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EXTREMELY THERMOPHILIC BACTERIA IN
NEW ZEALAND HOT SPRINGS

A thesis
submitted in partial fulfilment
of the requirements for the degree
of
Doctor of Philosophy in Biological Sciences
at the
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Dedicated to the memory of my late father Mr. Chimanlal J. Patel

ABSTRACT:

Pools in thermal areas were investigated by direct microscopic procedures, by indirect tracer methods and by conventional bacteriological methods of enrichment and isolation, in order to determine the diversity and distribution of extremely thermophilic bacteria.

The indirect isotope uptake tracer method was unsuccessful during the earlier part of the project. Although, problems with isotope methodology were largely solved, this did not occur early enough in the investigation to allow use of the method as a means of establishing a biomass activity index for pools. The immunofluorescence method worked well on laboratory grown isolates but was surprisingly unsatisfactory when used in situ. The method was abandoned because of the time required to correct possible problems which may have been due to the choice of the microorganisms used in the study. The implications of these results with regard to ecological studies are discussed in the thesis.

Electron microscopy was used to further investigate the morphology and diversity of bacteria in pools. Direct microscopic examination of pool water by phase contrast microscopy showed that over 90% of the pools contained bacteria. Gram staining of slides colonized in situ revealed that Gram negative bacteria were the only types present in most pools. New methods of grid preparation and handling were developed. In situ colonisation of grids showed that practically all the 38 pools over the temperature range of 70°C to 102°C and pH's from 3.0 to 9.5 contained bacteria. Pools varied as to diversity of species and numbers of cells of the same morphotype in each pool. Some pools contained an apparent monoculture whereas others had a diverse ecosystem.

In situ EM technique and subsequent cultural studies revealed that sulphur-respiring archaeobacteria were the dominant microorganisms present in springs with temperatures above 90°C with both coccal and rod-shaped forms present. Methanogens, another archaeobacterial group, were also isolated but they inhabited pools with lower temperatures (between 65 to 80°C).

Isolation and cultivation procedures were also successfully used to obtain cultures of more conventional anaerobes. These were chiefly obtained from neutral springs in the 70 to 85°C range and fermented carbohydrates to a variety of end products but primarily acetate, ethanol or lactate. The diversity of this group of caldoactive glycolytic anaerobes was demonstrated by isolating species which produced quite distinct ratios of the three major end products. Some of these isolates were very similar to glycolytic anaerobes described previously e.g. Thermoanaerobium brockii, Thermoanaerobacter ethanolicus. A number of isolates had properties different from the type strains.

Investigation of the aerobic flora of thermal springs were not as intensive as those on anaerobes, largely because these organisms have been well described elsewhere. Thermus and Bacillus species were the most dominant aerobic microflora in many of the New Zealand springs. Their distribution and characteristics conformed to expectations of overseas results. Thermothrix and Sulfolobus species were isolated from a few pools but Thermomicrobium species which have been isolated from the Yellowstone National Park thermal springs, USA, have not been detected in the New Zealand thermal springs.

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CHAPTER 1

INTRODUCTION

1.1 DEFINING AN EXTREME ENVIRONMENT

Organisms which grow in extreme environments are called "extremophiles". The word extreme is often used in the sense of stressful, severe, and even hostile. This anthropocentric view - as previously pointed out by Brock, (1969) - poses problems. Why should one consider anaerobiosis more stressful than aerobiosis considering the toxicity of oxygen itself, or the products of its utilization? And why should one consider the non halophiles and most terrestrial organisms, which have to deal with osmotic stress, as less stressed when compared to halophiles, which have their internal environments fairly isoosmotic with the external one? An appropriate definition of an extreme environment would be a condition under which some kinds of organisms can grow, whereas others cannot. If we accept this definition, it means that an environmental extreme must be defined taxonomically. Instead of looking at a single species or groups of related species, we must examine the whole assemblage of species, microbial or multicellular, living in various environments. When we do this, we find that there are environments with low species diversity and others with high species diversity. In some environments with low species diversity, we find that whole taxonomic groups are missing. For instance, in saline and thermal lakes there are no vertebrates and no vascular plants, although these

environments may be rich in microorganisms and have very high population densities of the species that are present. In many of the more extreme environments, we find conditions approaching the pure culture, with only a single species present.

The most commonly considered environmental extremes are factors such as temperature, redox potential (Eh), pH, salinity, hydrostatic pressure, water activity, nutrient concentration and electromagnetic and ionizing radiations.

For virtually all of these factors, it has been found that organisms with simple structures can grow under more extreme conditions than organisms with more complicated structures, hence the predominance of microorganisms in extreme environments. The capability of microorganisms to grow under such extreme conditions is either due to (i) evolutionary adaptation, that is, natural selection or (ii) their ability to respond and adapt to a range of environmental conditions. However, we should bear in mind that an organism may have to cope with more than one factor in an extreme environment. For instance, in a hot acidic thermal spring, an organism not only has to cope with high temperatures and temperature fluctuations but has to cope with a low pH and low nutrient content as well.

The most widely studied organisms from extreme environments to date are the halophiles and the thermophiles. The reasons for such an interest in the study of these groups of organisms, particularly the thermophiles, will be discussed in section 1.3.

1.2 ORIGIN AND DEVELOPEMENT OF THERMOPHILY

1.2.1 The Past And Present Status

The thermophilic bacteria are a ubiquitous group of microorganisms existing in a variety of thermal and non-thermal environments. The thermal environments include those heated by solar rays (soils or saline lakes), combustion processes which are either biological (self heating of hay) or non-biological (burning refuse piles), geothermal activity (thermal springs) and man made sources of heat (industrial heating).

That microorganisms live at high temperatures has been known since the early years of the nineteenth century, but work on them was limited to observations of more or less spectacular growth in hot springs. The first thermophilic bacterium, an aerobic sporeformer, however was not isolated from such a thermal environment but was isolated from river water by Miquel in 1888.

During these early days of thermophily, several theories were proposed to explain the origin of thermophiles. Arrhenius in 1927 proposed that thermophilic microorganisms had their origin on the planet Venus and were carried to the earth in a few days by radiation pressure from the sun. Allen, (1953) proposed that thermophiles developed from mesophiles by adaptation or mutation. The basis of this argument was the finding that mesophilic species could be adapted to grow at elevated temperatures and the ubiquitous occurrence of thermophilic species in non-thermal environments. For instance, Bacillus stearothermophilus had been isolated from soil, sea water, ocean depths, desert soil, decaying plant matter, air, snow, sewage and faeces of humans and animals. However, the evidence available today suggests a thermophilic origin for mesophiles. The most compelling support for this hypothesis comes from

the argument that evolution proceeded from an environment considerably warmer than the present one (Dickerson, 1978). The organisms growing under such a warm and reducing atmosphere present some 3-4 billion years ago, would have been structurally simple anaerobes. Evidence presented by geobiochemists indicates that kerogen and geochemical fossils in sediments contain isoprenoid hydrocarbons, the origin of which could be traced to hydrocarbons of certain species of bacteria, collectively called "archaebacteria" (Ourisson et al.,1982; Michaelis and Albrecht,1979; Hahn,1982; Tornabene et al.,1979 and Langworthy et al.,1982). Archaebacteria are a phenotypically varied group existing today in extreme environmental conditions and include methanogens, some extreme halophiles like Halobacterium and Halococcus species, the sulphur utilizing thermophiles like Thermoplasma, Sulfolobus, Pyrodictium, Thermofilum, Desulfurococcus and Thermoproteus species. The relationship of archaebacteria to eubacteria and eucaryotes will be discussed in chapter 5.

An understanding of the biology of thermophilic microorganisms has been greatly advanced in the past twelve years because of a most productive research effort. This golden era of thermophily has been largely due to the efforts of Brock and his co-workers who spent ten years from 1965 to 1975 working on the ecology and physiology of aerobic thermophiles in hot springs of Yellowstone National Park, U.S.A. Their studies yielded some new groups of bacteria. These included a new species of thermoacidophilic organism, Bacillus acidocaldarius, a sporeforming rod shaped bacterium and two new genera, namely Thermus and Sulfolobus. Thermus species were Gram negative rods to filaments occurring predominantly in slightly acidic to alkaline hot springs whereas Sulfolobus species were coccal shaped organisms existing

exclusively in hot acidic environments.

The study of anaerobic bacteria has always been late coming, either in the study of mesophilic or thermophilic anaerobes. This probably reflects to the fact that their physiology is less well understood and also the fact that limited techniques for culturing and isolating them exist. Hungate, (1944, 1950) pioneered the technique for culturing anaerobes, the principles of which are still used today. McBee (1948) became the first to isolate and study a thermophilic anaerobic cellulolytic Clostridium species, Clostridium thermocellum. Most of the work on thermophilic anaerobes was done on this organism until the isolation of Clostridium thermohydrosulfuricum, a glycolytic organism, by Klaushofer and Parkkinen, (1965) from an Austrian sugar factory. Since then, Clostridium thermohydrosulfuricum has been isolated from compost and soil, (Wiegel et al., (1979) and thermal springs (Zeikus et al., 1980). A methane producing organism, Methanobacterium thermoautotrophicum was isolated by Zeikus and Wolfe, (1972) from sewage sludge and subsequently strains of this organism have also been isolated from thermal springs (Zeikus et al., 1980) and from industrially polluted coastal sediment (Ronnow and Gunnarsson, 1981).

Recently there has been increasing interest in anaerobic thermophiles. The features of anaerobic thermophilic systems producing methane, ethanol and acetate show tremendous potential for the development of novel biotechnology due to a number of advantages. Again, a greater variety of isolates provides more diverse material with which a study of the nature of thermophily can be conducted. The importance of anaerobic thermophiles will be discussed more fully in section 1.3. Naturally, thermal springs are the most important habitat for isolating anaerobic thermophiles. Both the low solubility of oxygen at higher

temperatures and the presence of hydrogen sulphide create reducing conditions in the hot spring environments and an ideal place for the growth of anaerobes.

The most widely studied thermal ecosystem has been decomposing algal-bacterial mats. Radioactive tracer studies and laboratory culture work have shown the presence of methanogens (Ward, 1978; Zeikus et al., 1980; Sandbeck and Ward, 1982), glycolytic anaerobes (Zeikus et al., 1979, 1980; Wiegel and Ljungdahl, 1981; Ben-Bassat and Zeikus, 1981; Schink and Zeikus, 1983a) and sulphate-reducing bacteria (Zeikus et al., 1983) interacting in this ecosystem. Isolation and characterization of the organisms from this ecosystem has shown that the optimum temperature for growth is usually between 65-75°C.

Work on the isolation of organisms capable of growth optimally between 85-105°C has been undertaken by Zillig and coworkers during the past 2-3 years and a number of organisms have been described (Zillig et al., 1981a,b, 1982a,b, 1983a,b; Stetter, 1982; Stetter et al., 1983). These organisms were unique in a number of ways. All of them were capable of using sulphur as an electron acceptor, were strict anaerobes, did not grow at a temperature below 65-70°C and could be isolated from thermal environments only. They also belonged to a novel group of bacteria known as "archaebacteria". However, no studies have been performed to investigate growth rates and the mode of metabolism "in situ" as yet. More recently, Baross and Deming (1983) have cultured microbial communities from deep sea hydrothermal vents which were capable of growth at 250°C.

1.2.2 Definition Of Thermophiles

Since Bacillus species were the only known thermophiles until recently, thermophiles were loosely defined as organisms capable of growing above 55°C. However, this concept has changed due to the isolation of increasing numbers of organisms capable of growing at temperatures well above 55°C and up to 80 - 85 °C. The term "extreme thermophiles" has been used to describe organisms capable of growth above 65 °C (Williams, 1975). However, Heinen and Heinen, (1972) proposed that the term "caldo-active" be used instead of "extreme thermophiles". More recently, Kogut, (1980a) proposed to classify thermophiles on the basis of temperature ranges for growth. According to this definition, organisms with growth temperature minima of above 37°C and growth temperature maxima of above 70°C were classified as extreme thermophiles or caldoactive. Those organisms which were capable of growth at or below 37°C and with growth temperature maxima in the caldoactive or thermophilic range were classified as adaptable or facultative thermophiles. Hence, according to this scheme Bacillus caldotenax, Bacillus caldolyticus and Bacillus caldovelox should be termed adaptable or facultative thermophiles and not caldoactive as previously described. Similarly, Bacillus coagulans and Bacillus licheniformis should be redefined as adaptable thermophiles. Organisms such as Thermus, Thermomicrobium, Thermoanaerobium and Sulfolobus would remain in the caldoactive group.

However, it should be borne in mind that such a classification scheme will require that all strains be compared for their growth temperature maxima and minima on the same type of media since media composition has been shown to affect growth. For instance, glucose or pyruvate utilization at 70 and 80 °C by Bacillus caldolyticus is

dependent on the presence and concentration of Brain Heart Infusion broth (Heinen and Heinen, 1972).

The definition of thermophiles will require revision as more information is obtained. For instance, it would not be appropriate to allocate Pyrodictium to any of the existing categories since it does not grow below 80°C, a temperature at which almost all the existing extreme thermophiles are incapable of growth. Since there is no unified definition for thermophiles at present, I have considered organisms with an optimum growth temperature of above 60-65°C as extreme thermophiles. All reference to extreme thermophiles throughout this thesis, will be made in this context.

1.3 THERMOPHILES AN IMPORTANT GROUP OF BACTERIA

The study of thermophilic bacteria has recently become a topic of increasing interest. It is of interest to microbial ecologists since it has diversified their field of study. Biochemists have become intrigued with the fact that macromolecules such as proteins, membranes, DNA and RNA, which were thought to be thermolabile molecules, can withstand temperatures greater than 100°C. The study of archaebacteria has already provided a different perspective on the evolution of life and not unnaturally are now being intensively studied by evolutionary biologists. Industrial microbiologists and biotechnologists have put forward the proposal that novel processes can be developed by exploiting thermophilic microorganisms. It appears that there will be an overall rapid advancement in the field of thermophily in the near future.

1.3.1 A Model To Study An Ecosystem

We have already seen that in extreme environments organisms can reach a pure culture stage. This is especially true in thermal springs which have very high temperatures. This means that studies on this ecosystem can be carried out without any complications due to interaction with other organisms. This is in contrast to studying the soil ecosystem where a handful of soil can contain very many different species of organisms. Studies in thermal environments thus become much simpler. For example, studies on sulphur metabolism by bacteria in situ, could be ideally studied in the natural ecosystem of the thermal environment because the thermal areas are rich in sulphur compounds and at high temperatures only a few species of microorganism exist and in some thermal environments, for example only a single species can sometimes be isolated from some hot acidic springs,

1.3.2 A Model To Study Evolution

The presence of archaebacteria in hot springs has opened the door for studies on the evolution of life on earth, since it has been proposed that the thermal environment in which these organisms exist is reminiscent of the hot and reducing primitive world that existed billions of years ago. Refer chapter 5 for the present theories on evolution of life.

1.3.3 A Model To Study The Nature Of Thermophily

Several mechanisms have been proposed to explain adaptation of microorganisms to extreme environments. In studies to understand the nature of thermophily, Bacillus and Clostridium were until recently the

most commonly species studied. However, with the isolation of new extreme thermophiles including archaebacteria, the emphasis of such studies has shifted towards these organisms.

The word "adaptation" can have different meanings. An organism can adapt to an ecological niche by accumulated alterations in cellular properties - by mutation and genetic exchange - on which natural selection can act (inherent or intrinsic property), and by physiological modifications to cope with changing environments. Both types of adaptation occur in microorganisms in extreme environments. The mechanisms of adaptation have been reviewed recently (Kogut, 1980a, b).

1.3.3.1 The Role Of Membrane Lipids:- -

Elucidation of the structure of biological membranes (Singer and Nicholson, 1972; Welker 1976a, b), the composition of fatty acids in membranes (Kates and Baxter, 1962; Chan et al., 1971) and investigations on the thermotropic gel-liquid-crystalline phase transition of lipids (Welker, 1976b; McElhaney, 1976; Esser and Souza, 1976; Cronan, 1978) have helped in our understanding of the role played by membrane structure in adaptation of microorganisms to extreme environments.

Microbial membranes consist of a phospholipid bilayer containing loosely "peripheral" proteins and the integrally embedded proteins (Singer and Nicholson, 1972; Welker 1976a, b). A majority of the proteins found in biological membranes are "integral" proteins. Studies of enzymes associated with membranes (integral proteins), have shown that membrane lipid-integral protein interactions influence the thermostability of enzymes (Wisdom and Welker, 1973) and may affect the cardinal temperatures of a microorganism. However, recent evidence

suggests that the lipid bilayer rather than proteins may be directly involved in thermophily (McElhaney, 1976; Esser and Souza, 1976).

Brock (1967) suggested that the thermostability of the cell membrane was a factor determining the ability of thermophilic growth and that it, as influenced either directly or indirectly by the fatty acid composition of its lipid constituents, was perhaps the major factor in determining the growth range of microorganisms. The temperature span for growth of microorganisms is usually between 30 to 40°C. The observation that spheroplasts of Thermus (Ray and Brock, 1971) and Bacillus stearothermophilus (Bodman and Welker, 1969) did not undergo thermal lysis in boiling water lent further support to the hypothesis of Brock (1967).

An important feature of the lipid bilayer is the ability to undergo reversible phase transition from a gel to a liquid-crystalline phase. The temperature at which phase transition occurs is dependent to a large extent on the fatty acid composition of the bilayer. Lipid in a gel (rigid) state has a high degree of order and restricted intra- and intermolecular motion, but in a liquid-crystalline (fluid) state the hydrocarbon chains are partially melted, with an increase in fluidity. Straight chain saturated fatty acids have, as a class, the highest melting points and the melting point increases increasing carbon chain length. An extra methyl group at the penultimate carbon atom of the carbon chain (iso-branched) yields acids with only slightly lower melting temperatures than corresponding straight-chain acids. If a methyl group is present on other carbon atoms (ante-iso-branched), the melting temperatures of the acids are decreased considerably. Finally, acids with cis-double bonds have the lowest melting points of all fatty acids (McElhaney, 1976). Hence lipid layers containing straight-chain and

iso-branched acids have high phase transition temperatures, whereas membranes with ante-iso-branched acids and cis-double bonded acids have lower phase transition temperatures. Mixtures of the different types of acids yield membranes with intermediate phase transition temperatures. However, the transition temperature is not sharp due to the heterogeneity in the composition of the membrane and domains of gel and liquid-crystalline phase can exist simultaneously.

Studies on lipid composition of psychrophiles mesophiles and thermophiles belonging to the same genus, namely Bacillus (Shen et al., 1970), Clostridium (Chan et al., 1971) and Candida (Kates and Baxter, 1962) have revealed that a relationship exists between the fatty acid composition of the membrane lipids and the growth temperature range. In general, psychrophiles have high proportions of unsaturated or ante-iso-branched fatty acids compared to their mesophilic counterparts, whereas closely related thermophiles have high proportions of straight-chain saturated and iso-branched fatty acids. Additionally, the average length of the hydrocarbon chain increases as one proceeds from the psychrophilic members to the thermophilic species within the same genus. These studies have revealed that organisms which have evolved (or adapted) to grow at lower temperatures have fatty acids characterised by low melting points.

Lipids of Thermus species contain in greatest abundance iso-C₁₇- and iso-C₁₅- fatty acids, lesser amounts of ante-iso-C₁₇- and ante-iso-C₁₅ acids and very few straight chain saturated acids (Heinen et al., 1970; Ray et al., 1971a; Jackson et al., 1973; Oshima and Migagawa, 1974). Cho and Salton, (1966) analysed lipids from Bacillus stearothermophilus and found them to consist mainly of iso-C₁₅- and iso-C₁₇- fatty acids. Lipids of Bacillus caldotenax and Bacillus caldolyticus contained

iso-C₁₅-, iso-C₁₆- and iso-C₁₇- fatty acids as major constituents (Weerkamp and Heinen, 1972) but another sporulating bacterium PS-3 was found to contain only the saturated fatty acids iso-C₁₅-, iso-C₁₆- and iso-C₁₇- as the major components (Kagawa and Ariga, 1977).

Like most microorganisms, thermophiles can change their fatty acid composition with growth temperature (Daron, 1970; Ray et al., 1971a,b). Heinen et al., (1970) and Oshima and Miyagawa, (1974) observed that the ratios of C₁₇:C₁₅ in Thermus species increased with increasing temperature, indicating chain elongation of fatty acids. 70 percent of the lipids of Thermus thermophilus and Thermus flavus were found to contain a novel galactofuranose-containing glycolipid. The lipid component of the glycolipid increased with an increase in temperature (Oshima and Yamakawa, 1972; Oshima and Ariga, 1976).

Unusual lipids are present in archaeobacteria, in which, unlike eubacteria and eucaryotes, the polar glycerolipids contain ether linkages rather than ester linkages. The fatty acid chains of these lipids consists of identical pairs of isoprenoid branched, fully saturated C₂₀-phytane or C₄₀-biphytane (two-head-to-head linked phytanes) hydrocarbon skeletons. The isoprenyl glycerol ethers occur in archaeobacteria as two structural types diphytanylglycerol diether (Kates et al., 1965) or dibiphytanylglycerol tetraether (Langworthy, 1977a). Diethers contain two C₂₀-phytanol chains ether-linked to glycerol, whereas the tetraethers consist of two glycerol molecules bridged through ether-linkages by two identical pairs of C₄₀-biphytanyl terminal diols with the primary hydroxyls of the glycerol. More recently, diethers consisting of C₂₅ isoprenyl chains have been found in an archaeobacterial haloalkaliphile (De Rosa et al., 1982, 1983). The membrane of haloalkaliphilic strain MS3 consisted of equal proportions of C₂₀, C₂₀

and C_{20},C_{25} diether (De Rosa et al., 1982) whereas strain SP8 consisted of 85% C_{20},C_{25} diether, 9% C_{20},C_{20} diether and 6% C_{25},C_{25} diether in its lipids De Rosa et al., 1983). It has been suggested that the presence of such asymmetric diether structures formed due to the presence of C_{25} isopranyl chains gives rise to "zip" membranes which might be much thicker than other archaeobacterial membranes.

A considerable variation in the ratios of diether to tetraether membrane lipids exists amongst different genera in the archaeobacterial group. Thermoplasma and Sulfolobus contain 90 - 95 percent tetraether and 5 - 10 percent diether (Langworthy, 1979b); the coccal shaped methanogens contain 100 percent diether in their membranes compared to 40 - 70 percent diether found in the membranes of most of the rod shaped methanogens. Hence, the distribution of diethers and tetraethers can prove useful in establishing the relationship and taxonomy of archaeobacteria. The thermoacidophiles and methanogens also differ in the type of biphytanyl chains present. The thermoacidophiles contain from 1 to 4 cyclopentyl rings in their biphytanyl chains whereas methanogens contain acyclic biphytanyl chains. (De Rosa et al., 1977, 1980a,b). Within the tetraether, the identical pairs of cyclic biphytanes are in the antiparallel configuration. Thermofilum pendens (Zillig et al., 1983a), Thermococcus celer (Zillig et al., 1983b), Thermoproteus tenax (Zillig et al., 1981a) and Desulfurococcus mobilis and Desulfurococcus mucosus (Zillig et al., 1982a) all contain diethers and tetraethers in their lipids but the ratios of tetraethers to diethers and the type of biphytanyl chains present in the tetraether has not been investigated as yet. Pyrodictium occultum, an organism capable of optimal growth at 105°C has been found to have a novel lipid structure (Stetter, 1982). However, the structure has not been elucidated.

Due to the presence of tetraethers in lipids of archaebacteria, the structure of the membrane is in effect monolayered rather than bilayered. This concept is supported by observations that Thermoplasma, Sulfolobus and certain species of methanogens fail to freeze-fracture tangentially but do so perpendicularly, and hence inner and outer membrane faces are not revealed (Langworthy, 1977a, 1979a,b). Diethers, on the other hand bear a structural resemblance to the normal lipid bilayer.

It has been assumed that cyclization within the C₄₀-biphytanyl chains in thermoacidophilic tetraethers plays a role in the mechanism of survival and growth of these organisms at high temperatures. Since the alkyl chains are fixed at 40-carbons and linked across the membrane, ring formation would reduce rotational freedom in the chain, thereby increasing rigidity, regulating chain length and simultaneously controlling width and density. This hypothesis has gained support by the observation of increased cyclization in the biphytanyl chains of Sulfolobus, an organism requiring extremely high temperature for growth (De Rosa et al., 1980c). Further evidence for this hypothesis was obtained by Langworthy et al., (1982) who observed that in Thermoplasma the proportion of cyclic biphytanyl chains increased with an increase in temperature at the expense of acyclic biphytanyl chains. There was a complete reversal of this phenomenon as the temperature was decreased.

The neutral lipids of archaebacteria consist of isoprenoid and hydroisoprenoid hydrocarbons (Tornabene et al., 1979). A considerable variation in the ratio of isoprenoid to hydroisoprenoid exists among the archaebacterial group. These differences are of taxonomic importance. Additionally, these differences may express physiological differences in the cells. For example, the cellular ratio of hydroisoprenoid to isoprenoid decreased with a decrease in aeration rates in Halobacterium

cutirubrum (Tornabene, 1978). In addition to the acyclic isoprenoids, Sulfolobus and Thermoplasma contain a series of branched alkylbenzenes. The role of neutral lipids in archaeobacteria has not as yet been elucidated. A number of polar and neutral lipids of archaeobacteria have been detected in crude oils and sediments (Ourisson et al., 1982). The significance of this finding has been discussed in chapter 5.

Bacillus acidocaldarius, an acidophilic eubacterium isolated from the same type of environments as Sulfolobus, was found not to contain tetraethers in its lipids, indicating that the presence of tetraethers in archaeobacteria is not an environmental adaptation but is a physiological feature. Bacillus acidocaldarius possesses hopane-containing lipids collectively known as hopanoids. Tetrahydroxybacteriohopane (THB) and a glycolipid constitute 15 percent of the total lipids of Bacillus acidocaldarius (Langworthy et al., 1976). Both the hopanoid and glycolipid fractions are presumed to interact and bring about a temperature dependent condensation of cyclohexane and fluidisation of the membrane (Kannenbergh and Poralla, 1980). An additional feature of the membranes of Bacillus acidocaldarius is the presence of ω -cyclohexane fatty acids, which have not been observed in any other bacterial species (De Rosa et al., 1971; Oshima et al., 1978; Hippchen et al., 1981). The presence of such a unique group of fatty acids has been related to the property of these bacteria to withstand both an acidic and a hot environment. This hypothesis has gained further support with the observation that Bacillus coagulans, which is less acidophilic than Bacillus acidocaldarius (Belly and Brock, 1974), did not possess ω -cyclohexane fatty acids. More recently, a different type of ω -alicyclic fatty acid, called ω -cycloheptane fatty acid was isolated from a moderately thermoacidophilic soil bacillus (Poralla and Konig,

1983). The reason for the presence of such species of bacteria in neutral environments is yet to be resolved.

1.3.3.2 Inherent Structural Properties Of Proteins:- -

(a) The Protein Synthetic Machinery:

There is no apparent relationship between the thermal stability of DNA and the ability of cells to grow at high temperatures since the base composition and melting characteristics of thermophiles are comparable to those of mesophilic organisms (Marmur, 1960; Saunders and Campbell, 1966).

However, more recently, it was observed that RNAs of extreme thermophiles possessed unique thermal stability. (Zeikus et al., 1970; Watanabe et al., 1976). In the case of Thermus transfer RNA, the thermostability was attributable to the presence of an increased number of guanine plus cytosine bases at specific base-paired regions which as a result provided greater hydrogen bonding and an increased thermal stability. It was also observed that the base-paired region in transfer RNAs from Thermus contained other thiolated thymidine residues which provided a stronger stacking force inside the molecule and that the degree of thiolation increased with an increase in growth temperature.

It has also been noted that extreme thermophiles produce unique polyamines, thermine (Oshima, 1975) and thermospermine (Oshima, 1979). In addition, more novel polyamines have been isolated from the extremely thermophilic bacterium, Thermus thermophilus and have been studied by Oshima (1982, 1983). The function of these polyamines is to stabilize the protein synthetic machinery and also to help protect macromolecules from high temperatures.

Thermostability of bacteriophage infectious to Thermus thermophilus could be restored in the presence of water of the hot spring from which the phage had been isolated (Sakaki and Oshima, 1975). They proposed that the water contained a natural stabilizing factor or factors for the phage. The factor responsible for such a mechanism was not studied.

Similarly, initiator factors from Bacillus stearothermophilus (Kay, et al., 1976) and polypeptide chain release factor from Thermus thermophilus (Ota, et al., 1977) possessed inherent stability.

Studies on synthesis and turnover of proteins, and other macromolecules of thermophilic bacteria, have provided evidence that thermostability is an intrinsic property of proteins and the protein synthetic machinery in these organisms. This suggests that rapid resynthesis is unnecessary as a major mechanism of thermophily.

(b) Enzyme structure:

A number of mechanisms have been proposed to explain enzyme thermostability. Manning and Campbell, (1961), who were amongst the first to isolate and study a thermophilic α -amylase from Bacillus stearothermophilus, provided evidence that the increased thermostability of this molecule was due to its low molecular weight and suggested that all thermostable proteins might show the same characteristic. However, subsequent studies have shown that a number of enzymes from thermophilic organisms have the same molecular weight as their mesophilic counterparts (Ljungdahl and Sherod, 1976).

