactive glutamate (see above).

Competition and metabolic inhibitor experiments. Competitive substrates (amino acid) and metabolic inhibitors, tested as potential inhibitors of glutamate uptake, were added to the transport assay medium 5 min before

[^{14}C]glutamate. Unlabeled amino acids were added at a final concentration of 5 mM. Metabolic inhibitors were added at the final concentrations indicated in the text. All of the water-insoluble inhibitors were dissolved in 95% ethanol and compared with ethanol-treated controls. The results of competition experiments were expressed as the mean of three determinations and the level of inhibition was expressed as the percent inhibition of the initial rate of uptake compared to controls (nominally 100%) in the absence of competitive substrate.

Results

Growth of *Bacillus* strain TA2.A1. *Bacillus* strain TA2.A1 grew rapidly on minimal medium containing L-glutamate as the sole carbon and energy source. The optimal conditions for growth were pH 9.2 and 70°C, and the maximum specific growth rate was 0.35 h^{-1} (data not shown). Sodium was an absolute requirement for cell growth (Fig. 1a). Growth was barely evident below a concentration of 5 mM NaCl and the final optical density increased as the sodium concentration in the growth medium was increased from 5 to 100 mM. Sodium concentrations greater than 100 mM were inhibitory to cell growth. These results suggested that the growth of *Bacillus* strain TA2.A1 may depend on sodium for energy generation and bioenergetic processes (i.e., transport, motility, etc). To investigate this possibility in more detail, we tested the effect of specific metabolic inhibitors on *Bacillus* strain TA2.A1 to determine whether a Δp or ΔpNa was required for growth.

The Δp, and therefore the energized state of the membrane, can be abolished by proton conductors or uncouplers such as CCCP (11). Monensin is a carboxylic ionophore that disrupts sodium or potassium gradients or both across bacterial membranes (21). When monensin was added to exponential-phase cells growing on glutamate, there was an immediate cessation of growth, even when as little as 0.1 μM was added (Fig. 1b). Growth was completely inhibited, and cells did not become resistant to monesin even after prolonged incubation. Cells challenged with CCCP (100 μM) in the exponential phase of growth were not initially inhibited, but after 2 h of further incubation there was a significant decline in the growth rate (Fig. 1b). This effect was more dramatic if higher concentrations of CCCP (500 μM) were added. Because CCCP is a weak acid and causes a collapse of Δp by cycling between the protonated and unprotonated state, this process may be somewhat reduced at high pH and hence the small effect seen on growth. When amiloride (500 μM), an inhibitor of Na^+/H^+ antiporters (12), was added to exponentially growing cells there was an immediate cessation of growth (Fig. 1c). Similarly, DCCD (25 μM), an inhibitor of

the F_1F_0-ATPase, also caused growth to cease (Fig. 1c). Vanadate (500 μM), an inhibitor of V-type ATPases, had no effect on the growth of *Bacillus* strain TA2.A1 (Fig. 1c).

Figure 1a: Effect of sodium ion concentration on the growth of *Bacillus* strain TA2.A1 in minimal medium containing glutamate.

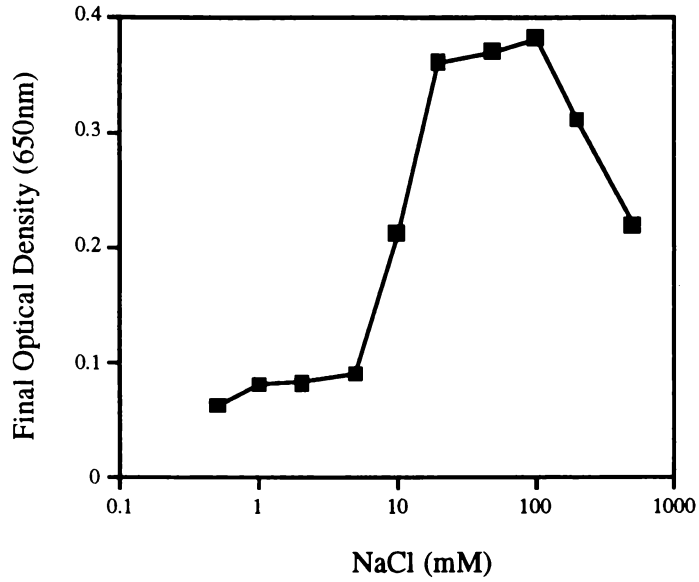
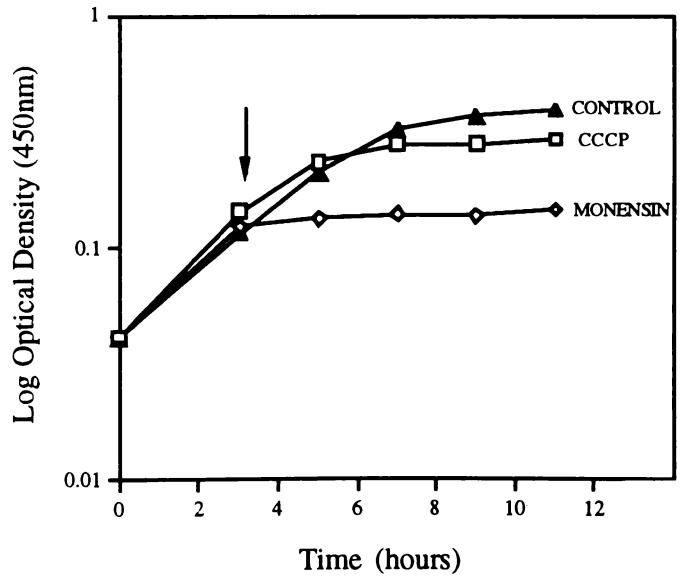
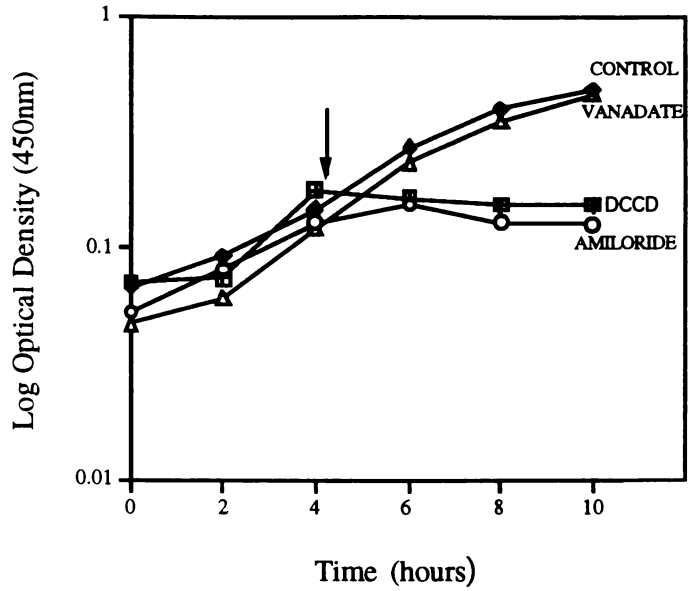


Figure 1b: Effects of monensin (0.1 μM) and CCCP (100 μM) on the growth of *Bacillus* strain TA2.A1 in minimal medium containing glutamate



100 μl of a 100% ethanol solution was added to control where inhibitor was dissolved in ethanol. Arrow indicates addition of inhibitor or ethanol.

Figure 1c: Effects of vanadate (500μM), DCCD (500μM), and amiloride (500μM) on the growth of *Bacillus* strain TA2.A1 in minimal medium containing glutamate

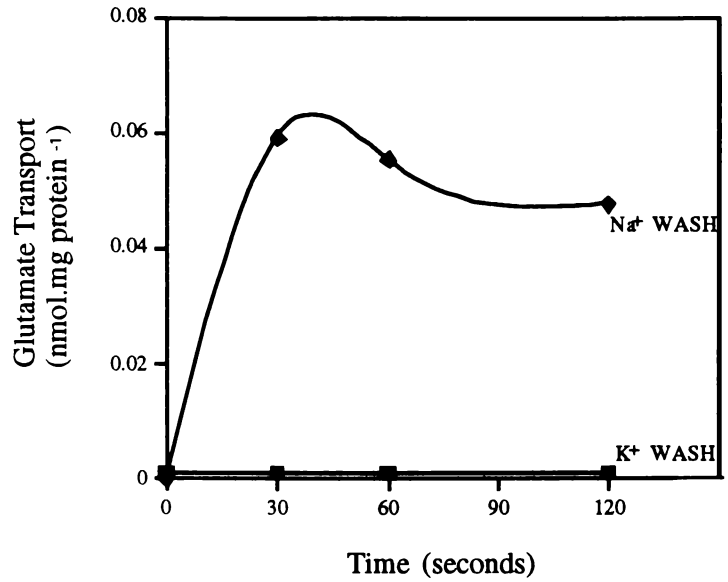


100 μl of a 100% ethanol solution was added to control where inhibitor was dissolved in ethanol. Arrow indicates addition of inhibitor or ethanol.

[^{14}C]glutamate transport. Cells grown in minimal medium containing glutamate and washed twice in Tris-HCl (100 mM, pH 9.0) containing 100 mM NaCl transported [^{14}C]glutamate at an initial rate of 0.12 nmol.min^{-1} mg of protein (Fig. 2a). However, if cells were washed in Tris-HCl (100 mM, pH 9.0) containing 100 mM KCl, little if any uptake was observed (Fig. 2a). When sodium-washed cells were preincubated with either monensin (10 μM) or amiloride (500 μM), the uptake of [^{14}C]glutamate was completely abolished (Fig. 2b). Preincubation with CCCP (10 μM) had no effect on [^{14}C]glutamate transport. To better understand this requirement for extracellular sodium, we performed experiments in which the extracellular concentration of sodium was varied. Cells resuspended in Tris-HCl (pH 9.0) containing 100 mM KCl did not transport [^{14}C]glutamate at a rate that could be differentiated from that of nonspecific [^{14}C]glutamate binding to cells (Fig. 2c). When the extracellular concentration of NaCl was increased to greater than 5 mM, the rate of [^{14}C]glutamate uptake increased and reached a maximum rate at 25 mM extracellular NaCl (Fig. 2c). Further increases in sodium concentration did not increase the rate of [^{14}C]glutamate transport. The K_m for sodium as calculated from an Eadie-Hofstee plot

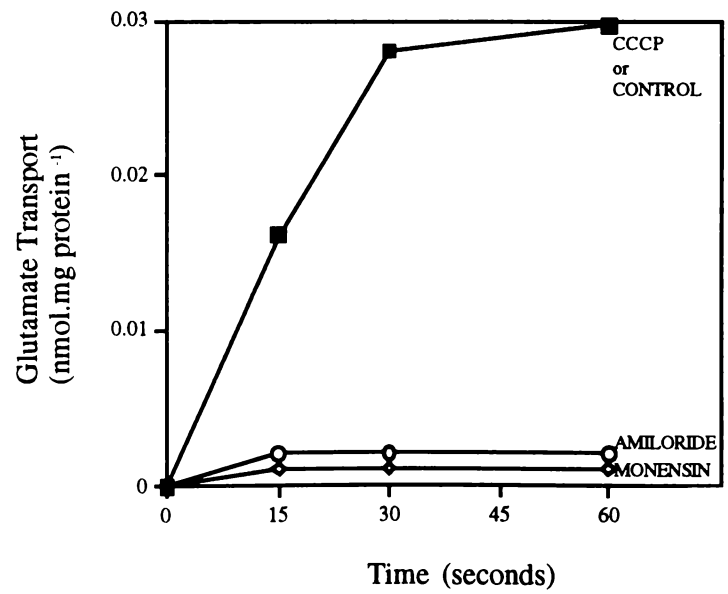
was 5.6 mM, and the slope of the Hill plot was approximately 1.0, suggesting that sodium binding was noncooperative (Fig. 2c, inset).

Figure 2a: [^{14}C]glutamate transport by washed cells of *Bacillus* strain TA2.A1 with either 100 mM NaCl or 100 mM KCl (without sodium)



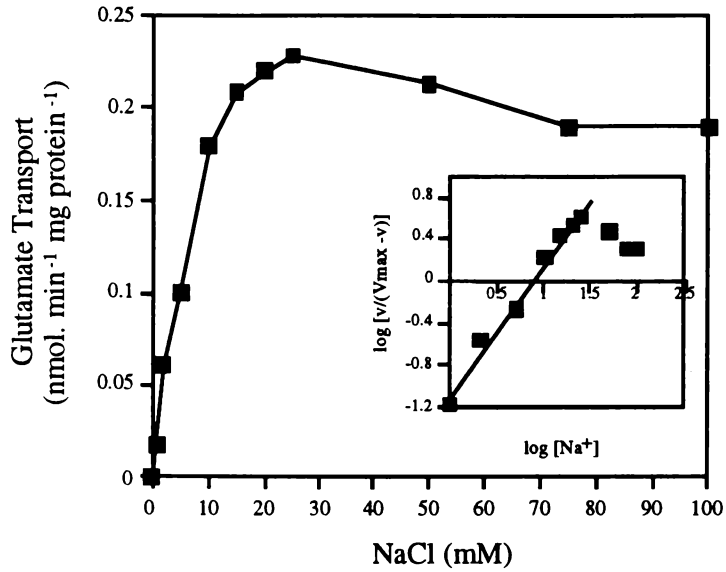
The final concentration of [^{14}C]glutamate was 1 μM

Figure 2b: Effects of monensin (10 μM), CCCP (100 μM), and amiloride (100 μM) on [^{14}C]glutamate (1 μM) transport by washed cells of *Bacillus* strain TA2.A1



The assay buffer contained 100 mM NaCl

Figure 2c: Effect of extracellular sodium chloride concentration on [^{14}C]glutamate (1 μM) uptake by washed cells of *Bacillus* strain TA2.A1

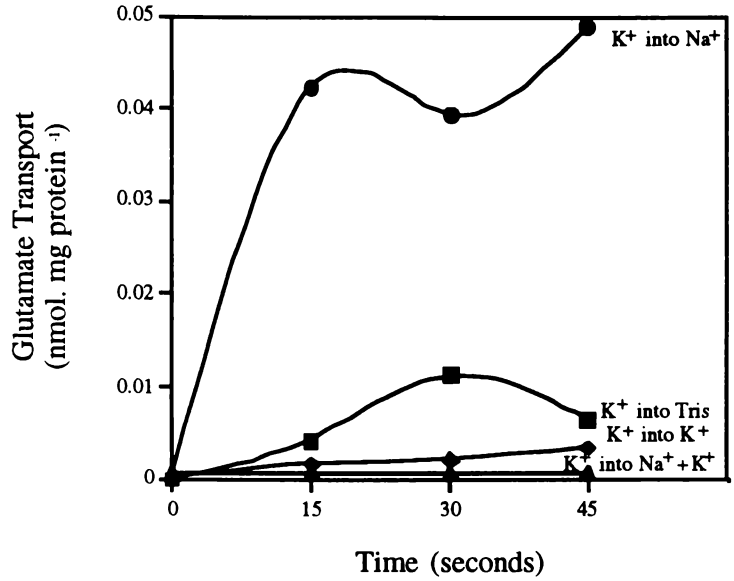


A Hill plot of the data is shown in the inset

To demonstrate that sodium and therefore a ΔpNa was the driving force for [^{14}C]glutamate transport, we used an artificially generated ΔΨ and ΔpNa to study [^{14}C]glutamate transport. When K^+-loaded cells were diluted in Tris-HCl containing 100 mM NaCl to create a ΔpNa and ΔΨ, the rate of [^{14}C]glutamate uptake was 0.16 nmol.min^{-1} mg of protein (Fig. 3a). K^+-loaded cells diluted in Tris-HCl containing both 100 mM NaCl and 100 mM KCl to create a ΔpNa in the absence of ΔΨ did not transport [^{14}C]glutamate, suggesting that ΔΨ was required for glutamate transport. An artificially generated ΔΨ (K^+-loaded cells diluted in Tris-HCl) in the absence of a ΔpNa was able to drive [^{14}C]glutamate transport, but the level was fourfold lower, indicating that sodium was required in addition to a ΔΨ. No [^{14}C]glutamate transport was observed when K^+-loaded cells were diluted into Tris-HCl containing KCl (no driving force). Other ions were tested for their ability to act as a coupling ion for [^{14}C]glutamate transport (Fig. 3b). Cells (K^+-loaded) diluted into buffer containing 100 mM NaCl transported [^{14}C]glutamate at a rate of 0.19 nmol.min^{-1} mg of protein

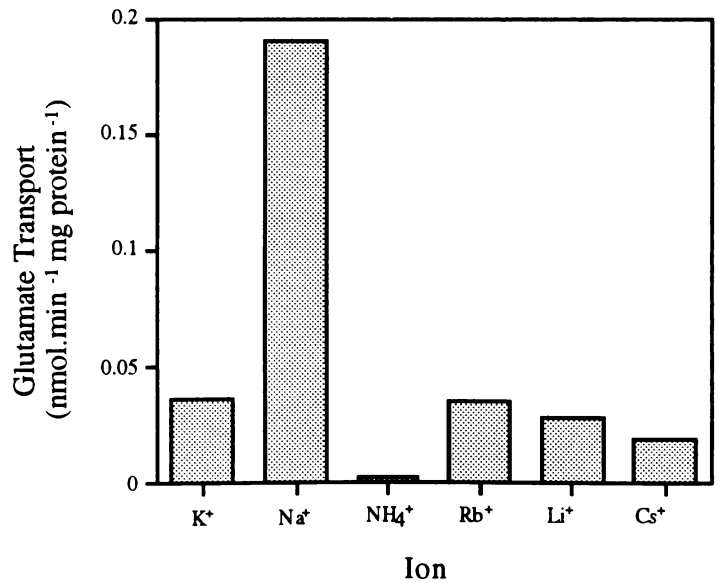
(Fig. 3b). The following ions; Rb^+, Li^+, Cs^+, NH_4^+ were ineffective in driving [^{14}C]glutamate transport (Fig. 3b).

Figure 3a: Transport of [^{14}C]glutamate by valinomycin-treated and potassium (100 mM KCl)-loaded TA2.A1 cells.



K^+-loaded cells were diluted into 100 mM Tris-HCl buffer (pH 9.2) to create a $\Delta\Psi$, Tris HCl buffer containing either 100 mM KCl (no driving force), 100 mM NaCl to create a $\Delta\Psi$ and a ΔpNa, or 100 mM NaCl and 100 mM KCl to create a ΔpNa in the absence of $\Delta\Psi$.

Figure 3b: Cation specificity of the glutamate uptake system of *Bacillus* strain TA2.A1



Transport of [^{14}C]glutamate (1 μM) was measured in valinomycin-treated, potassium-loaded cells, which were diluted into Tris-HCl (pH 9.2) containing 100 mM KCl, NaCl, NH_4Cl, RbCl, LiCl, or CsCl.

[^{14}C]glutamate transport versus glutamate concentration, temperature and pH. The rate of glutamate uptake was studied over a range of glutamate concentrations, pH values and temperatures. The pH range for glutamate uptake correlated well with the pH profile for growth, with greatest uptake at pH 9.5 and low levels of uptake at pH 7.5 and pH 11.0 (Fig. 4a). Maximum [^{14}C]glutamate uptake was observed at 60°C and the rate decreased rapidly above this temperature. Temperatures below 40°C decreased the rate by 50% (Fig. 4b). When the extracellular glutamate concentration was increased from 1 μM to 50 μM, the rate of glutamate transport increased rapidly and saturation kinetics were observed (Fig. 4c). The Eadie-Hofstee plot was linear; the K_m for glutamate was 2.90 μM and the V_{max} was 0.7 nmol.min^{-1} mg protein (Fig. 4c inset).

Figure 4a: Effect of extracellular pH on the rate of [^{14}C]glutamate (1 μM) transport by washed cells of *Bacillus* strain TA2.A1

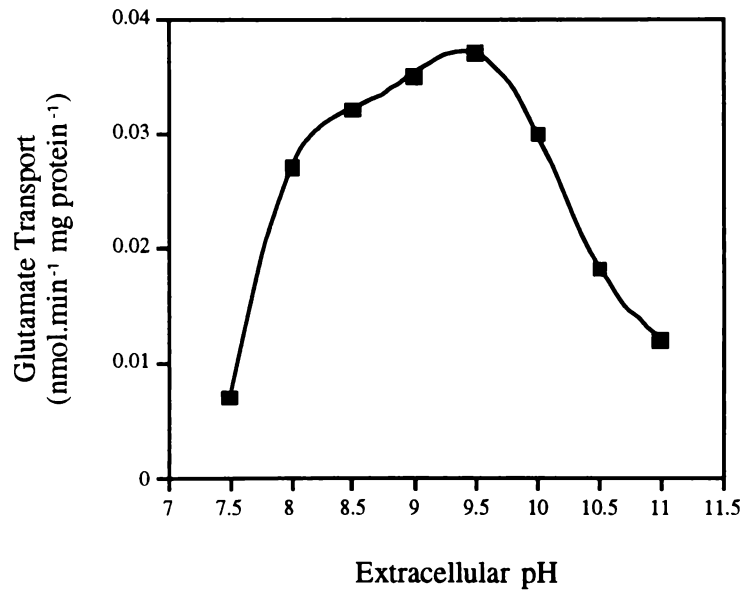


Figure 4b: Effect of temperature on the rate of [^{14}C]glutamate (1 μM) transport by washed cells of *Bacillus* strain TA2.A1

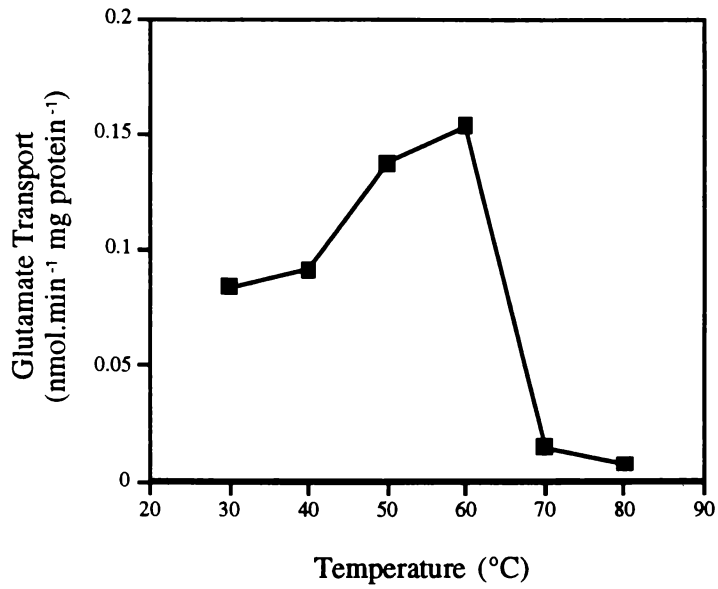
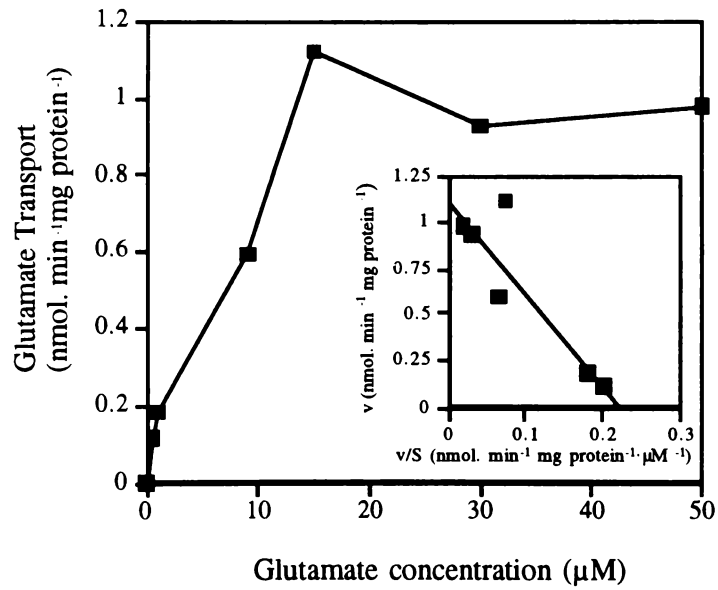


Figure 4c: Effect of glutamate concentration on the rate of [^{14}C]glutamate (1 μM) transport by washed cells of *Bacillus* strain TA2.A1



An Eadie-Hofstee plot of glutamate transport is shown in the inset

Competitive amino acids of [^{14}C]glutamate transport. The specificity of the glutamate transport system was determined by measuring the uptake of [^{14}C]glutamate in the presence of a 50-fold excess of other amino acids (Table 1). [^{14}C]Glutamate uptake was specifically inhibited by L-glutamate, D-proline, and the

glutamate analogues DL-α-methyl-glutamate, L-cysteate, and D- and L-aspartate (Table 1). D-glutamate had no effect on [^{14}C]glutamate uptake suggesting that the transporter was specific for the L isomer.

Table 1. Effects of competing amino acids on the uptake of [^{14}C]glutamate by *Bacillus* strain TA2.A1[a]

Competing amino acid (5 mM)	Inhibition (% of control level)
D-Aspartate	57
L-Aspartate	60
L-Cysteate	50
L-Glutamate	93
α-methyl–glutamate	45
D-Proline	38
L-Serine	50

[a] The following amino acids had no (<10%) effect on [^{14}C]glutamate uptake: D-arginine, L-arginine, DL-asparagine, D-glutamate, L-glutamine, L-proline, and D-serine.

Discussion

Bacteria that grow at extremes of pH and temperature are confronted by two bioenergetic problems. Firstly, at high growth temperatures, the cytoplasmic membrane becomes more permeable to ions, including protons, and therefore the use of protons as coupling ions for solute transport and ATP generation has limitations (20, 27, 28). The use of sodium as a coupling ion for bioenergetic processes can be an important energetic advantage because phospholipid membranes are 6 to 10 orders of magnitude less permeable for sodium compared to protons (5, 28). The second bioenergetic problem is that of alkaline pH. The acidification of cytoplasmic pH at alkaline extracellular pH creates special bioenergetic problems. If the pH gradient is reversed ($pH_{in} < pH_{out}$), the electrical potential must increase to prevent an overall decline in the total Δp. This chemiosmotically adverse pH gradient is bypassed by the

use of an electrochemical gradient of Na^+ rather than of protons to energize solute uptake and motility (1, 16, 17, 22). In this study, we describe the growth of an obligate aerobic, alkaliphilic, thermophilic *Bacillus* isolate, strain TA2.A1, which has a pH and temperature optimum of 9.2 and 70° C, respectively. Microbial growth under these conditions has not been previously described. Growth of *Bacillus* strain TA2.A1 was completely dependent on sodium (> 5 mM) and was inhibited by monensin and amiloride, inhibitors that collapse the ΔpNa and inhibit Na^+/H^+ antiporters, respectively (12, 21).

Because *Bacillus* strain TA2.A1 is an obligate aerobe and has a growth requirement for sodium, it was of further interest to determine what coupling ion this bacterium used to drive bioenergetic processes such as transport. *Bacillus* strain TA2.A1 grew on glutamate as the sole carbon and energy source in minimal medium. To study the precise nature of the driving force for glutamate transport, glutamate uptake in response to an artificially imposed ion gradient was used. Glutamate transport could be driven by an artificially created ΔpNa, but only in the presence of a $\Delta\Psi$. $\Delta\Psi$ alone was a weak driving force for glutamate transport. When both gradients, i.e. ΔpNa and $\Delta\Psi$, were applied, the highest rate of glutamate transport was observed. These results demonstrate that glutamate uptake is driven by $\Delta\Psi$ with sodium as a coupling ion. Because CCCP had no effect on glutamate transport, the transmembrane pH gradient ($Z\Delta pH$) does not seem to play an important role in glutamate uptake. The use of different monovalent cations to create an artificial membrane potential demonstrated that only sodium was able to couple effective glutamate uptake by *Bacillus* strain TA2.A1. Two glutamate transport mechanisms have been described for thermophilic bacteria. In *Bacillus stearothermophilus*, L-glutamate (or L-aspartate) transport proceeds via a sodium/proton symport mechanism with a 1:1:1 stoichiometry (3, 4, 8). Glutamate transport in other thermophilic bacteria has also been shown to depend on an electrochemical gradient of sodium. For example, *Clostridium fervidus*, a fermentative bacterium, has been shown to transport glutamate electrogenically ($\Delta\Psi$ driven) in symport with two Na^+ molecules and not by $Z\Delta pH$ (26). Glutamate transport by the aerobic thermophilic bacterium *Thermus thermophilus* was also shown to be catalysed by a sodium/glutamate symport mechanism (9).

Na^+ is the predominant ion for solute transport in obligately alkaliphilic bacteria, and often in these bacteria the Na^+-solute symporter has a low affinity for sodium (16). The Na^+-H^+-L-glutamate transport system in the thermophilic organism

B. stearothermophilus has a very high affinity for sodium (K_m < 5.5 μM) (8). In the present study, the effect of sodium ion concentration on glutamate transport followed Michaelis-Menten kinetics. The Hill plot suggested that the glutamate transporter had only one binding site for sodium. The K_m for sodium was 5.6 mM, and glutamate transport was completely abolished by 0.1 μM monensin, but not the protonophore CCCP. The results of this study indicate that *Bacillus* strain TA2.A1 transports glutamate electrogenically ($\Delta\Psi$ driven) in symport with sodium. Like other sodium/glutamate symporters (5), the affinity for sodium is low (>5 mM).

Initial rates of L-glutamate uptake in *B. stearothermophilus* have been shown to be strongly dependent on the medium pH. Glutamate uptake was highest at low external pH (5.5 to 6.0) and declined with increasing pH (4, 8). Glutamate transport by *Bacillus* strain TA2.A1 was also affected by the extracellular pH, and the maximum rate was observed at an extracellular pH of 9.5. The K_m for glutamate was 2.90 μM, and glutamate uptake by *Bacillus* strain TA2.A1 was specifically inhibited by the glutamate analogues DL-α-methylglutamate, L-cysteate, and D- and L-aspartate. The closely related amines L-glutamine and DL-asparagine did not inhibit glutamate transport. The glutamate uptake system of *C. fervidus* is not competitively inhibited by α-methylglutamate (26), suggesting that the glutamate transporter from *Bacillus* strain TA2.A1 is more like the glutamate transporter in *E. coli* (6, 7) that is specifically inhibited by this analogue (24). The glutamate transporter in *B. stearothermophilus* is specific for acidic amino acids and is also most similar to that of *E. coli* (7, 24).

Despite the inverted pH gradient and the reduced Δp in alkaliphiles, ATP synthesis occurs via completely proton coupled oxidative phosphorylation, but the mechanism for this remains unknown (14, 15). The Na^+-ATPase from the thermophile *C. fervidus* functions as a Na^+-extruding ATPase, stimulated to the same extent by both LiCl and NaCl (25). Speelmans *et al.* (25) found no evidence for an additional H^+-pumping ATPase. *Bacillus* strain TA2.A1 grows aerobically and presumably generates the bulk of its Δp by respiration. CCCP had no effect on glutamate transport, but growth did slow in the presence of this protonophore, suggesting that the collapse of the Δp causes a decrease in ATP synthesis and hence anabolic reactions such as growth. DCCD, an inhibitor of the F_1F_0-ATPase (H type), inhibited growth, but vanadate, an inhibitor of V-type ATPases, had no effect on the growth of *Bacillus* strain TA2.A1. Further studies are needed to determine the precise mode of ATP generation in *Bacillus* strain TA2.A1.

Acknowledgements

C. J. Peddie was supported by a University of Waikato Post-Graduate Fees Scholarship. G.M. Cook was supported by a travel grant from the School of Medical Sciences, University of Otago, Dunedin, New Zealand.

References

1. **Booth, I. R.** 1985. Regulation of cytoplasmic pH. Microbiol. Rev. **49**:359–378.

2. **Cook, G. M., J. B. Russell, A. Reichert, and J. Wiegel.** 1996. The intracellular pH of *Clostridium paradoxum* , an anaerobic, alkaliphilic, and thermophilic bacterium. Appl. Environ. Microbiol. **62**:4576–4579.

3. **de Vrij, W., G. Speelmans, R. I. R. Heyne, and W. N. Konings.** 1990. Energy transduction and amino acid transport in thermophilic aerobic and fermentative bacteria. FEMS Microbiol. Rev. **75**:183-200.

4. **de Vrij, W., R. A. Bulthuis, P. R. van Iwaarden, and W. N. Konings.** 1989. Mechanism of L-glutamate transport in membrane vesicles from *Bacillus stearothermophilus*. J. Bacteriol. **171**:1118-1125.

5. **Dimroth, P.** 1987. Sodium ion transport decarboxylases and other aspects of sodium cycling in bacteria. Microbiol. Rev. **51**:320-340.

6. **Fujimura, T., I. Yamato, and Y. Anraku.** 1983a. Mechanism of glutamate transport in *Escherichia coli B*. 1. Proton-dependent and sodium-ion dependent binding of glutamate to a glutamate carrier in the cytoplasmic membrane. Biochemistry **22**:1954-1959.

7. **Fujimura, T., I. Yamato, and Y. Anraku.** 1983b. Mechanism of glutamate transport in *Escherichia coli B*. 2. Kinetics of glutamate transport driven by artificially imposed proton and sodium ion gradients across the cytoplasmic membrane. Biochemistry **22**:1959-1965.

8. **Heyne, R. I. R., W. de Vrij, W. Crielaard, and W. N. Konings.** 1991. Sodium ion-dependent amino acid transport in membrane vesicles of *Bacillus stearothermophilus*. J. Bacteriol. **173**:791-800.

9. **Holtom, G. J., R. J. Sharp, and R. A. D. Williams.** 1993. Sodium-stimulated transport of glutamate by *Thermus thermophilus* strain B. J. Gen. Microbiol. **139**:2245-2250.

10. **Horikoshi, K.** 1991. Microorganisms in alkaline environments. Kodansha Ltd, Tokyo, Japan, and VCH Publishers Inc., Weinheim, Germany.

11. **Kaback, H. R., J. P. Reeves, S. A. Short, and F. J. Lombardi.** 1974. Mechanisms of active transport in isolated bacterial membrane vesicles. XVIII.

The mechanism of action of carbonylcyanide m-chlorophenylhydrazone. Arch. Biochem. Biophys. **160**:215- 222.

12. **Kleyman, T. R., and E. J. J. Cragoe.** 1988. Amiloride and its analogs as tools in the study of ion transport. J. Membr. Biol. **105**:1-21.

13. **Kroll, R. G.** 1990. Alkalophiles, p. 55-92. *In* C. Edwards (ed.), Microbiology of extreme environments. Mc Graw-Hill, New York, N.Y.

14. **Krulwich, T. A., M. Ito, R. Gilmour, M. G. Sturr, A. A. Guffanti, and D. B. Hicks.** 1996. Energetic problems of extremely alkaliphilic aerobes. Biochim. Biophys. Acta **1275**:21-26.

15. **Krulwich, T. A., and A. A. Guffanti.** 1992. Proton-coupled bioenergetic processes in extremely alkaliphilic bacteria. J. Bioenerg. Biomembr. **24:** 587-599.

16. **Krulwich, T. A., and A. A. Guffanti.** 1989. The Na^+ cycle of extreme alkalophiles: a secondary Na^+/H^+ antiporter and Na^+/solute symporters. J. Bioenerg. Biomembr. **21**:663-678.

17. **Krulwich, T. A.** 1986. Bioenergetics of alkalophilic bacteria. J. Membr. Biol. **89**:113-125.

18. **Li, Y., M. Engle, N. Weiss, L. Mandelco, and J. Wiegel.** 1994. *Clostridium thermoalcaliphilium* sp. nov., an anaerobic and thermotolerant facultative alkaliphile. Int. J. Syst. Bacteriol. **44**:111-118.

19. **Li, Y., L. Mandelco, and J. Wiegel.** 1993. Isolation and characterization of a moderately thermophilic anerobic alkalophile, *Clostridium paradoxum* sp. nov. Int. J. Syst. Bacteriol. **43**:450-460.

20. **Nichols, J. W., and D. W. Deamer.** 1980. Net proton-hydroxyl permeability of large unilamellar liposomes measured by an acid-base titration technique. Proc. Natl. Acad. Sci. USA **77**:2038-2042.

21. **Pressman, B. C.** 1976. Biological applications of ionophores. Annu. Rev. Biochem. **45**:501-530.

22. **Prowe, S. G., J. L. van de Vossenburg, A. J. Driessen, G. Antranikian, and W. N. Konings.** 1996. Sodium-coupled energy transduction in the newly isolated thermoalkaliphilic strain LBS3. J. Bacteriol. **178**:4099-4104.

23. **Saiki, T., K. Yasuhiko, and K. Kawagoe.** 1985. *Dictyoglomus thermophilum* gen. nov., sp. nov., a chemoorganotrophic, anaerobic, thermophilic bacterium. Int. J. Syst. Bacteriol. **35**:253-259.

24. **Schellenberg, G. D., and C. E. Furlong.** 1977. Resolution of the multiplicity of the glutamate and aspartate transport systems of *Escherichia coli.* J. Biol. Chem. **252**:9055-9064.

25. **Speelmans, G., B. Poolman, T. Abee, and W. N. Konings.** 1994. The F- or V-type Na^+-ATPase of the thermophilic bacterium *Clostridium fervidus.* J. Bacteriol. **176**:5160-5162.

26. **Speelmans, G., B. Poolman, and W. N. Konings.** 1993. Amino acid transport in the thermophilic anerobe *Clostridium fervidus.* is driven by an electrochemical sodium gradient. J. Bacteriol. **175**:2060-2066.

27. **Tolner, B., B. Poolman, and W. N. Konings.** 1997. Adaptation of microorganisms and their transport systems to high temperatures. Comp. Biochem. Physiol. A **118**:423-428.

28. **van de Vossenburg, J. L., T. Ubbink-Kok, M. G. Elferink, A. J. Driessen, and W. N. Konings.** 1995. Ion permeability of the cytoplasmic membrane limits the maximum growth temperature of bacteria and archaea. Mol. Microbiol. **18**:925-932.

Chapter 6 - Utilization of sucrose by *Bacillus* isolate TA2.A1

6.1 Introduction

Konings and others (de Vrij *et al.* 1990, 1989; Heyne *et al.* 1991; Speelmans *et al.* 1993, 1994; Tolner *et al.* 1997) and Holtom *et al.* (1993) have demonstrated high proton permeability of membranes at high temperature suggesting that bacteria that grow at high temperature may have membranes that are leaky to protons. Some thermophilic bacteria overcome this problem by using sodium as a coupling ion for solute transport especially in the case of amino acids (Speelmans *et al.* 1989; De Vrij *et al.* 1990; Heyne *et al.* 1991). It has been demonstrated earlier that *Bacillus* isolate TA2.A1 uses sodium as a coupling ion for the uptake of glutamate (Chapter 5). Since this isolate also utilizes sucrose as a carbon substrate, investigations were conducted into whether sodium was also required for sugar transport. Isolate TA2.A1 grows on sucrose and therefore the transport of this sugar was studied.

6.2 Materials and Methods

6.2.1 Chemicals and Growth

Chemicals were supplied as follows: [U-^{14}C]sucrose (442 mCi/mmol) (Du Pont NEN®); amiloride-HCl, carbonyl cyanide m-chlorophenylhydrazone (CCCP), monensin (Sigma); 2,4-dinitrophenol (2,4-DNP) (BDH).

Minimal medium (Section 3.3.1.2) was prepared and inoculated as outlined in Section 3.2.5.4. Sucrose was added from a filter sterilised (0.45μm) stock solution (10% w/v) immediately prior to inoculation to give a final concentration, unless otherwise stated, of 0.5 g.l^{-1}. Growth was assessed as outlined in Section 3.2.3.

6.2.2 Sucrose transport assays

Cells grown in sucrose supplemented minimal medium were harvested by centrifugation (12,000 x g, 5 min, 4°C) during exponential growth (0.22 mg protein/ml) and washed twice in Tris-HCl buffer (50mM, pH 9.2). The cell pellets were resuspended in the same buffer to achieve a final concentration of 14-20 mg protein per ml. Aliquots of 200 µl of cell suspension were placed into tubes in a shaking water bath (Julabo) at 60°C with a shaking speed of 70rpm and transport was initiated by the addition of [U-^{14}C]sucrose diluted 1:1 in 1mM cold sucrose giving a final sucrose concentration per assay of 12µM. Four microlitres of diluted [^{14}C]sucrose was used for each assay of 200µl washed cells. After 0 to 60 seconds, transport was terminated by the addition of ice cold LiCl (2 ml, 100 mM) and rapid filtration (0.45 µm pore size cellulose-nitrate filter). Preliminary experiments indicated that the transport rate was first order with respect to protein concentration from 0 to 20 seconds, and initial rates were always measured over this time interval. The filter was washed once with 2.0ml of LiCl, dried for 30 min at 105°C, and counted by liquid scintillation. Preliminary experiments indicated that virtually all of the [^{14}C]sucrose was either trapped in the cells or remained in the extracellular supernatant.

6.2.3 Competition and metabolic inhibitor experiments

Competitive substrates and metabolic inhibitors, tested as potential inhibitors of sucrose uptake, were added to the transport assay medium 5 min prior to initiating uptake. Unlabeled carbohydrates were added at a final concentration of 50µM. The following inhibitors were added at the final concentration: amiloride (5µM), CCCP (5µM), 2,4-DNP (500µM), monensin (10µM). All of the water-insoluble inhibitors were dissolved in 95 % ethanol and compared with ethanol-treated controls.

6.2.4 Direct and indirect measurement of glucose release

Culture samples were centrifuged and the resulting pellets resuspended in 50mM MOPS buffer (pH 7) before being sonicated at 4°C. Following centrifugation, sonicate supernatant samples were placed in 50mM MOPS (pH 7) containing 5mM sucrose and or 5mM trehalose. After pre-incubation at 65°C, glucose oxidase (GOD) assays (Boehringer Mannheim) were performed. Glucose release was also measured via HPLC (Dionex) using a Carbopac PA1 column and an isocratic 150mM NaOH solvent system as outlined in section 4.4.2.1.1. Indirect evidence of sucrase and trehalase activity was achieved through pNP (para-nitro phenyl) assays as outlined in Sections 4.4.2.1.1 and 4.4.2.2.1. The only substrate that gave a positive assay result, shown by para-nitro phenyl release, was p-Nitrophenyl-α–D glucopyranoside. The optimal conditions for the p-Nitrophenyl-α–D glucopyranoside assay were as outlined in Section 4.4.2.1.1.

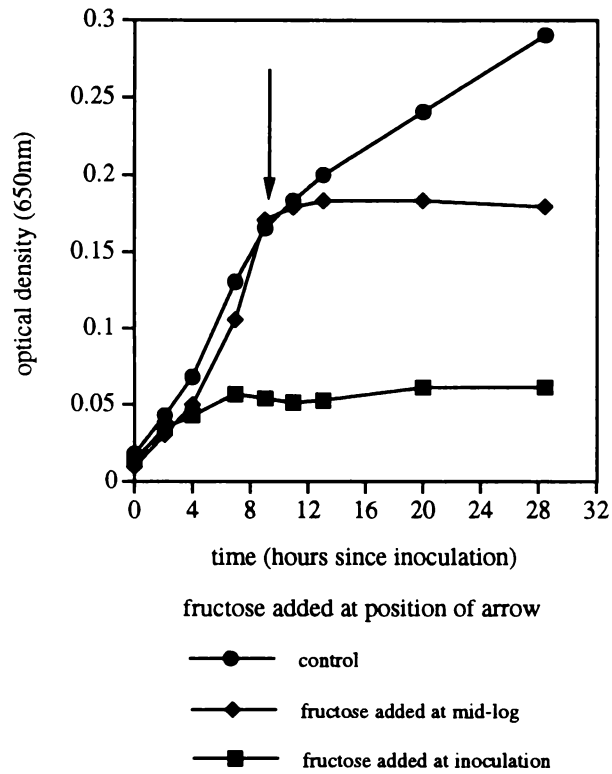
6.3 Results

6.3.1 Growth

Bacillus isolate TA2.A1 can utilize a number of di- and trisaccharides for growth (Table 4.9). Although it can grow on sucrose in the presence of $0.1 g.l^{-1}$ peptone and on trehalose it is unable to utilize the monosaccharide components of these disaccharides, namely fructose and glucose. The fact that strain TA2.A1 was unable to grow on glucose and fructose may be in part due to the instability of these compounds under the growth conditions of high pH and temperature (Figure 4.33). As illustrated earlier (Figure 4.35b) HPLC analysis of these compounds under growth conditions showed that they disappear from the growth medium at the same rate regardless of whether the medium is inoculated or not. This suggested that these compounds were unable to remain in the growth medium, under these conditions, for long enough to be metabolised. During growth in minimal medium with sucrose as the carbon source, no increase in glucose or fructose in the medium was detected. To ascertain whether monosaccharides had any effect on the growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with $0.5 g.l^{-1}$ sucrose, glucose or fructose ($2 g.l^{-1}$ final concentration) were added either at inoculation or mid log to a minimal medium and sucrose culture.

Fructose was inhibitory to growth of *Bacillus* isolate TA2.A1 on sucrose four hours after addition at inoculation or immediately after addition at mid-log phase (Figure 6.1a).

Figure 6.1a: Growth of *Bacillus* isolate TA2.A1 in sucrose supplemented minimal medium with fructose ($2g.l^{-1}$) added at inoculation or at mid-log



Glucose added at inoculation to the minimal medium and sucrose culture was not inhibitory for the first eight hours, after which growth was inhibited (Figure 6.1b). Glucose added to isolate TA2.A1 cells grown on sucrose in mid-log phase had no effect on growth, with growth resembling that achieved by the control sucrose culture, even twenty hours after glucose addition (Figure 6.1b). Glucose had a much weaker effect on the growth of *Bacillus* isolate TA2.A1 on sucrose than fructose. The glucose analogues, 2-deoxyglucose and α-methyl glucopyranoside had no effect on growth of isolate TA2.A1 cells on sucrose (data not shown).

Figure 6.1b: Growth of *Bacillus* isolate TA2.A1 in sucrose supplemented minimal medium with glucose ($2g.l^{-1}$) added at inoculation or at mid-log

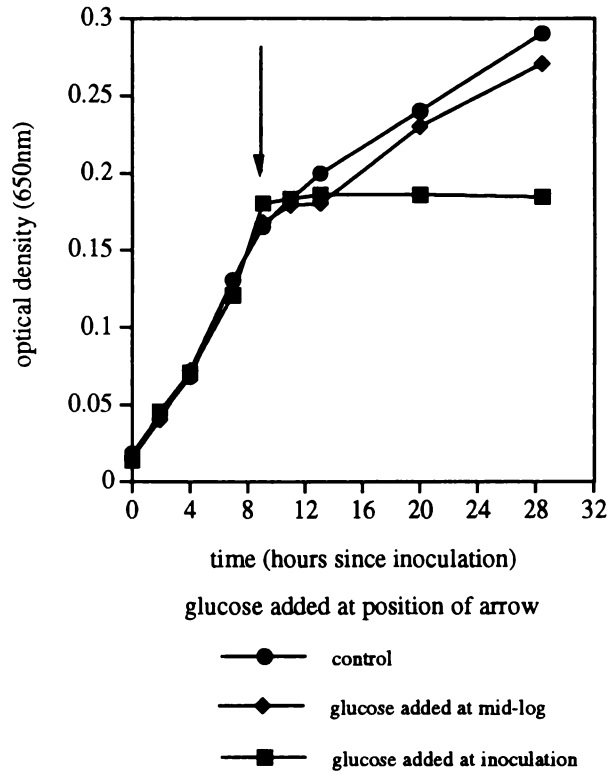
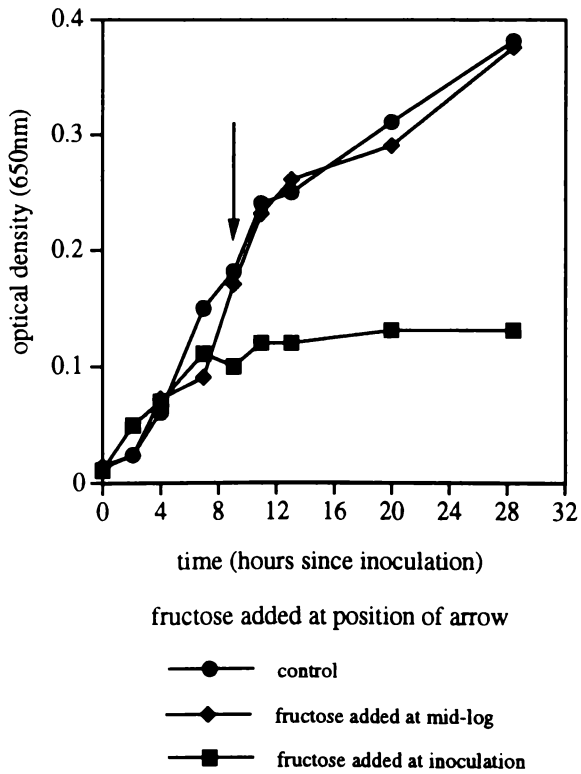
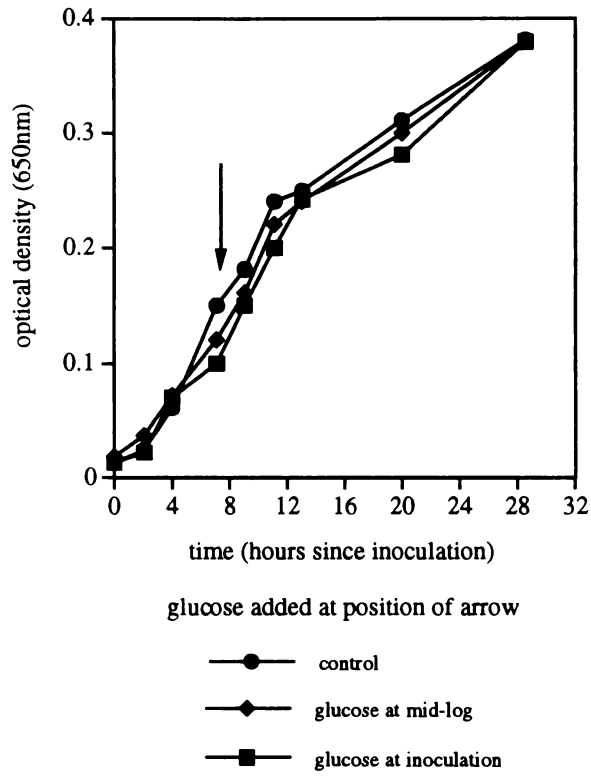


Figure 6.2a: Growth of *Bacillus* isolate TA2.A1 in trehalose supplemented minimal medium with fructose ($2g.l^{-1}$) added at inoculation or at mid-log



Minimal medium was supplemented with 0.5g.l^{-1} trehalose and had glucose or fructose (2g.l^{-1} final concentration) added at inoculation or mid log phase. Fructose was inhibitory to growth on trehalose eight hours after addition at inoculation however, the same time interval after fructose addition at mid-log phase growth was not inhibited (Figure 6.2a). Glucose added at inoculation or at mid-log phase had no effect on the growth of *Bacillus* isolate TA2.A1 on trehalose (Figure 6.2b).

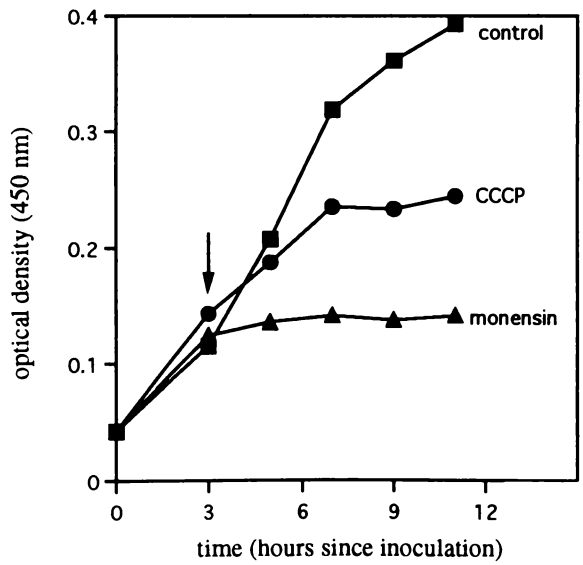
Figure 6.2b: Growth of *Bacillus* isolate TA2.A1 in trehalose supplemented minimal medium with glucose (2g.l^{-1}) added at inoculation or at mid-log



As reported earlier (Section 4.1.2.3) the presence of sodium ions was an absolute requirement for cell growth. Growth was barely evident below a concentration of 5mM NaCl and the yield and growth rate increased as the sodium ion concentration in the growth medium was increased from 5-100mM (Figure 4.4). Sodium concentrations greater than 100mM were inhibitory to growth. It is possible that strain TA2.A1 depends on sodium ions for energy generation and bioenergetic processes (i.e., transport, motility

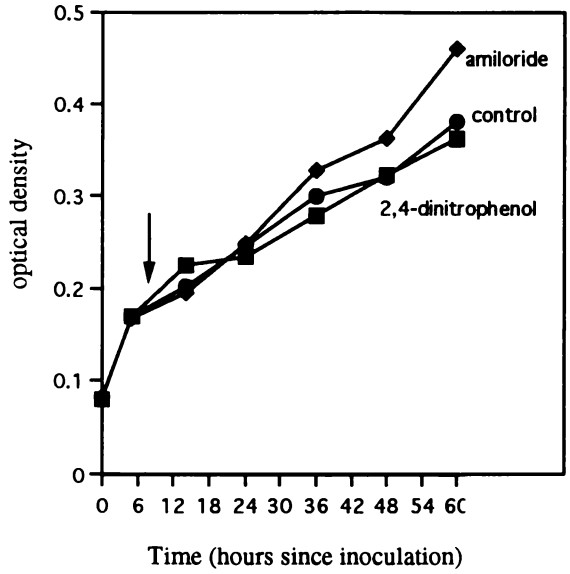
etc). To investigate this in more detail, the effect of various metabolic inhibitors on the growth of strain TA2.A1 was assessed to ascertain whether a proton-motive force ($\Delta\mu H^+$) or sodium motive motive force ($\Delta\mu Na^+$) was required for growth of strain TA2.A1 on sucrose. The Δp (and therefore the energized state of the membrane) can be abolished by proton conductors or uncouplers such as CCCP (Kaback *et al.* 1974). Monensin is a carboxylic ionophore that disrupts sodium ($\Delta\mu Na^+$) or potassium gradients or both across bacterial membranes. When monensin was added to cells growing (exponential phase) on sucrose there was an immediate cessation of growth, even when as little as 0.1μM was added (Figure 6.3). Growth was completely inhibited and cells did not become resistant to monesin even after prolonged incubation. Cell lysis was not evident. The addition of CCCP had an inhibitory effect on the growth of *Bacillus* isolate TA2.A1 on sucrose, although the effect was not as complete as the effect of monensin (Figure 6.3). Because CCCP is a weak acid and causes a collapse of Δp by cycling between the pronated and unpronated state, this process may be somewhat reduced at high pH and hence the effect on growth is not as pronounced.

Figure 6.3: The effect of addition of CCCP (100μM) or monensin (0.1μM) on the growth of *Bacillus* isolate TA2.A1 in sucrose supplemented minimal medium



100μl of 100% ethanol was added as a control. Arrow indicates addition of inhibitor or ethanol.