Brock, (1967) suggested that thermostable proteins would be relatively rigid and inflexible, having sacrificed efficiency and control for stability. However, there are now numerous examples of enzymes isolated from facultative and extreme thermophiles which are

allosterically functional and in some cases, function in an identical fashion to their mesophilic counterparts (Ljungdahl and Sherod, 1976). Recent evidence (Daniel et al., 1982) suggests that some thermophilic proteins do possess great structural rigidity, but that the changes in molecular flexibility do not significantly affect allosterism.

Similarly, the suggestion that differences in secondary structure allowed for increased or decreased thermostability in protein molecules was disproved by Stellwagen and Barnes, (1976) and Singleton, et al., (1977).

Wedler and Hoffmann, (1974) proposed that an increase in thermostability could be achieved by aggregation. They observed an increase in thermostability of glutamine synthetase when a calcium dependent dodecameric aggregation of the enzyme occurred. However, little evidence is available to suggest that aggregation is a significant mechanism of thermophily.

Suggestions that enhanced thermostability of proteins was due to the presence of an increased number of disulfide bonds in the molecule has been observed not to be an essential prerequisite for thermostability. Perutz and Raidt, (1975) and Perutz, (1978) stressed the influence of extra salt bridges on thermostability. Argos, et al., (1979) showed an increased internal hydrophobicity in the proteins from thermophilic organisms. An increased number of hydrogen bonds has been proposed by Barnes and Stellwagen, (1973) to account for thermostability.

Grutter, et al., (1979) observed that a single amino acid change altered the thermostability of bacteriophage T4 lysozyme. A similar observation was made by Langridge, (1968) when point mutations of β -galactosidase were studied. Evidence suggests that the answer or

thermostability does not lie in the amino acid composition and sequence of amino acids but lies in very subtle changes in the internal structure of the protein, which result in greater intramolecular interactions in the protein without any alteration to the amino acid composition. Walker et al., (1980) studied the tetrameric enzyme D-glyceraldehyde-3-phosphate dehydrogenase from Bacillus stearothermophilus and Thermus aquaticus and proposed that ionic bonds between the subunits, and the hydrophobic bonds located in the core of the molecule were equally important for the thermostability of the enzyme.

When the differences between mesophilic and thermophilic proteins are translated into energetic terms, then simple explanations based on single properties are insufficient to explain thermostability. Relatively small changes in the free energy of stabilization of a molecule can be responsible for substantial differences in thermostability. Often, the energy separation between mesophilic and thermophilic proteins is of the same order of magnitude as the stabilisation energies of the common intramolecular bonds. Thus it can be deduced, as found experimentally, that substitution of a few residues to provide a small number of additional linkages should be sufficient to account for the phenomenon of molecular thermophily.

1.3.4 Economically Important Group Of Bacteria

In the past few years it has become increasingly clear that the utilization of thermophilic bacteria, particularly the extreme thermophiles, may at least in some biotechnological processes offer some advantages over the traditional mesophiles which have been exhaustively used in these processes to date. This is especially true in the case of production of acetic acid or ethanol and to a smaller extent in the case

of production of biocatalysts. The reason for this is two-fold. (1) Thermophilic temperatures used for the growth of thermophiles can affect design parameters of the reactor vessels and so lead to a decrease in the initial cost of constructing a commercial plant. (2) Furthermore, the characteristics of the thermophiles may also provide advantages which would help further in decreasing production costs as described below.

1. The effects of increased temperatures on the physical and chemical properties of water are very large. Higher temperatures increase water vapour pressure, volume, diffusion, ionization and solubility of most organic and inorganic compounds, while it decreases water density, surface tension, dielectric constant, viscosity and gas (O_2 , H_2 , CH_4) solubility. The lower solubility of oxygen at high temperatures (the amount of oxygen dissolved in water at $70^\circ C$ is 5% of that dissolved at $20^\circ C$) is an advantage for cultivation of anaerobes but is an obvious disadvantage for thermophilic aerobes. However, this disadvantage can be overcome by greater mixing and aeration rates. This in turn would lead to increased water and medium evaporation thereby providing a situation where metabolite toxicity could occur. An efficient water condensation apparatus could solve this problem.
2. The energy requirements for cultivation of thermophilic bacteria and end product recovery will be less. The energy required to heat a reactor is less than that for cooling it. Heat exchange/refrigeration equipment will not be required for cooling the reactor after sterilization and during product recovery. For instance, the recovery of enzymes from mesophiles is done at $4^\circ C$ to prevent enzyme denaturation.

3. Increased solubility of organic compounds is also an important positive factor in thermophilic processes because substrate solubility is often a rate-limiting factor in the bioconversion of carbon substrates like biopolymers, hydrocarbons and aromatics.
4. The possibility of continuous distillation of volatile products (such as ethanol) directly from fermentation vessels (continuous process) during fermentation.
5. Compared to the use of mesophilic bacteria, sterile conditions are less essential. There are no obligate thermophilic pathogens known. This is of great importance in the bioconversion of some municipal wastes and in the production of single cell protein. However, the thermophilic bioconversion processes are not necessarily free from extraneous microbial contamination that may significantly alter product formation. The thermophilic endospore-forming bacterium, Bacillus stearothermophilus, was frequently observed as a contaminant of Thermus T-351 cultures, when Thermus T-351 cells were grown in 600 litre fermentors. Similar contamination problems have been cited by other workers. Zeikus, (1979) reported the overgrowth of Thermus aquaticus by Bacillus stearothermophilus in improperly sterilized media. Contamination problems have been also known to occur in the cultivation of anaerobic thermophiles. For instance, an autotrophic culture of Methanobacterium thermoautotrophicum was found to be contaminated by Clostridium thermohydrosulfuricum (Zeikus, 1979). A similar observation was made by Wiegel, (1980). He found that Clostridium thermohydrosulfuricum was unable to grow

without the presence of Methanobacterium thermoautotrophicum and concluded that Clostridium thermohydrosulfuricum utilized traces of lysed products of the cells of Methanobacterium thermoautotrophicum. It should be noted, however, that thermophilic spore-formers (Clostridium thermohydrosulfuricum and Bacillus stearothermophilus) appear to be the only group of bacteria ubiquitous in nature and hence an easy source of contamination in thermophilic processes (Allen, 1953, Wiegel et al., 1979). Thermophilic phages have been isolated from Thermus species (Sakaki and Oshima, 1975) and from Bacillus species (Farrell and Campbell, 1969; Sakaki and Oshima, 1976). It appears that the phages infective to Thermus species have a very narrow host range (refer chapter 8) and the problem of cultures collapsing due to phage infection appears to be minimal. However, data is still lacking on this subject.

6. Thermophiles exhibit high catabolic activities at temperatures optimal for growth. For instance, the generation time for growth of thermophilic Bacillus can be as low as 14 minutes compared to the mesophilic Bacillus species which have a generation time of about 28 minutes. A doubling time of 5 hours is normal for the mesophilic methanogens whereas a much faster generation time of 55 minutes has been observed with Methanococcus thermolithotrophicus. Faster growth rates result in shorter fermentation time and a high productivity and thus lead to a higher efficiency of industrial plants. These features are especially useful in large volume processes such as industrial, municipal and agricultural waste disposal and chemical feedstock and fuel production. However it should be

noted that very high growth rates are often gained at the expense of yield. Sonnleitner and Fiechter, (1983) observed that Thermus strains in continuous culture, either grew very rapidly and gave extremely low yields or grew slowly, utilized all the available substrate and thereby gave a much better yield. The reasons for such behaviour was not investigated. This could become a disadvantage in the production of enzymes, where such an unstable characteristic could give decreased enzyme yields; for example, in case of protease or β -galactosidase production.

In view of the general features listed above, relatively little biotechnological exploitation of thermophilic bacteria has been achieved. However, the potential benefits for a number of thermophilic processes have been explored and include areas such as bioconversion of municipal and agricultural residues (Glymph and Styetzenberger, 1977; Varel et al., 1977; Wise et al., 1978;), production of fuels and chemical feedstocks from wastes, biomass polymers and gasification products (Zertuche and Zall, 1982; Ng et al., 1981; Wiegel, 1982; Zeikus et al., 1981; Wang et al., 1983; Ferchak et al., 1982; Avgernios and Wang, 1981), single cell protein production from one-carbon and lignocellulosic substrates (Mateles et al., 1967; Snedecor and Cooney, 1974; Goto et al., 1977; Bellamy, 1977; Cooney et al., 1977), enzymatic hydrolysis of cellulosic substrates (Ng and Zeikus, 1981; Garcia-Martinez et al., 1980), and novel processes like microbial enhanced oil recovery (Energy research reports 1983a,b), extraction of metals from low-grade ores (Brierley, 1982; Torma and Bosecker, 1982;) and desulfurization of coal (Kargi and Robinson, 1982; Murr and Mehta, 1982).

(a) Production of biocatalysts

Enzymes are important biological catalysts. They have been used in food and leather industries and have also been used for clinical analysis. There are a number of forms in which enzymes have been used as biocatalysts including whole cells (metabolizing, non-metabolizing or immobilized) or isolated enzymes (free or immobilized).

The processes of some of the industries which use biocatalysts require high temperatures. For instance, in the starch industry, a temperature of between 55 to 110°C depending on the type of end product desired, is routinely used. A number of thermophilic enzymes from moderate thermophiles are already in use in these industries, but the half lives of these enzymes are short at such elevated temperatures, and this increases production costs. Much more thermostable enzymes are needed and the best source for isolating such enzymes appear to be from extreme thermophiles. A large number of enzymes from extreme thermophiles have been isolated and studied. These include pullulanase (Suzuki and Chishiro, 1983), acidophilic α -amylases (Buonocore et al., 1976; Uchino, 1982) and proteases (Taguchi et al., 1983; Matsuzawa et al., 1983; Cowan et al., 1982). Table 1-1 provides a comparison of half lives of some enzymes from mesophiles, moderate thermophiles and extreme thermophiles.

In addition to the property of thermostability, enzymes from extreme thermophiles have been found to be extremely resistant to denaturing agents (Taguchi et al., 1983; Cowan et al., 1982). Similarly, the specific activity of these enzymes appears to be very high (Zeikus, 1979).

Table 1.1 Thermal stability of enzymes from mesophiles, thermophiles and extreme thermophiles:

Enzyme	Temperature (°C)	Half life (Hours)		
		Mesophile	Thermophile	Extreme thermophile
Pectin esterase	58.5	0.5(a)	-	-
	70	-	-	0.5(b)
Asparaginase	55	0.3(c)	1.4(d)	-
	75	0.3(e)	-	70(f)
β -galactosidase	50	0.3(g)	-	-
	60	-	7.5(h)	-
	65	-	1.4(h)	>750(i)
	80	-	-	>20(i)
	85	-	-	55(j)
Protease	60	0.25(k)	-	-
	75	-	-	193(n)
	80	-	1(l)	30(n)
	85	-	0.2(m)	6(o)
α -amylase	90	0.005(p)	0.4(q)	0.3(r)
	95	-	-	1-2(s)

Organisms and growth temperatures:

- (a) *Coniophora cerebella* (25°C); Kulp, 1975.
- (b) *Cl. thermosulfurogenes* (60°C); Schink and Zeikus, 1983b.
- (c) *B. coagulans* (37°C); Doig, 1974.
- (d) *B. stearothermophilus* (55°C); Doig, 1974.
- (e) *E. coli* (37°C); Guy, 1980.
- (f) *Thermus* T-351 (75°C); Guy, 1980.
- (g) *N. crassa* (25°C); Johnson and De Busk, 1970.
- (h) *B. stearothermophilus* (65°C); Griffiths and Muir, 1978.
- (i) *Thermophile* 4-1A (75°C); Daniel et al., 1981.
- (j) *Caldariella acidophila* (87°C); Buonocore et al., 1980.
- (k) *B. subtilis* (37°C); McConn et al., 1964.
- (l) *Malbreacha pulchella* (45°C); Voorduow et al., 1974.
- (m) *B. thermoproteolyticus* (55°C); Ohta, 1967.
- (n) *Thermus* T351 (75°C); Cowan et al., 1982.
- (o) *Thermus* T351 (75°C); Cowan and Daniel, 1982b.
- (p) *B. subtilis* (37°C); Doig, 1974.
- (q) *B. stearothermophilus* (55°C); Doig, 1974.
- (r) *B. caldolyticus* (72°C); Heinen and Heinen, 1972.
- (s) *Bacillus* species (?°C); Sonnleitner and Fiechter, 1983.

Enzyme immobilization technology with enzymes (Schmid, 1979) or whole cells (Abbott, 1978) from mesophiles has been successfully used today. However, in the case of thermophiles, only a limited amount of information is available. In the case of immobilization of D-asparaginase (Guy, 1980) and protease (Cowan and Daniel, 1982b) an increase in the thermostability of these enzymes was observed. On the other hand, β -galactosidase from Caldariella (Buonocore et al., 1980) and Thermus 4-1A (Cowan, D.A., Daniel, R.M. and Morgan, H.W., unpublished results), showed no increase in thermostability after immobilization.

However, a higher degree of thermophilicity of an organism does not necessarily mean that pure enzymes derived from such organisms will always be more thermostable. For example, while glucose isomerases produced by thermophilic bacilli were quite stable, the most thermostable glucose isomerase was purified from a mesophilic Actinoplanes species. The activity was still retained after more than 20 minutes at an incubation temperature of 90°C. Another point worthy of noting was that with a few of the enzymes from thermophiles or extreme thermophiles, enzyme thermostability was dependent on presence of calcium ions (Heinen and Lauwers, 1975; Cowan and Daniel, 1982a; Cowan et al., 1982). The fact remains, however, that one has a better chance of finding more thermostable or more chemoresistant enzymes from extreme thermophiles than in moderate thermophiles. Obviously, the recently isolated thermophilic archaeobacteria, which have growth optima as high as 105°C, would be prime candidates for further investigation. A β -galactosidase from Caldariella (a thermophilic archaeobacterium capable of optimal growth at 85°C, was found to have a half life of 55 hours at 85°C. Furthermore, the enzyme was found to be constitutive rather than inducible (Buonocore et al., 1980; Drioli et al., 1981). An

extracellular protease from an archaebacterial coccus was found to have a half life of about 30 minutes at 100°C (Cowan, unpublished results).

(b) Thermophilic Ethanol Production

The production (and use) of ethanol from biological materials as an alternative fuel has become one of the hopes for solving the worsening energy crisis. The most important criterion for its use as an alternative fuel rests on the production cost. As with all industrial processes, the main factors which affect production cost are the availability (cost) of the substrate, the efficiency of the conversion organism being used, and plant design and product recovery.

It appears that cellulosic biomass such as agricultural, forestry and municipal residues are the potentially important substrates for the production of ethanol. This is because cellulose is abundant, renewable, inexpensive and at present poorly utilized. (Rabson and Rogers, 1981; Wilke et al., 1981; Flickinger, 1980). Chemically, cellulosic biomass consists of lignin, cellulose and hemi-cellulose. Hemicellulose consists of hexose and pentose polymers; the ratios of hexose and pentose polymers vary considerably in different biomass (Rosenberg, 1980; Zeikus, 1980). Cellulose is composed of D-glucose polymers.

At present, there are two major ways of producing ethanol from cellulose. These processes have been reviewed recently by Ladisch et al., (1983) and Wiegel, (1982).

1. The two-step process: Cellulose is converted enzymatically (Reese and Mandels, 1980) or by treatment with chemicals (Han and Callihan, 1974; Grant et al., 1978; Ladisch et al., 1978; Uprichard and Burton, 1982) to pentoses and hexoses. Hexoses are further fermented in a second stage to ethanol by yeasts

(Reese and Mandels, 1980) or by Zymomonas (Viikari et al., 1981).

2. The one step process: Cellulose is degraded anaerobically to ethanol by the cellulolytic thermophile, Clostridium thermocellum (Avgernios et al., 1981) or by co-cultures consisting of cellulolytic and glycolytic bacteria (Alexander et al., 1981; Cooney et al., 1978; Ng et al., 1981)

The two step process:

Saccharomyces strains have been used in the production of ethanol for a number of years. Numerous techniques including continuous process and rapid fermentation process have been tested to improve production (Gencer and Mutharasan, 1981; Robinson et al., 1981). In spite of all the rapid advances, a major disadvantage of ethanol production by Saccharomyces strains is their inability to utilise the pentose sugar, D-xylose. D-xylose is the major product of hemi-cellulose hydrolysis. However, it was observed recently that Saccharomyces strains could utilise D-xylulose (Wang et al., 1980). This feature has been exploited to convert D-xylose to D-xylulose using D-glucose isomerase or D-xylose isomerase and hence indirectly bring about the fermentation of D-xylose by Saccharomyces strains (Hsiao et al., 1982; Wang et al., 1980; Gong et al., 1981). Another yeast species, Pachysolen tannophilus was able to ferment D-xylose but the yield of ethanol produced was too little to be of commercial value. The rate of fermentation of D-xylose could be improved by allowing growth to occur semi-anaerobically (Maleszka and Schneider, 1981).

Out of the numerous anaerobic mesophilic eubacteria which have the capability to produce ethanol, some strains of Zymomonas mobilis have the potential for industrial ethanol production (Skotnicki et al., 1981; Lee et al., 1979; Rogers et al., 1979). The advantage Zymomonas mobilis strains hold over yeast strains is their higher uptake rates for glucose and hence the rate of ethanol production is higher than for yeasts. However, like yeasts, they too have the disadvantage of not being able to ferment D-xylose. In addition there is an associated problem with using Zymomonas. The bacterial fermentation requires pH values of above 5, a range which is suitable for the growth of other microorganisms and hence the necessity of extra precautions with sterility monitoring.

At present, strains are being genetically engineered for two purposes. (1) To enhance cellulase production for the initial hydrolysis step. (2) To clone the cellulase gene so that cellulose hydrolysis and ethanol production can simultaneously occur in one organism (Montenecourt, (1983; Cornet et al., 1983).

The one step process:

The advantages of thermophilic fermentations have already been discussed above. A number of anaerobic extreme thermophiles have been isolated and tested for production of ethanol. Thermoanaerobacter ethanolicus (Wiegel and Ljungdahl, 1981) Clostridium thermohydrosulfuricum (Wiegel et al., 1979), and Thermoanaerobium brockii (Zeikus et al., 1979) have been observed to ferment a wide range of carbohydrates including pentoses and biopolymers such as starch, cellobiose and lactose to ethanol. This nutritional versatility exhibited by extremely thermophilic anaerobic glycolytic anaerobes is a very important property and can be exploited for the fermentation of cellulose by cocultures. For instance, a stable coculture of Clostridium

thermocellum and Clostridium thermohydrosulfuricum was found to produce an ethanol yield that was twofold higher than that obtained by a monoculture of Clostridium thermocellum, when grown on cellulose (Ng et al., 1981).

Of all the extreme thermophiles so far tested, Thermoanaerobacter ethanolicus had some advantages over the other ethanol producing extreme thermophiles: (1) It exhibited a very broad pH optimum range and (2) Under some conditions it fermented glucose and other sugars quantitatively to ethanol as do yeast and Zymomonas. However, variations in the ethanol to lactate ratio occur for as yet an unknown reason.

Clostridium thermohydrosulfuricum isolated by Zeikus et al., (1980) produced more ethanol than Thermoanaerobacter ethanolicus. However, ethanol production, as with all Clostridium thermohydrosulfuricum strains, was pH dependent.

None of the strains under current investigation with the exception of Clostridium thermocellum, are capable of simultaneously hydrolysing cellulose and producing ethanol. This direct conversion of cellulose to ethanol, has been of interest to several groups of workers (Cooney et al., 1978; Garcia-Martinez et al., 1980; Avgerinos and Wang., 1981; Zertuche and Zall, 1982).

A drawback of these organisms, as is the case with a number of industrially important organisms, is product inhibition. Clostridium thermocellum was found to be inhibited by end products of fermentation, such as acetate, ethanol and lactate (Herrero and Gomez., 1981). Thermoanaerobacter ethanolicus was found to be inhibited by 0.5% ethanol (Carreiria and Ljungdahl, 1982) whereas, Thermoanaerobium brockii was observed to be inhibited by low concentrations of hydrogen (Lamed and

Zeikus, 1980). The mechanisms for product inhibition in Clostridium thermocellum has been under investigation by Herrero and Gomez, (1981) and Herrero, (1983). Similarly, mutants of Clostridium thermocellum (Herrero and Gomez, 1980) and Thermoanaerobacter ethanolicus (Carreiria and Ljungdahl, 1983; Carreiria et al., 1983) with increased ethanol tolerance have been isolated and studied.

Saccharide fermentation by thermophiles proceeds via the Embden-Meyerhof-Parnas pathway and hence they produced the same end products as mesophiles, namely ethanol, acetate, lactate, CO₂ and H₂, but in totally different proportions. The biochemical basis for such a difference in the end product ratios is related to subtle differences in the specific activities and regulatory properties of the enzymes which control carbon and electron flow during fermentation. Some of the enzymes involved in these fermentation processes have been studied recently (Lamed et al., 1981; Bryant and Ljungdahl, 1981; Mitchell et al., 1982; Lamed and Zeikus, 1980, 1981; Bryant and Wiegel, 1983) and are reviewed by Ljungdahl et al., (1981) and Zeikus et al., (1981).

Hot springs contain a wide spectrum of glycolytic anaerobes of which only those with immediate commercial potential have been studied. Our understanding of many of the others is still rudimentary and incomplete (Carreira et al., 1983; Biomass Digest, 1982; Schink and Zeikus, 1983a).

PART A

"IN SITU" STUDIES ON HOT POOL MICROBES

Isolation and characterization of bacteria in vivo is the most widely used method for gaining information on microorganisms. However, not all cultural techniques are suitable for isolation and cultivation of microorganisms. This means that some of the microorganisms in the microbial population may not be detected and hence not studied. Studies of microorganisms in situ can be an aid to a better understanding of the ecosystem.

Successful in situ studies are dependent on sensitive methods. To study the ecology of thermal springs in situ, three sensitive methods, namely, radio-isotope uptake, electron microscopy and immunofluorescence and were chosen. Radio-isotope uptake experiments were used in an attempt to determine growth rates, electron microscopy to determine morphological types of microorganisms present and immunofluorescence to determine the distribution and prevalence of some extremely thermophilic species and their serotypes in the thermal spring environment.

CHAPTER 2

MICROSCOPY OF HOT POOL BACTERIA

2.1 INTRODUCTION:

Initial studies on the ecology of the thermal pools were hampered due to lack of suitable culture methods. It could not be assumed that organisms cultured from the pool were necessarily the dominant ones, since they might merely reflect the selectivity of culture methods available. Hence, it was necessary to develop a suitable method to identify the dominant microbial flora for comparison with the pure cultures isolated in vivo.

Microscopic examination of pool water was a quick, simple and effective method of correlating the morphology of organisms in situ with those that were cultured. However, pool water, especially from pools at low pH contained a high concentration of particulate material which under the light microscope, made differentiating bacteria, especially coccal shaped cells, very difficult. This problem is not uncommon with many natural environments; for example, Tuovinen and Nurmiaho, (1979), examined ochreous sludge for the presence of iron bacteria and observed that interference due to presence of ferric hydroxide particles was so great that bacterial cells could not be detected under the light microscope at all.

Additionally, adsorbed bacteria are possibly more important in many pools (those pools with a fast runoff) than free living bacteria, so that observation of bacteria colonising a surface might more truly reflect the important bacterial species in the pool. Observations to this effect have been made in marine and fresh-water systems (Paerl, 1974).

In the present study it was found that observation of colonized glass slides by phase contrast illumination in most alkaline to moderately acidic thermal pools was possible but far more information could be obtained by using electron microscopy techniques in situ.

Two different approaches have been generally used to study the morphologies of bacteria in situ using electron microscopy. (1) The sample containing the microbial community to be studied is fixed and concentrated by means of filtration (Carlucci et al., 1976; Torrella and Morita, 1979; Paerl, 1975), or centrifugation (Moriarty and Hayward, 1982; Ewert and Paynter, 1980; Ackermann and Nguyen, 1983) or by an agar diffusion technique (Anderson and Doane, 1972). (2) Plastic films (Caldwell et al., 1981; Jordan and Staley, 1976), glass slides (Bott and Brock, 1970), parafilm (Brock, et al., 1971), nucleopore membrane filters (Jannasch and Wirsén, 1981) and carbon films (Chalcroft and Morgan, 1982) have been used as substrates for adsorption and colonization in the environment. Natural substrates which harbour and allow colonization of the microbial populations under investigation, have also been studied, using electron microscopy (Berry et al., 1978; Ducklow and Mitchell, 1979). These methods which use artificial or natural substrates for colonization also become an effective way for concentrating the organisms in the ecosystem. The samples from either of the procedures listed above are then processed for transmission electron microscopy using techniques of thin sectioning and /or negative staining, or scanning electron

microscopy.

It should be noted, however, that studies which use artificial substrates for colonization may not necessarily reflect the true population present in the ecosystem under study, since microbial attachment to a surface appears to be a more complex process than earlier envisaged, and colonization may reflect the type of material used for such in situ studies. Mills and Maubrey, (1981), used different substrates for colonization studies in aquatic environments and found that microbial colonization did vary with the type of substrate used.

In situ techniques involving the use of electron microscopy have not only demonstrated the presence of unique bacterial morphologies but have also been successfully used for determining the proportion of Gram positive to Gram negative bacteria in sediments and in determining generation times of bacteria in a number of environments, the examples of which are illustrated below.

Nikitin, (1973), used a "thermal adhesion method" for preparing an electron microscopy specimen from soil suspensions and observed a number of unusual bacterial morphologies and viruses in the samples, some of which were isolated in pure culture. Brock et al., (1971), observed coccal and rod shaped bacteria with unique cell wall structures in the thermal springs of Yellowstone National Park U.S.A., but were unable to culture them under laboratory conditions. Waid (cited by Uydess and Vishniac, 1976) demonstrated the presence of bacterial cells in arid ice-free Antarctic soil using a "soil film floatation method." This disproved earlier suggestions that such extreme environments were sterile; a notion which came about because earlier attempts to isolate microorganisms from these environments had failed. Subsequently, a number

of unique bacterial morphologies were isolated from these areas by Uydess and Vishniac, (1976).

Moriarty and Hayward, (1982), studied marine sediments using a thin sectioning method and observed bacteria with unique cell wall morphologies, some of which exhibited a honey comb structure and others which exhibited spiny structures. They also used this method to obtain a distribution profile of Gram positive and Gram negative bacteria in marine sediments. They observed that 10% of bacteria in the surface sediment (0 to 1 cm depth) were Gram positive whereas in the deeper anaerobic zone (20 to 21 cm depth), about 40% were Gram negative. This was in good agreement as determined earlier by muramic acid measurements (Moriarty, 1975, 1980).

Jordan and Stanley, (1976), used an "electron microscope grid immersion" method to study autogenic succession in freshwater (the succession from pioneering community with low species diversity toward an increasingly complex community with greater species diversity on approach of the climax). Caldwell et al., (1981), used a similar technique of grid immersion to determine microbial attachment and generation time of microorganisms in lakes. Generally, immersed glass slides have been used to determine the generation time of bacteria in natural ecosystems (Brock, 1971; Bott and Brock, 1969, 1970; Bott, 1975).

To study bacterial morphologies present in their natural environment of the hot springs of New Zealand, a number of studies were performed. These included (i) microscopic examination of pool water, (ii) determination of colonization rates of bacteria "in situ" by a slide colonization method and subsequent studies of colonized bacteria by Gram staining and electron microscopy and (iii) ultrastructural studies of bacteria colonising electron microscope grids and membrane filters.

SECTION 1

DISTRIBUTION OF BACTERIA IN NEW ZEALAND THERMAL SPRINGS-- A LIGHT MICROSCOPIC SURVEY

Microscopic examination of hot spring samples from a number of thermal regions of the world have shown the presence of bacteria (Brock and Brock, 1971a). That these bacteria are actively metabolising in such extreme environments has also been demonstrated (Brock and Brock, 1971b; Brock et al., 1971).

Although the presence of bacteria in some New Zealand thermal springs has been studied previously (Brock and Brock, 1971a), a much more thorough investigation of their distribution was attempted.

2.2 MATERIALS AND METHODS:

2.2.1 Location Of Field Sites And Sampling Procedure

Samples were collected from thermal regions of New Zealand and White Island. A number of these thermal areas, for example, Rotorua, Waimangu, Waiotapu, Tikiteri were visited on several occasions whereas others like White Island and Fiji were visited once. The details of the location and sites of the sampling areas have been provided in the inside back cover pocket.

Soil samples from fumaroles were scooped into sterile 30 ml universal bottles. Samples from hot springs were collected in sterile 30 ml universal bottles or 125 ml medical-flats which were held with a pair of tongs, or tied to plastic coated wire if samples were to be collected from inaccessible pools. The collection vessels were completely filled, usually with both sediment and pool water, sealed with a gas tight septum

and capped tightly. All samples were transported to the laboratory at ambient temperature.

The samples were usually used immediately for light microscopy, or stored at room temperature for future use, mainly for culturing.

2.2.2 Chemical And Physical Determinations

pH, temperature and conductivity measurements were performed "in situ" using a Digi-sense LED pH meter, a Digi thermometer and Triac Controls conductivity meter respectively.

The temperatures of the thermal springs ranged from 37 to 103°C with the majority being between 65-95°C. The temperatures of springs surveyed microscopically is provided in figure 2.1A.

The pH values ranged from as low as 1.9 to as high as 9.1. The pH profile of the springs studied is given in figure 2.1B. Two major types of springs, seen as peaks in the regions of pH 4 and pH 7 were predominantly studied, reflecting the actual pH and temperature profiles of New Zealand thermal springs.

Springs in different parts of the world do not always show the same range. The thermal springs of Yellowstone National Park, USA, have boiling temperatures at 93-94°C reflecting the altitude at which they occur. The New Zealand thermal springs are also different with respect to the relatively high amounts of sulphide present. This is evidenced by the presence of sulphur, formed due to oxidation of sulphide, around the rims of a majority of alkaline springs, especially in Whakarewarewa thermal area in Rotorua. The Fijian springs, especially those around the coastal areas, are high in chloride and sulphate ions due to mixing of thermal

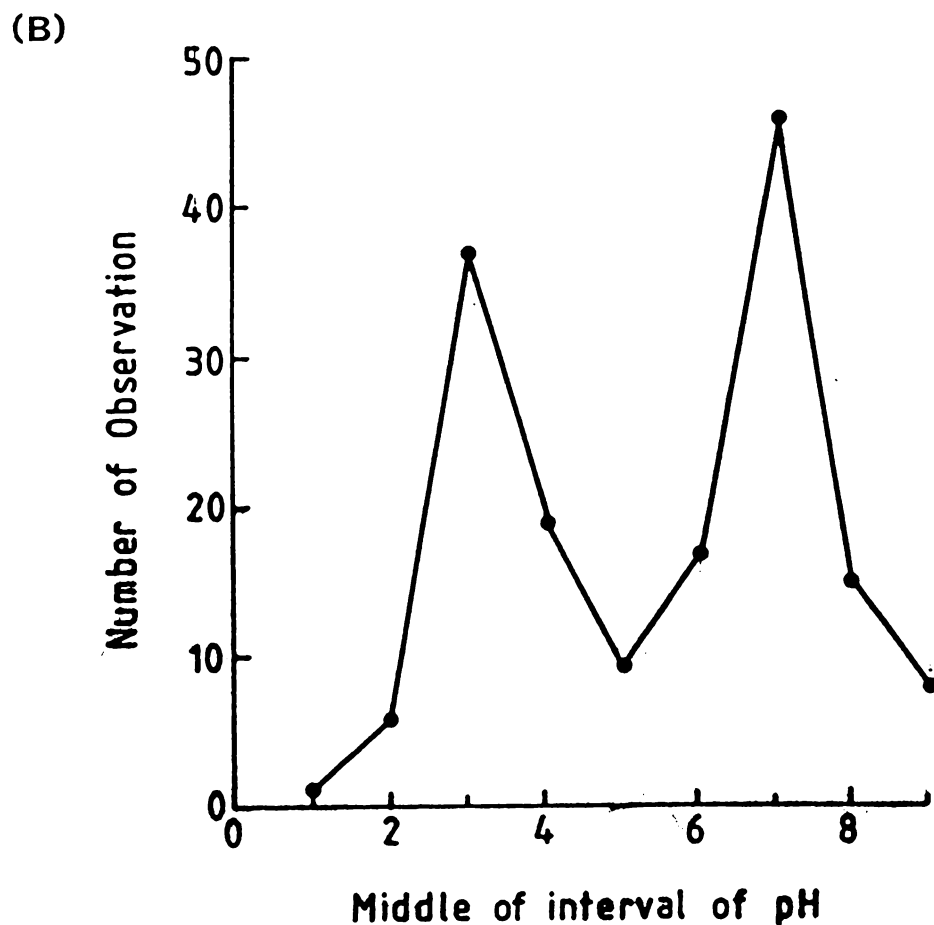
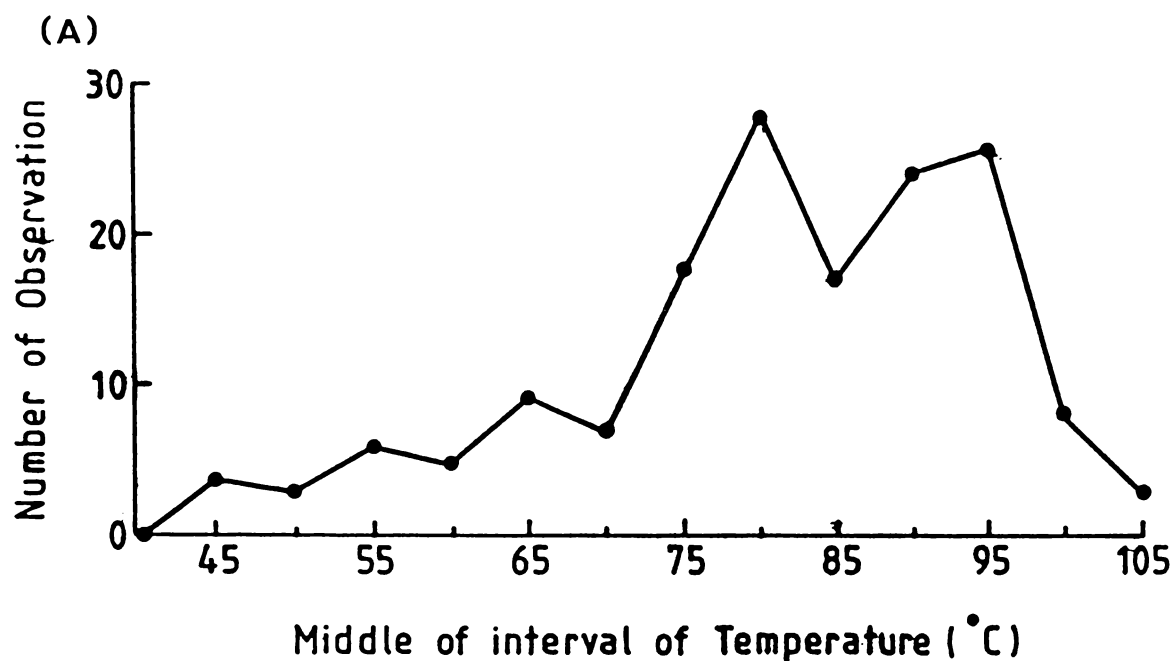


Figure 2.1. Temperature (A) and pH (B) profiles of 158 New Zealand thermal springs, samples of which were observed microscopically for bacterial presence. Data is obtained from Appendix I. Another 31 pools are not represented in the figure because of missing pH and/or temperature data.

water with sea water. The Fijian springs were all alkaline with no springs having pH below 6.8. Sulphur was not evident around the rims of any Fijian spring. On the other hand, the springs of Iceland are predominantly neutral to alkaline with very few springs occurring between pH 4 and 6.

2.2.3 Examination Of Pool Water

10 micro-litres of pool water was placed on a slide and a coverslip (22 X 22 mm, No. 1; Chance Propper Ltd., U.K.) pressed over it. The preparation was then observed under a phase contrast microscope (Leitz or Olympus). Approximately 30 fields were observed and a rough estimate of the morphological types of bacteria present noted.

For observation of bacteria in soil samples obtained from fumaroles, a soil suspension was prepared in acidified Allen's salts (Darland and Brock, 1971) and in alkaline Allen's media (Jackson et al., 1973). The particulate material was allowed to settle and the above procedure then followed for microscopy.

2.3 RESULTS AND DISCUSSION:

2.3.1 Field Observations

New Zealand thermal springs with an outflow and which were less than 70°C, usually had visible algal growth. In certain cases, visible bacterial mats were also observed. Fluctuations in temperature and pH was noted in several springs in the Rotorua thermal area during the course of this investigation. The pH of a spring is mainly due to the solubility of the silica present which is known to be dependent on the temperature. Hence, fluctuations in the temperature of a spring can alter the pH of the

spring, which may be reflected in alterations in the bacterial flora. Similarly, other chemicals present in the springs can also play an important part in determining the predominant microbial flora of a particular spring; for example sulphide. However, it was beyond the scope of this investigations to fully investigate these changes.

2.3.2 Distribution, Density And Morphotype Profile

Bacterial presence was demonstrated in 171 of the 190 thermal samples analysed microscopically (Appendix I) and bacterial absence was recorded in another 13 samples.

Analysis of a further 5 samples was hampered due to particulate interference. Of the 13 samples from which bacteria were absent, 4 were obtained from fumaroles. Additional samples from fumaroles (not recorded in the appendix), were also devoid of bacteria. Bacterial absence from fumaroles has also been noted by Brock, (1967). He concluded that liquid water, rather than steam was necessary for development of living organisms. Sulfolobus have been isolated from moisture saturated hot and acid soils. However the densities of these bacteria were so low that they were impossible to detect under the light microscope (Fliermans and Brock, 1972).

Bacterial absence in the 9 other (non-fumarole) samples cannot be explained. The samples had temperature and pH ranges similar to the samples in which bacteria were present. Chemical analysis of these springs would be necessary to determine whether toxic elements existed in these pools. As an example, Waiotapu which has high levels of arsenic was one region where bacterial presence was unusually low.

Morphologically diverse groups of bacteria were present in the thermal springs investigated and included filaments, rods, cocci, spirochetes, spirals and vibrioids. The predominant microbial morphology was rods which were present in 70% of the springs. This probably suggests a diverse physiology allowing colonization over such a range of pH and temperature (Figure 2.2).

Cocci were present in 55% of the springs. They were present in both the boiling acid pools and the vigorously boiling alkaline pools. Previously their presence has been primarily associated almost exclusively with hot and acidic springs, from which they could be readily isolated. Zillig et al., (1981a) isolated anaerobic cocci, whereas Brock et al., (1972) had earlier only isolated aerobic cocci from such hot and acidic environments. Brock et al., (1971) on the other hand did observe coccal shaped cells in an alkaline hot spring of Yellowstone National Park, USA. However, this organism was not cultured and nor was their presence in other alkaline springs studied.

Cocci were more dominant in the acidic springs than the alkaline ones (Figure 2.3). Again, the density and morphology of cocci in acidic environments was different to that found in alkaline hot springs. The coccal population was greater in acidic environment (as high as 10^7 cells/ml) and these cocci were much more phase dark compared to the cocci in alkaline environments, where the population was far less (10^4 cells/ml) and the cells phase light. The isolation and characterisation of these cocci is dealt with more fully in chapter 5.

Filaments were present in 42% of the springs. The presence of filaments was dependent on the pH of the spring, filaments being more prevalent in springs above pH 6.0 (Figure 2.4). The presence of filaments was independent of temperature and they were observed in springs covering

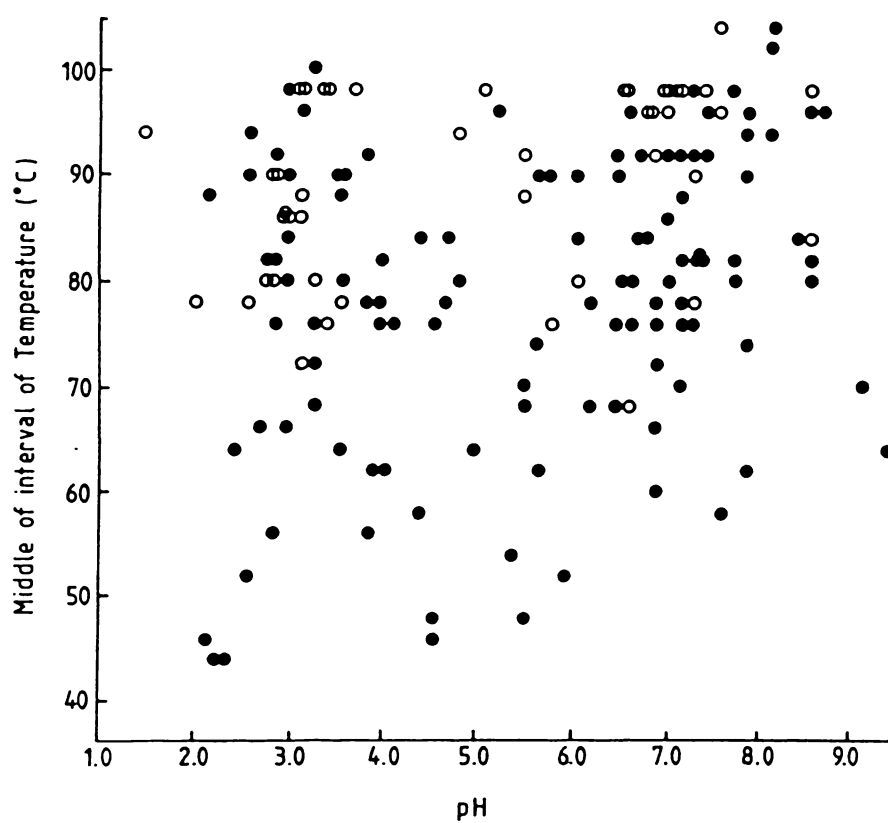


Figure 2.2. Distribution profile of rods in 158 New Zealand thermal springs. Data is obtained from Appendix I. 31 pools are not included in the distribution profile because of missing pH and/or temperature data. ● rods seen; ○ rods absent

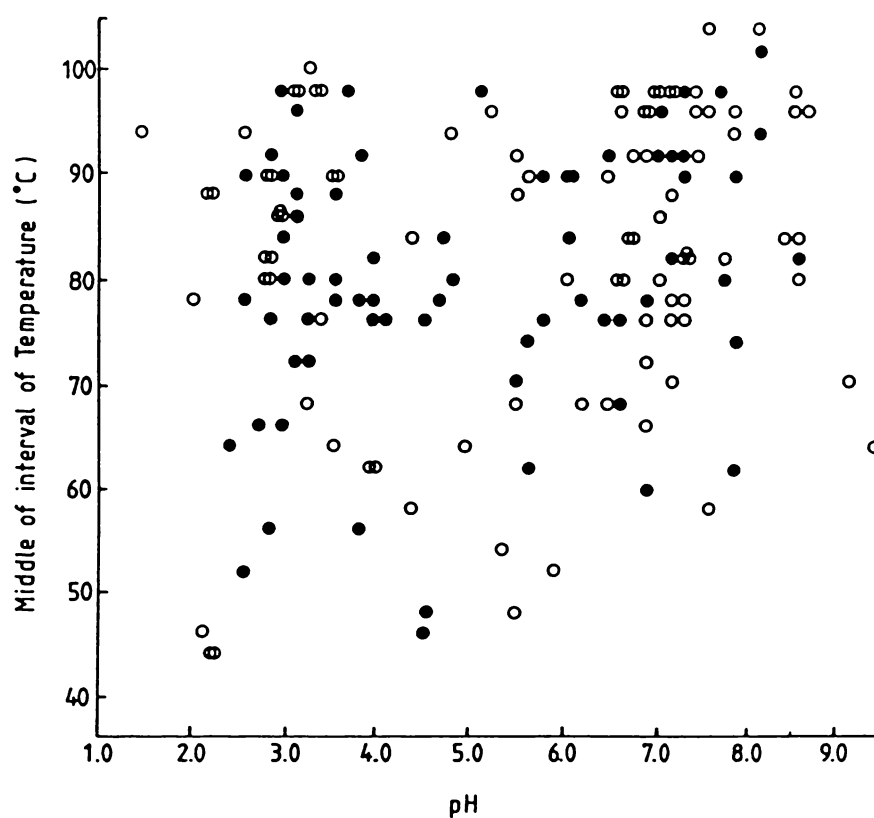


Figure 2.3. Distribution profile of cocci in 158 New Zealand thermal springs. Data of graph is from Appendix I. 31 pools are not included in the distribution profile because of missing pH and/or temperature data. ● = cocci seen; ○ = cocci absent

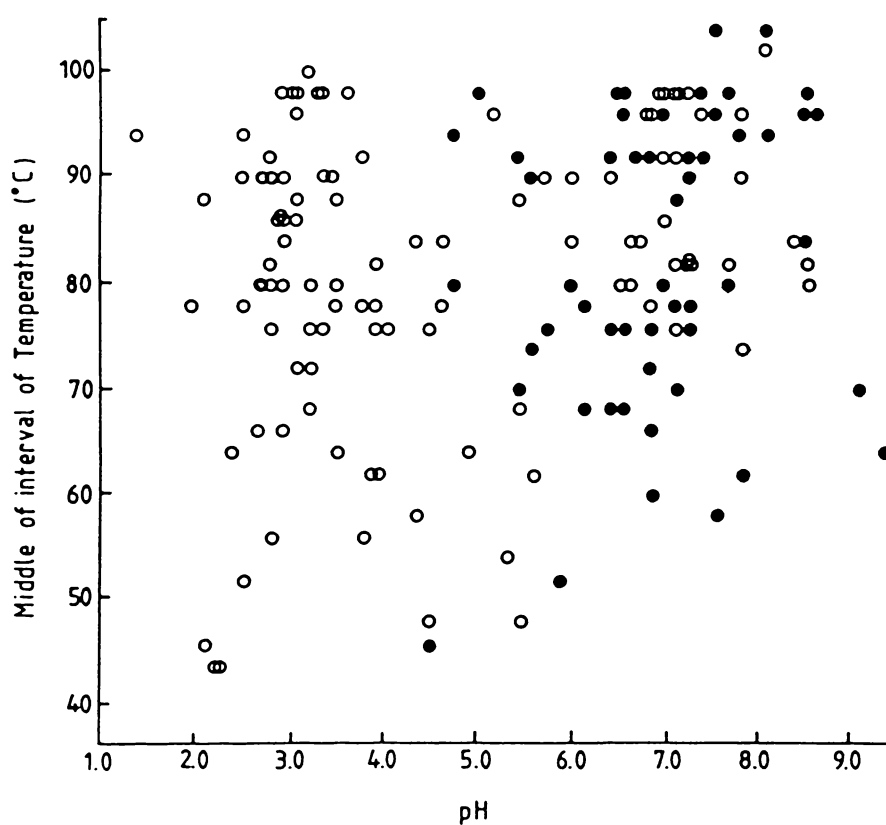


Figure 2.4. Distribution profile of filaments in 158 New Zealand thermal springs. Data of graph was obtained from Appendix I. 31 pools are not included in the distribution profile because of missing pH and/or temperature data.
 o=filaments seen; o=filaments absent

the whole temperature spectrum.

In only two springs was the presence of spirochetes demonstrated. These were Rt3 and Rt6, both of which were located in Kuirau Park, Rotorua. One of the spirochetes was isolated for further studies (Refer chapter 7). Spiral bacteria which were morphologically distinct from spirochetes were observed in three pools, namely Rt37 (Located in Kuirau Park thermal area) and Rt94 and Rt99 (located in Whakarewarewa thermal area). Vibrions were observed in two pools only. Both the pools were located in White Island (Refer appendix I). Unique spheroid producing rods were observed in pools Rt8, Rt12, Rt44 and Rt86. Caldoactive glycolytic anaerobes with a similar morphology as those above (spheroid producers) were isolated from these and some other pools and were found to constitute a new genus Fervidobacterium which is more fully described in chapter 7.

Repeated sampling of a number of pools for microscopy, yielded the same morphotype on each occasion indicating that the organisms were a stable component of the thermal environment. A number of springs were observed to fluctuate in temperature, pH and water level. However, reversal of these fluctuations resulted in the rapid reestablishment of the original flora.

During the course of these investigations, at least four springs underwent drastic changes in pool characteristics. In pools Rt1 and Rt3, both of which cooled considerably (from 85°C to 30°C and from 70°C to 50°C respectively), dramatic changes in microbial flora resulted. Pool Rt1 lost a coccal shaped archaebacterium designated AN1 (Morgan, 1979) and pool Rt3 lost a spirochete population. In a third pool, Rt8, which was runoff channel formed by a vent from a heat exchanger, closure of the vent resulted in drying of the channel and the decay of black gelatinous bacterial mats present. However when the vent was reopened the bacterial

mats re-established rapidly. Whether recolonization by the same organisms would occur in pools Rt1 and Rt3 if they return to original conditions remains to be seen. A fourth pool, Rt34 (DSIR no. 344) was devoid of bacteria when pool water was examined microscopically by Brock and Brock, (1971a). The temperature and the pH recorded by them was 95.5°C and 3.05 respectively. Recent microscopic examination revealed the presence of bacteria in this pool. The temperature of the pool was 75.7°C and pH 6.53 (appendix I). Hence, colonization of this pool must have occurred recently or alternatively, changes in the condition of the pool must have favoured a rapid growth of a previously more or less dormant flora to occur.

SECTION II

STUDIES ON BACTERIAL COLONIZATION OF GLASS SLIDES

2.4 MATERIALS AND METHODS:

The purpose of these studies was to determine colonization rates of bacteria present in situ from 5 springs. It was also necessary to determine the type of bacteria present in a number of springs. This was achieved by performing Gram staining and electron microscopy of the colonized slides. Twenty four pools were selected for these studies.

For colonization of bacteria, detergent washed and distilled water-rinsed glass slides in glass staining racks were suspended by plastic coated wire in hot pools. Slides were removed at intervals from these racks and immediately fixed in 4% formalin solution prepared in appropriate pool water.

These colonized slides were either used immediately if colonization rates and morphology of the bacteria was to be determined, or were dried

and stored in slide mailers for a period of not more than two weeks during which time they were used for studies on immunofluorescence (Chapter 4), electron microscopy and Gram staining.

2.4.1 Determination Of Colonization Rates

The selection of the 5 springs for studies on colonization rates was based on (a) the accessibility of the pools since the work was to be performed by day and night and (b) pH and temperature range of the pools. To determine colonization rates, bacteria in 100 fields per slide were counted for each incubation period with the aid of an Olympus Phase contrast microscope, and an average of the number of bacteria per field calculated. Colonization rate in this case is equal to the growth rate plus the attachment rate since no attempts were made to account for the two processes separately as done by Bott, (1975). It was assumed that if growth rates of adsorbed cells was rapid compared to attachment then colonization would be exponential. However, if the reverse was true, then colonization would be linear. Studies on colonization rates was compounded by the fact that the spring microorganisms did not grow as microcolonies due to the nature of these micro-organisms. The mode of division of such archaebacteria is presently unknown. This made it more difficult to use correction equations devised by Caldwell et al., (1981) to account for attachment rates and hence calculate the actual growth rates of the micro-organisms under study.

2.4.2 Gram Staining And Electron Microscopy Of Colonized Slides

Gram staining was performed using the method of Lillie, (1928) as modified by Preston and Morrell, (1962).

For electron microscopy, cells adhering to colonized slides were released by scraping the slide surface gently with a scalpel. Two drops of appropriate filter sterilized pool water was added to the scraped surface and a butvar-carbon coated electron microscope grid was floated on the surface of this suspension for 20 to 30 minutes (Refer Chapter 3 for preparation of butvar films). The excess liquid was blotted off and the grids stained with 1% uranyl acetate for 30-40 seconds. The grids were then blotted to remove excess stain and observed under a Phillips EM400 electron microscope at an accelerating voltage of 80kV.

2.5 RESULTS AND DISCUSSION:

2.5.1 Colonization Rates

Due to slow colonization rates, attempts at determining colonization rates in three pools, viz Rt30, Rt42 and Rt57 were abandoned. In the first two pools, typically less than 1-2 cells per field were observed after lengthy incubation periods. Incubation of slides was abandoned in pool Rt57 for the same reason where as few as 12 cells per 100 fields were observed after 24 hours incubation.

The colonization rate of bacteria present in pools Rt51 and Rt56 was 0.31 cells/field/hour and 8.1 cells/field/hour which was equivalent to a generation time of 16 hours and 12 hours respectively (Figure 2.5). The microbial population of Rt 51 consisted of filaments for the first 12 hours after which very few small rods, usually attached to the filaments were observed. The Rt56 population consisted of rods, with very few filaments present. Colonization rates of only the dominant bacterial flora present in both these pools was calculated.

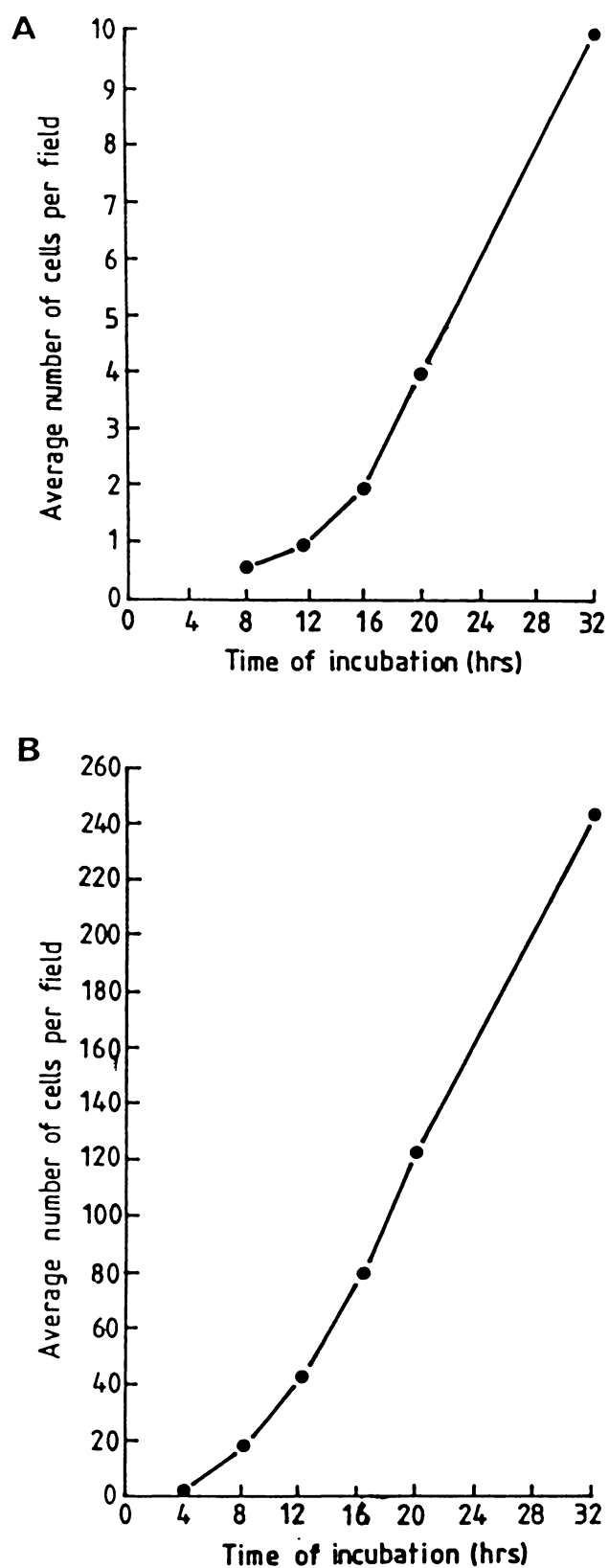


Figure 2.5. Colonisation rates of filamentous bacteria in pool Rt51(A) and rods in pool Rt56(B). Colonisation rates were determined as described in Materials and Methods in Section II.

2.5.2 Morphology, Gram Reaction And Electron Microscopy Of Colonized Slides

Microbial colonization of microscope slides occurred in 23 of the 24 hot springs analysed (Table 2.1). Filaments, rods and cocci were the usual morphotypes observed.

Filaments usually formed rosettes due to attachment to mineral particles, presumably sulphur or silica. This was a common occurrence in almost all of the hot alkaline springs.

Rod shaped bacteria were also observed in a few hot alkaline springs, for example, Rt2, Rt56, Rt68. In some of the springs, cell division of these rod shaped bacteria was also observed.

An abundant coccal population was observed in pool Rt71, which was an extremely hot, acid spring. Cocci were also observed in a number of alkaline springs (eg., Rt56, Rt60, Rt61) but were not as numerous.

Algal cells were also observed in a relatively cool and acid pool, Wai3 and a much hotter, alkaline pool, Wai19. The presence of algae in Wai19 (temp of 79°C) was not surprising, since cool spots existed in this pool, where algal mats thrived.

All the pools, except pools Rt8 and Wai19, had exclusively Gram negative microbial populations.

The cell wall of the filaments from pool Rt8 stained Gram negative however, portions within the wall stained Gram positive. Whether the organisms were sheathed bacteria or whether the Gram positive portions were storage granules of some sort within the cells, was not investigated.

In Gram stain preparation of Rt51, scutellum bearing filaments were observed (Refer scutellum bearing organisms in section III of this chapter). The scutellum stained a much darker pink compared to the filament which was much lighter in colour. The intensity of Gram stain on

Table 2.1: Morphological studies and Gram reaction of microbial population colonising microscopic slides:

Pool No.	Temp. (°C)	pH	Bacterial Morphology	
			Gram stained preparation	Electron microscopy
Rt2	82.5	7.66	Gram -ve short rods and filaments.	
Rt3	70	7.2	Gram -ve cocci and filaments.	
Rt6	64	9.4	Gram -ve filaments.	
Rt8	70	9.1	Gram -ve sheath enclosing Gram +ve cells; Gram +ve short rods.	
Rt12	66	6.9	Gram -ve rods (0.75um x 1.8um); Gram -ve curved rods (0.75um x 1.5um); Gram -ve filaments.	Rods with slime and pili; Dividing rods with pili; Few curved cells.
Rt13	98	7.4	Gram -ve filaments (0.75um in diameter).	
Rt14	80	7.0	Gram -ve small rods (0.5um x 0.75um) with slime layer surrounding the cells; Gram -ve filaments.	Rods with slime and pili.
Rt15	96.4	8.7	Gram -ve filaments.	Ribbon like ghost cells; Rods with pili and slime. Rods in association with flagellated cocci.
Rt30	97.0	3.69	Gram -ve cocci.	
Rt34	80	6.53	Gram -ve rods, some dividing (0.75 x 1 to 2 um); Gram -ve filaments.	
Rt42	97	7.3	Few Gram -ve filaments.	
Rt44	62	7.81	Gram -ve rods, some dividing (1.25um x 3.0um); Gram -ve long rods (1um x 10um); Gram -ve spheroid bearing rods.	Fat rods; Cells with slime, pili; Spheroid bearing rods.