Figure 6.4: The effect of addition of 2,4-dinitrophenol (500μM) or amiloride (500μM) on the growth of *Bacillus* isolate TA2.A1 in sucrose supplemented minimal medium



100μl of 100% ethanol was added as a control. Arrow indicates addition of inhibitor or ethanol

Amiloride, an inhibitor of Na^+/H^+ antiporters had no effect on the growth of *Bacillus* isolate TA2.A1 on sucrose (Figure 6.4). 2,4-DNP a protonophore which abolishes the $\Delta\mu H^+$ by catalysing the transfer of protons across the membrane (Pressman 1976) had no effect on the growth of isolate TA2.A1 on sucrose, which suggests that the isolate is not dependent on a Δp for energizing sucrose transport (Figure 6.4).

Figure 6.5a: Growth of *Bacillus* isolate TA2.A1 under fermenter conditions in minimal medium with 0.5g.l^{-1} sucrose

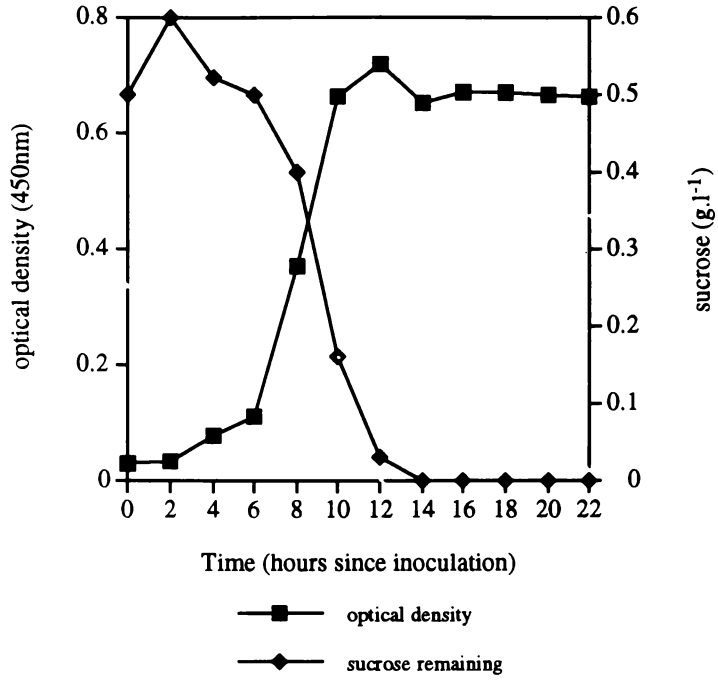


Figure 6.5b: Growth of *Bacillus* isolate TA2.A1 under fermenter conditions in minimal medium with 0.5g.l^{-1} trehalose

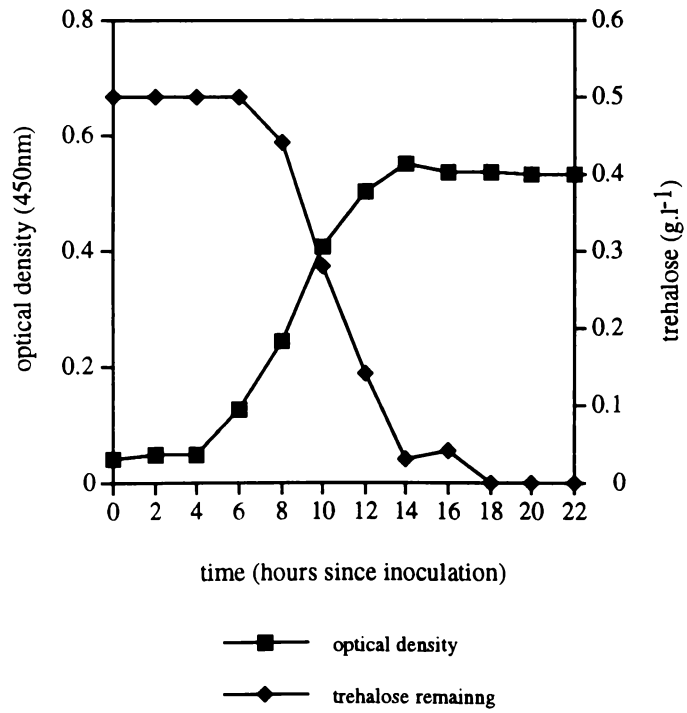
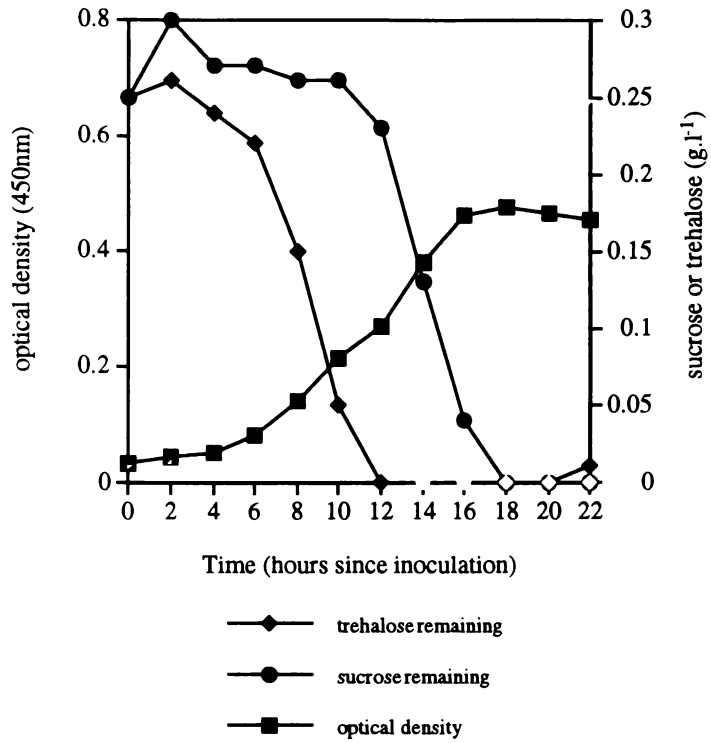


Figure 6.5c: Growth of *Bacillus* isolate TA2.A1 under fermenter conditions in minimal medium with 0.25g.l^{-1} sucrose and 0.25g.l^{-1} trehalose

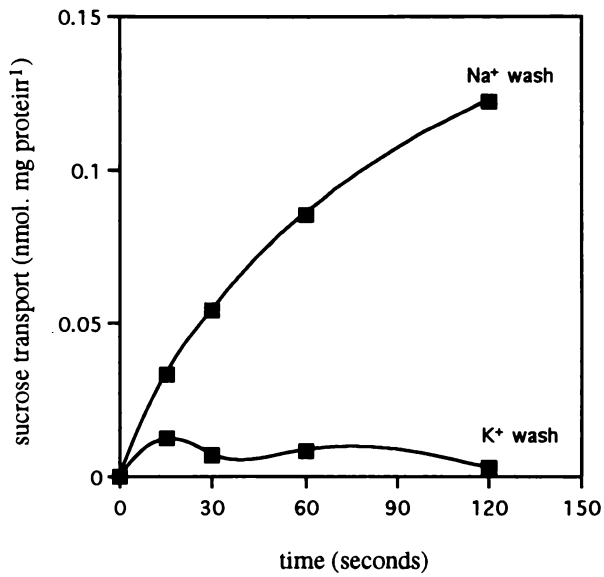


Strain TA2.A1 was grown under fermenter conditions in minimal medium with sucrose (Figure 6.5a) or trehalose (Figure 6.5b) as carbon substrates (0.5g.l^{-1}), a higher cell yield was obtained from growth on sucrose. When isolate TA2.A1 was grown under optimal conditions in minimal medium with equal concentrations of sucrose and trehalose (0.25g.l^{-1}) in a fermenter the cell yield was similar to that from growth on trehalose alone (Figure 6.5c). The doubling times (in minutes) were trehalose 78, sucrose 103, sucrose and trehalose 170 minutes. The disappearance of each carbohydrate from the medium during growth was monitored via HPLC (Dionex). The effect of enzymatic action of growth culture samples on sucrose or trehalose substrates measured by glucose oxidase assays supported the initial action of a trehalase enzyme, followed by the action of a sucrase enzyme.

6.3.2 Transport

The dependence of *Bacillus* isolate TA2.A1 on sodium for sucrose transport was proven when cells grown on minimal medium containing sucrose which were washed twice in Tris-HCl (100mM, pH 9.0) containing 100mM NaCl transported [^{14}C]sucrose whereas if the cells were washed in sodium free buffer (50mM Tris-HCl pH 10) [^{14}C]sucrose uptake was greatly reduced (Figure 6.6). Pre-incubation with CCCP (5µM) had no inhibitory effect on [^{14}C]sucrose transport - the uptake of [^{14}C]sucrose in fact increased (Figure 6.7).

Figure 6.6: [^{14}C]sucrose transport by washed cells of *Bacillus* isolate TA2.A1 with 100mM NaCl or without sodium (100mM KCl)



The specificity of the Na^+-dependent sucrose transport was determined by measuring the uptake of [^{14}C]sucrose in the presence of a 50-fold excess of other sugars. Sucrose uptake was inhibited by cold trehalose which suggests that both carbohyrates may be competing for the same uptake system. Cold maltose, glucose, fructose and 2-deoxy-glucose (Sigma) did not effect uptake (Table 6.1). Sucrose grown cells could transport [^{14}C]glucose at low concentrations but the rate was 10-fold lower than for [^{14}C]sucrose transport. No transport of [^{14}C]fructose by sucrose grown cells could be demonstrated.

Figure 6.7: The effect of monensin (10μM) and CCCP (5μM) on [^{14}C]sucrose transport by washed cells of *Bacillus* isolate TA2.A1

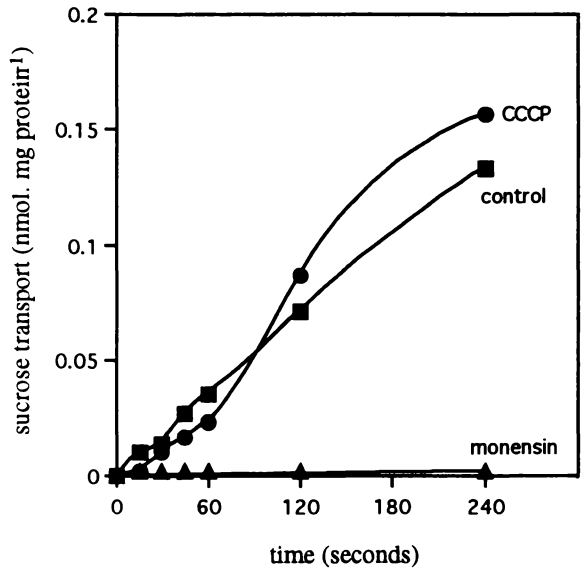


Table 6.1: The effect of carbohydrates and specific inhibitors on [^{14}C]sucrose uptake by *Bacillus* isolate TA2.A1 grown on sucrose.

Competing carbohydrate/inhibitor	% inhibition of control
Amiloride (5 μM)	87
CCCP (5 μM)	0 [1]
DNP (500 μM)	0
Melibiose	21
Monensin (10μM)	100
Raffinose	60
Trehalose	56
Turanose	0 [2]

[1] 75% inhibition after 30 seconds, no inhibition after 120 seconds (125% of control)

[2] 78% inhibition at 500μM

[^{14}C]sucrose was used at a concentration of 12μM and carbohydrates and inhibitors were added at a concentration of 50μM except where stated

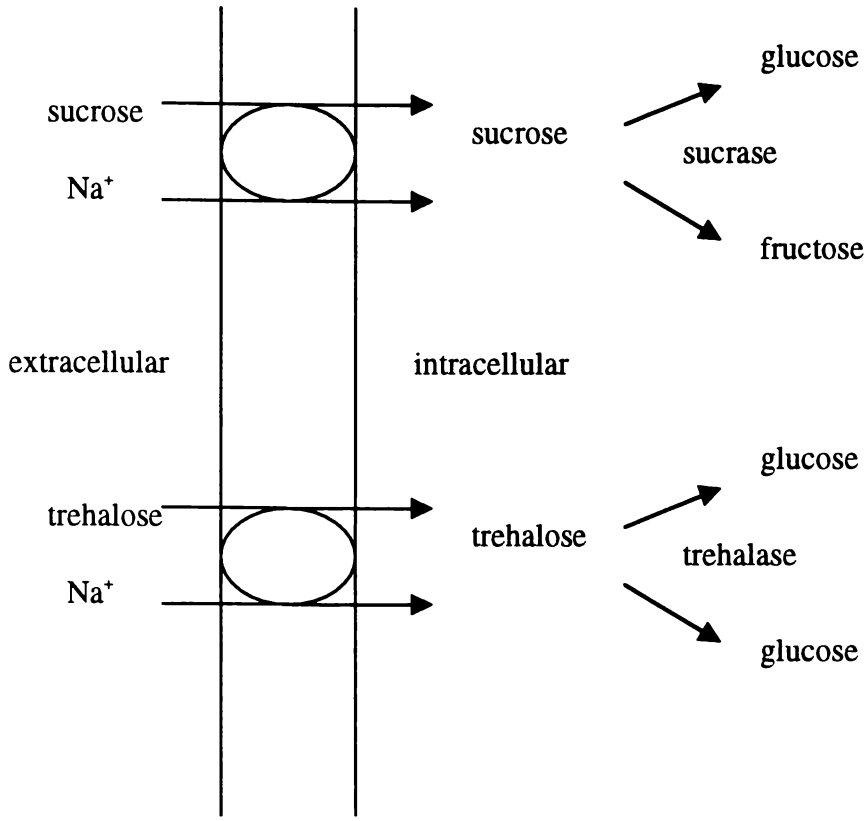
6.4 Discussion

A wide range of bacteria have the ability to grow on disaccharides. These include those living in extreme environments, for example, *Pyrococcus furiosus* which can grow on maltose and cellobiose (Fiala and Stetter 1986; Schäfer and Schönheit 1992; Kengen and Stams 1994a, 1994b). Like many hyperthermophiles *P. furiosus* can transport and metabolise glucose, but cannot grow on glucose or any other monomeric sugar (Usenko *et al.* 1993; Kengen *et al.* 1994; Schäfer *et al.* 1994). Kengen *et al.* (1996) stated that there was at present no explanation for this phenomenon which is observed for almost all hyperthermophiles. The ability of a microorganism to utilise disaccharides or complex carbohydrates but not their monosaccharide components has been shown over a range of species. Helaszek and White (1991) showed that a *Ruminococcus flavefaciens* isolate was able to utilize cellobiose, but not glucose, as a growth substrate. They showed that the organism could transport cellobiose in the presence of Na^+ or K^+, and uptake was inhibited by 2, 4-DNP (100%), monensin (61%) and CCCP (49%). Parker *et al.* (1997) proposed that competition for fructose transport plays an important role in the catabolism of glucose from sugar mixtures for the Gram negative bacterium *Zymomonas mobilis*. Since this bacterium uses the Entner-Doudoroff pathway anaerobically little energy is available for sugar transport systems. Glucose and fructose are therefore transported by a single low-affinity facilitated diffusion system (Parker *et al.* 1997; DiMarco and Romano 1985; Parker *et al.* 1995; Weisser *et al.* 1995). The only sugars that support growth are glucose, fructose and sucrose. Sucrose is cleaved outside the cell to provide free glucose and fructose (Preziosi *et al.* 1990). Kakinuma and Unemoto (1985) showed that sucrose uptake by *V. alginolyticus* was energized by glycerol and repressed by glucose. They suggested that sucrose uptake in *V. alginolyticus* was directly energized by the Na^+ electrochemical potential, possibly via a Na^+/sucrose symporter. A range of methods for monosaccharide uptake have also been documented. The use of a phosphotransferase system (Martin and Russell 1987) and the use of facilitated diffusion to transport glucose have been demonstrated in *Streptococcus bovis* (Russell 1990). Glucose uptake using a sodium symport mechanism, which could also use lithium, was reported for *Fibrobacter succinogenes* (Chow and Russell 1992). For

Clostridium thermocellum the disaccharide (cellobiose) is cleaved under phosphorylation during the uptake process, thereby conserving the energy of the sugar bond (Freier *et al.* 1988). The use of a phosphotransferase transport system is more common in anaerobic bacteria, whereas aerobic bacteria generally accumulate sugars by active transport. *Bacillus* isolate TA2.A1 is able to utilise sucrose, but not its component monosaccharides, as a growth substrate. The addition of fructose to a culture growing on sucrose caused inhibition of growth.

A number of different mechanisms of sucrose catabolism have been described. Studies with *Bacillus subtilis* (Kunst *et al.* 1974), *Streptococcus lactis* (Thompson and Chassy 1981) and *Streptococcus mutans* (Slee and Tanzer 1979; St. Martin and Wittenberger 1979) have reported that sucrose is accumulated in the cell by the phosphoenolpyruvate-dependent phosphotransferase system. Sucrose-6-phosphate is then hydrolysed to glucose-6-phosphate and fructose by sucrose-6-phosphate hydrolase. Kakinuma and Unemoto (1985) found that the initial step of the sucrose catabolic pathway in *V. alginolyticus* is different to that described above. They showed that sucrose was taken up by active transport driven by a Na^+ electrochemical potential, and then hydrolysed to glucose and fructose by sucrase. The evidence presented here suggests that sucrose is actively transported by *Bacillus* isolate TA2.A1 in the presence of sodium ions, probably by a sucrose/Na^+ symport. The sucrose is then hydrolysed by an intracellular sucrase into glucose and fructose (Figure 6.8).

Figure 6.8: Proposed mechanisms for the transport of the disaccharides sucrose and trehalose in *Bacillus* isolate TA2.A1



The evidence that supports this hypothesis is as follows: Growth of *Bacillus* isolate TA2.A1 in minimal medium supplemented with sucrose is dependant on the presence of sodium ions (Figure 6.6) and on a sodium gradient, as is deduced by the inhibitory effect of monensin (Figure 6.3). Amiloride and 2,4-DNP had no effect on growth of isolate TA2.A1 on sucrose (Figure 6.4) which suggests that Na^+/H^+ antiporters or a $\Delta\mu H^+$, i.e. a proton transport system is not involved. Growth of isolate TA2.A1 in minimal medium supplemented with trehalose is affected by ionophores/protonophores in the same manner as that described for sucrose, suggesting that perhaps trehalose is actively transported in the same manner (Figure 6.8). In fact both disaccharides may utilize the same symport since sucrose uptake was inhibited by cold trehalose which suggests competition for the same transporter. In addition to the intracellular sucrase produced by isolate TA2.A1 when growing on sucrose, an intracellular trehalase was detected in

trehalose grown cells, in both cases the two crude enzyme preparations (sonicated cell supernatant, prepared as outlined in section 4.4.2.1.1) had activity specifically detected by pNP-α-D-glucopyranoside and not by the β-form of the same substrate.

Paavilainen *et al.* 1995 suggested that the redox potential obtained from sugar catabolism was essential to the growth of alkaliphilic bacilli in sugar media. It was hypothesized that the most probable source of protons was from the dehydrogenation reactions of the sugar and that these protons drift out with the final introduction to oxygen. In a study of the starch or glucose catabolism and pH-dynamics of *Bacillus circulans* var. *alkalophilus* Paavilainen *et al.* (1995) reported that the drop in medium pH during growth did not happen at the same time as the production of acetic and formic acid but that the acids were detected several hours after the decrease in medium pH. During active growth the concentration of dissolved oxygen remained zero despite the aeration levels in the fermenter, which was cited as evidence that sugars were not metabolized through the tricarboxylic acid cycle, but that the reduction potential generated from dehydrogenation of sugars is the driving force for growth followed by very effective transfer of hydrogen atoms/protons onto molecular oxygen/oxygen anion. It was hypothesized that the rise in medium pH after the cessation of growth was from the re-equilibration of the redox state, and not from the consumption of acids or generation of basic compounds. During growth of *Bacillus* isolate TA2.A1 in medium supplemented with sucrose the pH altered half a pH unit during growth (Figure 4.13) and the concentration of dissolved oxygen, measured by oxygen probe (Section 3.3.3.1) remained at 100%; both of these results contrast with the work of Paavilainen *et al.* (1995) on *Bacillus circulans* var. *alkalophilus* where it was hypothesised that the reduction potential generated from dehydrogenation of sugars was the driving force for growth of alkaliphilic bacilli in sugar medium.

Chapter 7 - General Conclusion

This final chapter is intended to give an overview of some of the key points from preceding chapters and also suggest areas of future work that could be conducted to further characterize *Bacillus* isolate TA2.A1.

Wiegel (1998) stated that it was surprising that no true thermophilic alkaliphilic *Bacillus* species had been described, even though they should exist. The isolation and characterization of *Bacillus* isolate TA2.A1 proves that not only do these organisms exist but that, at least in the case of isolate TA2.A1, they are able to support growth using an unusual range of carbon sources. When characteristics of both temperature and pH are taken into account, *Bacillus* isolate TA2.A1, with a temperature optimum of 65°C and a pH optimum of 9.2 at 65 to 70°C is the most alkaliphilic thermophilic aerobic bacteria yet described.

Bacilli have been characterized that have temperature optima higher than isolate TA2.A1, for example, *B. caldotenax*, however, this species is alkali-tolerant (pH range 5.5-8.5). Similarly when the characteristic of pH optimum is considered in isolation there are a number of species with higher pH optima, for example *B. alkaliphilus* S-8 (pH 10-11.5) and *Bacillus* sp. strain TAR-1 (pH 10.5). It is difficult to directly compare pH values since the temperature at which they were obtained is often not reported, meaning that the actual pH at the growth temperature of the organism may be different to the reported value. The observation that all alkali-thermophiles require yeast extract or peptone as a supplement in the medium that could not be substituted by vitamins or trace elements (Wiegel 1998) was not true for all the carbon sources utilized by isolate TA2.A1. In the absence of peptone isolate TA2.A1 could utilize a number of substrates in minimal medium, for example, trehalose and L-glutamate (Tables 4.9 and 4.10). The addition of a small concentration of peptone, however, broadened the range of substrates used. The range of carbon sources utilized by isolate TA2.A1 has not been attributed to any alkali-thermophile characterized so far. Many alkali-thermophiles can utilize fructose or glucose as carbon substrates, however these monosaccharides, unlike sucrose and trehalose, are non-utilizable by isolate TA2.A1. The ability of a microorganism to

utilise disaccharide or complex carbohydrates but not their monosaccharide components has been shown over a range of species (Helaszek and White 1991). Sucrose grown cells of *Bacillus* isolate TA2.A1 could transport [^{14}C]glucose at low concentrations but the rate was 10-fold lower than for [^{14}C]sucrose transport. No transport of [^{14}C]fructose by sucrose-grown cells could be demonstrated. This characteristic is similar to that seen with many hyperthermophilic organisms which can transport and metabolise glucose, but cannot grow on glucose or any other monomeric sugar (Usenko *et al.* 1993; Kengen *et al.* 1994; Schäfer *et al.* 1994). No explanation has yet been reported for this phenomenon (Kengen *et al.* 1996).

The extreme growth conditions of isolate TA2.A1 meant that often a carbon substrate was degraded in uninoculated media which raises the possibility that the break-down products of carbon sources could be supporting the growth of isolate TA2.A1. The identification of these breakdown products was difficult since alkaline hydrolysis pathways for many compounds are unreported. The added factor of the high temperature of incubation served to further complicate the issue. As stated by Driskill *et al.* (1999) it is not completely clear what specific effects the intermediates generated by the thermochemical modifications of carbohydrates (Maillard reactions, caramelization) have on the nutritional value of sugars and how these effects are influenced by pH, temperature and salt concentrations. Certainly under the growth conditions of *Bacillus* isolate TA2.A1 the degradation of many substrates was associated with their inability to support growth. Similarly modifications of carbohydrates under the organism's growth conditions could not only effect their nutritional value but also whether they are available in a form that is utilizable by the organism.

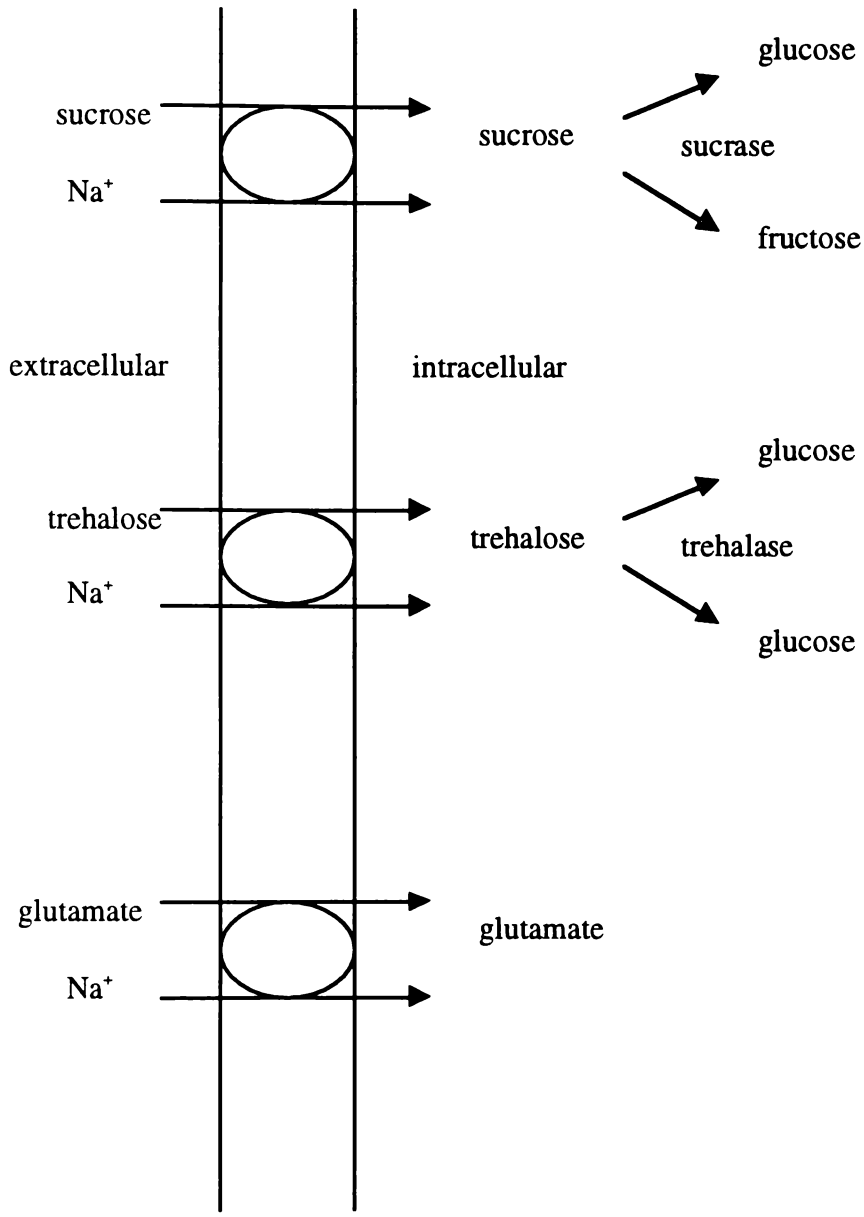
An exception to the degradation of substrates in the alkaline growth medium of isolate TA2.A1 was inulin which because of its polymeric nature was stable in minimal medium and not able to be utilized as a growth substrate. After acid hydrolysis of inulin at high temperature, the fructooligosaccharides or saccharinic acids produced were utilized by isolate TA2.A1 as a carbon source. The production of saccharinic acids from inulin would commence presumably with the alkaline degradation of the sucrose moiety thus exposing a reducing end. Further evidence to support the utilization of saccharinic acids by isolate TA2.A1 was the fact that the organism grew well on arabinogalactan as

a sole carbon source. In the high pH growth medium required for isolate TA2.A1, this compound would degrade extremely rapidly into oligosaccharides containing β-1-6 D-galactopyranose and / or α-1,3 L-arabinofuranose, and terminating in metasaccharinic acids (Manley-Harris 1997). The identification of saccharinic / meta saccharinic acids and their involvement as substrates for the growth of isolate TA2.A1 warrants further study. In conjuction with this it would interesting to ascertain whether this organism could grow on "Black liquor ", a waste product from the pulp and paper industry that is rich in saccharinic acid. If this was the case this could provide a commercial application for *Bacillus* isolate TA2A1.