Table 2.1: Morphological studies and Gram reaction of microbial population colonising microscopic slides:

Pool No.	Temp. (°C)	pH	Bacterial Morphology	
			Gram stained preparation	Electron microscopy
Rt51	82	7.34	Gram -ve filaments with scutellum; Gram -ve filaments; Gram -ve rods, single or dividing Few Gram -ve cocci.	Rods with subunit cell wall; Scutellum bearing filaments.
Rt56	90.2	7.87	Abundant Gram -ve short rods (1um x 1.8um); Few Gram -ve cocci and filaments.	
Rt57	97	7.2	Gram -ve filaments.	Rods with subunit cell wall.
Rt59	103	7.6	Gram -ve filaments and cocci; Gram -ve rods, single or dividing (0.75um x 1.25um).	
Rt60	97.7	7.16	Gram -ve cocci and filaments.	Rods with subunit cell wall.
Rt61	94.5	8.1	Good Gram -ve coccal population; Gram -ve filaments.	Small rods; Nonflagellated cocci; Ribbon like ghost cells; Filaments with slime and pili.
Rt68	95	7.52	Gram -ve rods, filaments and cocci.	Non flagellated cocci; Rods with slime and pili; Rods with subunit cell wall.
Rt70	98.2	3.38	Gram -ve cocci.	
Rt71	98.5	3.52	Abundant Gram -ve cocci.	
Rt72	97	6.64	Gram negative long rods.	
Rt84	ND	ND	Gram -ve filaments; Gram -ve rods (1um x 1.5um).	
Rt101	66	2.7	Negative.	Negative.
Wai3	48	5.5	Algae and Gram -ve filaments.	
Wai19	79	7.7	Algae, Gram -ve filaments and Gram +ve rods, single, in twos or in chains (0.75um x 1 to 1.5um).	

The time of incubation of slides was between 8 to 11 days.

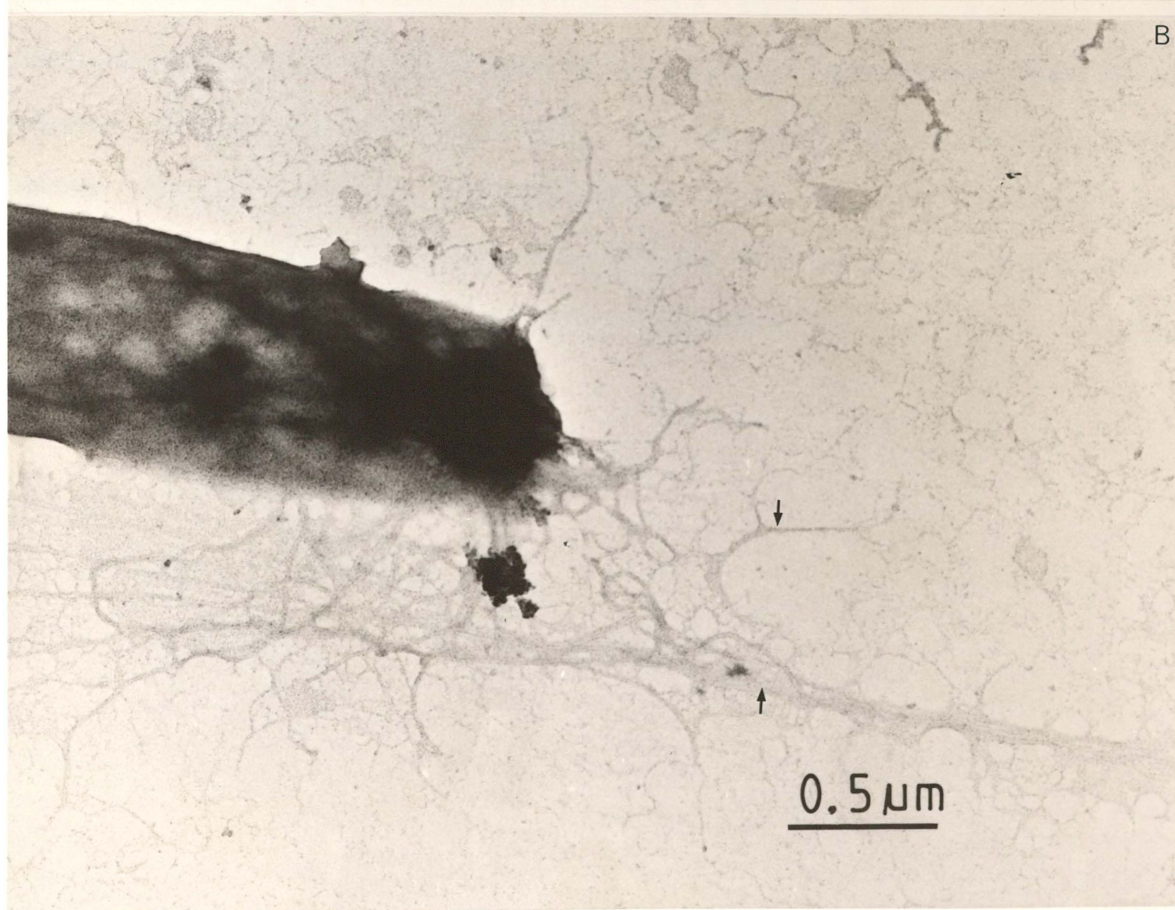
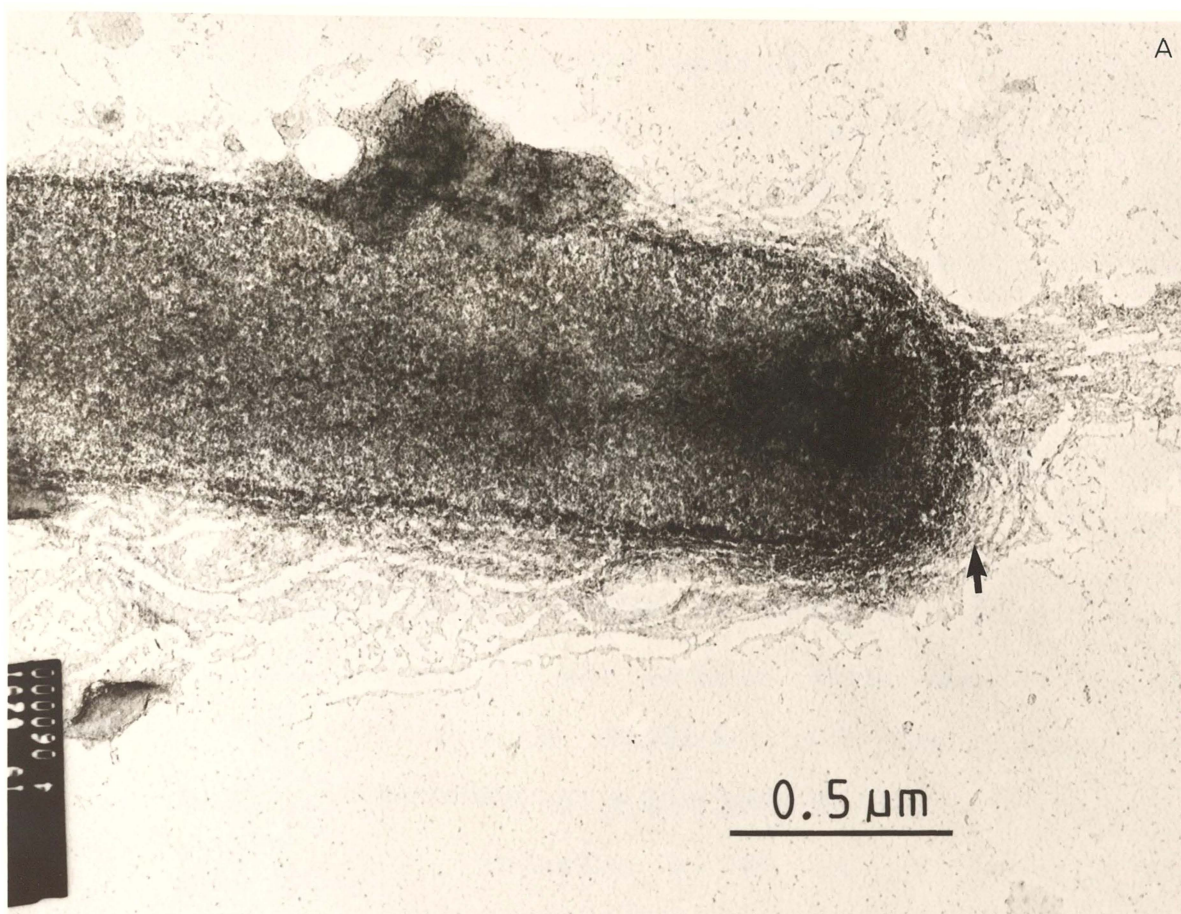
filaments from Rt13 decreased in slides which had been incubated in the pools for 7, 17 and 35 days respectively. The possibility that this was due to the degeneration of the colonized cells cannot be ruled out. However, since the number of cells colonising glass cylinders increased (Refer Chapter 3) as indicated by an increase in protein concentration, this seems unlikely. The more plausible explanation is the hindrance of stain uptake due to production of slime by bacterial cells due to adhesion.

Bacterial adhesion to a surface is known to play an important role in aquatic environments and a direct relationship exists between the surface properties of the substratum being colonized and the biological response of the colonising microbe, for example cell adhesion. It is likely that after adhering to glass slides, the bacteria produce more slime in response to the vigorously boiling and turbulent nature of the pool. The presence of extensive slime was noted in Gram stained preparations from pool Rt14. The presence of slime was a much more common phenomenon as revealed by electron microscopy of colonized slides (Table 2.1).

Electron microscopy of scrapings of colonized slides (Table 2.1) revealed the presence of rods, cocci and scutellum bearing filaments.

Cells from pools Rt12, Rt14, Rt15, Rt44, Rt61 and Rt68 possessed slime and/or pili at one end (Figures 2.6A, B). Cells from pools Rt57, Rt60 and Rt68, possessed a subunit cell wall structure similar to that of the sulphur respiring archaebacteria Thermoproteus tenax and Thermofilum pendens (Zillig et al., 1981a, 1983a). It has been known that the surface layer consisting of sub-units (S-layer) has evolved as a result of interactions between the cells and their environment and it is likely that under conditions that do not mimic the natural habitat, such as attachment

Figure 2.6. A cell from pool Rt68(A) showing subunit cell wall structure resembling Thermoproteus species. Note the electron dense material presumably slime around the outside of the cell and (B) showing extensive pili formation.



on artificial substrates, the cells produce slime. The slime produced may mask the subunit structure, so that the cells will not be recognised as archaebacteria. It is possible that in this study, cells of archaebacterial dimensions and morphology but not exhibiting wall subunit structure typical of the group were simply covered in a masking layer of slime.

SECTION III

ULTRASTRUCTURAL STUDIES OF BACTERIA COLONISING BUTVÅR AND MEMBRANE SURFACES

The gross ultrastructural studies of bacteria colonising butvar coated E.M. grids in situ were achieved using negative staining procedures. Thin sectioning of bacteria colonising polycarbonate membranes in situ was performed for a much more detailed investigation of bacterial ultrastructure. Initially, a lot of difficulties were encountered when sectioning bacteria concentrated by filtration. A great amount of chattering occurred during sectioning, presumably due to the presence of particulate material, such as silicates and iron complexes present in the pools. Similar difficulties were encountered when bacterial mats present in pools Rt6, Rt8 and Rt44 were sectioned and hence this work was abandoned and the above polycarbonate colonization method was used instead.

2.6 MATERIALS AND METHODS:

2.6.1 Chemicals

Chloroform, amyl acetate, uranyl acetate, osmium tetroxide, and ethanol were purchased from BDH, U.K.; butvar B-98 from Probing and Structure, Australia; formvar and Spurr's Embedding kit from Agar Aids, U.K.; phosphotungstic acid from TAAB, U.K.; sodium veronal, sodium acetate, anhydrous, sodium citrate, dihydrate, and sodium hydroxide from May and Baker Ltd., U.K., 25% glutaraldehyde solution from Polaron equipment Ltd., U.K., Parafilm from American Can Co., U.S.A., lead nitrate from Polysciences Inc., U.K., cellulose acetate (Industrial Lacquer, Duco, Dulex New Zealand Ltd, New Zealand) was a gift from Gary Leet, MRINZ, Hamilton, New Zealand.

2.6.2 Location Of Field Sites

The pools in which studies in this section were performed are listed in Table 2.2 (Refer section 2.7.1.2). The locations of the sampling sites are shown in the maps provided in the inside back pocket cover.

2.6.3 Chemical And Physical Determination

pH, temperature and conductivity measurements were performed "in situ" as described in section I.

Sulphide was determined by the method of Pachmayr as modified by Brock et al., 1971.

2.6.4 Grid Immersion And Floatation Technique

Butvar films were prepared as described by Handley and Olsen, (1979) with a minor modification. Microscope slides (Blue star microslides, Chance Proper Ltd, U.K.) were cleaned with tissue paper, made dust free and then used for casting films on.

For preparing formvar films, a film casting apparatus was used so as to obtain a consistent film thickness, especially at higher concentrations of formvar. For casting films, 40 to 50 ml of formvar solution was added to a separating cylinder. A clean slide (prepared as above) was placed in the cylinder. The stopcock was opened and the solution allowed to drain under gravity, after which the stopcock was closed, the top of the cylinder was fitted with the cap and the slide allowed to remain in the chloroform saturated chamber for one minute. The slide was then removed with a pair of forceps, and the film floated off in a trough filled with water.

Cellulose acetate films were prepared as described by Stolinski and Gross, (1969).

3.05 mm copper (200, 300 and 400 mesh) or nickel (200 and 300 mesh) grids (Polaron Equipment Ltd, U.K.) were dropped shiny side down onto the prepared film (as above). The grids plus film were picked up by putting a piece of parafilm on to them and drawing the parafilm out slowly. These grids were then carbon coated.

For the purpose of immersing grids into pools, double sided sellotape or autoclave tape was stuck onto glass slides. The edge of a grid was attached to the edge of the double sided sellotape or autoclave tape, and the slides were then suspended into pools in glass staining racks which

were tied to plastic coated wire.

After a suitable incubation period, grids were removed, blotted, and stained immediately with 1% uranyl acetate (pH 5.0) or 1% phosphotungstic acid (pH 7.0) for 40 to 60 seconds, after which the excess stain was blotted off.

The "floatation method" as described by Chalcroft and Morgan, (1982) was also used on occasions.

2.6.5 Membrane Immersion Technique

Polycarbonate membranes (0.2 μ m pore size and 25mm in diameter) were cut into four pieces and the edges stuck onto glass slides (usually two pieces per slide) by means of autoclave tape. The slides were then immersed into selected pools for colonization. After a fixed incubation period, normally 3 to 5 days, the polycarbonate membrane pieces were removed and fixed immediately in 2.5% glutaraldehyde (prepared in veronal-acetate buffer). The fixation process was terminated within two hours after which the membranes were washed twice in veronal acetate buffer, transported to the laboratory in fresh buffer and stored at 4°C before further processing usually on the following day.

Further fixation of the membrane pieces was achieved with 1% osmium tetroxide (prepared in veronal acetate buffer) for two hours after which they were rinsed in 2 changes of veronal acetate buffer and then dehydrated through a graded series of ethanol. The membrane pieces were then cut into approximately 2mm squares, infiltrated and polymerised in Beems Capsule with Spurr's resin. The protocol for the dehydration, infiltration and polymerization steps is given below:

1. 25% ethanol --5 to 8 minutes at room temperature.
2. 60% " --5 to 8 minutes at room temperature.
3. 95% " --5 to 8 minutes at room temperature.
4. 100% " --5 to 8 minutes at room temperature.
(two changes)
5. 100% ethanol + Spurr's
Embedding resin (1:1)-- 30 minutes at room temperature.
(two changes)
6. Spurr's resin -- 36 hrs. at 60°C or 9 hours at 70°C.

After polymerization, the specimen block was removed from the Beem capsules, trimmed and thin sections cut ^{vertically thru' the membrane} with glass knives in a Reichert microtome. Silver and grey sections were picked up on 300 mesh copper grids and stained using a double stain consisting of 1% uranyl acetate and lead citrate (Reynolds, 1963). The protocol for the staining procedure was as follows: (1) Grids were stained with 1% uranyl acetate for 60 seconds and then rinsed in distilled water.

(2) Further staining was achieved with lead citrate in a closed petri dish containing pellets of sodium hydroxide.

(3) After 3 to 5 minutes the grids were removed and submerged in 0.02N sodium hydroxide. They were then washed thoroughly in distilled water and blotted.

Observations of thin sections were made as previously described.

2.7 RESULTS AND DISCUSSION

2.7.1 Studies Of Bacteria Colonising EM Grids

2.7.1.1 Development Of A Method For Colonization Of E.M. Grids -

The major drawback with existing E.M. procedures was the instability of the coating materials, and on occasions of the grids themselves, in the hostile pool environment.

Formvar was found to be stable in pools with temperatures up to 68°C and pH's near neutrality but was unstable under more extreme conditions. Increasing formvar concentrations to 0.8%, or using chloroform or 1,2 dichloroethane as solvents had no beneficial effect on film stability.

Cellulose acetate films were time consuming to prepare and they were less stable than formvar films. They often cracked on drying in air, and difficulty was often encountered in adhering the grids to the film.

Butvar proved to be an extremely good support film being stable to conditions of up to 103°C or pH's from 2.8 to 9.2 for periods of over 60 hrs. No change in stability was observed when grids were carbon coated and on occasions this was routinely performed, especially as a comparison with the "floating carbon film method" devised by Chalcroft and Morgan, (1982).

Copper grids were generally unsatisfactory as film supports; in many pools they completely disintegrated within 2-3 hrs of incubation. In contrast nickel grids have been left in pool Rt56 (90C, pH 7.87) for over 60 hrs with no adverse effects.

Other features of importance were the greater reliability of autoclave tape over sellotape for attaching grids to supporting glass slides, and the use of glass staining racks as slide holders in the pool in preference to metal holders. Metal holders were particularly prone to corrosion, which besides possibly affecting the colonization of grids, also resulted on occasions in the complete loss of samples.

In general, better staining was achieved with the 1% uranyl acetate stain than with the 1% phosphotungstic acid.

Using the method described (butvar coated nickel E.M. grids attached to glass slides) the colonization of butvar films has been recorded in 38 thermal pools and the major morphotypes described. In some cases organisms resembling those observed in situ have been cultured and studied further, and will be reported in later sections.

2.7.1.2 Species Diversity In Hot Pools -

Electron microscopy was used to study bacterial morphotypes from 38 thermal pools, of which 23 pools were located in Rotorua, 4 in Tikiteri, 1 in Waiotapu, 7 in Waimangu, 1 in Wairakei and 2 in Fiji (Refer maps in back pocket for sampling sites). Table 2.2 lists the pools investigated by the butvar method with the pool characteristics and the types of bacterial morphologies present. Of the 38 pools listed covering temperatures from 56°C to boiling and a pH range from 2.96 to 9.2 only one pool produced a negative result with respect to bacterial presence. This pool (Rt55) was a very acidic hot pool. Pools of a similar pH but lower temperature e.g. pool WP9 (56°C and pH 2.96) had a population of flagellated rods. The absence of bacteria on grids cannot, of course, be taken as evidence of their absence from the pool. The population density

Table 2.2 A summary of microbes colonising butvar coated E.M. grids:

Pool No	Temperature (°C)	pH	Sulphide (ug/ml)	Description of pool	Morphotypes present		
					Rods and filaments	Cocci	Others
<u>ROTORUA (Kuiran Park)</u>							
Rt2	82.5	7.66	ND	Stagnant	Large and small rods, some dividing (2.34)	-	-
Rt3	63	7.5	3	Fluctuating	With cell-wall subunits; Spheroid bearing; with slime (2.42); with vesicles (2.43) with scutellum (2.16)	-	Spirochaetes (2.6) phages (2.13)
Rt6	70	9.2	ND	Flowing	With scutellum (2.15); Rosette forming (2.36)	-	-
Rt40	63	4.95	ND	Decaying matter	-	-	Spiral (2.11)
Rt58	103	7.6	6	Vig. boiling	Flagellated rods; with pili	-	-
Rt59	103	8.12	6	Vig. boiling	Filaments; flagellated rods	-	-
Rt60	97.7	7.16	ND	Boiling	Dividing rods (2.35); Non dividing rods; Flagellated rods	-	-
<u>ROTORUA (Whakarewarewa)</u>							
Rt12	66	6.9	12	Flowing	Polarly flagellated rods	-	-
Rt12A	72	6.34	ND	Flowing	Flagellated rods (2.39); with scutellum (2.17); Dividing rods (2.38); Filaments (2.52); Subunits	Flagellated (2.52)	-
Rt13	98	7.4	22	Boiling	With cell wall subunits and golf balls	-	-
Rt14	80	7.0	4	Stagnant	Rods in pairs; filaments	-	-
Rt30	97	3.69	6	Vig. boiling	Cocci, some dividing (2.48)	-	-
Rt34	75.7	6.53	ND	Fluctuating	Rods	-	-
Rt36	62	4.0	ND	Fluctuating	Flagellated and non-flagellated rods; filaments	-	-
Rt42	97	7.3	21	Waxy layer on surface - boiling	Curved cells (2.12)	-	-
Rt44	62	7.8	ND	Flowing	With intracellular material (2.40); With monopolar flagella (2.41)	Flagellated (2.50)	-
Rt51	82	7.34	19	Fluctuating	With subunits (2.51); Spiraling cells with swellings; with scutellum (2.19)	Non flagellated (2.51)	-
Rt52	68	6.16	ND	Flowing	Rods	-	-
Rt54	72.5	3.15	ND	Mud pool	Cocci	-	-

Table 2.2 A summary of microbes colonising butvar coated E.M. grids:

Pool No	Temperature (°C)	pH	Sulphide (µg/ml)	Description of pool	Morphotypes present		
					Rods and filaments	Cocci	Others
Rt55	80.9	2.89	20	Bubbling mud pool	-	-	-
Rt56	90.2	7.87	16	Flowing	With cell wall subunits (2.30, 2.31); Dividing rods (2.32); Polarly flagellated (2.33)	+	-
Rt57	97	7.2	23	Vig. boiling	With cell wall subunits	-	-
<u>ROTORUA (Government Gardens)</u>							
Rt8	70	9.1	25	Flowing	Spheroid bearing; rods with nipples; with scutellum (2.18); Filaments	-	-
<u>TIKITERI (Tourist Reserve)</u>							
Tk4b	86.9	6.17	ND	Mud pool	With cell wall subunits (2.28)	-	-
Tk8	80.5	3.2	ND	Mud pool	Rods	-	-
Tk14	80.4	5.85	ND	Mud pool	With cell wall subunits	-	-
Tk15	84.5	5.33	ND	Mud pool	With cell wall subunits	-	-
<u>WAIOTAPU</u>							
WP9	56	2.86	ND	Stagnant	Monopolarly flagellated and slime covered envelope (2.44)	-	-
<u>WAIMANGU THERMAL VALLEY</u>							
Wai12	95.5	7.38	ND	Boiling	With cell wall subunits	-	-
Wai17	75	7.75	ND	Stagnant	With cell wall subunits	-	-
Wai18	81.6	7.34	ND	Flowing	With scutellum (2.23, 2.24, 2.25, 2.26, 2.27); Subunits	-	-
Wai19	79	7.7	ND	Stagnant	With intracellular material (2.10); Filaments (2.37)	Cocci (2.37)	Spirochete (2.9)
Wai20	96	6.85	ND	Boiling mud	With cell wall subunits; Rods	-	-
Wai21	58	6.9	ND	Flowing	With scutellum (2.20, 2.21, 2.22)	Cocci (2.49)	Spirochete (2.7) Phage (2.14)
<u>WAIRAKEI</u>							
Wk1	97	3.15	ND	Boiling	-	Cocci (2.45 to 2.47)	Phage like particles (2.45, 2.46)
<u>FIJI</u>							
WgtTC	90.3	8	ND	Bubbling	Spiralizing cells with swellings (2.29)	-	-
SSFJ1	91	7.6	ND	Bubbling	Filaments and rods with square ends (2.53);	-	With flaps (2.54)

ND = not determined

Bracket indicates relevant figure numbers presented in this chapter.

might be too low to be easily detected by EM observation, or adsorption to the butvar surface might not have occurred. However bacterial absence in some acidic pools with high temperature in Yellowstone Park, USA does occur (Brock, et al., 1972). Bacteria were observed in all remaining pools. What is significant is that no two pools gave a similar density or distribution of morphotypes present even though in some pH, temperature and sulphide concentration were similar e.g. Rt42 and Rt57. Until a more detailed description of each pool is obtained with respect to the dominant microflora, it would be wise to regard each pool as a unique ecosystem.

An amazing range of morphologically diverse types of microorganisms was observed in the 37 pools examined. The morphological types included rods, cocci, and filaments which were more commonly observed, and more rarely observed scutellum bearing organisms, spirochetes, spiral and curved bacteria. Some of the rods and cocci possessed flagella and pili. Surprisingly, in many pools identifiable bacteriophages were observed and different phage types could be recognised.

The figures listed in Table 2.2 are representative of the most commonly observed morphotypes. Their association in Table 2.2 with a particular pool should not be taken as an indication that their distribution was restricted to that pool. Rather, the figures are provided to demonstrate the diversity of flora in hot springs and the effectiveness of the butvar coated grid procedure.

A description based on the morphological types of bacteria and phages present in the 38 pools surveyed is given below. It must be stressed that comparisons on the basis of colonization of E.M. grids could produce quite misleading results, but in the absence of other data this could provide an indication of where a more comprehensive study could be

fruitful.

(a) Spirochetes, spiral and curved bacteria:

Spirochetes were observed in pools Wai21 (Figure 2.7), Rt3 (Figure 2.8) and Wai19 (Figure 2.9). They were identified as spirochetes on the basis of presence of outer sheath (OS), protoplasmic cylinder (PC) and periplasmic filament (PF). The width of the cells was found to be approximately $0.2\mu\text{m}$. However, the number of turns (helix) appeared to vary considerably in each of the cells. For example, the spirochete from pool Wai19 had(b) more turns than the spirochetes from pool Rt3 or pool Wai21. Enrichment cultures of anaerobic spirochetes growing at the approximate pool temperatures have been obtained from pools Wai21 and Rt3. These have been subsequently isolated in pure culture and will be discussed fully in a paper in back pocket cover (Patel et al., 1985b).

Another spirochete-like organism was observed in pool Wai19 (Figure 2.10). However, this organism does not appear to possess typical features of a spirochete as discussed above. The width of this organism was approximately twice that of the spirochetes from Wai21 and Rt3 and it also possessed spherical globules. These spherical globules could be sulphur particles, either attached or produced by the cell, storage granules or gas vacuoles. The presence of such globules was also evident in rod shaped bacteria observed in the same pool, Wai 21 (Figure 2.10).

Spiral shaped organisms were observed in pool Rt40 (Figure 2.11), the width of which is very similar to the width of the spirochetes in figures 2.7, 2.8 and 2.9. Two spiral cells are shown in figure 2.11. One of cells is longer than the other and the cells appear to be attached to each other. Attempts to culture this organism using enrichment techniques

Figure 2.7. A spirochete present on butvar coated E.M. grids incubated in pool Wai21 for 3 hours. Note the tightly coiled nature of the organism and the presence of periplasmic filament.

Figure 2.8. Fine structure of a spirochete from pool Rt3, showing a periplasmic filament (PF) wound around the protoplasmic cylinder (PC) and the outer sheath (OS). Time of incubation of the E.M. grid in the pool was 1 hour.

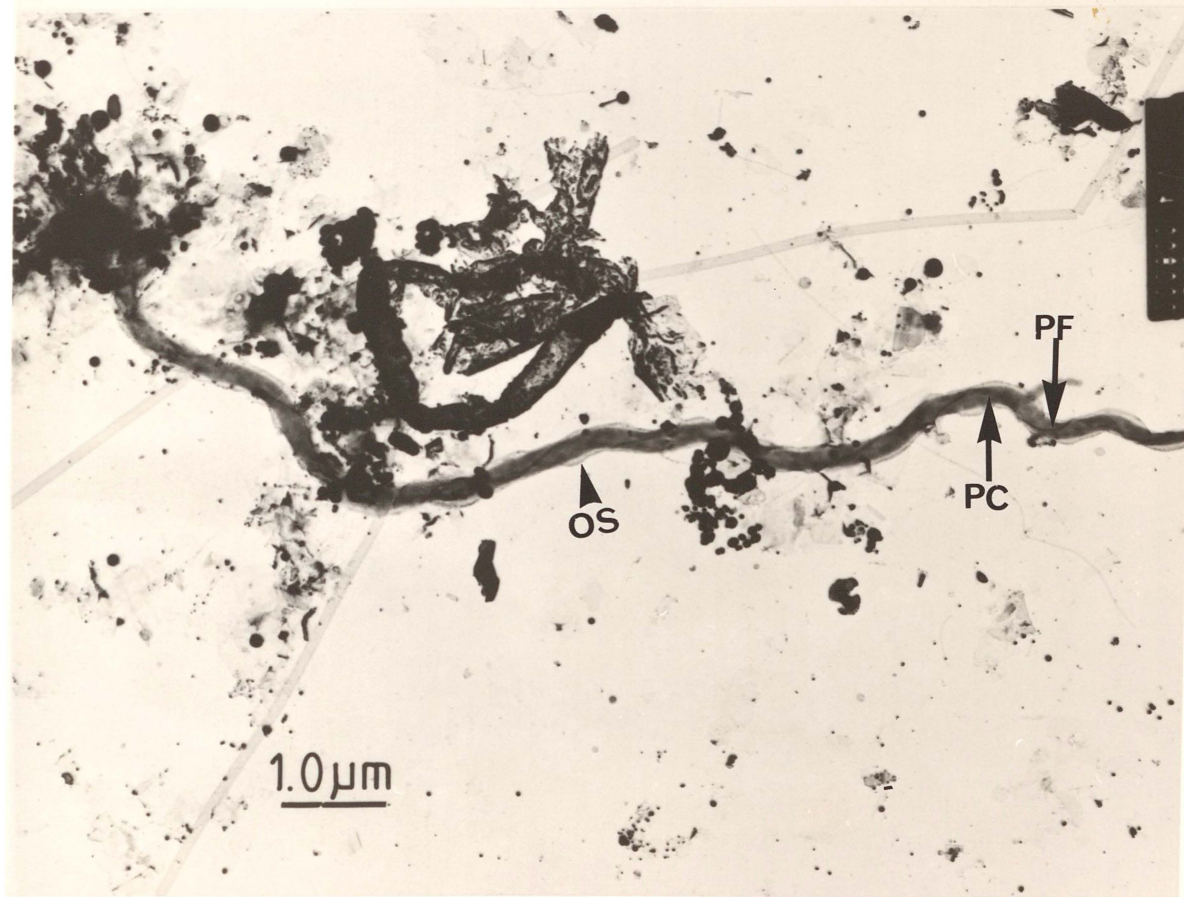
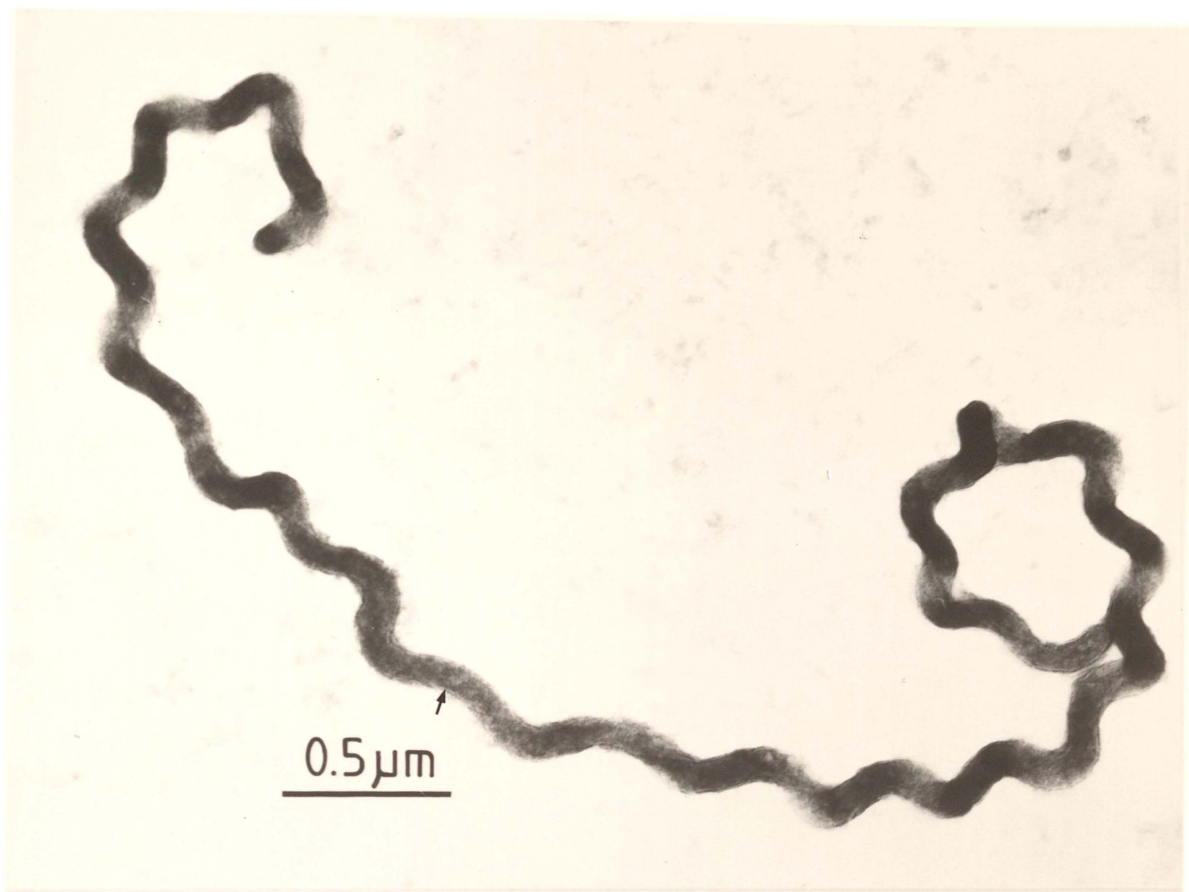


Figure 2.9. A tightly coiled spirochete similar to that depicted in Figure 2.8, present in pool Wai19. The E.M. grid was incubated in the pool for 4 hours.

Figure 2.10. A spiral bacterium present on the same grid as mentioned above. Note the electron light spherical particles in the bacterium.

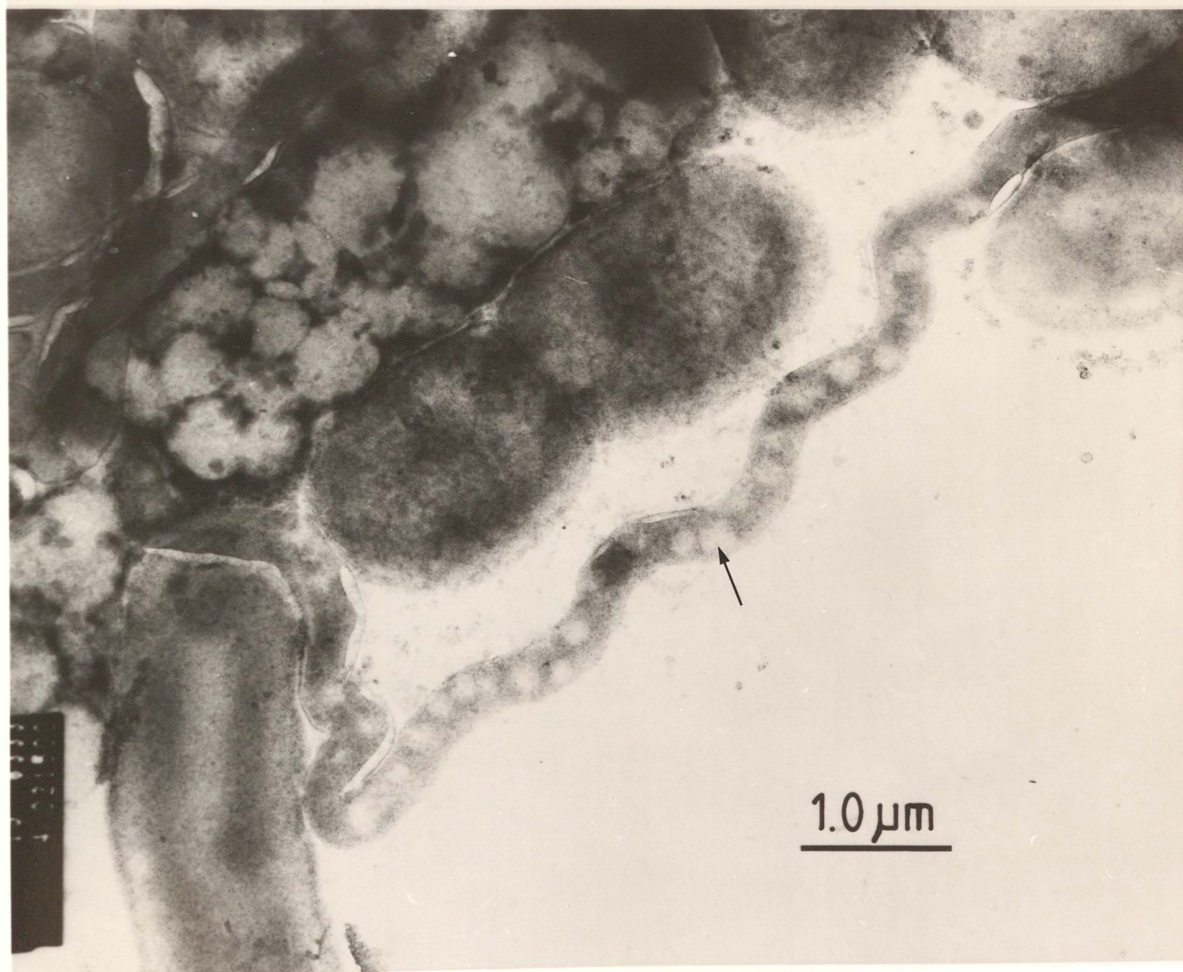
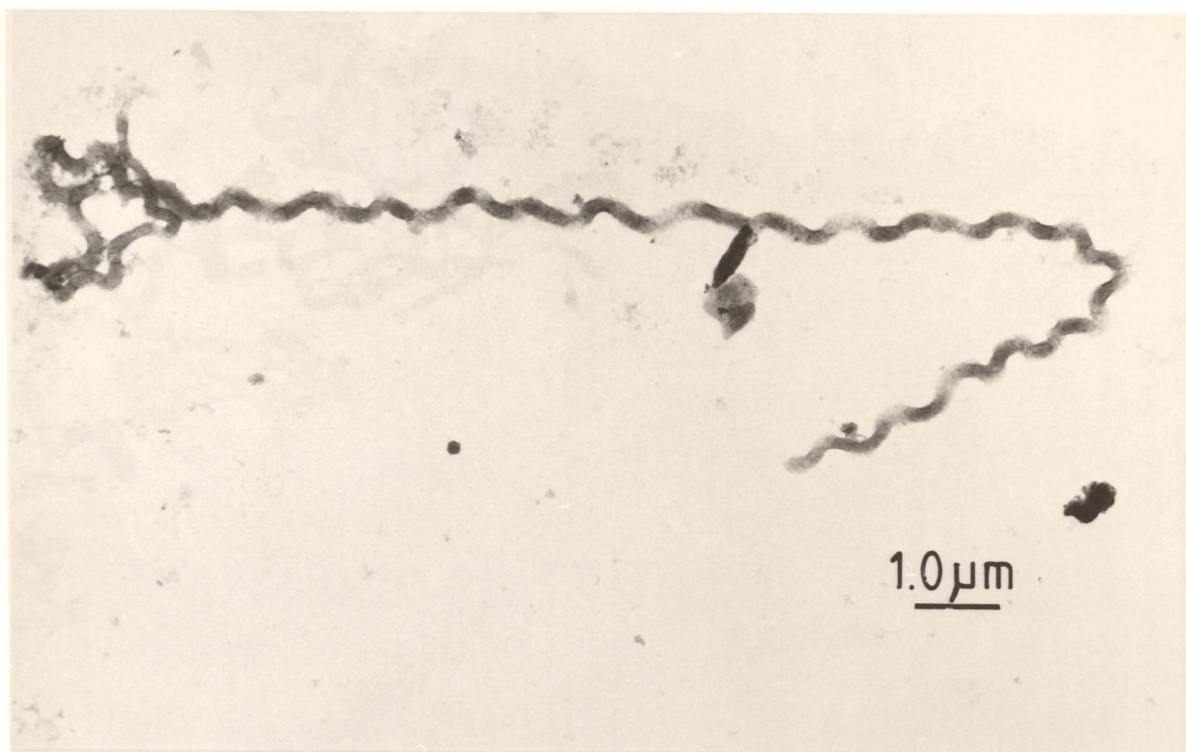
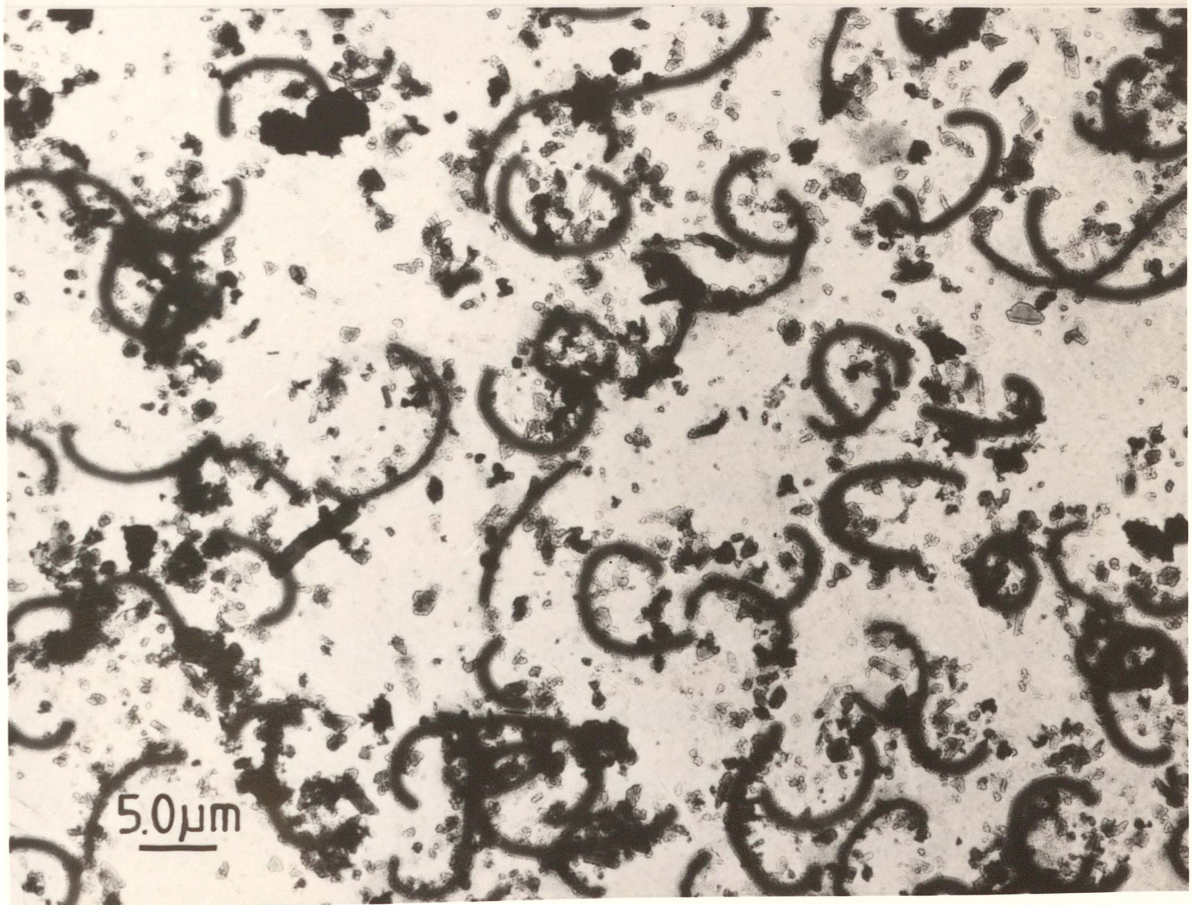
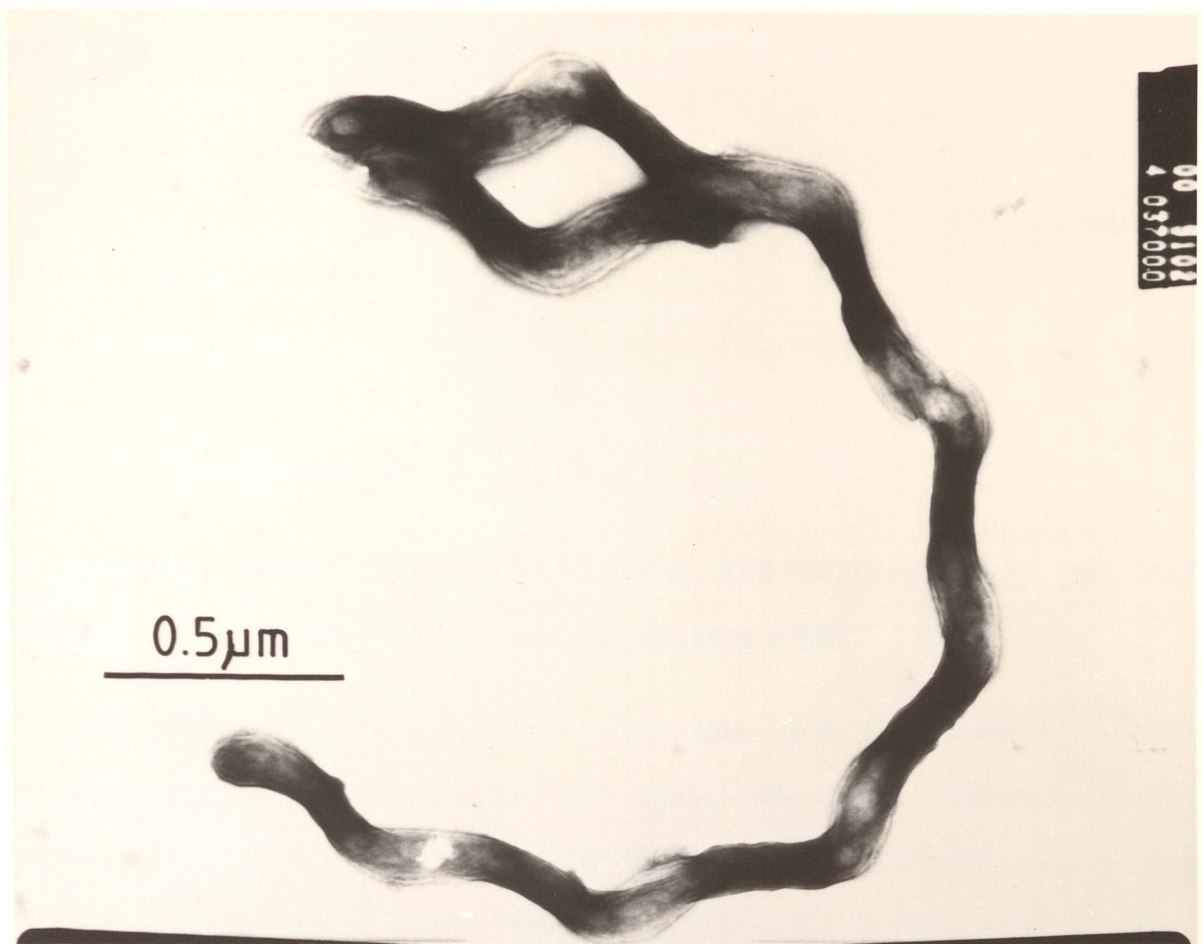


Figure 2.11. Spiral bacteria absorbed to butvar coated E.M. grid incubated in pool Rt40 for 1 hour. Note the difference in dimension and morphology when compared to the spiral bacterium in Figure 2.10.

Figure 2.12. Colonisation of butvar coated E.M. grid incubated in pool Rt42 for 2 hours by a single morphological type of bacterium.



successfully used for culturing spirochetes failed.

Curved rods were observed in pool Rt42 (Figure 2.12). This is a good example of heavy bacterial colonization/attachment to carbon coated butvar grids which occurred in some pools even after a short incubation period, in this case a period of 2 hrs. Attempts to culture this organism failed.

(b) Phage and Phage-like Particles:

Tailed phages were observed in pools Rt3 (Figure 2.13) and Wai21 (Figure 2.14). However, here the resemblance ended.

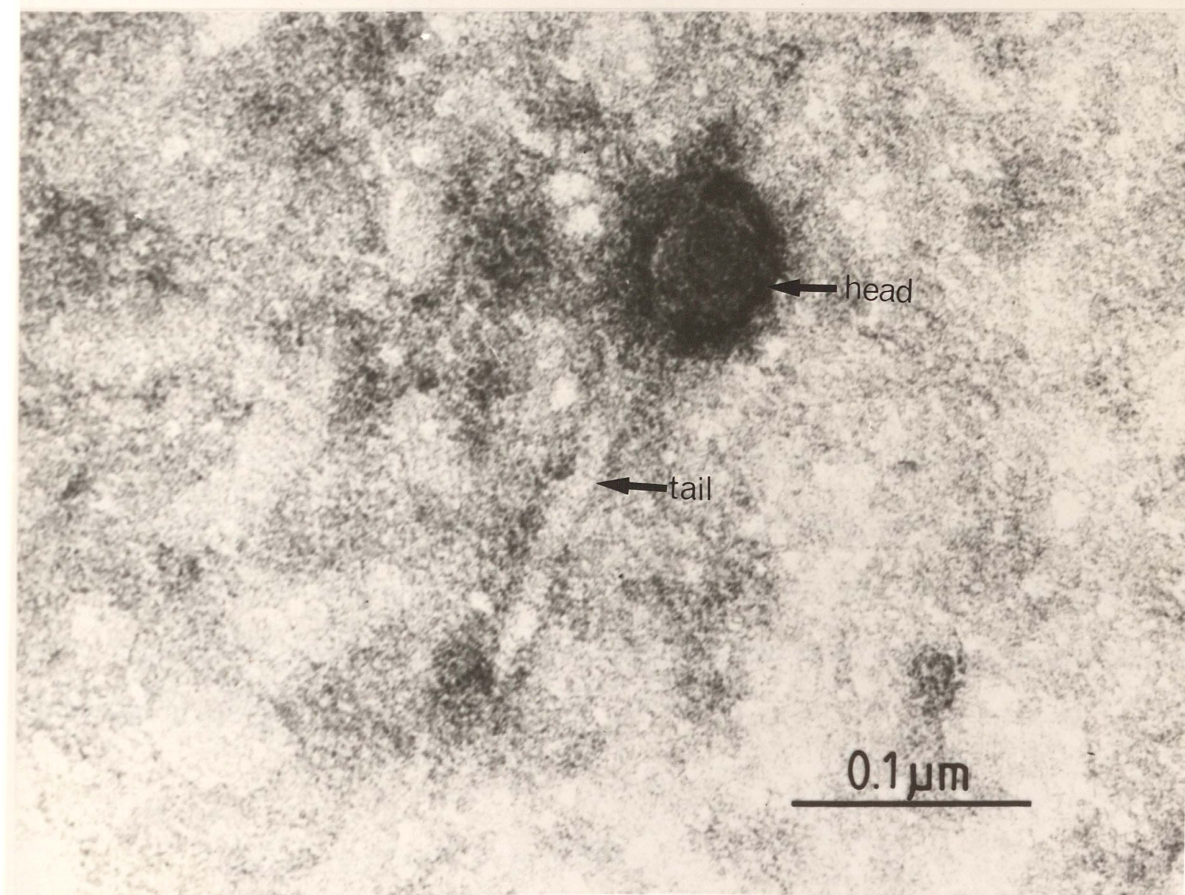
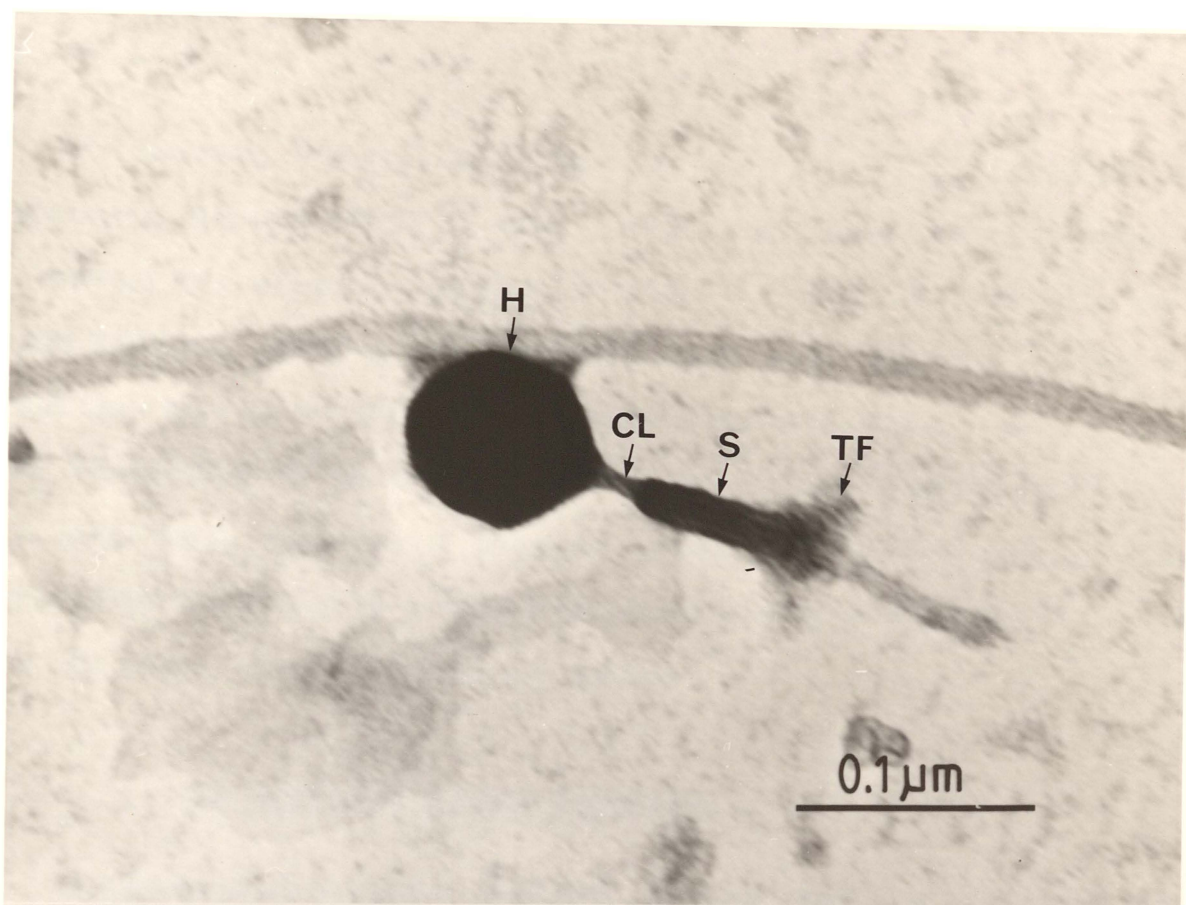
Rt3 phage was more complex in structure and possessed a head, a collar, a sheath enclosing the tail, and tail fibres. A phage morphologically similar to this phage was isolated from pool Wai19 using *Thermus* Wai19-AT1 as a host. Details of this will be discussed in chapter 8. The host organism for the phage from pool Rt3 is unknown.

The phage from pool Wai21 was simpler in morphology and possessed a head and a tail which appeared to be enclosed by a sheath. It is interesting to note that morphologically similar phages were observed attached to spirochetes in enrichment cultures from pool Rt3 (Figure 2.13). Since pool Wai21 was also colonized by spirochetes it would be of interest to determine the host specificity of the phages in pools Rt3 and Wai21. However, no serious attempts have been made at isolating these phages.

Tailless phage-like particles (Figures 2.45, 2.46) were observed in an extremely acid, boiling pool, Wk1, adsorbed to coccal cells, presumed to be Sulfolobus species since all attempts to enrich anaerobic organisms from this pool failed (Table 5.3). However, aerobic Sulfolobus

Figure 2.13. A viral particle observed on a butvar coated E.M. grid incubated in pool Rt3 for 1 hour. Note the presence of head (H), collar (CL), sheath (S) and tail fibres (TF).

Figure 2.14. A viral particle present in pool Wai21. The butvar coated E.M. grid was incubated in the pool for 3 hours. Note that this is morphologically distinct to that depicted in Figure 2.13.



species were isolated (Chapter 5).

A combined effort, involving the use of in situ studies and laboratory studies should be made to understand microbe-phage interaction in thermal environments. In situ studies of phage are on the whole unsatisfactory because staining of phage with negative stains can sometimes lead to artifacts. Such results obviously makes identification and classification of phage very difficult (Ackermann et al., 1974). On the other hand, isolation of phage by plaque purification method under laboratory conditions is a highly selective procedure which can not detect all the phage types present in the sample.

(c) Scutellum^{*} bearing organisms:

Filamentous cells with a central, swollen sac-like structure were observed in 7 of the 38 pools examined. We have termed this structure a "scutellum". Scutellum bearing cells were observed in pools Rt6 (Figure 2.15), Rt3 (Figure 2.16), Rt12A (Figure 2.17), Rt8 (Figure 2.18), Rt51 (Figure 2.19), located in the Rotorua thermal areas, and pools Wai21 (Figures 2.20, 2.21, 2.22) and Wai18 (Figures 2.23, 2.24, 2.25, 2.26, 2.27), located in Waimangu thermal valley.

The scutellum was a part of the cell in all the scutellum bearing organisms observed and were wider than the filamentous cells which bore them. The width of the scutella varied from 0.4 μm to 0.8 μm and the length varied from 1.0 μm to 1.63 μm whereas the width of the cells varied from 0.1 μm to 0.4 μm (Table 2.3).

* Scutellum (L. shield; plural= scutella)

Figure 2.15. A filamentous bacterium with a centrally situated "scutellum" observed on formvar coated E.M. grids incubated in pool Rt6. Note the holed appearance of the formvar film.

Figure 2.16. A filamentous bacterium with a sub-terminal scutellum observed in pool Rt3. The butvar coated E.M. grid was incubated in the pool for 1 hour.

Figure 2.17. A very similar morphotype to that of Figure 2.16 observed in a butvar coated E.M. grid incubated in pool Rt12A for 1.5 hours.