Bacillus isolate TA2.A1, like other obligately alkaliphilic organisms, for example *B. agaradhaerens* and *B. clarkii* (Nielsen *et al.* 1995) is dependent on sodium for growth. Since sodium is the main ion for solute transport in obligately alkaliphilic bacteria it is not surprising that the proposed mechanism for glutamate uptake in isolate TA2.A1 is a Na^+/glutamate symporter (Figure 7.1). The use of sodium-coupled uptake for amino acid uptake has been shown for other thermophilic microorganisms (Speelmans *et al.* 1989, 1993; Heyne *et al.* 1991), however the fact sucrose uptake in isolate TA2.A1 was also sodium dependent was unusual.

A number of different mechanisms of sucrose catabolism have been described. Studies with *Bacillus subtilis* (Kunst *et al.* 1974), *Streptococcus lactis* (Thompson and Chassy 1981) and *Streptococcus mutans* (Slee and Tanzer 1979; St. Martin and Wittenberger 1979) have reported that sucrose is accumulated in the cell by the phosphoenolpyruvate-dependent phosphotransferase system. Sucrose-6-phosphate is then hydrolysed to glucose-6-phosphate and fructose by sucrose-6-phosphate hydrolase. Kakinuma and Unemoto (1985) found that the initial step of the sucrose catabolic pathway in *V. alginolyticus* is different to that described above. They showed that sucrose was taken up by active transport driven by a Na^+ electrochemical potential, and then hydrolysed to glucose and fructose by sucrase. Sucrose is actively transported by *Bacillus* isolate TA2.A1 in the presence of sodium ions, probably by a sucrose/Na^+ symport. The sucrose is then hydrolysed by an intracellular sucrase into glucose and fructose. Preliminary investigations suggest that trehalose is taken up by the same symporter.

Figure 7.1: Proposed mechanisms for sucrose / trehalose and glutamate uptake by *Bacillus* isolate TA2.A1



Having described the system used by isolate TA2.A1 for glutamate and sucrose uptake the next stage would be to investigate the methods used by this organism for ATP generation. Despite the inverted pH gradient and the reduced Δp in alkaliphiles, ATP synthesis occurs via completely proton coupled oxidative phosphorylation, but the mechanism for this remains unknown (Krulwich *et al.* 1996; Krulwich and Guffanti

1992). The Na^+-ATPase from the thermophile *C. fervidus* functions as a Na^+-extruding ATPase. No evidence for an additional H^+-pumping ATPase has been found (Speelmans *et al.* 1994). *Bacillus* strain TA2.A1 grows aerobically and presumably generates the bulk of its Δp by respiration. CCCP had no effect on glutamate transport, but growth did slow in the presence of this protonophore, suggesting that the collapse of the Δp causes a decrease in ATP synthesis and hence anabolic reactions such as growth. DCCD, an inhibitor of the F_1F_0-ATPase (H type), inhibited growth, but vanadate, an inhibitor of V-type ATPases, had no effect on the growth of *Bacillus* strain TA2.A1 on glutamate. Future work could include investigations to confirm whether isolate TA2.A1 does use an H-type ATPase.

The 16S rDNA sequence of *Bacillus* isolate TA2.A1 contained a five base insert near the beginning of the sequence (Figure 4.65), bases 36-40 from the 5′ end of the isolate TA2.A1 sequence. Other *Bacillus* strains with an insert in the same position were *B. clarkii*, *B. horti*, *B. agaradhaerens*, and *B. vedderi*. The 16S rRNA gene of an undescribed species isolated from deep-sea mud (HTA454) (Takami *et al.* 1997) and a sequence fragment 'ab009' (complete 16S rDNA sequence not currently available) from a clone library from mesophilic sewage sludge also contained the insertion. The inserted fragment "CGTGA" is the same for both *Bacillus* isolate TA2.A1 and the fragment ab009. This inserted sequence could be incorporated into a genetic probe to screen for similar organisms. This was not attempted during this work due to a lack of time.

Ascertaining the phylogenetic position of *Bacillus* isolate TA2.A1 by generating a phylogenetic trees proved to be problematic because of the poor reliability of the branching pattern as shown by the low bootstrap values. Neither a single most parsimonious (MP) tree nor a maximum likelihood (ML) tree was significantly better at representing the sequence data. What can be deduced from the trees is that isolate TA2A1 is a member of the *Bacillus* group and is deeply branching.

Appendix – Clustal V alignment of *Bacillus* 16S rDNA sequences

```
TA2.A1          TGCTGGCGGCACGCCTAATACATGCAAGTCGAGCGCGTGAAGCTTCCAGA
clarkii         CGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCAGGAAACAGGCTGA
agaradhaer      CGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCAGGAAGCCGGCGGA
horti           CGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGTGAAACTGACGGA
HTA454          ----------GGCTAATACATGCAAGTCGAGCGAGTGAA-CAAACAGA
vedderi         --CTGGCGGCGTGCCTAATACATGCAAGTCGAGCG-GATCAATAAGAGCT
thermoalka      --CTGGCGGCGTTCCTAAXACATTCAAGTCGAXCG-----GACCGXAGG-
alcalophil      --CTGGCGGCGTGCCTAATACATGCAAGTCGAGCG-----GACAGA-AGG
thermophil      --CTGGCGGCGTGCCTAATACATGCAAGTCGAGCG-----AACCGAT-GA
horikoshii      --CTGGCGACGTGCCTAATACATGCAAGTCGAGCG-----GACGTTTTTG
subtilis        --CTGGCGGCGTGCCTAATACATGCAAGTCGAGCG-----GACAGAT-GG
                   *            ** **** *******  **

TA2.A1          AGCCTTCGGGCGGACGGGAGTGGATCGAGCGGCGGACGGGTGAGTAACAC
clarkii         TCCCTTCGGGGTGATGCCTGTGGAATGAGCGGCGGACGGGTGAGTAACAC
agaradhaer      TCCCTTCGGGGTGAAXCCGGTGGAATGAGCGGCGGACGGGTGAGTAACAC
horti           AGCCTTCGGGTGGAAGACAGTGGATCTAGCGGCGGACGGGTGAGTAACAC
HTA454          AGCCTTCGGGTGGACGTTTGTGGATCTAGCGGCGAACGGGTGAGTAACAC
vedderi         TGCTCTTA---TGAAG--------ATCAGCGGCGGACGGGTGAGTAACAC
thermoalka      GAGCTT-------CTCCT--TTAGGTTAACGGCGGACGGGTGAGTAACAC
alcalophil      GAGCTT-------GCTCC-CGGAAGTCAGCGGCGGACGGGTGAGTAACAC
thermophil      GTGCTT-------GCATTCCTGAGGTTAGCGGCGGACGGGTGAGTAACAC
horikoshii      AAGCTT-------GCTCCAXAAACGTTAGCGGCGGACGGGTGAGTAACAC
subtilis        GAGCTT-------GCTCCC-TGATGTTAGCGGCGGACGGGTGAGTAACAC
                     *                    * ***** * *************

TA2.A1          GTAGGCAACCTGCCTGTAAGACCGGGATAACCCCGGGAAACCGGGGCTAA
clarkii         GTGGGCAACCTGCCTTACAGACTGGGATAACTCCGGGAAACCGGGGCTAA
agaradhaer      GTGGGCAACCTACCTTGTAGACTGGGATAACTCCGGGAAACCGGGGCTAA
horti           GTAGGCAACCTGCCTGTAAGATGGGGATAACTCCGGGAAACCGGAGCTAA
HTA454          GTAGGCAACCTGCCTGTAAGACGGGGATAACTCCGGGAAACCGGAGCTAA
thermoalka      GTGGGCAACCTXCCCTXCAGACTGGGATAACTTCGGGAAACCGGAACTAA
alcalophil      GTAGGTAACCTGCCCCTTAGACTGGGATAACTCCGGGAAACCGGAGCTAA
thermophil      GTAGGCAACCTGCCTGTACGACCGGGATAACTCCGGGAAACCGGAGCTAA
horikoshii      GTGGGCAACCTACCTTATCGACTGGGATAACTCCGGGAAACCGGGGCTAA
vedderi         GTGGGCAACCTGCCTTACAGACTGGGATAACTCCGGGAAACCGGAGCTAA
subtilis        GTGGGTAACCTGCCTGCAAGACTGGGATAACTCCGGGAAACCGG-GCTAA
                ** ** ***** **     *   ********    ******     ****

TA2.A1          TACCGGATAGGACCTTCGGTCGCATGACCGTTGGTTGAAAGGTGGC-CGC
clarkii         TACCGGATGAXCAGAAGAACCGCATGGTTCTTCTGTAAAAGTTGGGACTT
agaradhaer      TACCGGATGATCATTTGGATCGCATGATCCGAATGTAAAAGTGGGGATTT
horti           TACCGAATAATCGTTTTGCTCGCATGAGCAAAAGGTGAAAGGTGGC--GC
HTA454          TACCGGATAATACGTAAGCCCGCATGGGCTAGCGTTGAAAGGTGGC--GC
thermoalka      TACCGGATAACACCGAAAACCGCATGGTTTTCGGTTGAAAGGCGG--CTT
alcalophil      TACGGGATAATAGAGAGAATCACCTGATTCTCTTTTGAAAGACGG--TTT
thermophil      TACCGGATAGGATGCCGGACCGCATGGTTCGGCATGGAAAGGC----CTT
horikoshii      TACCGGATAACATCTAGCACCTCCTGGTGCCGGATTAAAAGAGGG--CTT
vedderi         TACCGGATGACCXCTTGGACCGCATGGTCCGATTGTAAAAGTTGGGATTT
subtilis        TACCGGATGCTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGG--CTT
                ***   **              * **          ****
```

```
TA2.A1          AAGGCTACCGCTTACAGATGGGCCTGCGGCGCATTAGCTGGTTGGTGAGG
clarkii         GTTCCTAACACTGTAAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGG
agaradhaer      AT-CCTCACACTGCAAGATGGGCCCGCGGCGCATTAGCTAGTTGGTAAGG
horti           AAX--TACCGCTTACAGATGGGCCTGCGGCGCATTAGCTAGTTGGTGAGG
HTA454          AAG-CTACCACTTACAGATGGGCCTGCGGCGCATTAGCTAGTTGGTGAGG
vedderi         AT-CCTAACACTGTGAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGGGG
thermoalka      TTAGCTGTCACTGCAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGG
alcalophil      CG-GCTGTCACTA-GGGATGGGCCTGCGGCGCATTAGCTAGTTGGTAAGG
thermophil      TGAGC---CGCGTACAGATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGG
horikoshii      CTTGCTCTCACGATGAGATGGGCCCGCGGCGCATTAGCTAGTTGGAGAGG
subtilis        CG-GCTACCACTTACAGACGGACCCGCGGCGCATTAGCTAGTTGGTGAGG
                       * *     ** ** ** ***  ******* * *****   **

TA2.A1          TAACGGCTCACCAAGGCGACGATGCGTAGCCGGCCTGAGAGGGTGACCGG
clarkii         TAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG
agaradhaer      TAATGGCTTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG
horti           TAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG
HTA454          TAACGGCTCACCAAGGCGACGATGCGTACGCGACCTGAGAGGGTGATCGG
vedderi         TAACGGCCTACCAAGCCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG
thermoalka      TAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGACCGG
alcalophil      TAACGGCTTACCAAGGCGACGATGCGTAGCTGACCTGAGAGGGTGATCGG
thermophil      TAACGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGG
horikoshii      TAACGGCTCCCCAAGXCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG
subtilis        TAACGGCTCACCAAGGCAACGATGCGTACGCGACCTGAGAGGGTGATCGG
                *** ***   ***** *  ****** **   * * *********** ***

TA2.A1          CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
clarkii         CCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAG
agaradhaer      CCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAG
horti           CCACACTGGGACTGAGACACGGCCCAGACTC-TACGGGAGGCAGCAGTAG
HTA454          CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
vedderi         CCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAG
thermoalka      CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
alcalophil      CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
thermophil      CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
horikoshii      CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
subtilis        CCACACTGGGACTGAGACACGGCCCAGACTCCTACGG-AGGCAGCAGTAG
                ***** *** ************ *** **** ***** ************

TA2.A1          GGAATCTTCCGCAATGGGCGCAAGCCTGACGGAGCGATGCCGCGTGAGCG
clarkii         GGAATCATCCGCAATGGGCGAAAGCCTGACGGTGCAACGCCGCGTGAACG
agaradhaer      GGAATCATCCGCAATGGGCGAAAGCCTGACGGTGCAACGCCGCGTGAACG
horti           GGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTG
HTA454          GGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTG
vedderi         GGAATCATCCGCAATGGGCGAAAGCCTGACGGTGCAACGCCGCGTGAACG
thermoalka      GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCG
alcalophil      GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
thermophil      GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
horikoshii      GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACCCCGCGTGAGTG
subtilis        GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
                *** ** **  ****** ** *** ****  * ** *  ********  *
```

```
TA2.A1          AGGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTCAGGGAAGAACAAGTACC
clarkii         ATGAAGGTCTTCGGATTGTAAAGTTCTGTTGTCAGGGAAGAACACGTGCC
agaradhaer      ATGAAGGTTTTCGGATCGTAAAGTTCTGTTATGAGGGAAGAACAAGTGCC
horti           AAGAAGGTTTTCGGATTGTAAAACTCTGTTGTTAGGGAAGAACAAGTATC
HTA454          AGGAAGGTTTTCGGATTGTAAAACTCTGTTGTTAGGGAAGAACAAGTACC
vedderi         ATGACGGTCTTCGGATTGTAAAGTTCTGTTGTTAGGGAAGAATAAGTACC
thermoalka      AAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTCAGGGAAGAACAAGTACC
alcalophil      AGGAAGGCCTXXXXGTCGTAAAGCTCTGTTGTGAGGGAAGAACAAGTACC
thermophil      AGGAAGGTCTTCGGATCGTAAAACTCTGTTGTCAGGGAAGAAC---CGCC
horikoshii      ATGAAGGGTTTCGGCTCGTAAAGCTCTGTTATGAGGGAAGAACACGTACC
subtilis        ATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGCGCC
                  ** **  *     * *****  ******    * ** ***

TA2.A1          GTTCGACAAGGGCGGTACCTTGACGGTACCTGACGAGAAAGCCCCGGCTA
clarkii         GTTCGAACAGGGCGGCACCTTGACGGTACCTGACCAGAAAGCCCCGGCTA
agaradhaer      GTTCGAATAGGTCGGCACCTTGACGGTACCTCACGAGAAAGCCCCGGCTA
horti           GGTTGAATAAGCCGGTACCCTGACGGTACCTAACGAGAAAGCCCCGGCTA
HTA454          GTTTGAACAAGGCGGTACCTTGACGGTACCTAACGAGAAAGCCCCGGCTA
vedderi         GTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCCCGGCTA
thermoalka      GTTCGAACAGGGCGGTACCTTGACGGTACCTGACGAGGAAGCCACGGCTA
alcalophil      GGCGTAACTAC-CGGTACCTTGACGGTACCTCACCAGAAAGCCACGGCTA
thermophil      GGGATGACCTCCCGGT---CTGACGGTACCTGACGAGAAAGCCCCGGCTA
horikoshii      GTTCGAATAGGGCGGTACCTTGACGGTACCTCATCAGAAAGCCACGGCTA
subtilis        GTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTA
                          *     ******** *     ** ***** ******

TA2.A1          ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGA
clarkii         ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGA
agaradhaer      ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGA
horti           ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGA
HTA454          ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGA
vedderi         ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGA
thermoalka      ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA
alcalophil      ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA
thermophil      ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGA
horikoshii      ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA
subtilis        ACTACGTGCCAGCAGCCGCG-TAATACGTAGGTGGCAAGCGTTGTCCGGA
                ******************** *********** *** ****** ******

TA2.A1          ATCACTGGGCGTAAAGCGCGCGCAGGCGGCCTCTTAAGTCTGGTGTGAAA
clarkii         ATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAA
agaradhaer      ATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAA
horti           ATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAA
HTA454          ATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAA
vedderi         ATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAA
thermoalka      ATTATTGGGCGTAAAGCGCGCGCAGGCGGXTCCTTAAGTCTGATGTGAAA
alcalophil      ATTATTGGGCGTAAAGCGCGCGCAGGCGGCTTCTTAAGTCTGATGTGAAA
thermophil      ATTATTGGGCGTAAAGCGCGCGCAGGCGGCTTCTTAAGTCAGGTGTGAAA
horikoshii      ATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAA
subtilis        ATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAA
                ** * *********** ** ****** **    ******* * *******
```

```
TA2.A1          GCCCGCGGCTCAACCGTGGAGACGCACTGGAAACTGGGAGGCTTGAGTGC
clarkii         GCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGT
agaradhaer      GCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGT
horti           GCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGT
HTA454          GCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGT
vedderi         GCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGT
thermoalka      TCTCGCGGCTCAACCGCGAGCGGCCATTGGAAACTGGGGAACTTGAGTGC
alcalophil      TCTCGGXXCTCAACCCCGAGCGGCCATTGGAAACTGGGAAGCTTGAGTGC
thermophil      GCCCACGGCTCAACCGTGGAGGGCCATCTGAAACTGGGGAGCTTGAGTGC
horikoshii      TCTTGCGGCTCAACCGCAAGCGGCCATTGGAAACTGGGAGGCTTGAGTAC
subtilis        GCCCCCGGCTCAACCG--GAGGGTCATTGGAAACTGGGGAACTTGGATGC
                 *      ** ****          **   ********    ****  *

TA2.A1          AGGAGAGGGAAGCGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCG
clarkii         AGGAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGT
agaradhaer      AGGAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGT
horti           AGGAGAGGAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT
HTA454          AGGAGAGGGAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT
vedderi         AGGAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGT
thermoalka      AGGAGAGGGGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT
alcalophil      AGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGT
thermophil      AGGAGAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT
horikoshii      AGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGT
subtilis        AGAAGAGGAGAGTGGAATTCCACGTGTAGCGGCGAAATGCGTAGAGATGT
                ** ****   **  *******  ********* ************ **

TA2.A1          GGAGGAACACCAGTGGCGAAGG-CGGCTTCCTGGCCTGTAACTGACGCTG
clarkii         GGAGGAACACCAGTGGCGAAGG-CGACTTTCTGGCCTATAACTGACGCTG
agaradhaer      GGAGGAACACCAGTGGCGAAGG-CGACTTTCTGGCCTACAACTGACGCTG
horti           GGAGGAACACCAGTGGCGAAGG-CGGCTTTCTGGCCTATAACTGACGCTG
HTA454          GGAGGAACACCAGTGGCGAAGG-CGGCTTCCTGGCCTATAACTGACGCTG
vedderi         GGAGGAACACCAGTGGCGAAGG-CGACTTTCTGGCCTACAACTGACGCTG
thermoalka      GGAGGAACACCAGTGGCGAAGG-CGGCTCTCTGGCCTGTAACTGACGCTG
alcalophil      GGAGGAACACCAGTGGCGAAGG-CGACTCTCTGGTCTGTAACTGACGCTG
thermophil      GGAGGAACACCAGTGGCGAAGG-CGGCTCTCTGGCCTGTAACTGACGCTG
horikoshii      GGAGGAACACCAGTGGCGAAGG-CGACTCTCTGGTCTGTAACTGACGCTG
subtilis        GGAGGAACACCAGTGGTGAAGGGCGACTCTCTGGTCTGTAACTGACGCTG
                ******* ******** ***** ** **   *** **   ****** ***

TA2.A1          AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
clarkii         AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
agaradhaer      AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
horti           AGGCGCGAAAXXXTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
HTA454          AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
vedderi         AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
thermoalka      AGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC
alcalophil      AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
thermophil      AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
horikoshii      AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
subtilis        AGGAGCGAAAG---GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC
                *** ******    ******* **************************
```

```
TA2.A1          GCCGTAAACGATGAGTGCTAGGTGTTGGGGGTTTCGACACCCTCAGTGCT
clarkii         GCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCTTAGTGCC
agaradhaer      GCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCTTAGTGCC
horti           GCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCTTAGTGCC
HTA454          GCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATGCCCTTAGTGCC
vedderi         GCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCTTAGTGCC
thermoalka      GCCGXAAACGATGAGTGCTAAGTGTTAGAGGGTATCCACCCTTTAGTGCT
alcalophil      GCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATGCCCGTAGTGCC
thermophil      GCCGTAAACGATGAGTGCTAGGTGTTGGGGAGTCCAC-CTCCTCAGTGCC
horikoshii      GCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATGCCCGTAGTGCC
subtilis        GCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTXXX
                **** ********* ***** ***** * *          *   ***

TA2.A1          -GAAGGCAACCCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
clarkii         -GCAGTTAACACATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
agaradhaer      -GAAGTTAACACATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
horti           -GACGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
HTA454          -GACGTTAACACAATAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
vedderi         -GCAGTTAACACATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTG
thermoalka      -GCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
alcalophil      -GAAGTAAACACATTAAGCACTCCGCCTGGG-AGTACGGCCGCAAGGCTG
thermophil      -GCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
horikoshii      -GAAGTTAACACATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
subtilis        TGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTG
                 *     *** *  ****** ********** ******  ******  **

TA2.A1          AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
clarkii         AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTT
agaradhaer      AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTT
horti           AAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGCATGTGGTT
HTA454          AAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGCATGTGGTT
vedderi         AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTT
thermoalka      AAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGTATGTGGTT
alcalophil      AAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGCATGTGGTT
thermophil      AAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTT
horikoshii      AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTT
subtilis        AAACTCAAAGGAATTGACGGGGGCCCGCACAAG-GCTGGAGCATGTGGTT
                ********************** ** *******   ***** ********

TA2.A1          TAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCGGTGCT
clarkii         TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCT-CTGAC
agaradhaer      TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCT-CTGAC
horti           TAATTCGAAGCAACGCGAAGAACCTTACCAGGACTTGACATCCCTCTGAA
HTA454          TAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCGATGCC
vedderi         TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCT-CTGCC
thermoalka      TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCCT-GAC
alcalophil      TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATTCT-TTGAC
thermophil      TAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCGCTGAC
horikoshii      TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCT-TTGAC
subtilis        TAATTCGAAGCAACGCAGAGAACCTTACCAGGTCTTGACATCCT-CTGAC
                ** *************  ************** ******** *    *
```

```
TA2.A1          ACCTCCAGAGATGGAG-GGTTCTCTTCGGAGA-CACCGGTGACAGGTGGT
clarkii         AACTCTGGAGACAGAGCGTTCCCCTTCGGGGG-ACAGAGTGACAGGTGGT
agaradhaer      ACCTCTGGAGACAGAGCGTTCCCCTTCGGGGG-ACAGAGTGACAGGTGGT
horti           TCCTCTAGAGATAGAG-GCGAC-CTTCGG-GA-CAGAGGTGACAGGTGGT
HTA454          CCCTCTAGAGATAGAG-GTTTCTCTTCGGAGA-CATCGGTGACAGGTGGT
vedderi         ACTCCTAGAGATAGGACGTTCCCCTTCGGGGG-ACAGAGTGACAGGTGGT
thermoalka      AACCCTAGAGATAGGGCGTTCCCCTTTGGGGGGACAGGGTGACAGGTGGT
alcalophil      CACTCTGGAGACAGAGC-TTXCC-TTCGGGGG--CAAAGTGACAGGTGGT
thermophil      CCCTCCAGAGATGGAG-GTTTCCTTC---GGGACAGCGGTGACAGGTGGT
horikoshii      CACTCTGGAGACAGAGC-TTCCCCTTCGGGGG--CAAAGTGACAGGTGGT
subtilis        AATCCTAGAGATAGGAC---TCCCTTCGGG----CAGAGTGACAGGTGGT
                      ****            *            ************

TA2.A1          GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
clarkii         GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
agaradhaer      GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
horti           GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
HTA454          GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
vedderi         GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
thermoalka      GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
alcalophil      GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
thermophil      GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
horikoshii      GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
subtilis        GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
                **************************************************

TA2.A1          CGAGCGCAACCCTTGGCCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
clarkii         CGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
agaradhaer      CGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
horti           CGAGCGCAACCCTTAATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
HTA454          CGAGCGCAACCCTTAATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
vedderi         CGAGCGCAACCCTTGACCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAG
thermoalka      CGAGCGCAACCCTTGACCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
alcalophil      CGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGG-CACTCTAAG
thermophil      CGAGCGCAACCCTTGTCCTTTGTTGCCAGCATTCAGTTGGGCACTCTAAG
horikoshii      CGAGCGCAACCCTTGACCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAG
subtilis        CGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
                **************     * ******* **** ****** *******

TA2.A1          GCGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC
clarkii         GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC
agaradhaer      GTGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC
horti           GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC
HTA454          GTGACTGCCGGTGACAAACCGGAG-AAGGTGGGGATGACGTCAAATCATC
vedderi         GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC
thermoalka      GTGACTGCCGGCTAXAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATC
alcalophil      -TGACTGCCGGTGACAAACCGGAGXAAGGTGGGGACXXCGTCXAATCATC
thermophil      GAGACTGCCGTCGACAAGACGGAGGAAGGTGGGGATGACGTCAAATCATC
horikoshii      GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC
subtilis        GTGACTGCCGGTGACAAACCG--AGAAGGTGGGGATGACGTCAAATCATC
                  ********     **  **    **********   **** *******
```

```
TA2.A1          ATGCCCCTTATGCCCTGGGCTACACACGTGCTACAATGGGCGGTACAAAG
clarkii         ATGCCCCTTATGACCTGGGCTTCACACGTGCTACAATGGGTGGTACAAAG
agaradhaer      ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAG
horti           ATGCCCCTTATGTCCTGGGCTACACACGTGCTACAATGGGCAGTACAAAG
HTA454          ATGCCCCTTATGTCCTGGGCTACACACGTGCTACAATGGGCAGTACAAAG
vedderi         ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGTGGTACAAAG
thermoalka      ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCGGTACAAAG
alcalophil      ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAG
thermophil      ATGCCCCTTATGTCCTGGGCTACACACGTGCTACAATGGACGGTACAACG
horikoshii      ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAG
subtilis        ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAG
                ************  ******* *****************     ***

TA2.A1          GGT-TGCGAACCCGCGAGGGGGAGCTAATCCCAAAAAGCCGCTCTCAGTT
clarkii         GGC-AGCAAAGCCGCGAGGCCGAGCGAATCCCATAAAGCCACTCTCAGTT
agaradhaer      GGC-AGCGAGACCGCGAGGTTAAGCGAATCCCATAAAGCCATTCTCAGTT
horti           GGT-AGCGAAGC-GCGAGGTGGAGCCAATCCCATAAAGCTGCTCTCAGTT
HTA454          GGT-CGCGAAGCCGCGAGGTGGAGCCAATCCCATAAAGCTGCTCTCAGTT
vedderi         GGC-AGCAAAGCCGCGAGGCCGAGCGAATCCCATAAAGCCACTCTCAGTT
thermoalka      GGC-TGCGAAACCGCGAGGTGGAGCGAATCCCAAAAAACCACTCTCAGTT
alcalophil      GGC-AGCGAAACCGCGAGGTGGAGCCAATCCCATAAAGCCATTCTCAGTT
thermophil      GGCGTGCCAACCCGCGAGGGTGAGCCAATCCCTAAAAACCGTTCTCAGTT
horikoshii      GGT-TGCGAAGCCGCGAGGTGAAGCCAATCCCATAAAGCCATTCTCAGTT
subtilis        GGCA-GCGAAACCGCGAG-TTAAGCCAATCCCACAAATCTGTTCTCAGTT
                **   ** *    ** **     ** ****    ***      * *****

TA2.A1          CGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATC
clarkii         CGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATTGCTAGTAATC
agaradhaer      CGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATTGCTAGTAATC
horti           CGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATTGCTAGTAATC
HTA454          CGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATTGCTAGTAATC
vedderi         CGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATTGCTAGTAATC
thermoalka      CGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATC
alcalophil      CGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATTGCTAGTAATC
thermophil      CGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATC
horikoshii      CGGATTGTAGGCTGCAACTCGCCTGCATGAAGCTGGAATTGCTAGTAATC
subtilis        CGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATC
                ***** * ** ******  ** ** * *****  ***** **********

TA2.A1          GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
clarkii         GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
agaradhaer      GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGC
horti           GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGC
HTA454          GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGC
vedderi         GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
thermoalka      GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGC
alcalophil      GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGC
thermophil      GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGC
horikoshii      GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
subtilis        GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
                ************** * ****** ********** **************
```

```
TA2.A1          CCGTCACACCACGAGAGTCTGTAACACCCGAAGTCGGTGAGGTAACCCGC
clarkii         CCGTCACACCACGAGAGCTTGTAACACCCGAAGTCGGTGAGGTAACCTTT
agaradhaer      CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGCGGTAACCTTT
horti           CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTT
HTA454          CCGTCACACCACGAGAGCTTGTAACACCCGAAGTCGGTGAGGTAACCTTT
vedderi         CCGTCACACCACGAGAGCTTGTAACACCCGAAGTCGGTGAGGTAACCTTT
thermoalka      CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCCTT
alcalophil      CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTT
thermophil      CCGTCACACCACGAGAGTTTGCAACACCCGAAGTCGGTGAGGTAACCTTC
horikoshii      CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTT
subtilis        CCGACACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTT
                *** *************  ** *******

TA2.A1          AAGGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAA
clarkii         T--GGAGCCAGCCGCCGAAGGTGGGACAGGTGATTGGGGTGAAGTCGTAA
agaradhaer      T--GGAGCCAGCCGXCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAA
horti           T--GGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAA
HTA454          TA-GGAGCCAGCTGCCTAAGGTGGGACAGGTGATTGGGGTGAAGTCGTAA
vedderi         T--GGAGCCAGGCACCGAAGGTGGGACAGGTGATTGGGGTGAAGTCGTAA
thermoalka      ACGGGAGCCAGCCGCCGAAGGTGGGACAAATGATTGGGGTGAAGTCGTAA
alcalophil      T--GGAGCCAGCCGCCTAAGGTGGGACAAATGATTGGGGTGAAGTCGTAA
thermophil      T--GGAGCCAGCCGCCGAAGGTGGGGCAGATGATTGGGGTGAAGTCGTAA
horikoshii      T--GGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAA
subtilis        AT-GGAGCCAGCCGCCGAAG-TGG-ACAGATGATTGGGGTGAAGTCGTAA
                   ********    * *** ***   *  ********************
```

Clustal V (Thompson *et al.* 1994)

References

Achenbach-Richter, L., R. Gugta, K. O. Stetter, and C. R. Woese. 1987. Were the original eubacteria thermophiles? Syst.Appl, Microbiol. **9:**34.