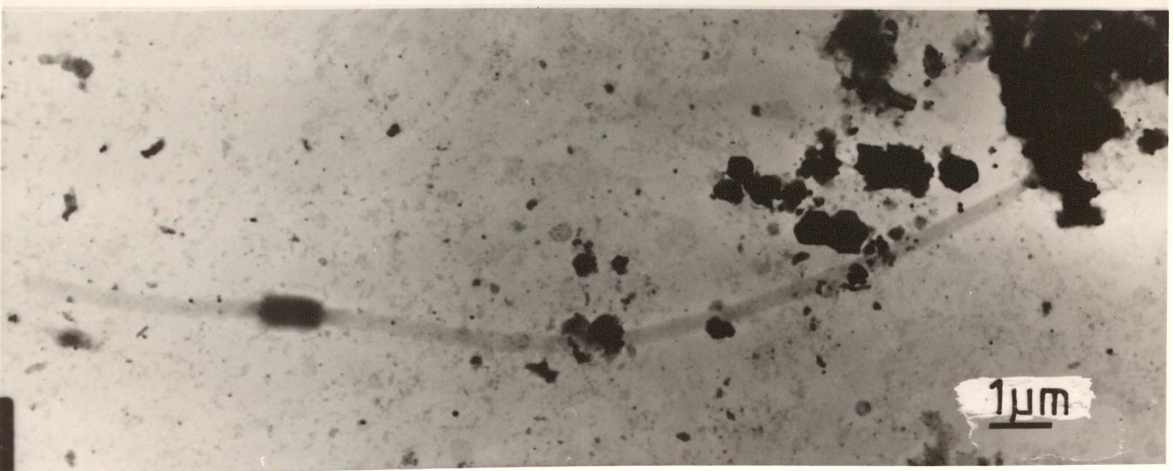
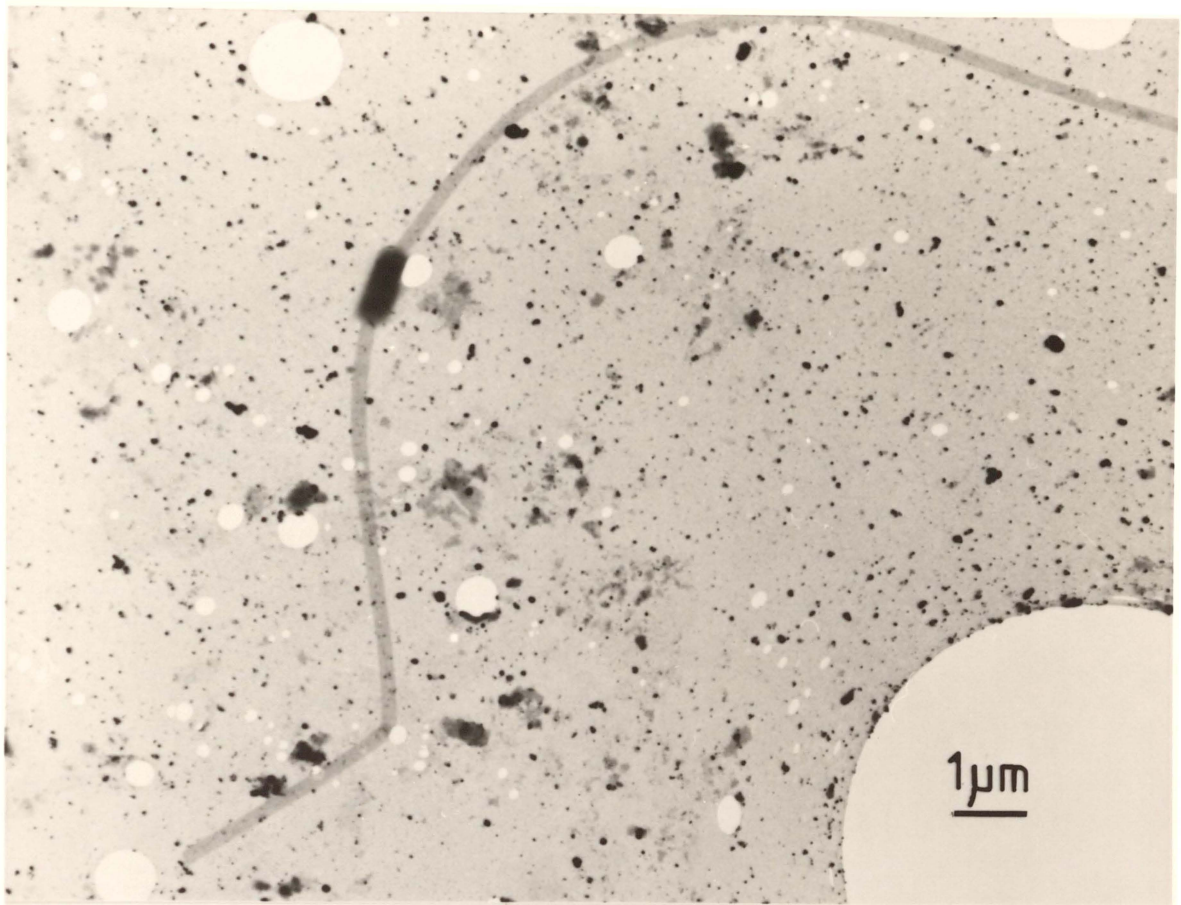


Figure 2.18. A scutellum bearing filamentous bacterium observed in pool Rt8. Note that the scutellum possesses square ends and is positioned sub-centrally on the cell. The E.M. grid was incubated in the pool for 1 hour.

Figure 2.19. A distinctly different scutellum bearing organism to those depicted in Figures 2.15 through to 2.18 observed in pool Rt51. Time of incubation of butvar coated E.M. grid was 1.75 hours.

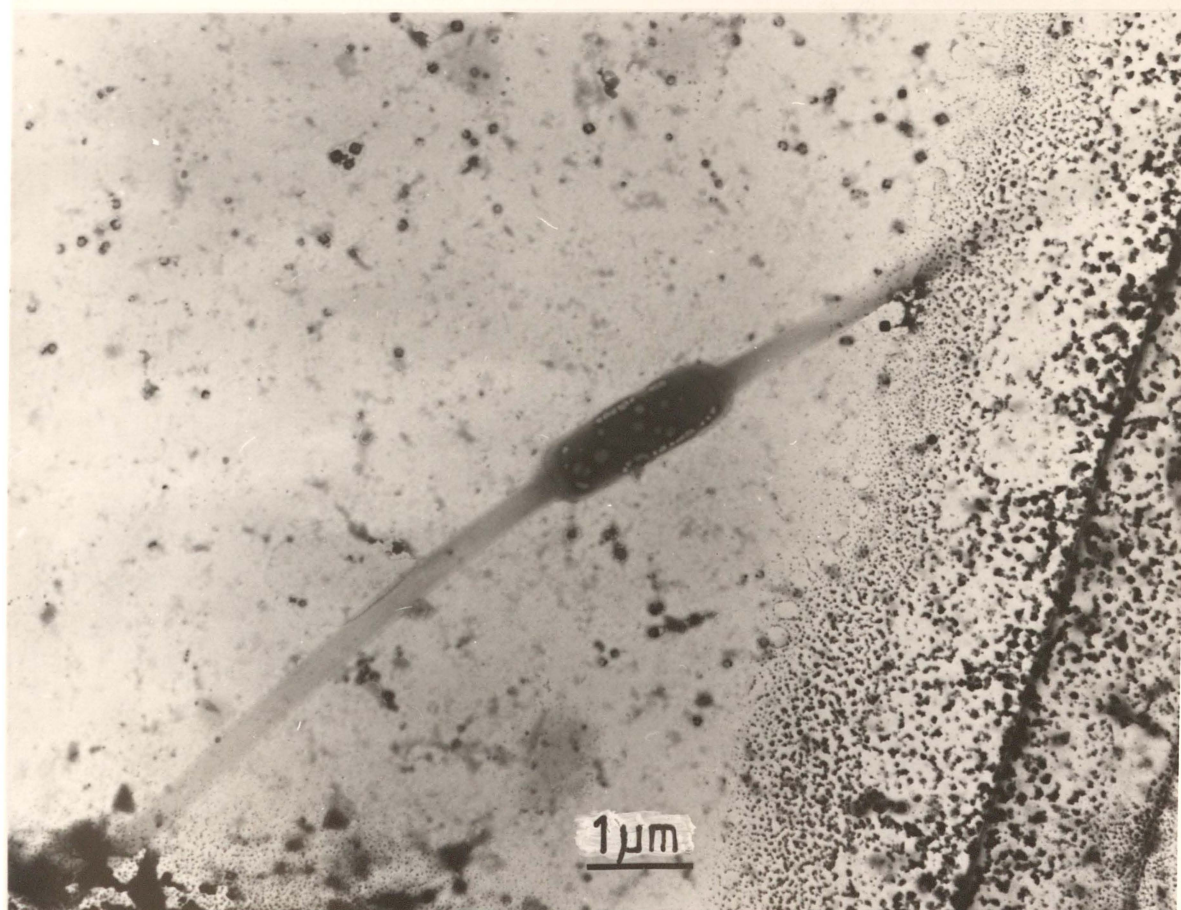
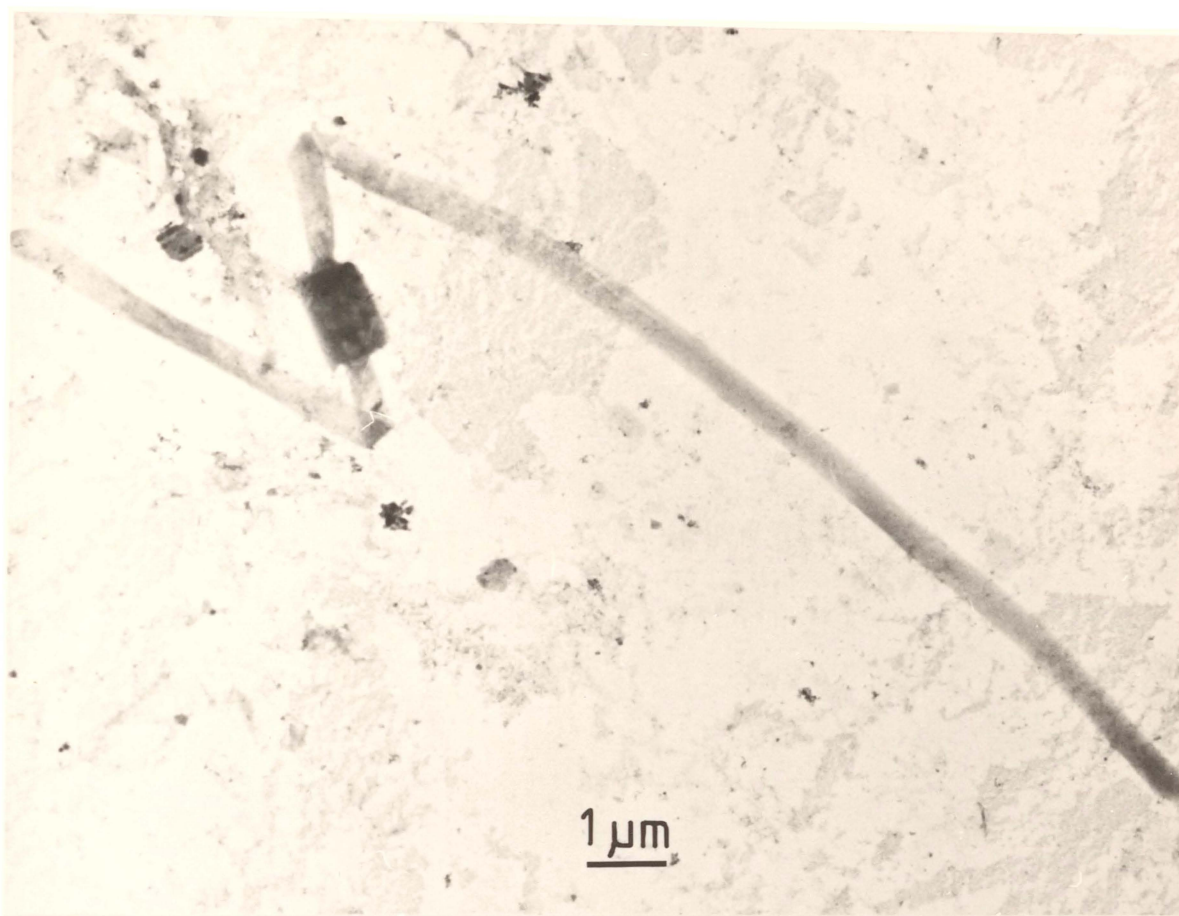


Figure 2.20. Scutellum present on a cell from pool Wai21. Note that it is situated almost terminally on the cell. The butvar coated E.M. grid was incubated in the pool for 3 hours.

Figure 2.21. A morphologically distinct scutellum bearing organism present in the same pool as above. Note the sheath covering the scutellum and part of the cell. The time of incubation of the butvar coated E.M. grid was 3 hours.

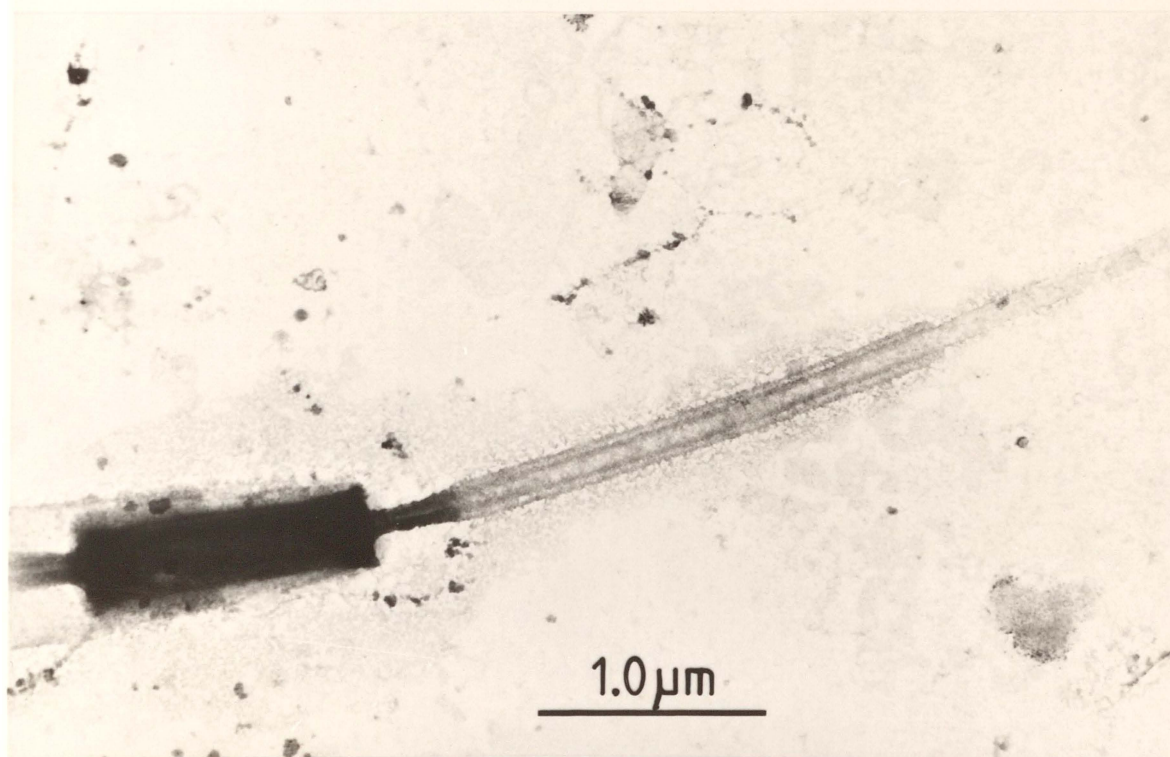
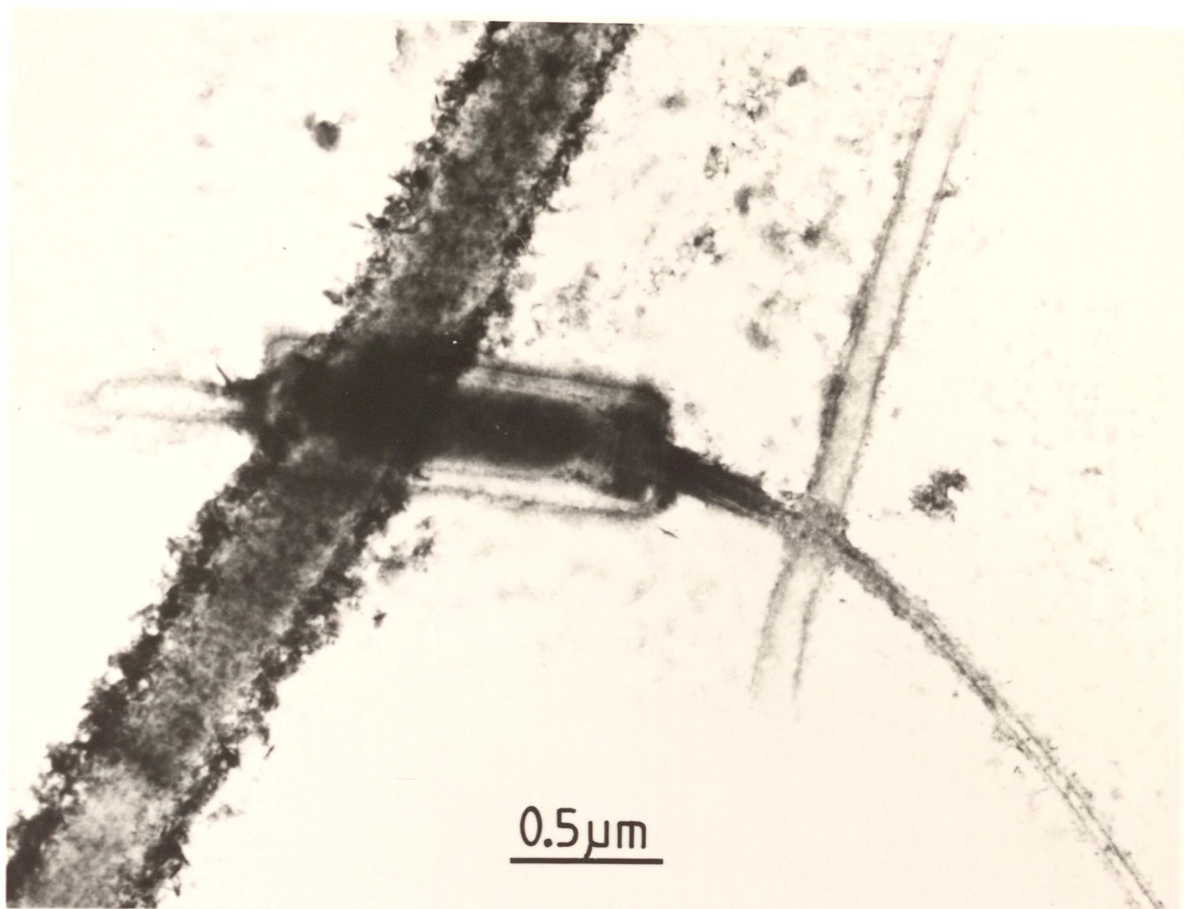


Figure 2.22. Detail of the scutellum structure of Figure 2.21 showing the sheath in detail.

Figure 2.23. A cylindrical scutellum present in pool Wai18. The butvar coated E.M. grid was incubated in the pool for 2.75 hours.

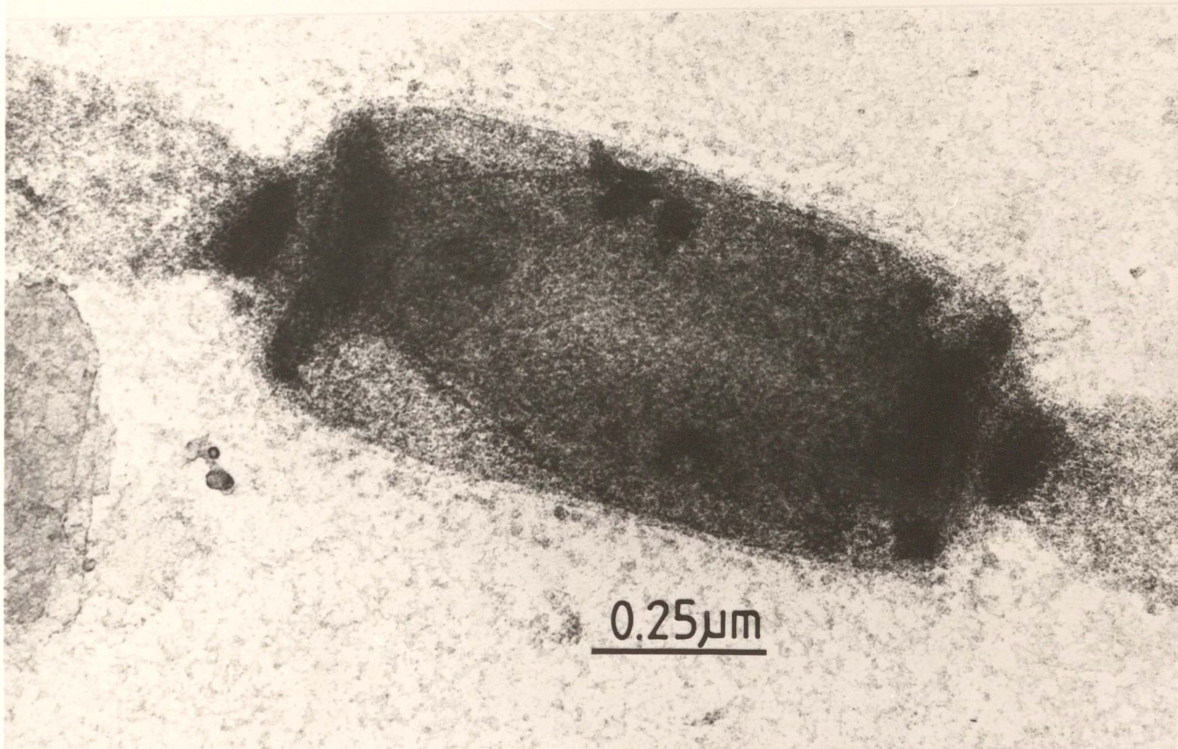
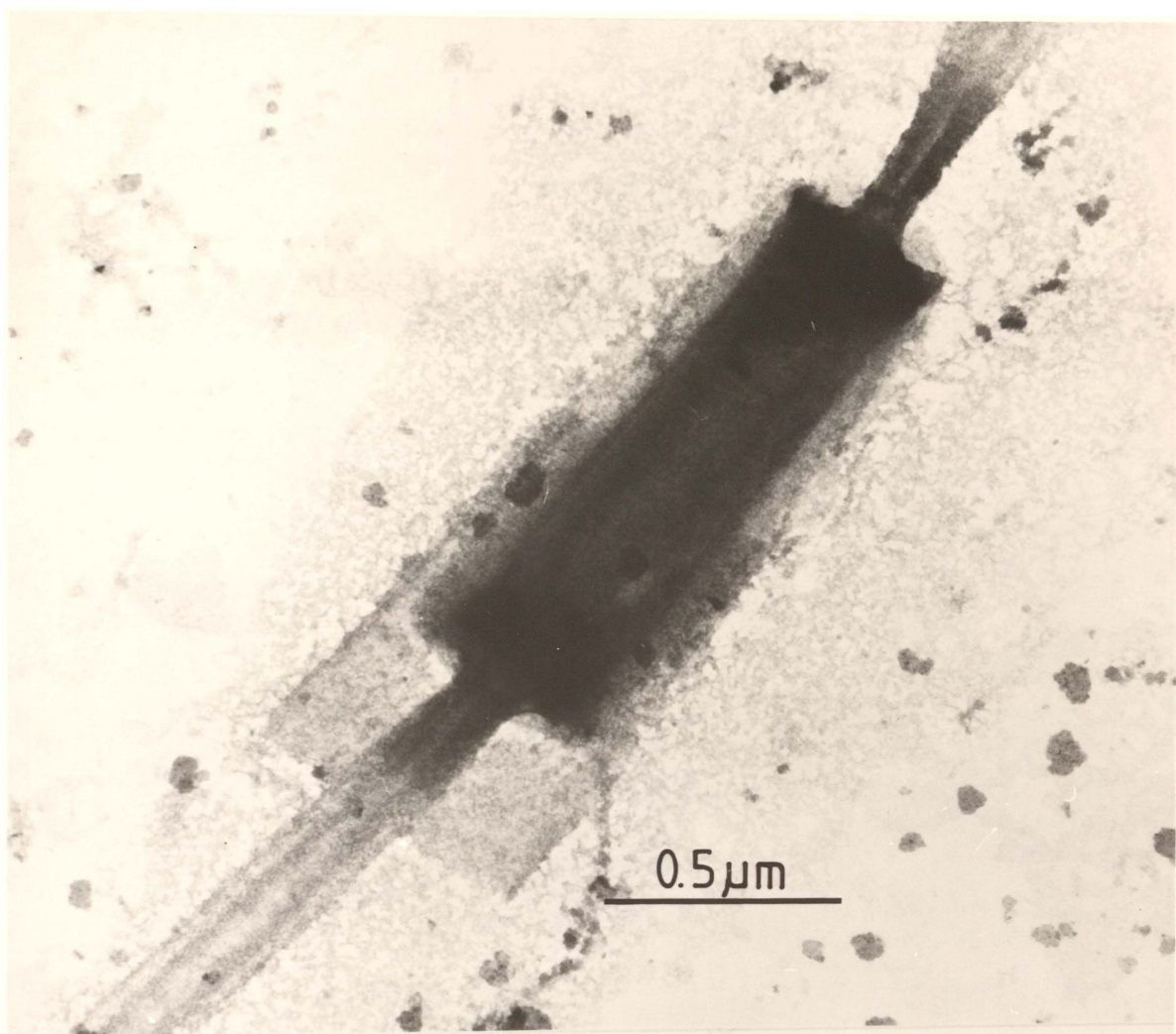


Figure 2.24. Another cylindrical scutellum bearing filament present in Wai18. The length of incubation of the butvar coated E.M. grid was 2.75 hours. Note the "flap" like structure present on the scutellum. (Refer text for details)

Figure 2.25. A third scutellum morphotype present in pool Wai18. Note the slime surrounding the scutellum and the filament. The incubation schedule was as mentioned above. (Refer text for details)