Adams, M. W. W. 1993. Enzymes and proteins from organisms that grow near and above 100°C. Annual Review Microbiology **47:**627-658.

Agnew, M. D., S. F. Koval, and K. F. Jarrell. 1995. Isolation and characterization of novel alkaliphiles from bauxite-processing waste and description of *Bacillus vedderi* sp. nov., a new obligate alkaliphile. Systematic and Applied Microbiology **18:**221-230.

Akiba, T., and K. Horikoshi. 1980. A further separation and some properties of beta-lactamase I. Agric. Biol. Chem. **44:**2741-2742.

Akiba, T., and K. Horikoshi. 1988. Xylanases of Alkalophilic Thermophilic *Bacillus* . Methods in Enzymology. **160:** 655-659.

Alexiev, U., R. Mollaaghababa, P. Scherrer, H. G. Khorana, and M. P. Heyn. 1995. Rapid long-range proton diffusion along the surface of the purple membrane and delayed proton transfer into the bulk. Proc. Natl. Acad. Sci. USA **92:**372-376.

Alfredson, G. A., J. K. Kristjansson, S. Hjorleifsdottir, and K. O. Stetter. 1988. *Rhodothermus marinus*, gen. nov., sp. nov., a thermophilic halophilic bacterium from submarine hot springs in Iceland. J. of Gen. Microbiol. **134:**299-306.

Ames, G. F.-L., and C. F. Higgins. 1983. The organisation, mechanism of action, and evolution of periplasmic transport systems. Trends. Biochem. Sci. **8:**97-100.

Aono, R. 1987. Characterization of structural component of cell walls of alkalophilic strain of *Bacillus* C-125. J. Biochem. **245:**467-472.

Aono, R. 1995. Assignment of facultatively alkaliphilic *Bacillus* sp. strain C-125 to *Bacillus lentus* Group 3. Int. J. System. Bacteriol. **45:**582-585.

Aono, R., M. Hashimoto, A. Hayakawa, S. Nakamura, and K. Horikoshi. 1992. A novel gene required for the alkaliphily of the facultative alkaliphile *Bacillus* sp. strain C-125. Bioscience Biotechnology Biochemistry **56:**842-844.

Aono, R., A. Hayakawa, M. Hashimoto, H. Kaneko, S. Nakamura, and K. Horikoshi. 1993. Cloning of a gene required for the alkaliphily of alkaliphilic *Bacillus* sp. strain C-125. Nucleic Acids Symp. Ser. **29:**139-140.

Aono, R., and K. Horikoshi. 1983. Chemical composition of cell walls of alkalophilic *Bacillus* strains. Journal of General Microbiology **129:**1083-1087.

Aono, R., M. Ito, and K. Horikoshi. 1993a. Occurrence of teichurono-peptide in cell walls of group 2 alkaliphilic *Bacillus* sp. J. Gen. Microbiology **139:**2738-2744.

Aono, R., M. Ito, and K. Horikoshi. 1997. Measurement of cytoplasmic pH of the alkaliphile *Bacillus lentus* C-125 with a fluorescent pH probe. Microbiology **143:**2531-2536.

Aono, R., M. Ito, K. Joblin, and K. Horikoshi. 1994. Genetic recombination after cell fusion of protoplasts from the facultative alkaliphile *Bacillus* sp. C-125. Microbiology **140:**3085-3090.

Aono, R., M. Ito, K. Joblin, and K. Horikoshi. 1995a. A high cell wall negative charge is necessary for the growth of the alkalophile *Bacillus lentus* C-125, at elevated pH. Microbiology **141:**2955-2964.

Aono, R., H. Kaneko, and K. Horikoshi. 1996. Alkaline growth pH-dependent increase of respiratory and NADH-oxidation activities of the facultatively alkaliphilic strain *Bacillus lentus* C-125. Bioscience Biotechnology Biochemistry **60:**1243-1247.

Aono, R., H. Ogino, and K. Horikoshi. 1992a. pH-dependent flagella formation by facultative alkaliphilic *Bacillus* sp. C-125. Biosci.Biotech.Biochem. **56:**48-53.

Aono, R., and M. Ohtani. 1990. Loss of alkalophily in cell-wall-component defective mutants derived from alkalophilic *Bacillus* C-125. Biochemistry Journal **266:**933-936.

Ara, K., K. Igarashi, K. Saeki, S. Kawai, and S. Ito. 1992. Purification and some properties of an alkaline pullulanase from alkaliphilic *Bacillus* sp. KSM-1876. Bioscience Biotechnology Biochemistry **56:**62-65.

Ara, K., K. Saeke, and S. Ito. 1993. Purification and characterization of an alkaline isoamylase from an alkalophilic strain of *Bacillus*. J. Gen Microbiol. **139:**781-786.

Aragno, M. 1992. Aerobic, chemolithoautotrophic, thermophilic bacteria, 77-103. *In* J. K. Kristjansson (ed.), Thermophilic Bacteria, chapter 5. CRC Press Inc, USA.

Ash, C., J. A. E. Farrow, S. Wallbanks, and M. D. Collins. 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequence. Lett.Appl.Microbiol. **13:**202-206.

Ash, C., F. G. Priest, and M. D. Collins. 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. Antonie van Leeuwenhoek **64:**253-260.

Atsumi, T., S. Sugiyama, E. F. j. Cragoe, and Y. Imae. 1990. Specific inhibition of the Na^+-driven flagellar motors of alkalophilic *Bacillus* strains by the amiloride analog phenamil. J. Bact. **172:**1634-1639.

Aunstrup, K., H. Outtrup, O. Andresen, and C. Dambmann. 1972. Proteases from alkalophilic *Bacillus* species, 299-305. *In* Fermentation Technology Today; Proceedings of the 4th International Fermentation symposium. Society for Fermentation Technology, Osaka, Japan.

Avetisyan, A. V., P. A. Dibrov, A. L. Semeykina, V. P. Skulachev, and M. V. Sokolov. 1991. Adaptation of *Bacillus* FTU and *Escherichia coli* to alkaline conditions: the Na^+-motive respiration. Biochimica et Biophysica Acta **1098:** 95-104.

Azarkina, N. V. 1995. Induction of Na^+-motive terminal oxidase in facultatively alkalophilic bacterium *Bacillus halodurans* grown under alkaline conditions. Biochemistry (Moscow) **60:**211-216.

Ballou, C. E. 1954. Alkali-sensitive glycosides. Adv. Carbohydrate Chem. **9:**59-95.

Baross, J. A. 1998. Do the geological and geochemical records of the early earth support the prediction from global phylogenetic models of a thermophilic cenancestor?, *In* J. Wiegel and M. W. W. Adams (ed.), Thermophiles – The keys to molecular evolution and the origin of life?, p3-18. Taylor and Francis, London/Philadelphia.

Bassilana, M., E. Damiano, and G. Leblanc. 1984. Kinetic properties of Na^+-H^+ antiport in *Escherichia coli* membrane vesicles : effects of imposed electrical potential, proton gradient, and internal pH. Biochemistry **23:**5288-5294.

Bath, A. H., N. Christofi, C. Neal, J. C. Philp, M. R. Cave, I. G. McKinley, and U. Berner. 1987. Trace element and microbiological studies of alkaline groundwaters in Oman, Arabian Gulf: A natural analogue for cement pore waters.

Beeder, J., T. Torsvik, and T. Lien. 1995. *Thermodesulforhabdus norvegicus* gen. nov., sp. nov., a novel thermophilic sulfate-reducing bacterium from oil field water. Arch. Microbiol. **164:**331-336.

Beffa, T., M. Blanc, and M. Aragno. 1996. Obligately and facultatively autotrophic, sulfur- and hydrogen-oxidizing thermophilic bacteria isolated from hot composts. Arch. Microbiol. **165:**34-40.

Berendes, F., G. Gottschalk, E. Heinedobbernack, E. R. B. Moore, and B. J. Tindall. 1996. *Halomonas desiderata* sp nov, a new alkaliphilic, halotolerant and denitrifying bacterium isolated from a municipal sewage works. System. Appl. Microbiol. **19:**158-167.

Biely, P. 1985. Microbial xylanolytic systems. Trends Biotechnol. **3:**286-290.

Blanc, M., L. Marilley, T. Beffa, and M. Aragno. 1999. Thermophilic bacterial communities in hot composts as revealed by most probable number counts and molecular (16S rDNA) methods. FEMS Microbiology Ecology **28:**141-149.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Biochem. J. **83:**606-614.

Blöchl, E., R. Rachel, S. Burggraf, D. Hafenbradl, H. W. Jannasch, and K. O. Stetter. 1997. *Pyrolobus fumarii*, gen. and sp.nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C . Extremophiles **1:**14-21.

Bollet, C., M. J. Gevaudan, X. de Lamballerie, C. Zandotti, and P. de Micco. 1991. A simple method for the isolation of chromosomal DNA from Gram positive or acid-fast bacteria. Nucleic Acids Research (Oxford University Press) **19:**1955.

Bouthier de la Tour, C., C. Portemer, R. Huber, P. Forterre, and M. Duguet. 1991. Reverse gyrase is present in thermophilic eubacteria. J. Bact. **173:**3921-3923.

Boyer, E. W., and M. B. Ingle. 1972. Extracellular alkaline amylase from a *Bacillus* species. J. Bact. **110:**992-1000.

Boyer, E. W., M. B. Ingle, and G. D. Mercer. 1973. *Bacillus alcalophilus* subsp. *halodurans* subsp. nov. : an alkaline amylase producing alkalophilic organism. Int. J. System. Bacteriol. **23:**238-242.

Brierley, J. A. 1978. Thermophilic iron-oxidising bacteria found in copper leaching dumps. Applied and Environmental Microbiology **36:**523.

Brierley, J. A., and S. J. Lockwood. 1977. The occurance of thermophilic iron-oxidising bacteria in a copper leaching system. FEMS Microbiol. Letters **2:**163.

Brock, T. D. 1971. Bimodal distribution of pH values of thermal springs of the world. Geol.Soc.Am.Bull. **82:**1393.

Brock, T. D. 1978. Thermophilic microorganisms and life at high temperature. M. P. Starr (ed.). Springer-Verlag, New York, Heildberg.

Brock, T. D. 1986a. An overview of the Thermophiles, *In* T. D. Brock (ed.), Thermophiles, vol. 1. John Wiley & Sons, New York.

Brock, T. D., and L. Boyen. 1973. Presence of thermophilic bacteria in laundry and domestic hot-water heaters. Applied Microbiology **88:**72-76.

Brock, T. D., and H. Freeze. 1969. *Thermus aquaticus* gen. n. and sp. n., a nonsporulating extreme thermophile. J.Bacteriol. **98:**289-297.

Buck, D. P., and G. D. Smith. 1995. Evidence for a Na^+/H^+ electrogenic antiporter in an alkaliphilic cyanobacterium *Synechocystis*. FEMS Microbiol. Letters **128:**315-320.

Buck, J. D. 1982. Nonstaining (KOH) method for determination of Gram reactions of marine bacteria. Appl. Env. Microbiol. **44:**992-993.

Caldwell, D. E., S. J. Caldwell, and J. P. Laycock. 1976. *Thermothrix thioparus* gen. et sp. nov. a facultatively anaerobic facultative chemolithotroph living a neutral pH and high temperature. Canadian Journal of Microbiology **22:**1509-1517.

Castenholz, R. W. 1979. Evolution and ecology of thermophilic organisms, 373. *In* M. Shilo (ed.), Strategies of Microbiol Life In Extreme Environments. Verlag Chemie, Weinheim.

Cheng, J., A. A. Guffanti, and T. A. Krulwich. 1994. The chromosomal tetracycline resistance locus of *Bacillus subtilis* encodes a Na^+/H^+ antiporter that is physiologically important at elevated pH. The Journal of Biological Chemistry **269:**27365-27371.

Cheng, J., A. A. Guffanti, W. Wang, T. A. Krulwich, and D. H. Bechhofer. 1996a. Chromosomal *tet*A(L) gene of *Bacilus subtilis*:regulation of expression and physiology of a *tet*A(L) deletion strain. J. Bacteriol. **178:**2853-2860.

Cheng, J., D. B. Hicks, and T. A. Krulwich. 1996b. The purified *Bacillus subtilis* tetracycline efflux protein TetA(L) reconstitutes both tetracycline-cobalt/H^+ and Na^+ (K^+)/H^+ exchange. Proc. Natl. Acad. Sci. USA **93:**14446-14451.

Chislett, M. E., and D. J. Kushner. 1961. A strain of *Bacillus circulans* capable of growing under highly alkaline conditions. Journal of General Microbiology **24:**187-190.

Chow, J. M., and J. B. Russell. 1992. Effect of pH and monensin on glucose transport by *Fibrobacter succinogenes,* a cellulolytic ruminal bacterium. Appl. Environ. Microbiol. **58:**1115-1120.

Clarke, M. A., L. A. Edye, and G. Eggleston. 1997. Sucrose decomposition in aqueous solution, and losses in sugar manufacture and refining, 441 – 470. *In* D. Horton (ed.), Advances in Carbohydrate Chemistry and Biochemistry. 52 Academic Press,

Claus, D., and R. C. W. Berkeley. 1986. Genus *Bacillus* Cohn 1872., 1105-1140. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (ed.), Bergey's Manual of Systematic Bacteriology. 2 Williams and Wilkins, Baltimore.

Clejan, S., and T. A. Krulwich. 1988. Permeability studies of liquid vesicles from alkalophilic *Bacillus firmus* showing opposing effects of membrane isoprenoid and diacylglycerol fractions and suggesting a possible basis for obligate alkalophily. Biochim. Biophys. Acta. **946:**40-48.

Clejan, S., T. A. Krulwich, K. R. Mondrus, and D. Seto-Young. 1986. Membrane lipid composition of obligately and facultatively alkalophilic strains of *Bacillus* spp. J. Bact. **169:**4469-4478.

Cook, G. M. 1988. Carbohydrate metabolism of thermophilic glycolytic anaerobic bacteria. M.Sc thesis, University of Waikato.

Cook, G. M. 1992. Control of glucose and xylose utilization by *Clostridium thermohydrosulfuricum* Rt8.B1. Ph.D. thesis, University of Waikato.

Darland, G., and T. D. Brock. 1971. *Bacillus acidocaldarius* sp.nov., an acidophilic thermophilic spore-forming bacteria. Journal of General Microbiology **67:**9-15.

Dawson, R. M. C., D. C. Elliott, W. H. Elliott, and K. M. Jones. 1986. Methods for the detection of biochemical compounds on paper and thin layer chromatograms with some notes on separation, 470. *In* R. M. C. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones (ed.), Data for Biochemical Research. Oxford Science Publications, Oxford.

De Ley, J. 1978. Modern molecular methods in bacterial taxonomy: evolution, application, prospects. *In* Proceedings of the fourth international conference on plant pathology and bacteriology p347 – 357.

De Rosa, M., A. Trincone, B. Nicolaus and A. Gambacorta. 1991. Archaebacteria: lipids, membrane structures, and adaptations to environmental stresses. In G. di Prisco (ed.), Life under extreme conditions. p61-87. Springer, Berlin Heidelberg New York.

De Rosa, M., A. Gambacorta, B. Nicolaus, and W. D. Grant. 1983. A C25, C25 diether core lipid from archaebacterial haloalkaliphiles. Journal of General Microbiology **129:**2333-2337.

De Rosa, M., A. Gambacorta, B. Nicolaus, H. N. M. Ross, W. D. Grant, and J. D. Bu'Lock. 1982. An asymmetric archaebacterial diether lipid from alkaliphilic halophiles. Journal of General Microbiology **128:**343-348.

de Vrij, W., R. A. Bulthuis, and W. N. Konings. 1988. Comparative study of energy-transducing properties of cytoplasmic membranes from mesophilic and thermophilic *Bacillus* species. J. Bacteriol **170:**2359-2366.

de Vrij, W., R. A. Bulthuis, P. R. van Iwaarden, and W. N. Konings. 1989. Mechanism of L-glutamate transport in membrane vesicles from *Bacillus stearothermophilus*. Journal of Bacteriology **171:**1118-1125.

de Vrij, W., G. Speelmans, R. I. R. Heyne, and W. N. Konings. 1990. Energy transduction and amino acid transport in thermophilic aerobic and fermentative bacteria. FEMS Microbiol. Rev. **75:**183-200.

Deinhard, G., P. Blang, K. Poralla, and E. Altan. 1987a. *Bacillus acidoterrestris* sp. nov., a new thermotolerant acidophile isolated from different soils. Syst. Appl. Microbiol. **10:**47-53..

Deinhard, G., J. Saar, W. Krischke and, K. Poralla. 1987b. *Bacillus cycloheptanicus* sp. nov., a new thermoacidophile containing ω-cycloheptane fatty acids. Syst. Appl. Microbiol. **10:**68-73.

Delobbe, A. H., H. Chalumeau, J.-M. Claverie, and P. Gay. 1976. Phosphorylation of intracellular fructose in *Bacillus subtilis* mediated by phosphoenolpyruvate-1-fructose phosphotransferase. Eur. J. Biochem.**66:**485-491.

Demharter, W., and R. Hensel. 1989. *Bacillus thermocloaceae* sp.nov. A new thermophilic species from sewage sludge. System. Appl. Microbiol. **11:**272-276.

Denariaz, G., W. J. Payne, and J. Le Gall. 1989. A halophilic denitrifier, *Bacillus halodenitrificans* sp. nov. Int. J. System. Bacteriol. **39:**145 –151.

Dey, D., J. Hinge, A. Shendye, and M. Rao. 1992. Purification and properties of extracellular endoxylanases from alkalophilic thermophilic *Bacillus* sp. Canadian Journal of Microbiology **38:**436-442.

Dills, S. S., A. Apperson, M. R. Schmidt, and M. H. Saier, Jr. 1980. Carbohydrate transport in bacteria. Microbiol. Rev. **44:**385-418.

DiMarco, A. A. and A. H Romano. 1985. D-glucose transport system of *Zymomonas mobilis*. Appl. Environ. Microbiol. **49:**151-157.

Donk, P. J. 1920. A highly resistant thermophilic organism. Journal of Bacteriology: **5:**373-374.

Driessen, A. J. M., J. L. C. M. van de Vossenberg, and W. N. Konings. 1996. Membrane composition and ion-permeability in extremophiles. FEMS Microbiol. Rev. **18:**139-148.

Driskill, L. E., K. Kusy, M. W. Bauer, and R. M. Kelly. 1999. Relationship between Glycosyl Hydrolase Inventory and Growth Physiology of the Hyperthermophile *Pyrococcus furiosis* on Carbohydrate-Based Media. Appl. Environ. Microbiol. **65:**893-897.

Duckworth, A. W., S. Grant, W. D. Grant, B. E. Jones, and D. Meijer. 1998. *Dietzia natronolimnaios* sp. nov., a new member of the genus *Dietzia* isolated from an east african soda lake. Extremophiles **2:**359-366.

Duckworth, A. W., W. D. Grant, B. E. Jones, and R. van Steenbergen. 1996. Phylogenetic diversity of soda lake alkaliphiles. FEMS Microbiology Ecology **19:**181-191.

Dunkley, E. A. J., A. A. Guffanti, S. Clejan, and T. A. Krulwich. 1991. Facultative alkaliphiles lack of fatty acid desaturase activity and lose their ability to grow at near-neutral pH when supplemented with an unsaturated fatty acid. J. Bacteriol. **173:**1331.

Durham, D. R., D. B. Stewart, and E. J. Stellwag. 1987. Novel alkaline- and heat-stable serine proteases from alkalophilic *Bacillus* sp. strain GX6638. J. of Bacteriol. **169:**2762-2768.

Edlund, A., P. D. Nichols, R. Roffey, and D. C. White. 1985. Extractable and lipopolysaccaride fatty acid and hydroxy acid profiles from *Desulfovibrio species*. J. Lipid Research. **26:**982-988.

Edmonds, C. G., P. F. Crain, R. Gupta, T. Hashizume, C. H. Hocart, J. A. Kowalak, S. C. Pomerantz, K. O. Stetter, and J. A. McCloskey. 1991. Post transcriptional modification of tRNA in thermophilic archaea (Archaebacteria). J. Bact. **173:**3138-3148.

Eggleston, G., J. R. Vercellotti, L. Edye, and M. A. Clarke. 1995. Behavior of water structure-breaking and structure-enhancing solutes on the thermal degradation of concentrated solutions of sucrose. Journal of Carbohydrate Chemistry **14:**1035-1042.

Emanuilova, E., M. Kambourova, M. Dekovska, and R. Manolov. 1993. Thermoalkaliphilic lipase-producing *Bacillus* selected by continuous cultivation. FEMS Microbio. Letters **108:**247-250.

Engle, M., Y. Li, F. Rainey, S. DeBlois, V. Mai, A. Reichert, F. Mayer, P. Messner, and J. Wiegel. 1996. *Thermobrachium celere* gen. nov., sp. nov., a rapidly growing thermophilic, alkalitolerant, and proteolytic obligate anaerobe. Int. J. System. Bacteriol. **46:**1025-1033.

Engle, M., Y. Li, C. Woese, and J. Wiegel. 1995. Isolation and characterization of a novel alkalitolerant thermophile, *Anaerobranca horikoshii* gen. nov., sp. nov. Int. J. System. Bacteriol. **45**:454-461.

Fahmy, F., J. Flossdorf, and D. Claus. 1985. The DNA base composition of type strains of the genus *Bacillus*. Syst. Appl. Microbiol. **6**:60-65.

Farrell, J and L.L. Campbell. 1969. Thermophilic bacteria and bacteriophages. Advances in Microbial Physiology. **3**:83-109.

Farrow, J. A. E., S. Wallbanks, and M. D. Collins. 1992. Phylogenetic analysis of the genera *Planococcus*, *Marinococcus* and *Sporosarcina* and their relationship to members of the genus *Bacillus*. FEMS Microbiol.Lett. **93:**167-172.

Feng, D.-F., and R. F. Doolittle. 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J. Mol. Evolution **25:**351-360.

Fiala, G., and K. O. Stetter. 1986. *Pyrococcus furiosis* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Archives of Microbiology **145:**56-61.

Forster, A., R. Daniel, and V. Muller. 1995. The Na(+)-translocating ATPase of *Acetobacterium woodii* is a F_0F_1-type enzyme as deduced from the primary structure of its beta, gamma and epsilon subunits. Biochim. Biophys. Acta. **1229:**393-397.

Forterre, P. 1995a. Looking for the most "primitive" organism(s) on Earth today : the state of the art. Planet. Space Sci. **43:**167-177.

Forterre, P. 1995b. Thermoreduction, a hypothesis for the origin of prokaryotes. Life Sciences **318:**415-422.

Forterre, P., F. Charbonnier, E. Marguet, and G. Henckes. 1992. Chromosone structure and DNA topology in extremely thermophilic archaea. Biochemical Society Symposium **58:**99-112.

Freier, D., C. P. Mothershed, and J. Wiegel. 1988. Characterisation of *Clostridium thermocellum* JW 20. Appl. Environ. Microbiol. **54**:204-211.

Fritze, D. 1996. *Bacillus haloalkaliphilus* sp. nov. Int. J. System. Bacteriol. **46:**98-101.

Fritze, D., J. Flossdorf, and D. Claus. 1990. Taxonomy of alkaliphilic *Bacillus* strains. Int. J. System. Bacteriol. **40:**92-97.

Fujiwara, N., A. Masui, and T. Imanaka. 1993. Purification and Properties of the highly thermostable alkaline protease from an alkaliphilic and thermophilic *Bacillus* sp. Journal of Biotechnology **30:**245-256.

Fujiwara, N., and K. Yamamoto. 1987. Decomposition of gelatin layers on X-ray film by the alkaline protease from *Bacillus* sp. B21. Journal of Fermentation Technology **65:**531-534.

Fujiwara, N., and K. Yamamoto. 1987a. Production of alkaline protease in a low-cost medium by alkalophilic *Bacillus* sp. and properties of the enzyme. J. Ferment. Technol. **65:** 345-348.

Fujiwara, N., K. Yamamoto, and A. Masui. 1991. Utilization of thermostable alkaline protease from an alkalophilic thermophile for the recovery of silver from used X-ray film. Journal of Ferment. Bioeng. **72:**306-308.

Gabriel, B., and J. Teissie. 1996. Proton long-range migration along protein monolayers and its consequences on membrane coupling. Proc.Natl.Acad.Sci.USA **93**:14521-14526.

Garcia, M. L., A. A. Guffanti, and T. A. Krulwich. 1983. Characterization of the Na^+ / H^+ antiporter of alkalophilic bacilli *in vivo* : a Ψ-dependent $^{22}Na^+$ efflux from whole cells. J. Bact. **156:** 1151-1157.

Gatje, G., V. Muller, and G. Gottschalk. 1991. Lactic acid excretion via carrier-mediated facilitated diffusion in *Lactobacillus helveticus*. Appl. Microbiol. Biotechnol. **34**:778-782.

Gee, G., B. M. Lund, G. Metcalf, and J. L. Peel. 1980. Properties of a new group of alkaliphilic bacteria. Journal of General Microbiology **117**:9-17.

Georganta, G., T. Kaneko, N. Nakamura, T. Kudo, and K. Horikoshi. 1993. Isolation and partial properties of cyclomaltodextrin glucanotransferase-producing alkaliphilic *Bacillus* spp. from a deep-sea mud sample. Starch **45**:95-99.

Gibson, T., and R. E. Gordon. 1974. The Genus Bacillus, 520-529. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's Manual of Determinant Bacteriology. Williams and Wilkins, Baltimore.

Gilmour, R., and T. A. Krulwich. 1996. Purification and characterization of the succinate dehydrogenase complex and CO-reactive *b*-type cytochromes from the facultative alkaliphile *Bacillus firmus* OF4. Biochim.Biophys.Acta **1276**:57-63.

Gilmour, R., and T. A. Krulwich. 1997. Construction and characterization of a mutant of alkaliphilic *Bacillus firmus* OF4 with a disrupted *cta* operon and purification of a novel cytochrome *bd* . J. Bacteriol. **179**:863-870.

Golovacheva, R. S., and G. I. Karavaiko. 1978. A new genus of thermophilic spore-forming bacteria, *Sulfobacillus*. Mikrobiologija **47**:658.

Gordon, R. E., and J. L. Hyde. 1982. The *Bacillus firmus* - *Bacillus lentus* complex and pH 7.0 variants of some alkalophilic strains. Journal of General Microbiology **128:** 1109–1116.

Gordon, R. E., and N. R. Smith. 1949. Aerobic sporeforming bacteria capable of growth at high temperatures. J. Bacteriol. **58**:327 – 341.

Gottschalk, G. 1986. Bacterial Metabolism. Springer-Verlag, New York.

Grant, W. D. 1989a. *Natronobacterium*, *In* J. T. Staley (ed.), Bergey's Manual of Systematic Bacteriology, vol. 3. Williams and Williams, Baltimore.

Grant, W. D. 1989b. *Natronococcus*, 2232-2233. *In* J. T. Staley (ed.), Bergey's Manual of Systematic Bacteriology, vol. 3. Williams and Williams, Baltimore.

Grant, W. D., and H. Larsen. 1989. Halobacteriales, 2216-2218. *In* J. T. Staley (ed.), Bergey's Manual of Systematic Bacteriology, vol. 3. Williams and Williams, Baltimore.

Grant, W. D., A. A. Mills, and A. K. Schofield. 1979. An alkalophilic species of Ectothiorhodospira from a Kenyan soda lake. J.Gen. Microbiol. **110**:137-142.

Grant, W. D., W. E. Mwatha, and B. E. Jones. 1990. Alkaliphiles: ecology, diversity and applications. FEMS Microbiol. Rev. **75**:255-270.

Grant, W. D., and B. J. Tindall. 1980 The isolation of alkalophilic bacteria, 27-38. *In* G. W. Gould and J. E. L. Corry (ed.), Society for Applied Bacteriology Technical Series 15: Microbial Growth and Survival in Extremes of Environment. Academic Press, London.

Grant, W. D., and B. J. Tindall. 1986. The alkaline, saline environment., 22-54. *In* R. A. Herbert and G. A. Codd (ed.), Microbes in Extreme Environments. Academic Press, London.

Grønstad, A., E. Jaroszewicz, M. Ito, M. G. Sturr, T. A. Krulwich, and A.-B. Kolsto. 1998. Physical map of alkaliphilic *Bacillus firmus* OF4 and detection of a large endogenous plasmid. Extremophiles **2:**447-453.

Guffanti, A. A., R. Blanco, R. A. Benenson, and T. A. Krulwich. 1980. Bioenergetic properties of alkaline-tolerant and alkalophilic strains of *Bacillus firmus*. J. Gen. Microbiol **119:**79-86.

Guffanti, A. A., D. E. Cohn, H. R. Kaback, and T. A. Krulwich. 1981. Relationship between the Na^+/H^+ antiporter and Na^+/substrate symport in *Bacillus alcalophilus*. Proc. Natl. Acad. Science USA **78:**1481-1484.

Guffanti, A. A., O. Finkelthal, D. B. Hicks, L. Falk, A. Sidhu, A. Garro, and T. A. Krulwich. 1986. Isolation and characterization of new facultatively alkalophilic strains of *Bacillus*. J. Bacteriol. **167:**766-773.

Guffanti, A. A., R. T. Fuchs, M. Schneier, E. Chiu, and T. A. Krulwich. 1984. A $\Delta\Psi$ generated by respiration is not equivalent to a diffusion potential of the same magnitude for ATP synthesis by *Bacillus firmus* RAB. Journal of Biological Chemistry **259:**2971-2975.

Guffanti, A. A., and D. B. Hicks. 1991. Molar growth yields and bioenergetic parameters of extremely alkalophilic *Bacillus* species in batch cultures and growth in a chemostat at pH 10.5. Journal of General Microbiology **137:**2375-2379.

Guffanti, A. A., and T. A. Krulwich. 1992. Features of an apparent non-chemiosmotic energization of oxidative phosphorylation by alkaliphilic *Bacillus firmus* OF4. Journal of Biological Chemistry **267:**9580-9588.

Guffanti, A. A., and T. A. Krulwich. 1994. Oxidative phosphorylation by ADP + Pi-loaded membrane vesicles from alkaliphilic *Bacillus firmus* OF4. The Journal of Biological Chemistry **269:**21576-21582.

Guffanti, A. A., P. Susman, R. Blanco, and T. A. Krulwich. 1978. The proton motive force and α-aminoisobutyric acid transport in an obligately alkalophilic bacterium. J. Biol. Chem **253:**708-715.

Gupte, S. S., C. B. Leesnitzer, C. R. Hackenbrock. 1991. Two-dimensional diffusion of F_1F_0-ATP synthase and ADP/ATP translocator. Testing a hypothesis for ATP synthesis in the mitochondrial inner membrane. Biochim. Biophys. Acta **1069:**131-138.

Gutman, M., and E. Nachliel. 1995. The dynamics of proton exchange between bulk and surface groups. Biochim. Biophys. Acta **1231:**123-138.

Hakamada, Y., K. Koike, T. Yoshimatsu, H. Mori, T. Kobayashi, and S. Ito. 1997. Thermostable alkaline cellulase from an alkaliphilic isolate, *Bacillus* sp. KSM-S237. Extremophiles **1:**151-156.

Hamamoto, T., M. Hashimoto, M. Hino, M. Kitada, Y. Seto, T. Kudo, and K. Horikoshi. 1994. Characterization of a gene responsible for the Na^+ / H^+ antiporter system of alkalophilic *Bacillus* species strain C-125. Molecular Microbiology **14:**939-946.

Hammer, B. W. 1915. Bacteriological studies on the coagulation of evaporated milk. Res.Bull.Iowa Agric.Exp.Stn. **19:**119-131.

Hasegawa, M., H. Kishino, and T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **21:**160-174.

Hashimoto, M., T. Hamamoto, M. Kitada, M. Hino, T. Kudo, and K. Horikoshi. 1994. Characteristics of alkali-sensitive mutants of alkaliphilic *Bacillus* sp. strain C-125 that show cellular morphological abnormalities. Biosci. Biotech. Biochem **58:**2090-2092.

Heberle, J., J. Riesle, G. Thiedemann, D. Oesterhelt, and N. A. Dencher. 1994. Proton migration along the membrane surface and retarded surface to bulk transfer. Nature **370:**379-382.

Heinen, U. J., and W. Heinen. 1972. Characteristics and properties of a caldo-active bacterium producing extracellular enzymes and two-related strains. Arch. Mikrobiol. **82:**1-23.

Heinen, W., A. M. Lauwers, and J. W. M. Mulders. 1982. *Bacillus flavothermus*, a newly isolated facultative thermophile. Antonie van Leeuwenhoek **48:**265-272.

Helaszek, C. T., and B. A. White. 1991. Cellobiose uptake and metabolism by *Ruminococcus flavefaciens*. Appl. Environ. Microbiol. **57:**64-68.

Hendy, N. A. 1987. Isolation of thermophilic iron-oxidizing bacteria from sulfidic waste rock. J. Ind. Microbiology **1:**389.

Hensel, R., W. Demharter, O. Kandler, R. M. Kroppenstedt, and E. Stackenbrandt. 1986. Chemotaxonomic and molecular-genetic studies on the genus *Thermus:* Evidence for a phylogenetic relationship of *Thermus aquaticus* and *Thermus ruber* to the genus *Deinococcus*. Int. J. System. Bact. **36:**444-453.

Heyndrickx, M., L. Lebbe, K. Kersters, P. De Vos, G. Forsyth, and N. A. Logan. 1998. Virgibacillus: a new genus to accommodate *Bacillus pantothenticus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenticus*. Int. J. System. Bacteriol. **48:**99-106.

Heyndrickx, M., L. Lebbe, M. Vancanneyt, K. Kersters, P. De Vos, N. A. Logan, G. Forsyth, S. Nazli, N. Ali, and R. C. W. Berkeley. 1997. A polyphasic reassessment of the Genus *Aneurinibacillus*, reclassification of *Bacillus thermoaerophilus* (Meier-Stauffer *et al.* 1996) as *Aneurinibacillus thermoaerophilus* comb. nov., and emended descriptions of *A. aneurinilyticus* corrig., *A. migulanus* and *A. thermoaerophilus*. Int.J.System.Bacteriol. **47:**808-817.

Heyndrickx, M., K. Vandemeulebroecke, P. Scheldeman, B. Hoste, K. Kersters, P. De Vos, N. A. Logan, A. M. Aziz, N. Ali, and R. C. W. Berkeley. 1995. *Paenibacillus* (formerly *Bacillus*) *gordonae* (Pichinoty *et al.* 1986) Ash *et al.* 1994 is a later subjective synonym of *Paenibacillus* (formally *Bacillus*) *validus* (Nakamura 1984) Ash *et al.* 1994: emended description of *P. validus*. Int.J.System.Bacteriol. **45:**661-119.

Heyndrickx, M., K. Vandemeulebroecke, P. Scheldeman, K. Kersters, P. de Vos, N. A. Logan, A. M. Aziz, N. Ali, and R. C. W. Berkeley. 1996b. A polyphasic reassessment of the Genus *Paenibacillus*, reclassification of *Bacillus lautus* (Nakamura 1984) as *Paenibacillus lautus* comb. nov. and of *Bacillus peoriae* (Montefusco *et al.* 1993) as *Paenibacillus peoriae* comb. nov., and emended descriptions of *P. lautus* and *P. peoriae*. Int. J. Systemat. Bacteriol. **46:**988-1003.

Heyne, R. I. R., W. de Vrij, W. Crielaard, and W. N. Konings. 1991. Sodium ion-dependent amino acid transport in membrane vesicles of *Bacillus stearothermophilus*. J. Bact. **173:** 791-800.

Hicks, D. B. 1995a. Purification of three catalase isozymes from facultatively alkaliphilic *Bacillus firmus* OF4. Biochim. Biophys. Acta **1229:**347-355.

Hicks, D. B., and T. A. Krulwich. 1990. Purification and reconstitution of the F_0F_1 ATP synthase from alkaliphilic *Bacillus firmus* OF4 : evidence that the enzyme translocates H^+, but not Na^+. Journal of Biological Chemistry **265:**20547-20554.

Hicks, D. B., and T. A. Krulwich. 1995. Review: The respiratory chain of alkaliphilic bacteria. Biochim. Biophys. Acta **1229:**303-314.

Hicks, D. B., R. J. Plass, and P. G. Quirk. 1991. Evidence for multiple terminal oxidases, including cytochrome *d*, in facultatively alkaliphilic *Bacillus firmus* OF4. J. Bacteriol. **173:**5010-5016.

Higashibata, A., T. Fujiwara, and Y. Fukumori. 1998. Studies on the respiratory system in alkaliphilic *Bacillus* , a proposed new respiratory mechanism. Extremophiles **2:**83-92.

Hirota, N., and Y. Imae. 1983. Na^+-driven flagellar motor of an alkalophilic *Bacillus* strain YN-1. J. Biol. Chem. **258:**10577-10581.

Hoffmann, A., and P. Dimroth. 1991a. The ATPase of *Bacillus alcalophilus* Reconstitution of energy-transducing functions. Eur. Journal of Biochemistry **196:**493-497.

Hoffmann, A., and P. Dimroth. 1991b. The electrochemical proton potential of *Bacillus alcalophilus*. Eur. J. Biochem. **201:**467-473.

Hollaus, F., and H. Klaushofer. 1973. Identification of hyperthermophilic obligate anaerobic bacteria from extraction juices of beet sugar factories. International Sugar Journal **75:**237 – 241.

Holm, N. G. 1992. Marine hydrothermal systems and the origin of life. Origin of Life and Evolution of the Biosphere. **22:**1-242.

Holtom, G. J., R. J. Sharp, and R. A. D. Williams. 1993. Sodium-stimulated transport of glutamate by *Thermus thermophilus* strain B. Journal of General Microbiology **139:** 2245-2250.

Honda, H., T. Kudo, and K. Horikoshi. 1985a. Molecular cloning and expression of xylanase gene of alkaliphilic strain C-125 in *Escherichia coli*. J. Bact. **161:**784-785.

Honda, H., T. Kudo, and K. Horikoshi. 1986. Extracellular production of alkaline xylanase of alkalophilic *Bacillus* sp. by *Escherichia coli* carrying pCX311. Journal of Fermenation Technology **64:**373-377.

Horikoshi, K. 1971a. Production of alkaline enzymes by alkalophilic micoorganisms : Part I. Alkaline protease produced by *Bacillus* No. 221. Agricultural and Biological Chemistry **35:**1407-1414.

Horikoshi, K. 1971b. Production of alkaline enzymes by alkalophilic micoorganisms : Part II. Alkaline amylase produced by *Bacillus* No. A-40-2. Agric. Biol. Chem. **35:**1783-1791.

Horikoshi, K. 1990c. Microorganisms in alkaline environments. Kodansha, Tokyo.

Horikoshi, K. 1991b. Microorganisms in Alkaline Environments. K. Horikoshi (ed.). Chapter 1 : Introduction (1–4), Chapter 2 : Isolation and Classification of alkalophilic microorganisms (5–24), Chapter 3 : Physiology (25–82). Kodansha Ltd,VCH Publishers Inc, Tokyo (Japan), (USA and Federal Republic of Germany).

Horikoshi, K. 1996. Alkaliphiles - from an industrial point of view. FEMS Microbiol. Rev. **18:**259-270.

Horikoshi, K., and T. Akiba. 1982. Alkalophilic Microorganisms - A New Microbial World. K. Horikoshi and T. Akiba (ed.). Japan Scientific Societies Press and Springer-Verlag, Tokyo , New York.

Horikoshi, K., and Y. Atsukawa. 1973a. Xylanase produced by alkalophilic *Bacillus* No. C-59-2. Agric. Biol. Chem. **37:**2097-2103.

Horikoshi, K. and W.D. Grant. 1991. Super Bugs - Microorganisms in Extreme Environments. K. Horikoshi and W. D. Grant (ed.). Japan Scientific Societies Press, Springer-Verlag, Tokyo.

Huang, X.-P. 1998. Characterization of a novel aerobic cellulolytic thermophilic bacterium and its cellulases. Doctor of Philosophy thesis, University of Waikato.

Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. Koenig, R. Rachel, I. Rockinger, H. Frickle, and K. O. Stetter. 1992. *Aquifex pyrophilus*, new genus new species, represents a novel group of marine hyperthermophilic hydrogen-oxidising bacteria. Syst. Applied Microbiology **63:**945-950.

Hudson, J. A., and R. M. Daniel. 1988. Enumeration of thermophilic heterotrophs in geothermally heated soils from Mount Erebus, Ross Island, Antarctica. Appl. Environ. Microbiol. **54:**622-624.

Hudson, J. A., R. M. Daniel, and H. W. Morgan. 1989. Acidophilic and thermophilic *Bacillus* strains from geothermally heated antarctic soil. FEMS Microbio. Letters **60:**279-282.

Igarashi, K., K. Ara, K. Saeki, K. Ozaki, S. Kawai, and S. Ito. 1992. Nucleotide sequence of the gene that encodes a neopullulanase from an alkalophilic *Bacillus*. Bioscience Biotechnology Biochemistry **56:**514-516.

Ikeda, K., K. Nakajima, and I. Yumoto. 1994. Isolation and characterization of a novel facultatively alkaliphilic bacterium, *Corynebacterium* sp., grown on *n*-alkanes. Arch. Microbiol. **162:**381-386.

Ikura, Y., and K. Horikoshi. 1977. Isolation and some properties of alkalophilic bacteria utilizing rayon waste. Agricultural and biological chemistry **41:**1373-1377.

Imhoff, J. F., H. G. Sahl, G. S. H. Soliman, and H. G. Truper. 1979. The Wadi Natrun chemical composition and microbial mass developments in alkaline brines of eutrophic desert lakes. Geomicrobiol. J. **1:**219-234.

Imming, R., Stendal, B. R., A. Lehnberger, and K.-M. Bliesener. 1996. A new approach to the kinetics of colour formation in concentrated carbohydrate solutions. Starch **48:**163-166.

Ito, M., B. Cooperberg, and T. A. Krulwich. 1997c. Diverse genes of alkaliphilic *Bacillus firmus* OF4 that complement K^+ uptake-deficient *Escherichia coli* include an *fts*H homologue. Extremophile **1:**22-28.

Ito, M., A. A. Guffanti, and T. A. Krulwich. 1998. *mrp* : a multigene, multiple resistance-pH adaptation locus in *Bacillus subtilis*. Abstr. #1975

Ito, M., A. A. Guffanti, J. Zemsky, D. M. Ivey, and T. A. Krulwich. 1997b. Role of the *nhaC*-encoded Na^+/H^+ antiporter of alkaliphilic *Bacillus firmus* OF4. J. Bacteriol. **179:**3851-3857.

Ito, M., K. Tabata, and R. Aono. 1994. Construction of a new teichuronopeptide-defective derivative from alkaliphilic *Bacillus* sp. C-125 by cell fusion. Biosci. Biotech. Biochem **58:**2275-2277.

Ito, S., S. Shikata, K. Ozaki, S. Kawai, K. I. Okamoto, S. A. Takei, Y. Ohta, and T. Satoh. 1989. Alkaline cellulase for laundry detergents : Production by *Bacillus* sp. KSM-635 and enzymatic properties. Agric. Biol. Chem. **53:**1275-1281.

Ivey, D. M., A. A. Guffanti, J. S. Bossewitch, E. Padan, and T. A. Krulwich. 1991. Molecular cloning and sequencing of a gene from alkaliphilic *Bacillus firmus* OF4 that functionally complements an *Escherichia coli* strain carrying a deletion in the *nha* A Na^+ / H^+ antiporter gene. The Journal of Biological Chemistry **266:**23483-23489.

Ivey, D. M., A. A. Guffanti, and T. A. Krulwich. 1993. The Na^+ cycle in alkaliphilic *Bacillus* species, 101 – 124. *In* E. P. Bakker (ed.), Alkali Cation Transport Systems in Prokaryotes. CRC Press Inc,

Ivey, D. M., M. Ito, R. Gilmour, J. Zemsky, A. A. Guffanti, M. G. Sturr, D. B. Hicks, and T. A. Krulwich. 1998. Alkaliphile bioenergetics, *In* K. Horikoshi and W. D. Grant (ed.), Extremophiles, Microbial Life in Extreme Environments. John Wiley & Sons, New York.

Ivey, D. M., and T. A. Krulwich. 1991b. Organization and nucleotide sequence of the *atp* genes encoding ATP synthase from alkaliphilic *Bacillus firmus* OF4. Mol. Gen. Genet. **229:**292-300.

Ivey, D. M., and T. A. Krulwich. 1992. Two unrelated alkaliphilic *Bacillus* species possess identical deviations in sequence from those of conventional prokaryotes in regions of F_0 genes implicated in proton translocation through the ATP synthase. Res. Microbiol. **143:**467-470.

Jackson, T. J., R. F. Ramaley, and W. G. Meinschein. 1973. *Thermomicrobium* a new genus of extremely thermophilic bacteria. Int. J. System. Bacteriol. **23:**28-36.

Janssen, P. H., M. C. R., and M. H. W. 1994. A thermophilic, lipolytic *Bacillus* sp., and continuous assay of its *p*-nitrophenol-palmitate esterase activity. FEMS Microbio. Letters **120:**195-200.

Jarrell, K. F., T. Vydykhan, P. Lee, M. D. Agnew, and N. A. Thomas. 1997. Isolation and characterization of bacteriophage BCJA1, a novel temperate bacteriophage active against the alkaliphilic bacterium, *Bacillus clarkii*. Extremophiles **1:**199-206.

Jones, B. E., W. D. Grant, A. W. Duckworth, and G. G. Owenson. 1998. Microbial diversity of soda lakes. Extremophiles **2:**191-200.

Kaback, H. R. 1974. Transport in isolated bacterial membrane vesicles. Methods Enzymol **31:**698-709.

Kaback, H. R. 1986. Active transport in *E. coli* passage to permease. Ann. Rev. of Biophysics and Biophysical Chemistry **15:**279-319.

Kaback, H. R. J. P. Reeves, S. A. Short, and F. J. Lombardi. 1974. Mechanisms of active transport in isolated bacterial membrane vesicles XVIII. The mechanism of action of carbonylcyanide m-chlorophenylhydrazone. Arch. Biochem. Biophys. **160:**215-222.

Kaim, G., and P. Dimroth. 1995. A double mutation in subunit c of the Na(+)-specific F_1F_0-ATPase of *Propionigenium modestrum* results in a switch from Na^+ to H(+)-coupled ATP stnthesis in the *Escherichia coli* host cells. J. Mol.Biol. **253:**

Kakinuma, Y., and T. Unemoto. 1985. Sucrose uptake is driven by the Na^+ electrochemical potential in the marine bacterium *Vibrio alginolyticus*. J. Bact. **163:**1293-1295.

Kalisz, H. M. 1988a. Microbial proteinases. Adv.Biochem.Eng.Biotech. **36:**1-65.

Kalisz, H. M. 1988b. Microbial proteinases, 3-61. *In* A. Fietcher (ed.), Advances in Biochemical Engineering / Biotechnology, vol. 36, Enzyme studies. Springer, Heidelberg New York.

Kampfer, P. 1994. Limits and possibilities of total fatty acid analysis for classification and identification of *Bacillus* species. Sys. Appl. Microbiol. **17:**86-98.

Kandler, O. 1998. The early diversification of life and the origin of the three domains: A proposal, *In* J. Wiegel and M. W. W. Adams (ed.), Thermophiles – The keys to molecular evolution and the origin of life?, p19-31. Taylor and Francis, London/Philadelphia.

Kaneda, T. 1977. Fatty acids in the genus *Bacillus* : an example of branched-chain preference. Bacteriology Review **41:**391-418.

Kaneda, T. 1991. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol. Rev. **55:**288-302.

Kang, S.-K., T. Kudo, and K. Horikoshi. 1992. Molecular cloning and characterization of an alkalophilic *Bacillus* sp. C125 gene homologous to *Bacillus subtilis sec* Y. J. Gen. Microbiol. **138:**1365-1370.

Kates, M. 1993. Membrane lipids in archaea. *In* M. Kates, D. J. Kushner and A. T. Matheson (ed.), The biochemistry of archaea (archaebacteria), p261-295. Elsevier Science, Amsterdam.

Kato, C., T. Kudo, K. Watanabe, and K. Horikoshi. 1983. Extracellular production of *Bacillus* penicillinase by *Escherichia coli* carrying pEAP2. Eur. J. Appl. Microbiol. Biotechnol. **18:**339-343.

Kelly, C. T., and W. M. Fogarty. 1978. Production and properties of polygalacturonate lyase by an alkalophilic microorganism, *Bacillus* sp. RK9. Canadian Journal of Microbology **24:**1164-1172.

Kelly, C. T., F. O'Reilly, and V. M. Fogarty. 1983. Extracellular alpha-glucosidase of an alkalophilic microorganism, *Bacillus* sp. ATCC 21591. FEMS Microbio. Letters **20:**55-59.

Kelly, F. H. C., and D. W. Brown. 1978 Sugar Technol. Rev. **6:**1-48.

Kembhavi, A. A., A. Kulkarni, and A. Pant. 1993. Salt tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No. 64. Appl. Biochem. and Biotechnol. **38:**83-92.

Kemper, M. A., M. M. Urrutia, T. J. Beveridge, A. L. Koch, and R. J. Doyle. 1993. Proton motor force may regulate cell wall associated enzymes of *Bacillus subtilis*. J. Bact. **175:**5690-5696.

Kengen, S. W. M., F. A. M. De Bok, N.-D. Van Loo, C. Dijkema, A. J. M. Stams, and W. M. de Vos. 1994. Evidence for the operation of a novel Embden-Meyerhof pathway that involves ADP dependent kinases during sugar fermentation by *Pyrococcus furiosis*. J. Biol. Chem. **269:**17537-17541.

Kengen, S. W. M., and A. J. M. Stams. 1994a. Fermentation of L-alinine as a reduced end product in carbohydrate fermentation by the hyperthermophilic archeon *Pyrococcus furiosis*. Archives of Microbiology. **161:**168-175.

Kengen, S. W. M., and A. J. M. Stams. 1994b. Growth and energy conservation in batch cultures of *Pyrococcus furiosis*. FEMS Microbiol. Lett. **117:**305-310.

Kengen, S. W. M., A. J. M. Stams, and W. M. de Vos. 1996. Sugar metabolism of hyperthermophiles. FEMS Microbiol. Rev. **18:**119-137.

Kevbrin, V. V., T. N. Zhilina, F. A. Rainey, and G. A. Zavarzin. 1998. *Tindallia magadii* gen. nov., sp. nov - an alkaliphilic anaerobic ammonifier from soda lake deposits. Current Microbiology **37:**94-100.

Khasin, A., I. Alchanati, and Y. Shoham. 1993. Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus* T-6. Appl. Environ. Microbiol. **59:**1725-1730.

Khyami-Horani, H. 1996a. Thermotolerant strain of *Bacillus licheniformis* producing lipase. World Journal of Microbiology and Biotechnology **12:**399-401.

Kimura, H., A. Okamura, and H. Kawaide. 1994. Oxidation of 3-, 7-, and 12-hydroxyl groups of cholic acid by an alkalophilic *Bacillus* sp. Biosci. Biotech. Biochem **58:**1002-1006.

Kishino, H., and M. Hasegawa. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from sequence data and the branching order in the Hominoidea. J. Mol. Evol. **29:**170-179.

Kitada, M., Y. Dobashi, and K. Horikoshi. 1989b. Enzymatic properties of purified D-xylose isomerase from a thermophilic alkalophile *Bacillus* TX-3. Agric. Biol. Chem. **53:**1461-1468.

Kitada, M., M. Hashimoto, T. Kudo, and K. Horikoshi. 1994. Properties of two different Na^+/H^+ antiport systems in alkaliphilic *Bacillus* sp. stain C-125. J. Bact. **176:**6464-6469.

Kitada, M., and K. Horikoshi. 1976. Effect of pH on the production of alkaline proteinase by alkalophilic *Bacillus* sp. J. Ferment. Technol. **54:**579-586.

Kitada, M., and K. Horikoshi. 1982b. Energy coupling reactions of the active transport system for gamma-amino-isobutyric acid in alkalophilic *Bacillus* No. 8-1. Agric.Biol.Chem. **46:**1115-1120.

Kitada, M., and K. Horikoshi. 1987b. Bioenergetic properties of alkalophilic *Bacillus* sp. strain C-59 on an alkaline medium containing K_2CO_3. J. Bacteriol. **169:**5761-5765.

Kitada, M., and K. Horikoshi. 1992. Kinetic properties of electrogenic Na^+/H^+ antiport in membrane vesicles from an alkalophilic *Bacillus* sp. J. Bact. **174:**5936-5940.