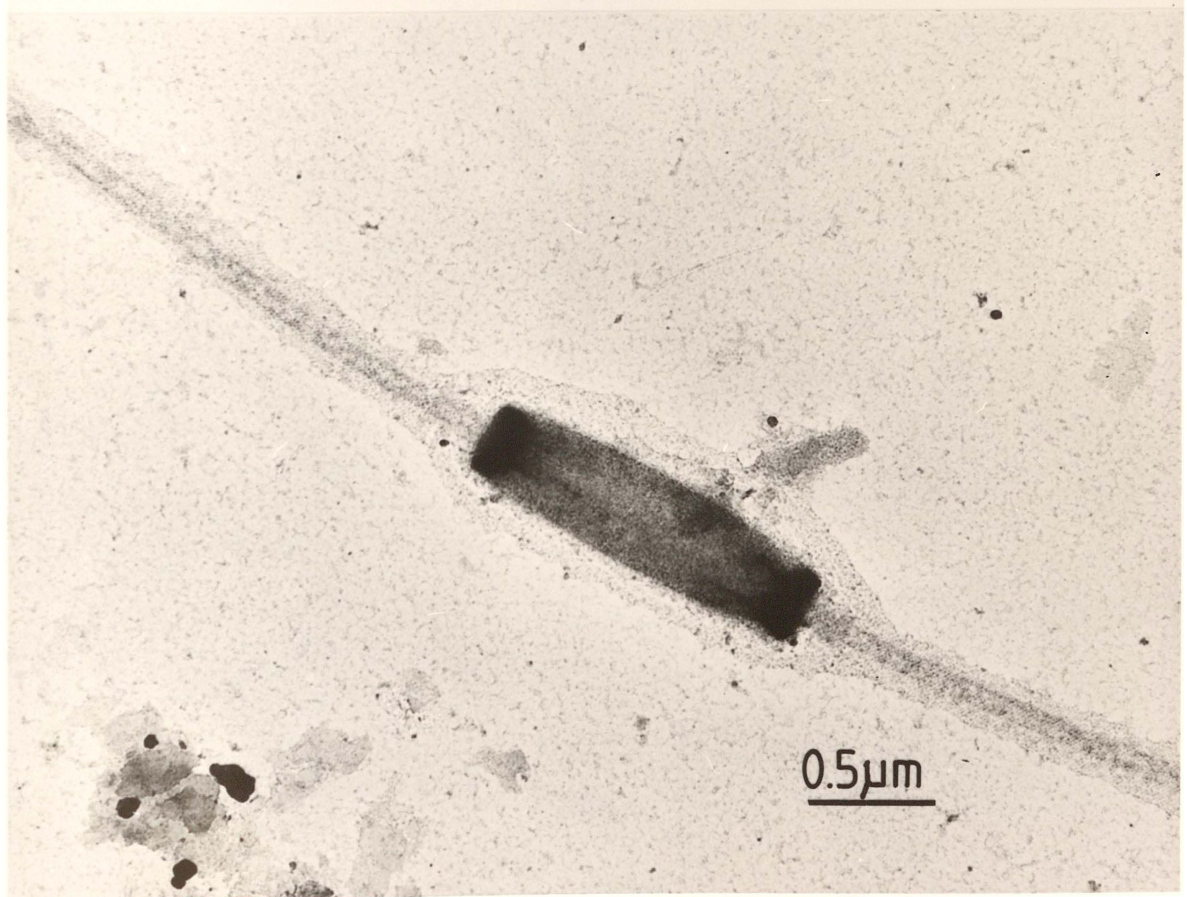
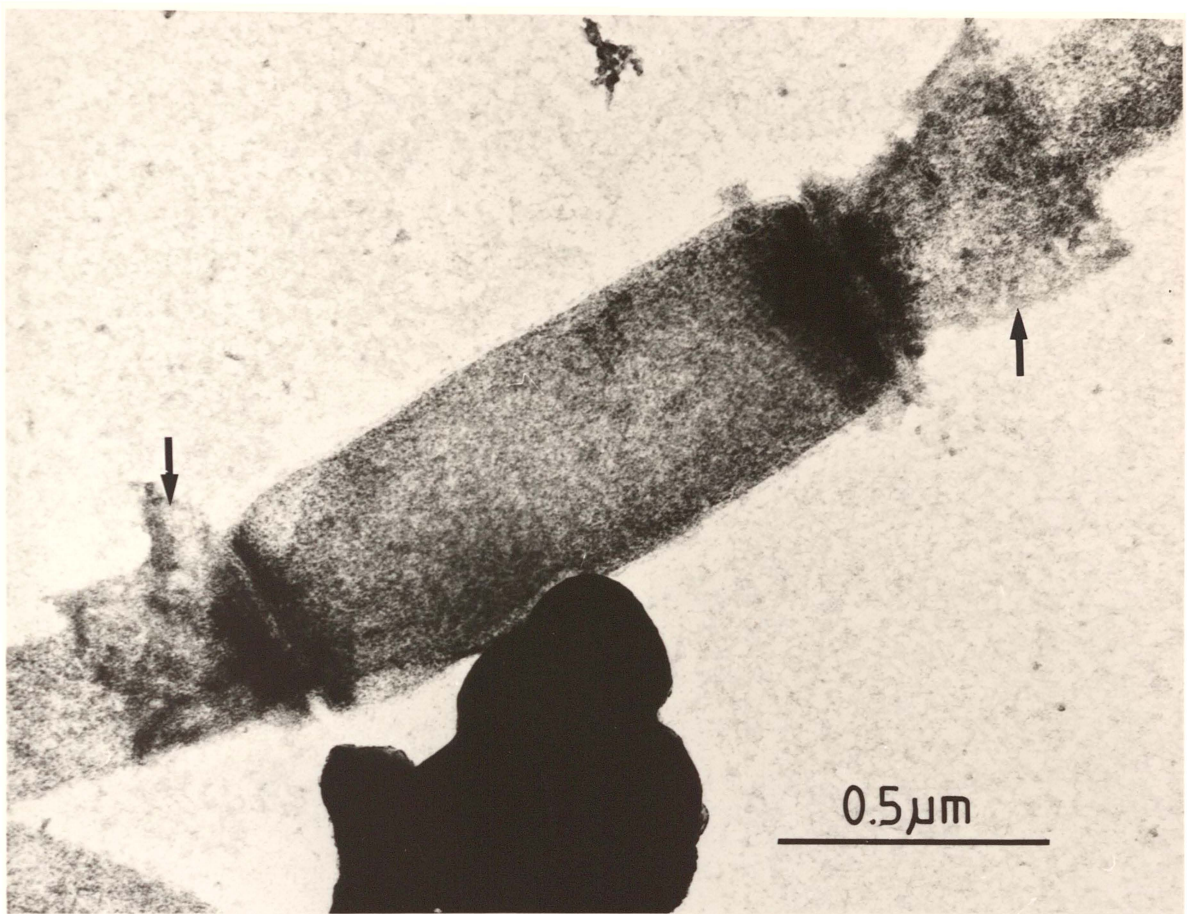
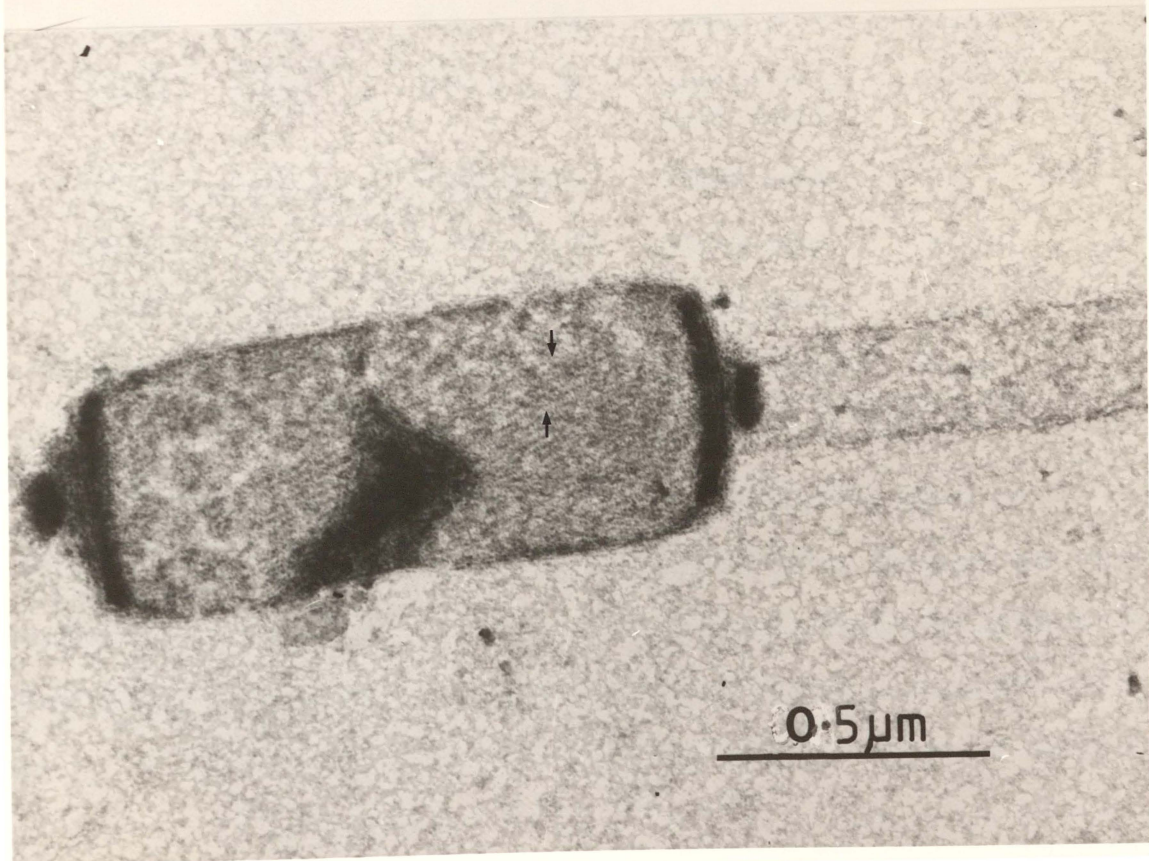
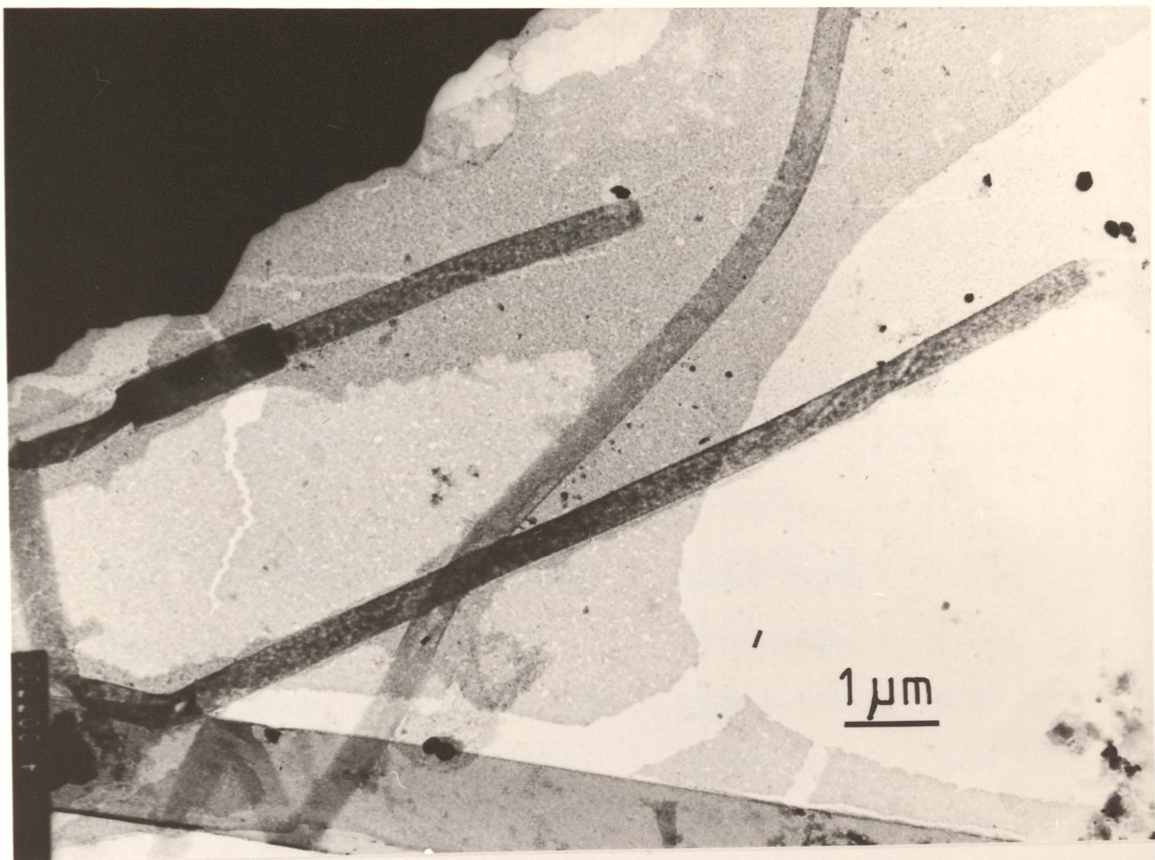


Figure 2.26. A fourth distinctly different scutellum morphotype present in pool Wai18. The incubation period for the butvar coated E.M. grid was 2.75 hours.

(Refer text for details)

Figure 2.27. Detail of the scutellum structure of Figure 2.26. Note the cell pattern on the scutellum.



Considerable variation in the position and structure of the scutellā was observed. Scutellā were positioned sub-terminally in the organisms from pools Rt3 (Figure 2.16), Rt12A (Figure 2.17), Rt8 (Figure 2.18), Wai21 (Figure 2.20) and Wai 18 (Figure 2.26) whereas the scutellum in pool Rt6 cells (Figure 2.15) were positioned sub-centrally.

Table 2.3 Size and structure of scutellum bearing organisms:

Pool Nos.	Figure Nos.	Features of Scutellum		Cell diameter (um)
		Size(um)	Structure	
Rt3	2.16	0.63 × 1.4	Square ends.	0.3
Rt6	2.15	0.4 × 1.1	Round ends.	0.2
Rt8	2.18	0.67 × 1.63	Square ends.	0.3
Rt12A	2.17	0.5 × 1.0	Square ends.	0.25
Rt51	2.19	0.8 × 1.25	Round ends.	0.4
Wai18	2.23	0.5 × 1.03	Round ends.	0.3
Wai18	2.24	0.5 × 1.15	Square ends with flaps.	0.28
Wai18	2.25	0.38 × 1.4	Round ends.	0.1
Wai18	2.27	0.47 × 1.17	Round ends.	0.22
Wai21	2.20	0.43 × 1.3	Round ends.	0.13
Wai21	2.21	0.47 × 1.17	Square ends.	0.18

The shape of the scutellā varied from ellipsoidal (pool Wai18, Figure 2.24) to cylindrical (pool Wai18 in Figure 2.25). Some of the scutellā had round ends (for example organisms from pool Wai21 in Figure 2.20) while others had square ends (for example from Rt8 in Figure 2.18). The types of scutellā observed in the pools has been summarised in Table 2.3.

Scutella of the organisms in the Rotorua region were simpler in structure (Figures 2.15 to 2.19) than those observed in the Waimangu region (Figures 2.20 to 2.27). For example organisms from pool Wai18 in figure 2.24 possessed "flap"-like structures on the scutellum. A sheath-like structure was present on the scutellum (and the cell bearing the scutellum) from pool Wai21 (Figures 2.21, 2.22). The scutellum of an organism from pool Wai18 (Figure 2.26, 2.27) possessed a definite subunit pattern in its surface envelope, which is characteristic of archaeobacterial cell envelopes, and may give a clue to the taxonomy of these organisms.

It is also interesting to note that pool Wai18 possessed four distinct scutellum morphotypes (Figures 2.23, 2.24, 2.25 and 2.27) compared to pools such as Rt8 (Figure 2.18) and Rt6 (Figure 2.15) which contained only one, but distinctly different scutelli morphotype.

(d) Rods and filaments:

Rods and filaments were the most common morphotypes observed in the 38 pools investigated. Table 2.2 summarises the distribution of rods and filaments present in the pools as observed by the grid colonization method.

A number of the rods and filaments possessed characteristics features typical of the extremely thermophilic archaeobacterial species, Thermoproteus tenax and Thermofilum pendens (Zillig et al., 1981a, 1983a). These features are discussed below.

Some of the rods observed in pools in Tk4b (Figure 2.28), Rt56 (Figures 2.30, 2.31), Rt51 (Figure 2.51), Rt3, Rt 12A, Wai17, Wai18, Wai19, possessed cell wall subunit patterns, also known as S-layer which is a characteristic feature of T. tenax and T. pendens. Numerous functions have been attributed to S-layers. They act as barriers against internal or external factors, maintain the cell shape or act as promoters for cell adhesion (Sleytr and Messner, 1983).

Association of rods with cocci is another feature which is a common archaeobacterial characteristic. This association can be possibly due to the presence of subunits on the cell wall, for example, in pool Rt51 (Figure 2.51) or due to the presence of slime as shown in figure 2.52 observed in pool Rt12A.

Spiralization of ghost cells and production of bulges within the cells is commonly observed in Thermoproteus tenax and Thermofilum pendens (Zillig et al., 1981a, 1983a). Such cells were observed in pools Wai12, and in Wg11C (Figure 2.29), a pool located in the Fiji Islands.

Another feature of archaeobacterial rods is the presence of "golf balls" or spherical bodies at the ends of the cells. Such a bacterial morphology was observed in pool Rt13.

T. tenax and T. pendens do not divide by the usual binary fission and nor do they possess flagella. Hence, the rods observed in pools Rt2 (Figure 2.34), Rt60 (Figure 2.35), Rt56 (Figure 2.32), Rt12A (Figure 2.38), Rt8 and Wai18 are clearly distinct. Morphologically the rods were diverse. Additionally, the dimensions of all these rods was different and ranged from 0.5-1 μ m by 1-8 μ m.

Figure 2.28. A rod shaped bacterium from pool TK46 with a subunit structure evident in the cell envelope. The butvar coated E.M. grid was incubated in the pool for 1 3/4 hours.

Figure 2.29. A bacterium showing disintegration of the cell, contraction of the cytoplasm and presence of electron light swollen areas, features typical of T. tenax. The E.M. grid was incubated in pool Wg11C for 2 hours.

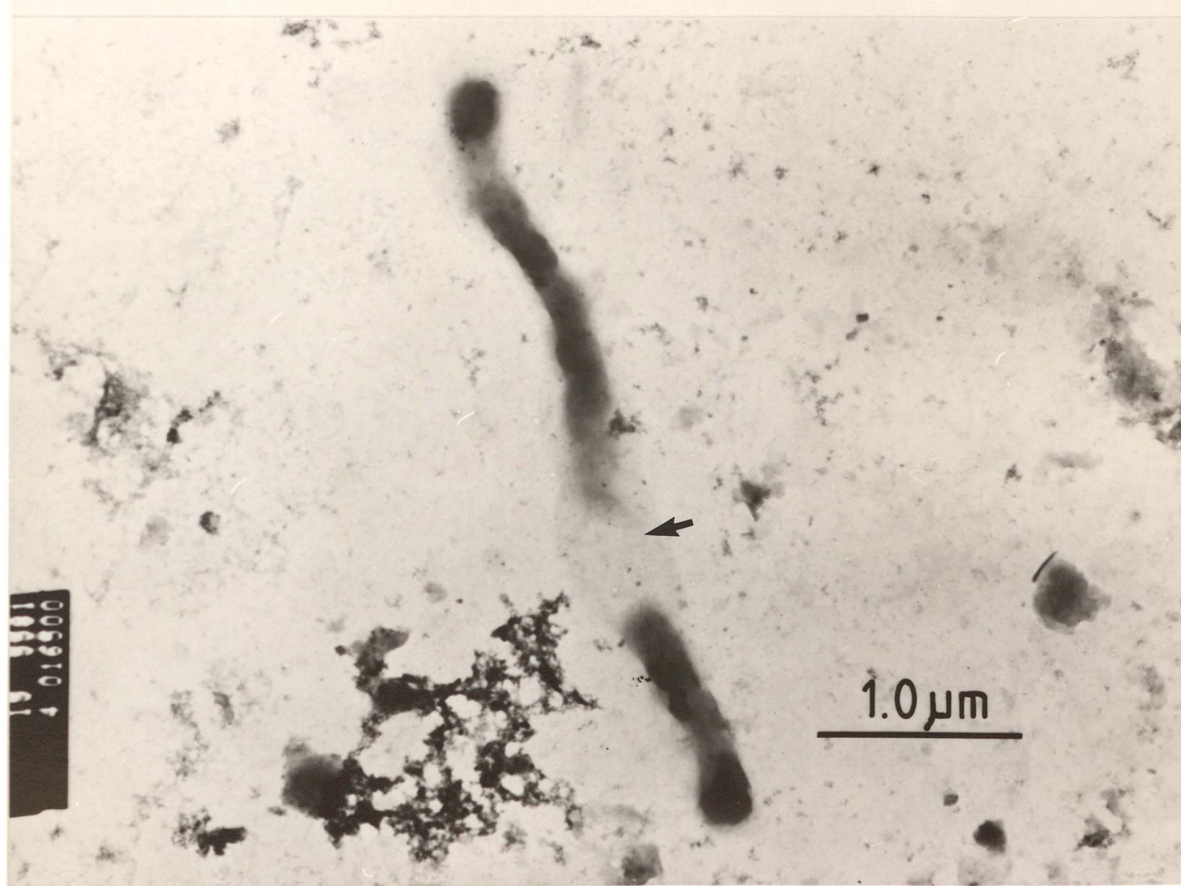
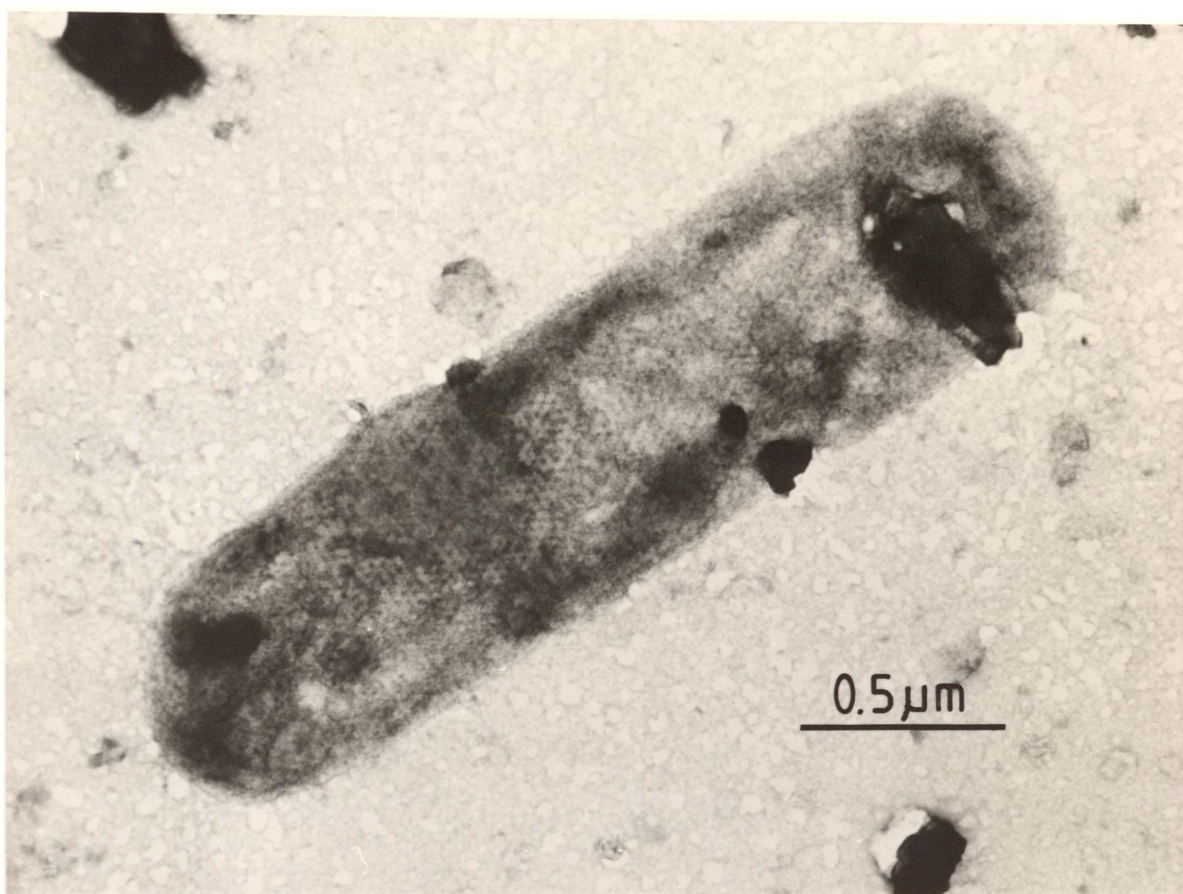


Figure 2.30. Another rod shaped bacterium exhibiting a cell-wall characteristic of the archaebacterial Thermoproteales. The time of incubation of grid was 8 hours in pool Rt56.

Figure 2.31. Detail of cell wall structure from bacterium in Figure 2.30.

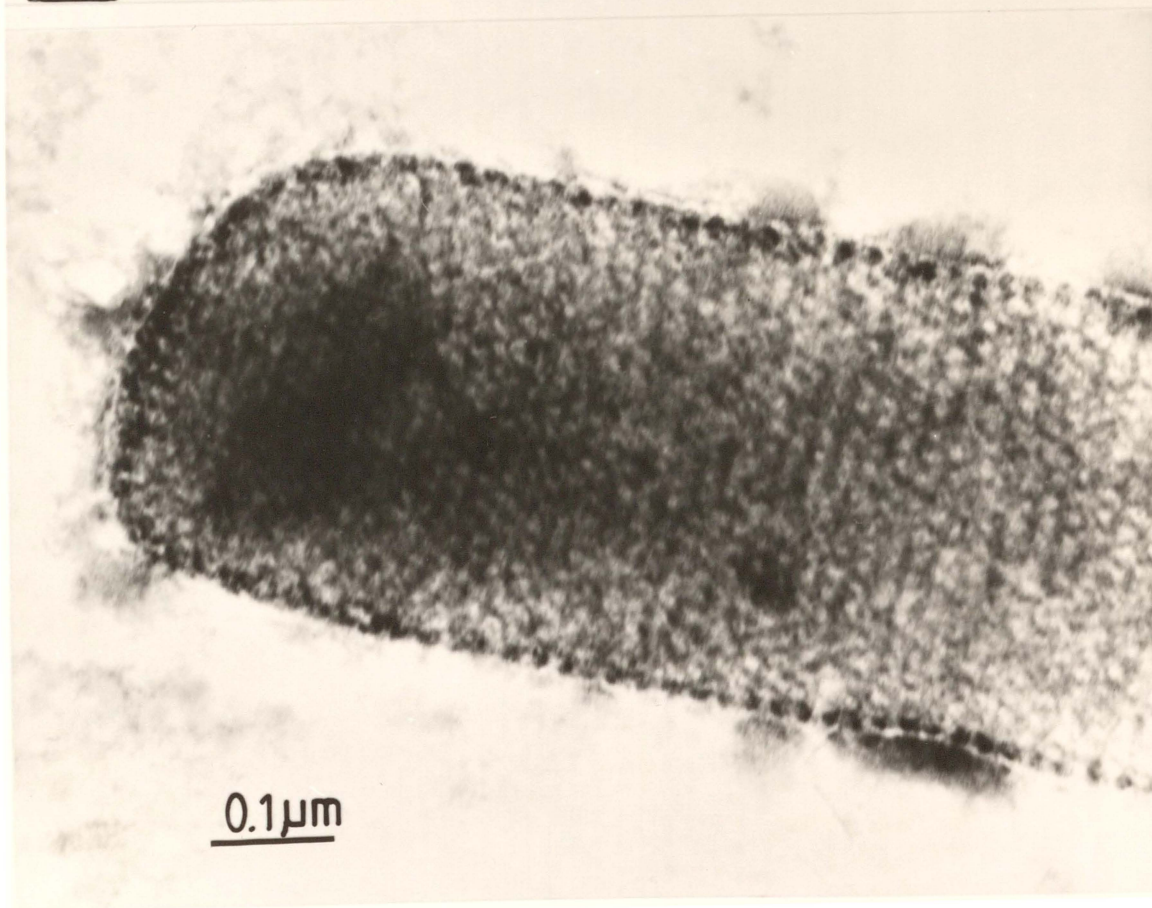
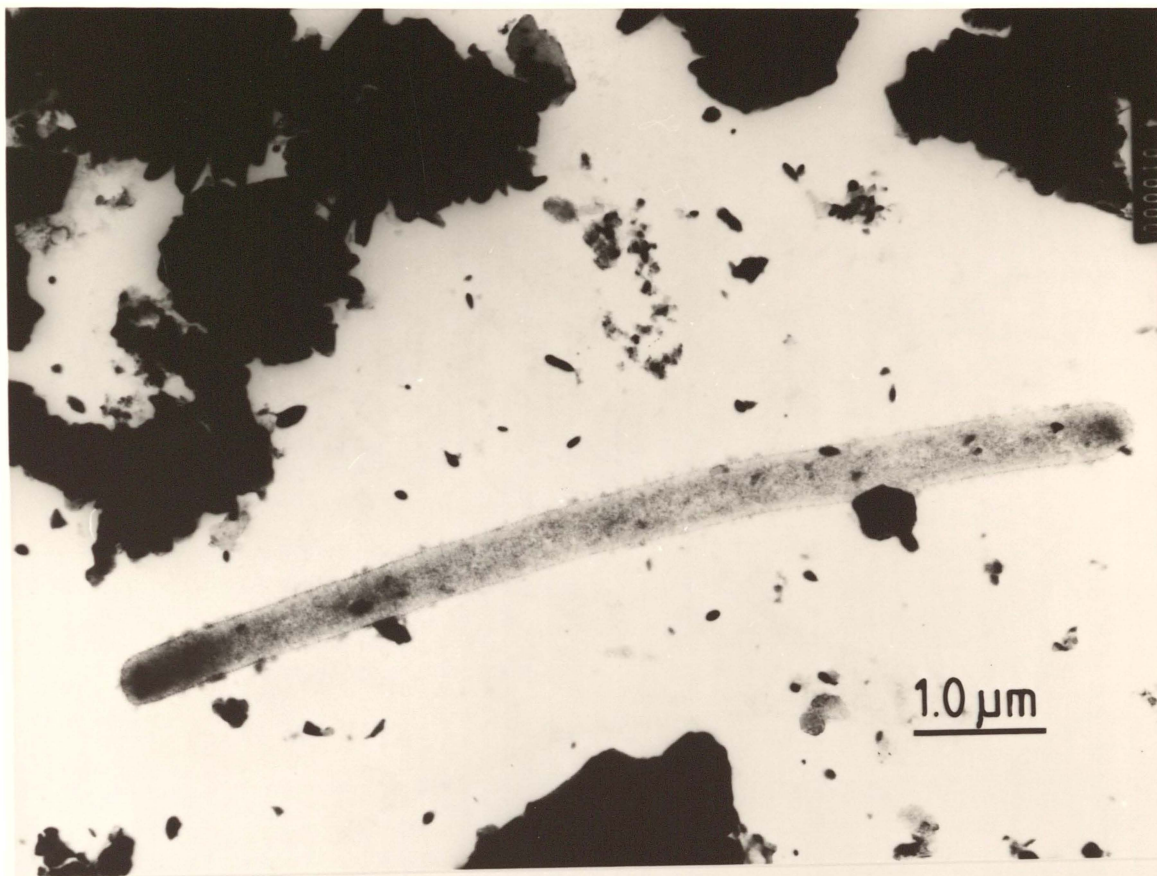


Figure 2.32. A dividing rod shaped bacterium from pool Rt56. The time of incubation of E.M. grid was 8 hours.

Figure 2.33. A polarly flagellated rod from pool Rt56. The time of incubation of the E.M. grid was 8 hours.