Kitada, M., R. J. Lewis, and T. A. Krulwich. 1983. Respiratory chain of the alkalophilic bacterium *Bacillus firmus* RAB and its non-alkalophilic mutant derivative. J. Bact. **154:**330-335.

Kitada, M., S. Morotomi, K. Horikoshi, and T. Kudo. 1997. K^+/H^+ antiporter in alkaliphilic *Bacillus* sp. No 66 (JCM 9763). Extremophile **1:**135-141.

Kitada, M., K. Onda, and K. Horikoshi. 1989. The sodium / proton antiport system in a newly isolated alkalophilic *Bacillus* sp. J. Bact. **171:**1879-1884.

Kitada, M., L. Wijayanti, and K. Horikoshi. 1987. Biochemical properties of a thermophilic alkalophile. Agric. Biol. Chem **51:**2429-2435.

Kitamoto, N., T. Kimura, Y. Kito, and K. Ohmiya. 1992. Cloning and sequencing of the gene encoding cyclodextrin glucanotransferase from Bacillus sp. KL201. Journal of Fermentation Bioengineering **74:** 345-351.

Kluge, C., W. Laubinger, and P. Dimroth. 1992. The Na(+)-translocating ATPase of *Propionigenium modestrum.* Biochem. Soc. Trans. **20:**572-577

Kolstø, A.-B. 1997. Dynamic bacterial genome organization. Mol. Microbiol. **24:**241-248.

Kometani, T., Y. Terada, T. Nishimura, H. Takii, and S. Okada. 1994a. Purification and characterization of cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species and transglycosylation at alkaline pHs. Biosci. Biotech. Biochem **58:**517-520.

Kovalenko, E. V., and P. T. Malahova. 1983. The spore-forming iron-oxidizing bacterium *Sulfobacillus thermosulfidooxidans.* Mikrobiologija **52:**962.

Koyama, N., and Y. Nosoh. 1985. Effect of potassium and sodium ions on the cytoplasmic pH of an alkaliphilic *Bacillus*. Biochim. Biophys.Acta. **812:**206-212.

Koyama, N., K. Wakabayashi, and Y. Nosoh. 1987. Effect of K^+ on the membrane fuctions of an alkalophilic *Bacillus*. Biochim. Biophys.Acta **898:**293-298.

Kristjánsson, J. K. 1989. Thermophilic organisms as sources of thermostable enzymes. Trends Biotechnol. **7:**349.

Kristjánsson, J. K., and G. A. Alfredsson. 1983. Distribution of *Thermus* spp. in Icelandic hot springs and a thermal gradient. Appl. Environ. Microbiol. **45:**1785-1789.

Kristjánsson, J. K., S. Hjörleifsdóttir, V. T. Marteinsson, and G. A. Alfredsson. 1994. *Thermus scotoductus,* sp. nov., a pigment-producing thermophilic bacterium from hot tap water in iceland and including *Thermus* sp. X-1. System. Appl. Microbiol. **17:**44-50.

Kristjánsson, J. K., and K. O. Stetter. 1992. Thermophilic bacteria, 1-13. *In* J. K. Kristjansson (ed.), Thermophilic Bacteria. CRC Press Inc, USA.

Kroll, R. G. 1990. Alkalophiles, 55-92. *In* C. Edwards (ed.), Microbiology of extreme enrivonments. McGraw-Hill, New York.

Krulwich, T. A. 1983. Na^+ / H^+ antiporters. Biochimica et Biophysica Acta **726:**245-264.

Krulwich, T. A. 1986. Bioenergetics of alkalophilic bacteria. J. Membr. Biol **89:**113-125.

Krulwich, T. A., R. Agus, M. Schneier, and A. A. Guffanti. 1985a. Buffering capacity of Bacilli that grow at different pH ranges. J. Bact. **162:**768-772.

Krulwich, T. A., J. Cheng, and A. A. Guffanti. 1994. The role of monovalent cation / proton antiporters in Na^+-resistance and pH homeostasis in *Bacillus* : an alkaliphile versus a neutralophile. Journal exp. Biology **196:**457-470.

Krulwich, T. A., J. G. Federbush, and A. A. Guffanti. 1985b. Presence of a nonmetabolizable solute that is translocated with Na^+ enhances Na^+-dependent pH homeostasis in an alkalophilic *Bacillus*. The Journal of Biological Chemistry **260:**4055-4058.

Krulwich, T. A., and A. A. Guffanti. 1989a. Alkalophilic bacteria. Annual Review Microbiology **43:**435-463.

Krulwich, T. A., and A. A. Guffanti. 1989b. Mini Review : The Na^+ cycle of extreme alkalophiles: a secondary Na^+/H^+ antiporter and Na^+/solute symporters. Journal of Bioenergetics and Biomembranes **21:**663-677.

Krulwich, T. A., and A. A. Guffanti. 1992. Proton-coupled bioenergetic processes in extremely alkaliphilic bacteria. J. Bioenerg. Biomembr **24:**587-599.

Krulwich, T. A., A. A. Guffanti, R. F. Bornstein, and J. Hoffstein. 1982. A sodium requirement for growth, solute transport, and pH homeostasis in *Bacillus firmus* RAB. The Journal of Biological Chemistry **257:**1885-1889.

Krulwich, T. A., A. A. Guffanti, M. Y. Fong, L. Falk, and D. B. Hicks. 1986. Alkalophilic *Bacillus Firmus* RAB generates variants which can grow at lower Na^+ concentrations than the parental strain. J. Bacteriol. **165:**884-889.

Krulwich, T. A., A. A. Guffanti, and D. Seto-Young. 1990. pH homeostasis and bioenergetic work in alkalophiles. FEMS Microbiol. Rev. **75:** 271-278.

Krulwich, T. A., M. Ito, R. Gilmour, and A. A. Guffanti. 1997. Mechanisms of cytoplasmic pH regulation in alkaliphilic strains of *Bacillus*. Extremophiles **1:**163 – 169.

Krulwich, T. A., M. Ito, R. Gilmour, D. B. Hicks, and A. A. Guffanti. 1998. Energetics of alkaliphilic *Bacillus* species : physiology and molecules, 401-438. *In* R. K. Poole (ed.), Advances in Microbial Physiology, vol. 40. Academic Press, San Diego, California, USA; London, UK.

Krulwich, T. A., M. Ito, R. Gilmour, M. G. Sturr, A. A. Guffanti, and D. B. Hicks. 1996. Energetic problems of extremely alkaliphilic aerobes. Biochim. Biophys. Acta. **1275:**21-26.

Krulwich, T. A., M. Ito, D. B. Hicks, R. Gilmour, and A. A. Guffanti. 1998b. pH homeostasis and ATP synthesis - studies of two processes that necessitate inward proton translocation in extremely alkaliphilic *Bacillus* species. Extremophiles **2:**217-222.

Krulwich, T. A., and D. A. Ivey. 1990. Bacterial Energetics - Bioenergetics in extreme environments, 417-447. *In* T. A. Krulwich (ed.), The Bacteria, vol. 12. Academic Press., New York.

Kudo, T., M. Hino, M. Kitada, and K. Horikoshi. 1990. DNA sequences required for the alkalophily of *Bacillus* sp. strain C-125 are located close together on its chromosomal DNA. J. Bact. **172:**7282-7283.

Kudo, T., and K. Horikoshi. 1979. The environmental factors affecting sporulation of an alkalophilic *Bacillus* species. Agric. Biol. Chem. **43:**2613-2614.

Kudo, T., C. Kato, and K. Horikoshi. 1983c. Excretion of the penicillinase of an alkalophilic *Bacillus* sp. through the *Escherichia coli* outer membrane. J. Bacteriol. **156:**949-951.

Kulkarni, N., and M. Rao. 1996. Application of xylanase from alkaliphilic thermophilic *Bacillus* sp NCIM 59 in biobleaching of bagasse pulp. Journal of Biotechnology **51:**167-173.

Kunst, F., M. Pascal, J. Lepesant, J. Walle, and R. Dedonder. 1974. Purification and some properties of an endocellular sucrase from a constitutive mutant of *Bacillus subtilis* Marburg 168. Eur. J. Biochem **42:**611-620.

Kushner, D. J. 1964. Microbial Life in extreme environments. *In* D.J. Kushner (ed.), (1978) University of Ottawa, Academic Press London

Kwon, Y. T., J. O. Kim, S. Y. Moon, H. H. Lee, and H. M. Rho. 1994. Extracellular alkaline proteases from alkalophilic *Vibrio metschnikovii* strain HH530. Biotechnology Letters **16:**413-418.

Lai, Y. Z., and D. E. Ontto. 1979. Carbohydrate Research **75:**51-59.

Lake, J. A., R. Jain, J. Moore and M. C. Rivera 1998. Hyperthermophilic and mesophilic origins of the eukaryotic genome, *In* J. Wiegel and M. W. W. Adams (ed.), Thermophiles – The keys to molecular evolution and the origin of life?, p147-161. Taylor and Francis, London/Philadelphia.

Langworthy, T. A. 1978. Microbial life in extreme pH values. *In* D. J. Kushner (ed.), Microbial life in extreme environments. Academic Press, London, New York.

Lauraeus, M., T. Haltia, M. Saraste, and M. Wikstrom. 1991. *Bacillus subtilis* expresses two kinds of haem-A-containing terminal oxidases. Eur. J. Biochem. **197:**699-705.

Lewis, R. J., S. Belkina, and T. A. Krulwich. 1980. Alkalophiles have much higher cytochrome contents than conventional bacteria and their own non-alkalophilic mutant derivatives. Biochem. Biophys. Res. Commun. **85:**857-863.

Lewis, R. J., R. Prince, P. L. Dutton, D. Knaff, and T. A. Krulwich. 1981. The respiratory chain of *Bacillus alcalophilus* and its non-alkaliphilic mutant derivative. J. Biol. Chem. **256:**10543-10549.

Li, Y., M. Engle, N. Weiss, L. Mandelco, and J. Wiegel. 1994. *Clostridium thermoalcaliphilium* sp. nov., an anaerobic and thermotolerant facultative alkaliphile. Int. J. Syst. Bacteriol **44:**111-118.

Li, Y., L. Mandelco, and J. Wiegel. 1993. Isolation and charcaterisation of a moderately thermophilic anerobic alkaliphile , *Clostridium paradoxum* sp. nov. Int. J. Syst. Bacteriol **43:**450-460.

Lillie, R. D. 1928. The Gram stain. I. A quick method for staining Gram-positive organisms in the tissues. Archives of Pathology **5:**828.

Lin, E. C. C. 1976. Glycerol dissimilation and its regulation in bacteria. Ann. Rev. Microbiol. **30:**535-578.

Lin, L.L., M. R. Tsau, and W. S. Chu. 1994. General characteristics of thermostable amylopullulanases and amylases from the alkalophilic *Bacillus* sp. TS-23. Appl Microbiol Biotechnol **42:**51-56.

Lin, L. L., M. R. Tsau, and W. S. Chu. 1996. Purification and properties of a 140 kda amylopullulanase from thermophilic and alkaliphilic *Bacillus* sp strain TS-23. Biotechnology and Applied Biochemistry **24:**101-107.

Lindsay, J. A. 1988. Characterisation of bacterial spores from high temperature growth, transformants of *Bacillus subtilis*. Current Microbiology **16:**265.

Lindsay, J. A. 1995. Is thermophily a transferrable property in bacteria? Critical Reviews in Microbiology **21:**165-174.

Ljungdahl, L. G. 1979. Physiology of thermophilic bacteria. Adv. Microb. Physiol. **19:**149-243.

Logan, N. A., and R. C. W. Berkeley. 1984. Identification of *Bacillus* strains using the API system. Journal of General Microbiology **130:**1871-1882.

López-García, P. 1998. DNA topoisomerases, temperature adaptation, and early diversification of life, *In* J. Wiegel and M. W. W. Adams (ed.), Thermophiles – The keys to molecular evolution and the origin of life?, p201-216. Taylor and Francis, London/Philadelphia.

Lu, B., M. Stefansson, and D. Westerlund. 1995. Indirect detection of saccharides in reversed-phase liquid chromatography with highly alkaline mobile phases. Journal of Chromatography A **697:**317-327.

Manachini, P. L., M. G. Fortina, C. Parini, and R. Craveri. 1985. *Bacillus thermoruber* sp. nov., nom. rev., a red-pigmented thermophilic bacterium. Int. J. System.Bacteriol. **35:**493-496.

Mandel, K. G., A. A. Guffanti, and T. A. Krulwich. 1980. Monovalent cation / proton antiporters in membrane vesicles from *Bacillus alcalophilus*. J. Biol. Chem **255:** 7391-7396.

Mandel, M., and Marmur, J. 1973. Use of ultraviolet absorbance-temperature profile for determining the Guanine plus Cytosine content of DNA, p. 195-206. *In*: Methods in Enzymology XII Part B, Grossman, L. and Moldave, K. (eds.). Academic Press, London.

Manley-Harris, M. 1997. Structural studies by NMR spectroscopy of the major oligomers from alkali degraded arabinogalactin from *Larix occidentalis*. Carbohydrate Polymers **34:**243-249.

Manley-Harris, M., and G. N. Richards. 1981. Carbohydrate Research **90:**27-40.

Mannino, S., M. S. Cosio, and P. Zimei. 1996. Microdialysis sampling and high performance liquid chromatography with amperometric detection for sugar analysis in milk products. Electroanalysis **8:**353-355.

Markwell, M. A. K., S. M. Haas, N. E. Tolbert, and L. L. Bieber. 1981. Protein determination in membrane and lipoprotein samples. Methods Enzymology **72:**296-303.

Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. Journal of Molecular Biology **5:**109-118.

Marsh, R. N., and P. R. Norris. 1983a. The isolation of some thermophilic, autotrophic, iron- and sulphur-oxidising bacteria. FEMS Microbiol. Letters **17:**311.

Marsh, R. N., and P. R. Norris. 1983b. Mineral sulphide oxidation by moderately thermophilic acidophilic bacteria. Biotechnology Letters **5:**585.

Marteinsson, V. T., J.-L. Birrien, C. Jeanthon, and D. Prieur. 1996. Numerical taxonomic study of thermophilic *Bacillus* isolated from three geographically separated deep-sea hydrothermal vents. FEMS Microbiology Ecology **21:**255-266.

Marteinsson, V. T., J.-L. Birrien, J. K. Kristjánsson, and D. Prieur. 1995. First isolation of thermophilic aerobic non-sporulating heterotrophic bacteria from deep-sea hydrothermal vents. FEMS Microbiology Ecology **18:**163-174.

Martin, S. A., and J. B. Russell. 1987. Transport and phosphorylation of disaccharides by the ruminal bacterium *Streptococcus bovis*. Appl. Environ. Microbiol. **53:**2388-2393.

Mathrani, I. M., and B. K. Ahring. 1992. Thermophilic and alkalophilic xylanases from several *Dictyoglomus* isolates. Applied Microbiology Biotechnology **38:**23-27.

Mathrani, I. M., D. R. Boone, R. A. Mah, G. E. Fox, and P. P. Lau. 1988. *Methanohalophilus zhilinae* sp. nov., an alkaliphilic, halophilic, methylotrophic methanogen. Int. J. Syst. Bacteriol **38:**139-142.

Mathrani, I. M., P. Nielsen, J. Sonne-Hansen, J. K. Kristjánsson, and B. K. Ahring. 1993. Influence of pH and temperature on enumeration of cellulose- and hemicellulose-degrading thermophilic anaerobes in neutral and alkaline icelandic hot springs. Appl. Environ. Microbiol. **59:**1963-1965.

Matsukura, H., and Y. Imae. 1987. Na^+ modulates the K^+ permeability and the membrane potential of alkalophilic *Bacillus*. Biochim. Biophys.Acta. **904:**301-308.

Matsunaga, A., N. Koyama, and Y. Nosoh. 1974. Purification and properties of esterase from *Bacillus stearothermophilus*. Arch. Biochem. Biophys. **160:**505-513.

Matsuzawa, M., M. Kawano, N. Nakamura, and K. Horikoshi. 1975. An improved method for the production of schardinger beta-dextrin on an industrial scale by cyclodextrin glycosyltransferase of an alkalophilic *Bacillus* sp. Die Staerke **27:**410-413.

McLaggan, D., M. H. Selwyn, and A. P. Dawson. 1984. Dependence on Na^+ of control of cytoplasmic pH in a facultative alkalophile. FEBS Lett. **165:**254-258.

Meadow, N. D., D. K. Fox, and S. Roseman. 1990. The bacterial phosphoenolpyruvate:glycose phosphotransferase system. Ann. Rev. Biochem. **59:**497-542.

Meadow, N. D., R. Revuelta, V. N. Chen, R. R. Colwell, and S. Roseman. 1987. Phosphoenolpyruvate:glycose phosphotransferase system in species of *Vibrio*, a widely distributed marine bacterial genus. J. Bacteriol. **169:**4893-4900.

Meier-Stauffer, K., H.-J. Busse, F. A. Rainey, J. Burghardt, A. Scheberl, F. Hollaus, B. Kuen, A. Makristathis, U. B. Sleytr, and P. Messner. 1996. Description of *Bacillus thermoaerophilus* sp. nov., to include sugar beet isolates and *Bacillus brevis* ATCC 12990. Int. J. System. Bacteriol. **46:**532-541.

Meijer, D., R. Van Steenbergen, and B. Jones. 1996. Isolation and characterisation of a novel alkaliphilic thermophilic bacterium from the soda lake environment, belonging to the order *Thermotogales*. Abstracts Thermophiles '96, International Conference on The biology, ecology and biotechnology of thermophilic microorganisms.

Michels, P. A. M., J. P. J. Michels, J. Boonstra, and W, N, Konings. 1979. Generation of an electrochemical gradient in bacteria by the excretion of metabolic end-products. FEMS Lett. **5**:357-364.

Miller, S. L., and J. L. Bada. 1988. Submarine hot springs and the origin of life. Nature **334**:609-611.

Miller, S. L., and A. Lazcano. 1998. Facing up to chemical realities: life did not begin at the growth temperatures of hyperthermophiles, *In* J. Wiegel and M. W. W. Adams (ed.), Thermophiles – The keys to molecular evolution and the origin of life?, p127-133. Taylor and Francis, London/Philadelphia.

Miquel, P. 1888. Monograpki d'un bacille vicant au-dela de 70°C. Ann. Micrograph **1**:3-10.

Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature **191:**144-148.

Mitchell, P. 1963. Molecule, group, and electron translocation through natural membranes. Biochem. Soc. Symp. **22:**142-168.

Mitchell, P. 1973. Performance and conservation of osmotic work by proton-coupled solute porter systems. Journal of Bioenergetics. 4:63-91

Mizusawa, K., E. Ichishima, and F. Yoshida. 1964. Studies on the proteolytic enzymes of thermophilic *Streptomyces* Part 1 . Purification and some properties. Agr. Biol. Chem. **28:**884-895.

Mizusawa, K., E. Ichishima, and F. Yoshida. 1969. Production of thermostable alkaline proteases by thermophilic *Streptomyces*. Applied Microbiology **Mar.:**366-371.

Munster, M. J., A. P. Munster, J. R. Woodrow, and R. J. Sharp. 1986. Isolation and preliminary taxonomic studies of *Thermus* strains isolated from Yellowstone National Park,USA. J.Gen. Microbiol. **132:**1677.

Nakamura, L. K. 1984. *Bacillus amylolyticus* sp. nov., nom. rev., *Bacillus lautus* sp. nov., nom. rev., *Bacillus pabuli* sp. nov., nom. rev., and *Bacillus validus* sp. nov., nom. rev. Int.J.Syst.Bacteriol. **34:**224-226.

Nakamura, L. K., I. Blumenstock, and D. Claus. 1988. Taxonomic study of *Bacillus coagulans* Hammer 1915 with a proposal for *Bacillus smithii* sp.nov. Int. J. System. Bacteriol. **38:**63-73.

Nakamura, N., and K. Horikoshi. 1976a. Purification and properties of neutral-cyclodextrin glycosyl-transferase of an alkalophilic *Bacillus* sp. Agric. Biol. Chem. **40:**1785-1791.

Nakamura, N., and K. Horikoshi. 1976b. Characterization of acid-cyclodextrin glycosyl-transferase of an alkaliphilic *Bacillus* sp. Agric. Biol. Chem. **40:**1647-1648.

Nakamura, N., and K. Horikoshi. 1976c. Characterization and some culture conditions of a cyclodextrin glycosyltransferase-producing alkalophilic *Bacillus* sp. Agric. Biol. Chem. **40:**753-757.

Nakamura, N., and K. Horikoshi. 1976d. Purification and properties of cyclodextrin glycosyltransferase of an alkalophilic *Bacillus* sp. Agric. Biol. Chem. **40:**935-941.

Nakamura, N., K. Watanabe, and K. Horikoshi. 1975. Purification and some properties of alkaline pullulanase from a strain of *Bacillus* No. 202-1, an alkalophilic microorganism. Biochimica et Biophysica Acta **397:**188-193.

Nakamura, S., R. Nakai, K. Wakabayashi, Y. Ishiguro, R. Aono, and K. Horikoshi. 1994. Thermophilic alkaline xylanase from newly isolated alkaliphilic and thermophilic *Bacillus* sp. strain TAR-1. Biosci. Biotech. Biochem **58:**78-81.

Nakamura, S., K. Wakabayashi, R. Nakai, R. Aono, and K. Horikoshi. 1993. Production of alkaline xylanase by a newly isolated *Bacillus* sp. YIM-1. World Journal Microbiology Biotechnology **9:**221-224.

Newmark, P. 1988. Two European Companies Market Lipases. Bio / Technology **369:**134-138.

Nicolaus, B., L. Lama, E. Esposito, M. Manca, G. di Prisco, and A. Gambacorta. 1996. *Bacillus thermoantarcticus* sp. nov., from Mount Melbourne, Antarctica: a novel thermophilic species. Polar Biology **16:**101-104.

Nicolaus, B., M. C. Manca, L. Lama, E. Esposito, and A. Gambacorta. 1995. Effects of growth temperature on the polar lipid pattern and fatty acid composition of seven thermophilic isolates from the antarctic continent. Journal of Systematic and Applied Microbiology **18:**32-36.

Nielsen, P., D. Fritze, and F. G. Priest. 1995. Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. Microbiology **141:**1745-1761.

Nielsen, P., F. A. Rainey, H. Outtrup, F. G. Priest, and D. Fritze. 1994. Comparative 16S rDNA sequence analysis of some alkaliphilic bacilli and the establishment of a sixth rRNA group within the genus *Bacillus*. FEMS Microbio. Letters **117:**61 – 66.

Niimura, Y., E. Koh, F. Yanagida, K.-I. Suzuki, K. Komagata, and M. Kozaki. 1990. *Amphibacillus xylanus* gen. nov., sp. nov., a facultatively anaerobic sporeforming xylan-digesting bacterium which lacks cytochrome, quinone and catalase. Int. J. System. Bacteriol. **40:**297-301.

Nishihara, M., T. Kyuragi, N. Sone and Y. Koga. 1998. *Sn*-glycerol-1-phosphate dehydrogenase: a key enzyme in the biosynthesis of ether phospholipids in archaea, *In* J. Wiegel and M. W. W. Adams (ed.), Thermophiles – The keys to molecular evolution and the origin of life?, p281-285. Taylor and Francis, London/Philadelphia.

Nomoto, M., D. Shew, S. Chen, T. Yen, C. Liao, and C. Yang. 1984. Cyclodextrin glucanotransferase from alkalophilic bacterium of Taiwan. Agric. Biol. Chem. **48:**1337-1338.

Nystrand, R. 1984. *Saccharococcus thermophilus* gen. nov. sp. nov. isolated from beet sugar extraction. System. Appl. Microbiol. **5:** 204-219.

Ohkoshi, A., T. Kudo, T. Mase, and K. Horikoshi. 1985. Purification and three types of xylanases from an alkalophilic *Aeromonas* sp. Agric. Biol. Chem. **49:**3037-3038.

Okazaki, W., T. Akiba, K. Horikoshi, and R. Akahoshi. 1984. Production and properties of two types of xylanases from alkalophilic thermophilic *Bacillus* sp. Applied Microbiology Biotechnology **19:**335-340.

Okazaki, W., T. Akiba, K. Horikoshi, and R. Akahoshi. 1985. Purification and characterization of xylanases from alkalophilic thermophilic *Bacillus* sp. Agric. Biol. Chem **49**:2033-2039.

Olendzenski, L., and J. P. Gogarten 1998. Deciphering the molecular record for the early evolution of life: gene duplication and horozontal gene transfer, *In* J. Wiegel and M. W. W. Adams (ed.), Thermophiles – The keys to molecular evolution and the origin of life?, p165-176. Taylor and Francis, London/Philadelphia.

Oshima, M., and A. Miyagawa. 1973. Comparative studies on the fatty acid composition of moderately and extremely thermophilic bacteria. Lipids **9**:476-479.

Overend, W. G. 1972. *In* W. Pigman and D. Horton (ed.), The carbohydrates : chemistry and biochemistry, vol. 1A. Academic Press, New York.

Paavilainen, S., P. Helistö, and T. Korpela. 1994. Conversion of carbohydrates to organic acids by alkaliphilic bacilli. Journal of Fermentation and Bioengineering **78**:217-222.

Paavilainen, S., M. Mäkelä, and T. Korpela. 1995. Proton and carbon inventory during the growth of an alkaliphilic *Bacillus* indicates that protons are independent from acid anions. Journal of Fermentation and Bioengineering **80**:429-433.

Padan, E., and S. Schuldiner. 1996. Bacterial Na^+/H^+ antiporters - molecular biology, biochemistry, and physiology., *In* W. N. Konings, H. R. Kaback and J. Lolkema (ed.), The handbook of biological physics.- Transport processes in membranes, vol. II. Elsevier Science, Amsterdam.

Parker, C., N. Peekhaus, X. Zhang, and T. Conway. 1997. Kinetics of sugar transport and phosphorylation influence glucose and fructose cometabolism by *Zymomonas mobilis*. Applied and Evironmental Microbiology **63**:3519-3525.

Parker, C., T., W. O. Barnell, J. L. Snoep, L. O. Ingram, and T. Conway. 1995. Characterization of the *Zymomonas mobilis* glucose facilitator gene product (*glf*) in recombinant *Escherichia coli*: examination of transport mechanism, kinetics, and the role of glucokinase in glucose transport. Mol. Microbiol. **15**:795-802.

Parker, K. J. 1970. La Sucrerie Belge **89**:119-126.

Parker, K. J. 1971. Chemical Problems in the Sucrose Industry, 85-92. *In* J. Yudkin, J. Edelman and L. Hough (ed.), Sugar - Chemical, Biological and Nutritional Aspects of Sucrose, chapter 9. Butterworths, London.

Pask-Hughes, R., and R. A. D. Williams. 1975. Extremely thermophilic Gram-negative bacteria from hot tap water. J. Gen. Micribiol. **88**:321.

Patel, B. K. C., J. H. Skerratt, and P. D. Nichols. 1991. The phospholipid ester-linked fatty acid composition of thermophilic bacteria. Syst. Appl. Microbiol. **14**:311-316.

Peteranderl, R., J. E. B. Shotts, and J. Wiegel. 1990. Stability of antibiotics at growth conditions of thermophilic anaerobes. Appl. Environ. Microbiol. **56**:1981-1983.

Peterson, G. L. 1979. Review of the Folin Phenol Quantitation Method of Lowry, Rosebrough, Farr and Randall. Anal.Biochem. **100**:201-220.

Peterson, G. L. 1983. Determination of total protein. Methods in Enzymology **91**:95-121.

Phillips, W. E., and J. J. Perry. 1976. *Thermomicrobium fosteri* sp. nov. a hydrocarbon utilising obligate thermophile. Int. J. System. Bacteriol. **26**:220-225.

Prado, A., M. S. Da Costa, and V. M. C. Madeira. 1988. Effect of growth temperature on the lipid composition of two strains of Thermus sp. J. Gen Microbiology **134:**1653-1660.

Pressman, B. C. 1976. Biological applications of ionophores. Ann. Rev. Biochem. **45:**501-530.

Preston, N. W., and A. Morrell. 1962. Reproducible results with the Gram stain. Journal of Pathology and Bacteriology **84:**241-243.

Preziosi, L., G. P. F. Michel, and J. Baratti. 1990. Characterization of sucrose-hydrolysing enzymes of *Zymomonas mobilis*. Arch. Microbiol. **153:**181-186.

Quirk, P. G., D. B. Hicks, and T. A. Krulwich. 1993. Cloning of the *cta* operon from alkaliphilic *Bacillus firmus* OF4 and characterization of the pH-regulated cytochrome*caa3* oxidase it encodes. J. Biol. Chem. **268:**678-685.

Qureshi, M. H., T. Fujiwara, and Y. Fukumori. 1996. Succinate:quinone oxidoreductase (complex II) containing a single heme b in facultative alkaliphilic *Bacillus* sp. Strain YN-2000. J. Bacteriol. **178:**3031-3036.

Qureshi, M. H., I. Yumoto, T. Fujiwara, Y. Fukumori, and T. Yamanaka. 1990. A novel *aco*-type cytochrome-c oxidase from a facultative alkaliphilic *Bacillus* : purification, and some molecular and enzymatic features. J. Biochem. **107:**480-485.

Rainey, F. A., D. Fritze, and E. Stackebrandt. 1994. The phylogenetic diversity of thermophilic members of the genus *Bacillus* as revealed by 16S rDNA analysis. FEMS Microbio. Letters **115:**205-211.

Reade, S. M., and D. H. Northcote. 1981. Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. Anal.Biochem. **116:**53-64.

Reizer, J., N. Grossowicz, and Y. Barenholz. 1985. The effect of growth temperature on the thermotropic behavior of the membranes of a thermophilic *Bacillus*. Composition –structure-function relationships. Biochim. Biophys. Acta **815:**268 – 280.

Rhode, M., F. Mayer, D. B. Hicks, and T. A. Krulwich. 1989. Immunoelectron microscopic localization of the F_0F_1 ATPase (ATP synthase) on the cytoplasmic membrane of alkalophilic *Bacillus firmus* RAB. Biochim, Biophys, Acta **985:**233-235.

Richards, G. N. 1986. Initial steps in thermal degradation of sucrose. International Sugar Journal **88:**145-148.

Ross, H. M. N., and W. D. Grant. 1985. Nucleic acid studies on halophilic archaebacteria. Journal of General Microbiology **131:**165-173.

Rottenberg, H. 1990. Decoupling of oxidative phosphorylation and photophosphorylation. Biochim. Biophys. Acta **1018:**1-17.

Russell, J. B. 1990. Low affinity, high capacity system of glucose transport in the ruminal bacterium *Streptococcus bovis*: evidence for a mechanism of facilitated diffusion. Appl. Environ. Microbiol. **56:**3304-3307.

Russell, J. B., H. J. Strobel, and S. A. Martin. 1990. Strategies of nutrient transport by ruminal bacteria. Journal of Dairy Science **73:**2996-3012.

Russell, N. J., and N. Fukunaga. 1990. A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. FEMS Microbiol. Rev. **75:**171-182.

Saheb, S. A. 1972. Permeation du glycerol et sporulation chez *Bacillus subtilis*. Can. J. Microbiol. **18:**1307-1313.

Saier, M. H., Jr. 1985. Mechanisms and regulation of carbohydrate transport in bacteria. Academic Press, Inc., New York.

Saier, M. H., Jr. and A. M. Chin. 1990. Energetics of the bacterial phosphotransferase system in sugar transport and the regulation of carbon metabolism. p.273-300. *In* T. A. Krulwich (ed.), Bacterial energetics. Academic Press, Inc., San Diego.

Saiki, T., K. Yasuhiko, and K. Kawagoe. 1985. *Dictyoglomus thermophilum* Gen. nov., sp. nov., a chemoorganotrophic, anaerobic, thermophilic bacterium. Int. J. Syst. Bacteriol. **35:**253-259.

Sakai, Y., C. Moritani, M. Tsuda, and T. Tsuchiya. 1989. A respiratory-driven and an artifically driven ATP synthesis in mutants of *Vibrio parahaemolyticus* lacking H^+-translocating ATPase. Biochim.Biophys.Acta **973:**450-456.

Sarkar, A. 1991. Isolation and characterization of thermophilic, alkaliphilic, cellulose-degrading *Bacillus thermoalcaliphilus* sp. nov. from termite (*Odontotermes obesus*) mound soil of a semiarid area. Geomicrobiology Journal **9:**225-232.

Sashihara, N. 1991. Extracellular invertase of alkaliphilic *Bacillus* strain, 137-142. *In* K. Horikoshi and W. D. Grant (ed.), Super-Bugs, Microorganisms in Extreme Environments, chapter 4. Japan Scientific Societies Press, Springer-Verlag, Tokyo.

Saul, D. J., A. Rodrigo, R. A. Reeves, L. C. Williams, and P. L. Bergquist. 1993. Taxonomy of twenty *Thermus* isolates based on the complete 16S ribosomal RNA gene sequences. Int. J. System. Bacteriol. **43:**754-760.

Schafer, T., and P. Schonheit. 1992. Maltose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic archaen *Pyrococcus furiosis*: evidence for the operation of a novel sugar fermentation pathway. Archives of Microbiology. **158:**188-202.

Schafer, T., K. B. Xavier, H. Santos, and P. Schonheit. 1994. Glucose fermentation to acetate and alanine in resting cell suspensions of *Pyrococcus furiosis:* proposal of a novel glycolytic pathway based on ^{13}C labelling data and enzyme activities. FEMS Lett. **121:**107-114.

Scherrer, P., U. Alexiev, T. Marti, H. G. Khorana, and M. P. Heyn. 1994. Covalently bound pH-indicator dyes at selected extracellular or cytoplasmic sites in bacteriorhodopsin. 1. Proton migration along the surface of bacteriorhodopsin micelles and its delayed transfer from surface to bulk. Biochemistry **33:**13684-13692.

Scholz, T., W. Demharter, R. Hensel, and 0. Kandler. 1988 *Bacillus pallidus* sp. nov. A new thermophilic species from sewage. System. Appl. Microbiol. **9:**91-96.

Seto, Y., M. Hashimoto, R. Usami, T. Hamamoto, T. Kudo, and K. Horikoshi. 1995. Characterization of a mutation responsible for an alkali-sensitive mutant, 18224, of alkaliphilic *Bacillus* sp. stain C-125. Biosci. Biotech. Biochem **59:**1364-1366.

Sharp, R. J., K. J. Bown, and A. Atkinson. 1980. Phenotypic and genotypic characterization of some thermophilic species of *Bacillus*. J. Gen. Microbiol. **117:**201-210.

Shida, O., H. Takagi, K. Kadowaki, L. K. Nakamura, and K. Komagata. 1997a. Transfer of *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the Genus *Paenibacillus* and emended description of the genus *Paenibacillus*. Int.J.Syst.Bacteriol. **47:**289-298.

Shida, O., H. Takagi, K. Kadowaki, L. K. Nakamura, and K. Komagata. 1997b. Emended description of *Paenibacillus amylolyticus* and description of *Paenibacillus illinoisensis* sp. nov. and *Paenibacillus chibensis* sp. nov. Int.J.Syst.Bacteriol. **47:**299-306.

Shida, O., H. Takagi, K. Kadowaki, S. Udaka, L. K. Nakamura, and K. Komagata. 1995. Proposal of *Bacillus reuszeri* sp, nov., *Bacillus formosus* sp. nov., nom. rev., and *Bacillus borstelensis* sp. nov., nom. rev. Int. J. Systemat. Bacteriol. **45:**93-100.

Shikata, S., K. Saeki, H. Okoshi, T. Yoshimatsu, K. Ozaki, S. Kawai, and S. Ito. 1990. Alkaline cellulase for laundry detergents : Production by alkalophilic strains *Bacillus* and some properties of the crude enzymes. Agricultural and Biological Chemistry **54:**91-96.

Shock, E. L., T. McCollom and M. D. Schulte. 1998. The emergence of metabolism from within hydrothermal systems, *In* J. Wiegel and M. W. W. Adams (ed.), Thermophiles – The keys to molecular evolution and the origin of life?, p59-76. Taylor and Francis, London/Philadelphia.

Sinensky, M. 1974. Homeoviscous adaptation - a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc Natl Acad Sci USA **71:**522-525.

Sinha, N., and T. Satyanarayana. 1991. Alkaline Protease Production by Thermophilic *Bacillus licheniformis*. Indian J. Microbiol. **31:** 425-430.

Skulachev, V. P. 1991. Chemiosmotic systems in bioenergetics : H^+ cycles and Na^+ cycles., 387-444. *In* P. Mitchell and C. A. Pasternak (ed.), Perspectives in vectorial metabolism and osmochemistry. Glynn Research Foundation, Bodmin, Cornwall P130 4AV , UK.

Slater, E. C. 1987. The mechanism of the conservation of energy of biological oxidations. Eur. J. Biochem. **166:**489-504.

Slaughter, L. H., and D. P. Livingston. 1994. Separation of fructan isomers by high performance anion exchange chromatography. Carbohydrate Research **253:**287-291.

Slee, A. M., and J. M. Tanzer. 1979. Phosphoenolpyruvate-dependent sucrose phosphotransferase activity in *Streptococcus mutans* NCTC 10449. Infect. Immun **24:**821-828.

Smith, N. R., R. E. Gordon, and F. E. Clark. 1946. Aerobic mesophilic sporeforming bacteria. USDA miscellaneous publication no. 559. U.S. Department of Agriculture, Washington D.C.

Sorokin, D. Y., G. Muyzer, T. Brinkhoff, J. G. Kuenen, and M. S. M. Jetten. 1998. Isolation and characterization of a novel facultatively alkaliphilic *Nitrobacter* species, *N. alkalicus* sp. nov. Arch. Microbiol. **170:**345-352.

Souza, K. A., P. H. Deal, H. M. Mack, and C. E. Turnbill. 1974. Growth and reproduction of microorganisms under extremely alkaline conditions. Applied Microbiology **28:**1066-1068.

Spanka, R., and D. Fritze. 1993. *Bacillus cohnii* sp.nov., a new obligately alkaliphilic, oval-forming *Bacillus* species with ornithine and aspartic acid instead of diaminopimelic acid in the cell wall. International Journal of Systematic Bacteriology **43:**150 – 156.

Speelmans, G., W. de Vrij, and W. Konings. 1989. Characterization of amino acid transport in membrane vesicles from the thermophilic fermentative bacterium *Clostridium fervidus*. Journal of Bacteriology **171:**3788-3795.

Speelmans, G., B. Poolman, T. Abee, and W. N. Konings. 1994. The F- or V-type Na^+-ATPase of the thermophilic bacterium *Clostridium fervidus*. J. Bact. **176:**5160-5162.

Speelmans, G., B. Poolman, and W. N. Konings. 1993. Amino acid transport in the thermophilic anaerobe *Clostridium fervidus* is driven by an electrochemical sodium gradient. J. Bact. **175:**2060-2066.

Spring, S., W. Ludwig, M. C. Marquez, A. Ventosa, and K.-H. Schleifer. 1996. *Halobacillus* gen. nov., with description of *Halobacillus litoralis* sp. nov. and *Halobacillus truperi* sp. nov., and transfer of *Sporosarcina halophilia* to *Halobacillus halophilus* comb. nov. Int.J.Syst.Bacteriol. **46:**492-496.

Sprott, G. D., M. Meloche, and J. C. Richards. 1991. Proportions of diether, macrocyclic diether and tetraether lipids in *Methanococcus jannaschii* grown at different temperatures. J. Bact. **173:**3907-3910.

St. Martin, E. J., and C. L. Wittenberger. 1979. Characterisation of of a phosphoenolpyruvate-dependent sucrose phosphyl transferase system in *Streptococcus mutans*. Infect. Immun **24:**865-868.

Stahl, S. R., and O. Olsson. 1977. Temperature range variance of *Bacillus megaterium*. Arch. Microbiol. **113:**221.

Stetter, K. O. 1992. Life at the upper temperature border., *In* Frontiers of Life. Editions Frontières, Gif-sur-Yvette, France.

Stramer, S. L. and M. J. Starzyk. 1981. The occurrence and survival of *Thermus aquaticus*. Microbios **32:** 99-110.

Strom, P. F. 1985a. Effect of temperature on bacterial species diversity in thermophilic solid waste composting. Appl. Environ. Microbiol. **50:**899-905.

Strom, P. F. 1985b. Identification of thermophilic bacteria in solid waste composting. Appl. Environ. Microbiol. **50:**906-913.

Sturr, M. G., A. A. Guffanti, and T. A. Krulwich. 1994. Growth and bioenergetics of alkaliphilic *Bacillus firmus* OF4 in continuous culture at high pH. J. Bact. **176:**3111-3116.

Sugiyama, S. 1995. Na^+-driven flagellar motors as a likely Na^+ re-entry pathway in alkalophilic bacteria. Molecular Microbiology **15:**591–592.

Sugiyama, S., E. J. J. Cragoe, and Y. Imae. 1988. Amiloride, a specific inhibitor for the Na^+-driven flagellar motors of alkalophilic *Bacillus*. J. Biol. Chem. **263:**8215-8219.

Sunaga, T., T. Akiba, and K. Horikoshi. 1976. Production of penicillinase by an alkalophilic *Bacillus*. Agric. Biol. Chem. **40:**1363-1367.

Sunaga, T., T. Akiba, and K. Horikoshi. 1979. Separation and properties of penicillinase of an alkalophilic *Bacillus*. Agric. Biol. Chem. **43:**477-480.

Sundaram, T. K. 1986. Physiology and growth of thermophilic bacteria, *In* T. D. Brock (ed.), Thermophiles - General, Molecular and Applied Microbiology. John Wiley and Sons Inc, USA.

Sutherland, K. J., M. Hashimoto, T. Kudo, and K. Horikoshi. 1993. A partial physical map for the chromosome of alkalophilic *Bacillus* sp strain C-125. J. Gen. Microbiol. **139:**661-667.

Suzuki, T., and K. Yamasato. 1994. Phylogeny of spore-forming lactic acid bacteria based on 16S rRNA gene sequences. FEMS Microbiol.Lett. **115:**13-18.

Suzuki, Y., T. Kishigami, K. Inoue, Y. Mizoguchi, N. Eto, M. Takagi, and S Abe. 1983. *Bacillus thermoglucosidasius* sp. nov., a new species of obligately thermophilic bacilli. Syst. Appl. Microbiol. **4:**487-495.

Svetlitshnyi, V., F. Rainey, and J. Weigel. 1996. *Thermosyntropha lipolytica* gen. nov., sp. nov., a lipolytic, anaerobic, alkalitolerant, thermophilic bacterium utilizing short- and long-chain fatty acids in syntrophic coculture with a methanogenic archaeum or organoheterotrophic alkalitolerant thermophile utilising short and long chain fatty acids in syntrophic co-culture with a methanogen. International Journal of Systematic Bacteriology 46: 1131-1137. Int. J. System. Bacteriol. **46:**1131 – 1137.

Svobodová, J., J. Julák, J. Pilar, and P. Svoboda. 1988. Membrane fluidity in *Bacillus subtilis*. Validity of homeoviscous adaptation. Folia Microbiol (Praha) **33:**170-177.

Swofford, D. L. 1990. PAUP: phylogenetic anaylysis using parsimony, version 3.0. Complete program and manual. Natural History Survey, Champaign., Illinois.

Swofford, D. L., G. J. Olsen, P. J. Waddell, and D. M. Hillis. 1996. Phylogenetic inference, 407-514. *In* D. M. Hillis, C. Moritz and B. K. Mable (ed.), Molecular Systematics. Sinauer, Sunderland, MA.

Tabernero, C., P. M. Coll, J. M. Fernández-Abalos, P. Pérez, and R. I. Santamaría. 1994. Cloning and DNA sequencing of *bgaA*, a gene encoding and Endo-ß-1,3-1,4-glucanase, from an alkalophilic *Bacillus* strain (N137). Appl. Environ. Microbiol. **60:**1213-1220.

Takagi, H., O. Shida, K. Kadowaki, K. Komagata, and S. Udaka. 1993. Characterization of *Bacillus brevis* with descriptions of *Bacillus migulanus* sp. nov., *Bacillus choshinensis* sp. nov., *Bacillus parabrevis* sp. nov and *Bacillus galactophilus* sp. nov. Int. J. Syst. Bacteriol. **43:**221-231.

Takahara, T., and O. Tanabe. 1960. Studies on the reduction of indigo in industrial fermentation vat(VII). J. Ferment. Technol. **38:**329-331.

Takahara, T., and O. Tanabe. 1962. Studies on the reduction of indigo in industrial fermentation vat (XIX). Taxonomic characteristics of strain No. S-8. J. Ferment. Technol. **40:**77-80.

Takahara, Y., Y. Takasaki, and O. Tanabe. 1961. Studies on the reduction of indigo in industrial fermentation vat (XVIII). On the growth factor of the strain No. S-8 (4). J. Ferment.Technol. **39:**183-187.

Takami, H., A Inoue, F. Fuji, and K. Horikoshi. 1997. Microbial Flora in the deepest sea mud of the Mariana Trench. FEMS Microbiolog. Lett. **152:**279-285.

Tanabe, H., K. Yoshihara, K. Tamura, Y. Kobayashi, I. Akamatsu, N. Niyomwan, and P. Footrakul. 1987. Pretreatment of peptic wastewater from orange canning process by an alkalophilic *Bacillus* sp. Journal of fermentation technology **65:**243-246.

Thompson, J., and B. M. Chassy. 1981. Uptake and metabolism of sucrose by *Streptococcus lactis*. J. Bacteriol **147:**543-551.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. Clustal W-Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research **22:**4673-4680.

Tindall, B. J., H. N. M. Ross, and W. D. Grant. 1984. *Natronobacterium* gen. nov, and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaebacteria. System. Appl. Microbiol. **5:**41-57.

Tolner, B., B. Poolman, and W. N. Konings. 1997. Adaptation of microorganisms and their transport systems to high temperatures. Comp. Biochem. Physiol. A. Physiol. **118**:423-428.

Tomb, J.-F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzgerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and C. Venter. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-547.

Tsuchiya, K., Y. Nakamura, H. Sakashita, and T. Kimura. 1992. Purification and characterization of a thermostable alkaline protease from alkaliphilic *Thermoactinomyces* sp. HS682. Bioscience Biotechnology and Biochemistry **56:**246-250.

Tsuchiya, K., H. Sakashita, Y. Nakamura, and T. Kimura. 1991. Production of thermostable alkaline protease by alkalophilic *Thermoactinomyces* sp. HS682. Agric. Biol. Chem. **55:**3125-3127.

Ueyama, H., and K. Horikoshi. 1981. JPN Kokai 56-186153. Patent.

Usenko, I. A., L. O. Severina, and V. K. Plakunov. 1993. Uptake of sugars and amino acids by extremely thermophilic archaea – and eubacteria. Microbiology (Mikrobiologiya) **62:**272-277.

van de Vossenberg, J. L. C. M., A. J. M. Driessen and W. N. Konings. 1998. The essence of being extremophilic: the role of the unique archaeal membrane lipids. Exremophiles. **2**:163-170.

van de Vossenberg, J. L. C. M., T. Ubbink-Kok, M. G. L. Elferink, A. J. M. Driessen, and W. N. Konings. 1995. Ion permeability of the cytoplasmic membrane limits the maximum growth temperature of bacteria and archaea. Mol Microbiol **18:**925-932.

van der Laan, J. C., G. Gerritse, L. J. S. M. Mulleners, R. A. C. von der Hoek, and W. J. Quax. 1991. Cloning, characterization and multiple chromosomal integration of a *Bacillus* alkaline protease gene. Appl. Environ. Microbiol. **57:**901-909.

Vartanyan, N. S., T. A. Pivovarova, I. A. Tsaplina, A. M. Lysenko, and G. I. Karavaiko. 1988. New thermoacidophilic bacterium of the genus *Sulfobacillus*. Mikrobiologiya **57**:268.

Vedder, A. 1934. *Bacillus alcalophilus* n. sp. ; benevens enkele ervaringen met sterk alcalische voedingsbodems. Antonie van Leeuwenhoek. J. Microbiol. Serol **1**:143 – 147.

Ventosa, A., M. T. Garcia, M. Kamekura, H. Onishi, and F. Ruiz-Berraquero. 1990. *Bacillus halophilus* sp. nov., a moderately halophilic Bacillus species. International Journal of Systematic Bacteriology: **40**:105.

Viikari, L., A. Kantelinen, J. Sundquist, and M. Linko. 1994. Xylanases in bleaching: From an idea to the industry. FEMS Microbiol. Rev. **13**:335-350.

Viljoen, J. A., E. B. Fred, and W. H. Peterson. 1926. The fermentation of cellulose by thermophilic bacteria,. Journal Agri. Sci. **16**:1-17.

Vukov, K. 1965. Kinetic aspects of sucrose hydrolysis. Int. Sugar J. **67**:172-175.

Walker, P. D., and J. Wolf. 1971. The taxonomy of *Bacillus stearothermophilus*, 247-262. *In* A. N. Barker, G. W. Gould and J. Wolf (ed.), Spore Research. Academic Press, London.

Wang, Y., and B. C. Saha. 1993. Purification and characterization of thermophilic and alkalophilic tributyrin esterase from *Bacillus* strain A30-1 (ATCC 53841). Journal of American Oil Chemists' Society **70**:1135-1138.

Waterborg, J. H., and H. R. Matthews. 1994. Methods in Molecular Biology, 1-4. *In* J. M. Walker (ed.), Basic Protein and Peptide Protocols, vol. 32. Humana Press Inc, Totowa, NJ.

Weisser, J. 1983. Ein neuer haloalkaliphilic *Bacillus* and Egyptischen Sodaseen : Untersuchungen zum Osmoregulation. Diplom. Arbeit Universitat., Bonn.

Weisser, P., R. Kramer, H. Sahm, and G. A. Springer 1995. Functional expression of the glucose transporter of *Zymomonas mobilis* leads to restoration of glucose and fructose uptake in *Escherichia coli* mutants and provides evidence for its facilitator action. J. Bacteriol. **177**:3351-3354.

Westerhoff, H., B. A. Melandri, G. Venturoli, G. F. Azzone, and D. B. Kell. 1984. A minimal hypothesis for membrane-linked free-energy transduction. Biochim.Biophys.Acta **768**:257-292.

White, D. C., R. J. Bobbie, J. S. Herron, J. D. King, and S. J. Morrison. 1979. Biochemical measurements of microbial biomass and activity from environmental samples, *In* J. W. Costerton and R. R. Colwell (ed.), Native Aquatic Bacteria: Enumeration, Activity and Ecology, p69-81. ATM STP 695, American Society for Testing and Materials, Philadelphia.

White, D., R. J. Sharp, and F. G. Priest. 1993. A polyphasic taxonomic study of thermophilic bacilli from a wide geographical area. Antonie van Leeuwenhoek **64**:357-386.

Wiegel, J. 1998. Anaerobic alkalithermophiles, a novel group of extremophiles. Extremophiles **2**:257-267.

Wiegel, J. 1998b. Lateral gene exchange, an evolutionary mechanism for extending the upper or lower temperature limits for growth of microorganisms ? A hypothesis, *In* J. Wiegel and M. W. W. Adams (ed.), Thermophiles – The keys to molecular evolution and the origin of life?, p177-185. Taylor and Francis, London/Philadelphia.

Wiegel, J. 1990. Temperature spans for growth: hypothesis and discussion. FEMS Microbiology Reviews. **75**: 155-170.

Wisotzkey, J. D., P. J. Jurtshuk, G. E. Fox, G. Deinhard, and K. Poralla. 1992. Comparative sequence analysis on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus *Alicyclobacillus* gen. nov. Int. J. System.Bacteriol. **42:**263-269.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. **51:**221-271.

Woese, C. R., O. Kandler, and M. L. Wheelis. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucaria. Proc. Natl. Acad. Sci. USA. **87:**4576-4579.

Wolin, M. J. 1963. Fructose-1,6-diphosphate requirement of streptococcal lactate dehydrogenases. Science **146:**775-777.

Yamagata, Y., and E. Ichishima. 1993. Interaction of α_2-macroglobulin with alkaline proteinase from an alkalophilic *Bacillus* sp. grown in an extraordinarily alkaline environment. Current Microbiology **27:**205-209.

Yamamoto, A., S. Adachi, T. Kitani, Y. Shinji, K. Seki, T. Nasu, and M. Nishikawa. 1971. Drug-induced lipidosis in human cases and in animal experiments. Accumulation of an acid glycerophospholipid. J. Biochem. **69:**613-615.

Yamauchi, K., and M. Kinoshita. 1995. Highly stable lipid membranes from archaebacterial extremophiles. Prog. Polym. Sci. **18**:763-804.

Yang, V. W., Z. Zhuang, G. Elegir, and T. W. Jeffries. 1995. Alkaline-active xylanase produced by an alkaliphilic *Bacillus* sp isolated from kraft pulp. Journal of Industrial Microbiology **15:**434-441.

Yim, D. G., H. H. Sato, Y.-H. Park, and Y. K. Park. 1997. Production of cyclodextrin from starch by cyclodextrin glycosyltransferase from *Bacillus firmus* and characterization of purified enzyme. J.Ind.Microbiol.Biotechnol. **18.**

Yoon, J.-H., D. K. Yim, J.-S. Lee, K.-S. Shin, H. H. Sato, S. T. Lee, Y. K. Park, and Y.-H. Park. 1998. *Paenibacillus campinasensis* sp. nov., a cyclodextrin producing bacterium isolated in Brazil. Int.J.Syst.Bacteriol. **48:**833-837.

Yoshihara, K., and Y. Kobayashi. 1982. Retting of mitsumata bast by alkalophilic *Bacillus* in paper making. Agric. Biol. Chem. **46:**109-117.

Yum, D. Y., H. C. Chung, D. H. Bai, D. H. Oh, and J. H. Yu. 1994. Purification and characterization of alkaline serine protease from an alkalophilic *Streptomyces* sp. Bioscience Biotechnology and Biochemistry **58:**470-474.

Yumoto, I., Y. Fukumori, and T. Yamanaka. 1991. Purification and characterization of two membrane-bound c-type cytochromes from a facultatively alkalophilic *Bacillus*. J. Biochem. **110:**267-273.

Yumoto, I., Y. Fukumoto, and T. Yamanaka. 1990. Purification and characterization of catalase from a facultative alkalophilic *Bacillus*. J. Biochem. **108:**583-587.

Yumoto, I., S. Takahashi, T. Kitagawa, Y. Fikumori, and T. Yamanaka. 1993. The molecular features and catalytic activity of Cu_a-containing aco_3-type cytochrome c oxidase from a facultative alkalophilic *Bacillus*. J. Biochem. **114:**88-95.

Yumoto, I., K. Yamazaki, T. Sawabe, K. Nakano, K. Kawasaki, Y. Ezura, and H. Shinano. 1998. *Bacillus horti* sp. nov., a new Gram-negative alkaliphilic bacillus. Int. J. System. Bacteriol. **48:**565-571.

Zhilina, T. N., E. N. Detkova, F. A. Rainey, G. A. Osipov, A. M. Lysenko, N. A. Kostrikina, and G. A. Zavarzin. 1998. *Natronoincola histidinovorans* gen. nov., sp. nov., a new alkaliphilic acetogenic anaerobe. Current Microbiology **37:**177-185.

Zillig, W., 1980b. Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List No. 5. Int. J. System. Bacteriol. **30:**676-677.

Zillig, W., K. O. Stetter, S. Wunderl, W. Schulz, H. Priess, and J. Scholz. 1980a. The *Sulfolobus-"Caldariella"* group: taxonomy on the basis of the structure of DNA-dependent RNA polymerases. Arch.Microbiol. **125:**259-269