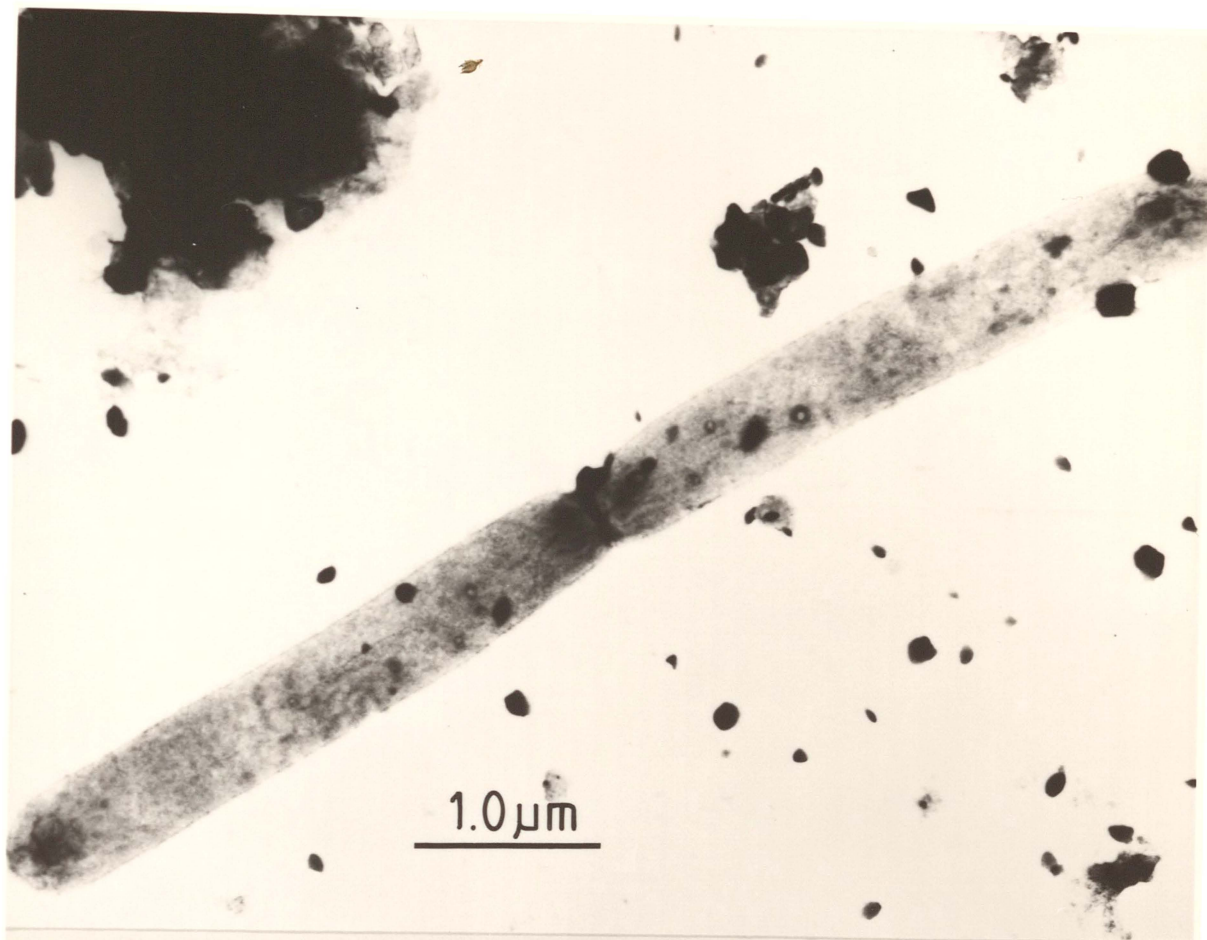
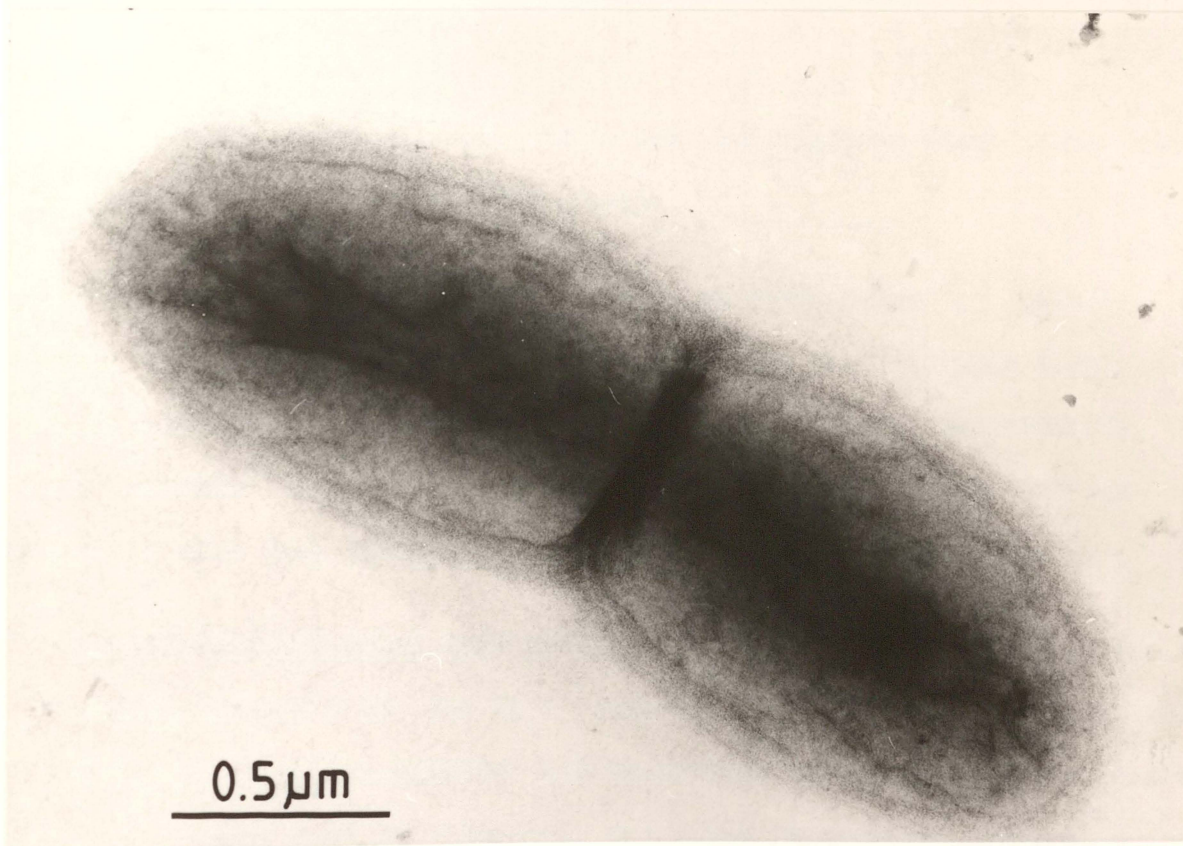
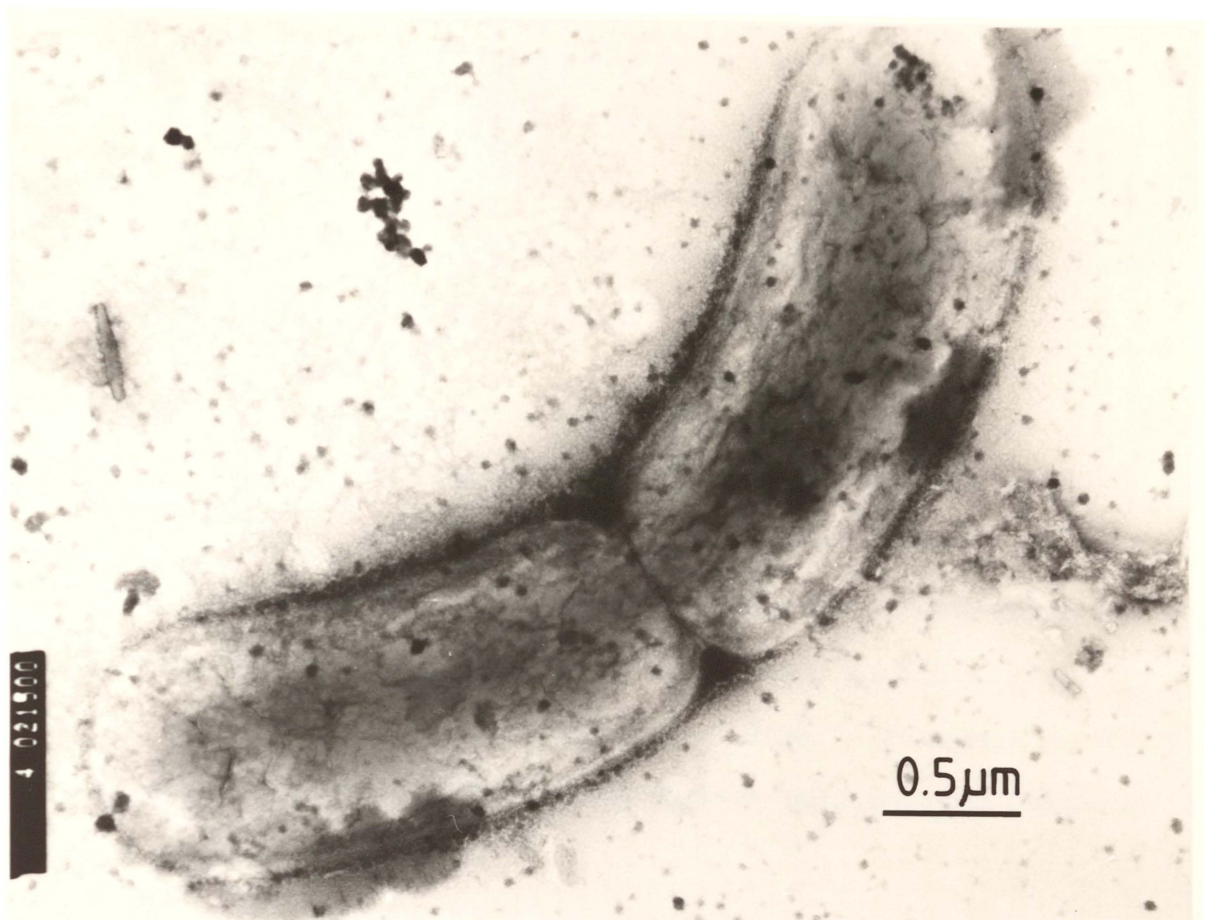


Figure 2.34. A dividing rod shaped bacterium adsorbed to a butvar coated E.M. grid incubated in pool Rt62 for 2 hours.

Figure 2.35. A dividing rod shaped bacterium morphologically distinct to that from shown in Figure 2.34 present in pool Rt60. The time of incubation of the E.M. grid was 2 hours.



Flagellated rods were observed in a number of pools including Rt59, Rt12, Rt12A (Figure 2.39), Rt44 (Figure 2.41), Rt56 (Figure 2.33), Rt3 (Figure 2.43) and WP9 (Figure 2.44). Bacteria from all these pools except those from pools Rt56 and Rt12A were monopolarly flagellated. Bacteria from pools Rt56 and Rt12A had a tuft of polar flagella.

Slime was associated with a number of bacterial cells for instance, it was clearly visible around the cells in pool Rt3 (Figure 2.42) and pool WP9 (Figures 2.44). Cells containing intracellular material were observed in pool Rt3 (Figure 2.43) and pool Rt44 (Figure 2.40). The nature of this material was not determined and could have been anything from storage vacuoles, gas vacuoles, to sulphur particles. Filaments attached to central particulate matter and thereby forming rosettes were observed in pool Rt6 (Figure 2.36).

(e) Cocci:

Even though cocci were generally more difficult to stain than rods, different types were easily identifiable.

Two distinct morphological groups existed based on presence or absence of flagella. The cocci present in pool Wai21 (Figure 2.49), Rt44 (Figure 2.50) and Rt12A (Figure 2.52) were flagellated. Additionally, the coccus present in pool Wai21 (Figure 2.49) possessed pili and was much smaller in size when compared to the rest of the flagellated cocci.

Non-flagellated cocci were present in pools Wk1 (Figures 2.45 to 2.47), Rt30 (Figure 2.48) and Rt51 (Figure 2.51). Two types of cocci were evident in Wk1 based on diameter and morphology of the cells. The coccus shown in Figure 2.45 was spherical and 1.5 μ m in diameter compared to the coccus in Figure 2.47 which was ellipsoidal and 0.35 μ m in diameter. The

Figure 2.36. Rosette formation of filamentous bacteria attached to mineral particles. Butvar coated grids were incubated in pool Rt6 for 1 hour.

Figure 2.37. Filamentous and a coccal shaped bacteria unadsorbed onto butvar coated E.M. grids incubated in pool Wai19 for 4 hours. Note the variation in diameters of the filaments.

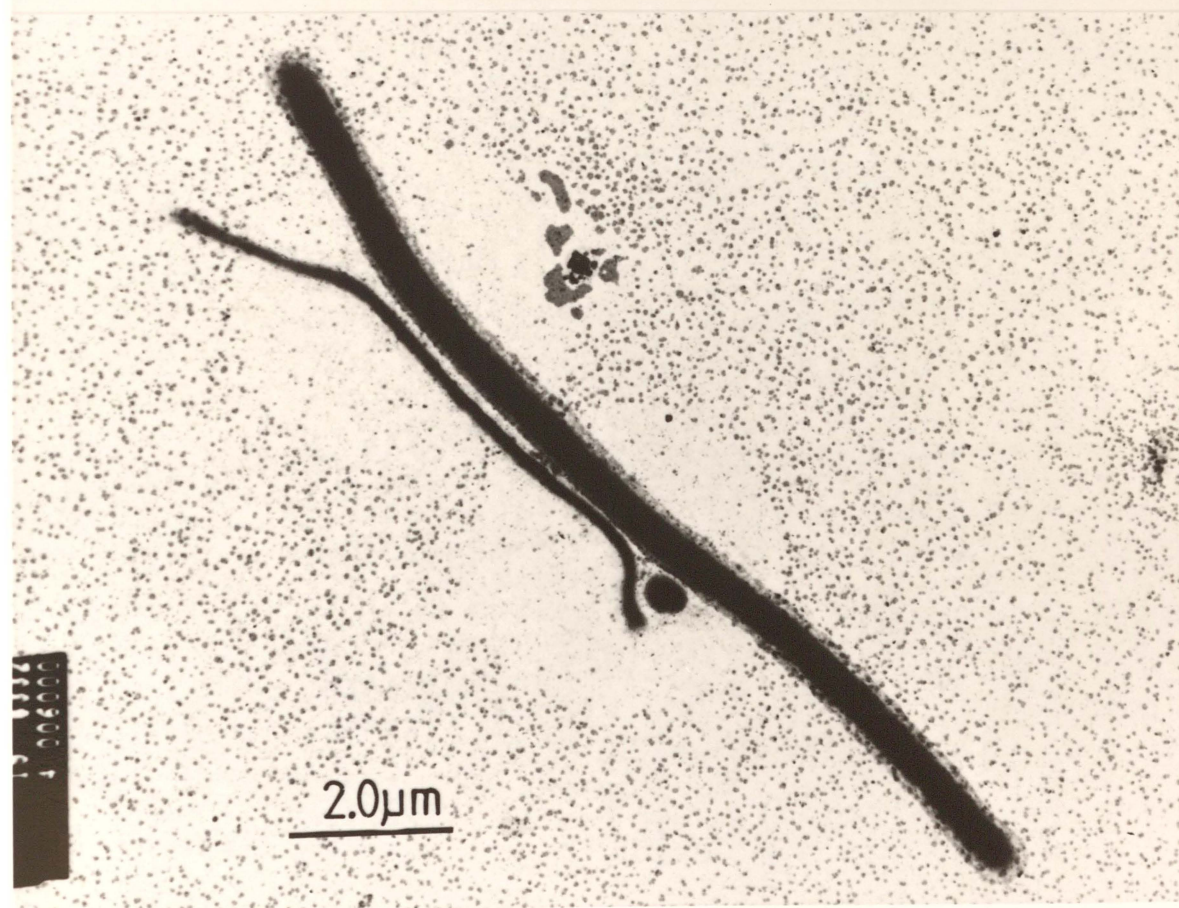
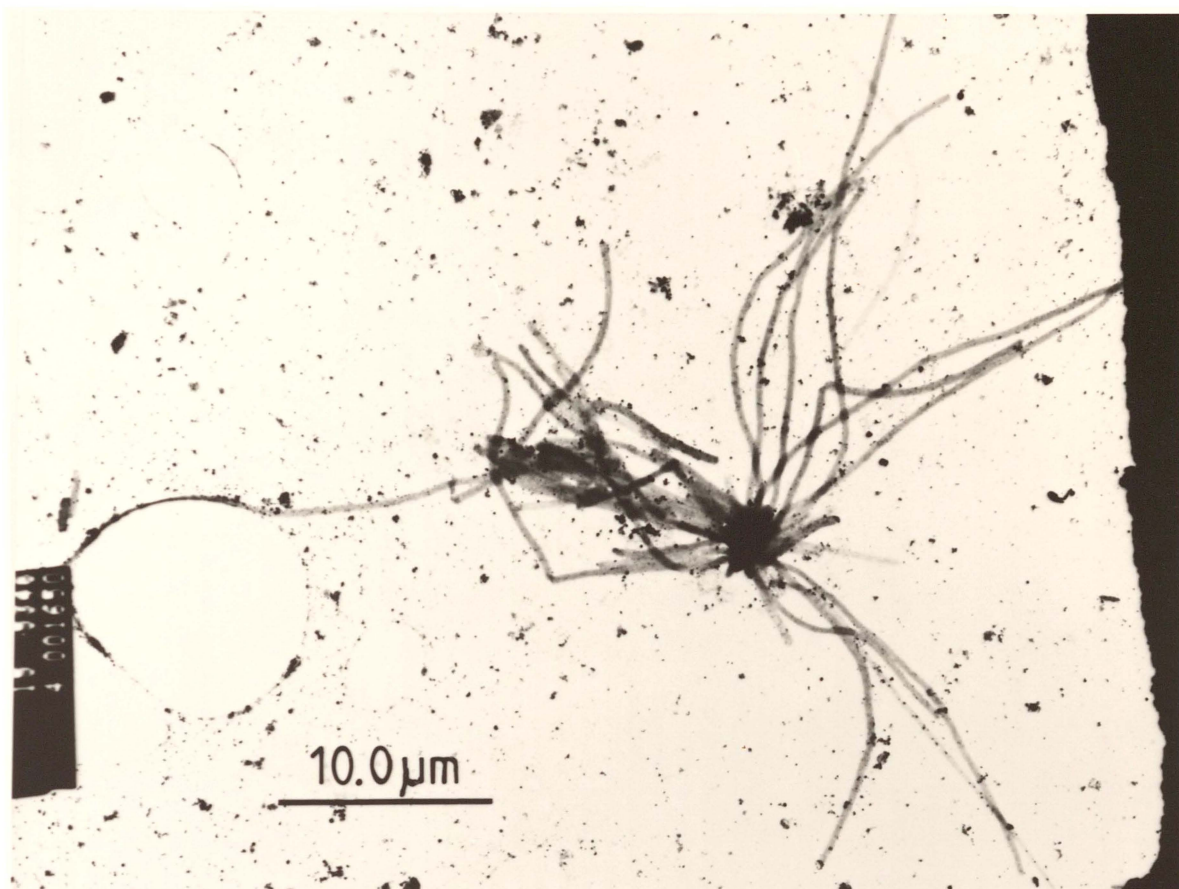


Figure 2.38. A dividing rod from pool Rt12A. The time of incubation of butvar coated E.M. grid was 1.5 hours.

Figure 2.39. A morphologically different rod from pool Rt12A. Note the presence of flagella. The time of incubation of butvar coated E.M. grid was 1.5 hours.

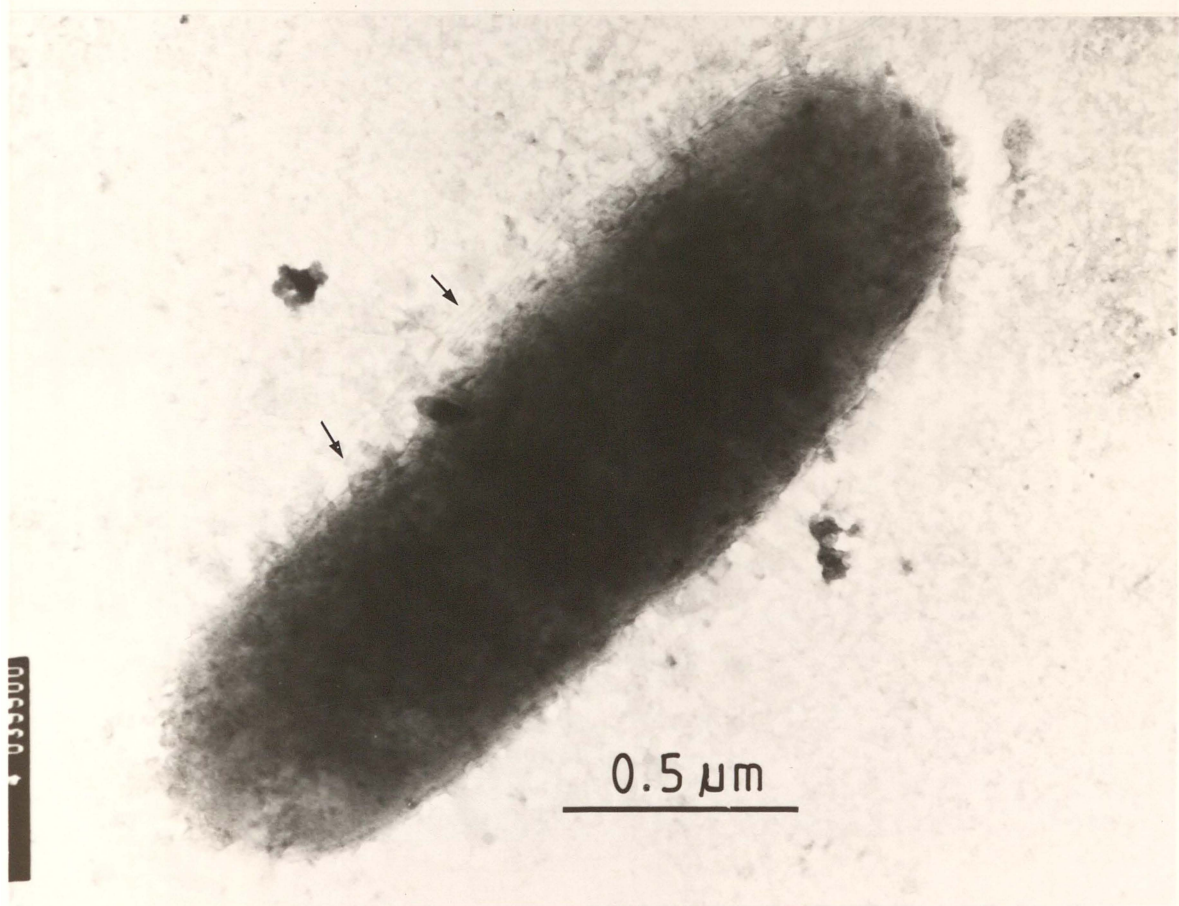
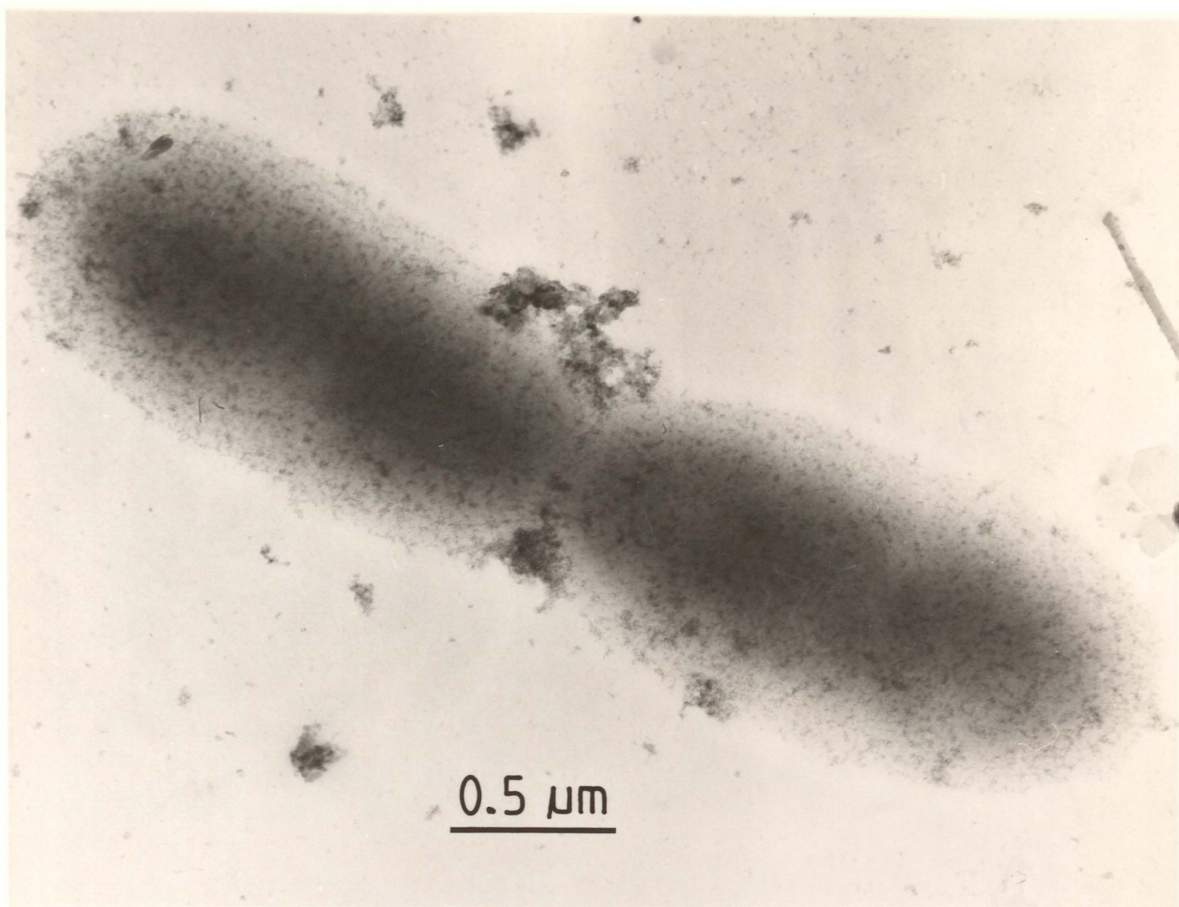


Figure 2.40. A 'V' shaped rod present on butvar coated E.M. grid incubated in pool Rt44 for 3 hours. Note the presence of electron dense and electron light globules.

Figure 2.41. A flagellated rod present in the same pool as mentioned above. The time of incubation of the butvar coated E.M. grid was 3 hours. ,

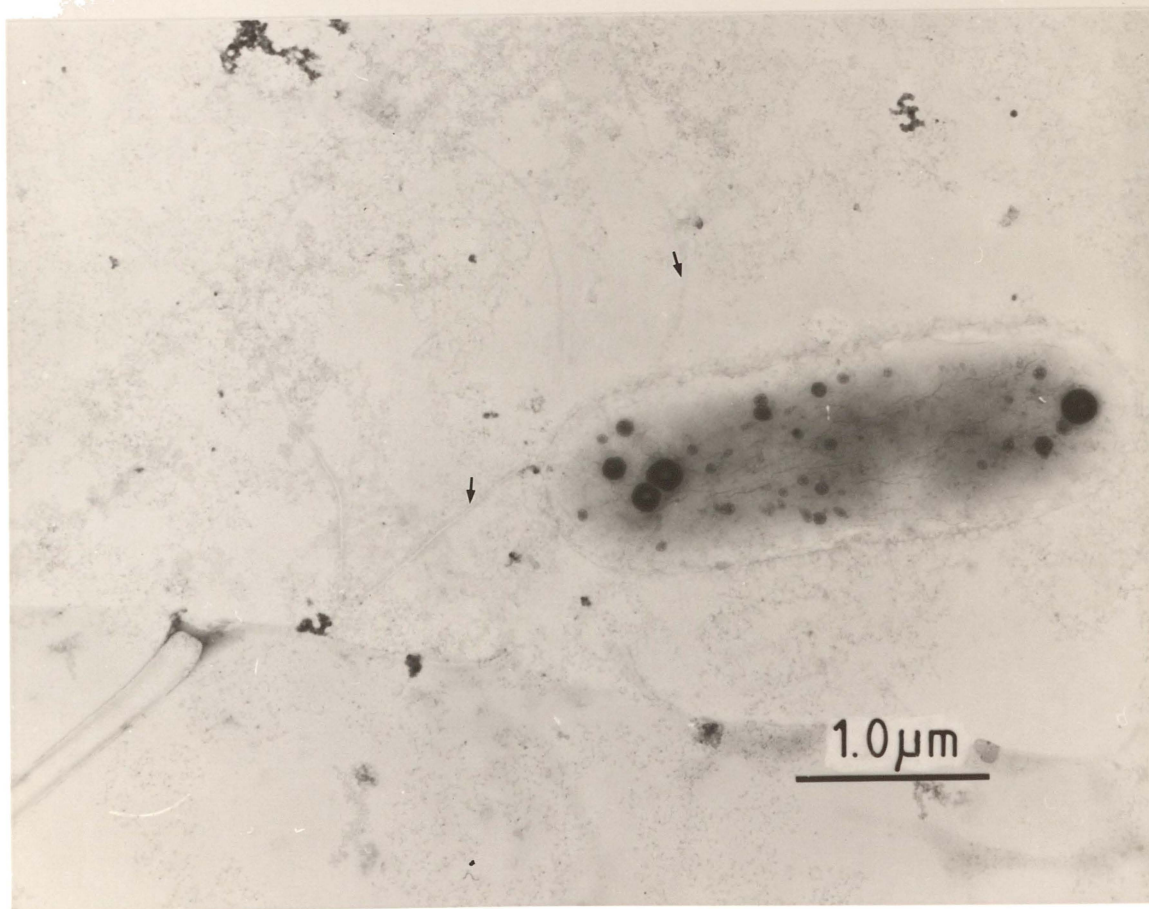
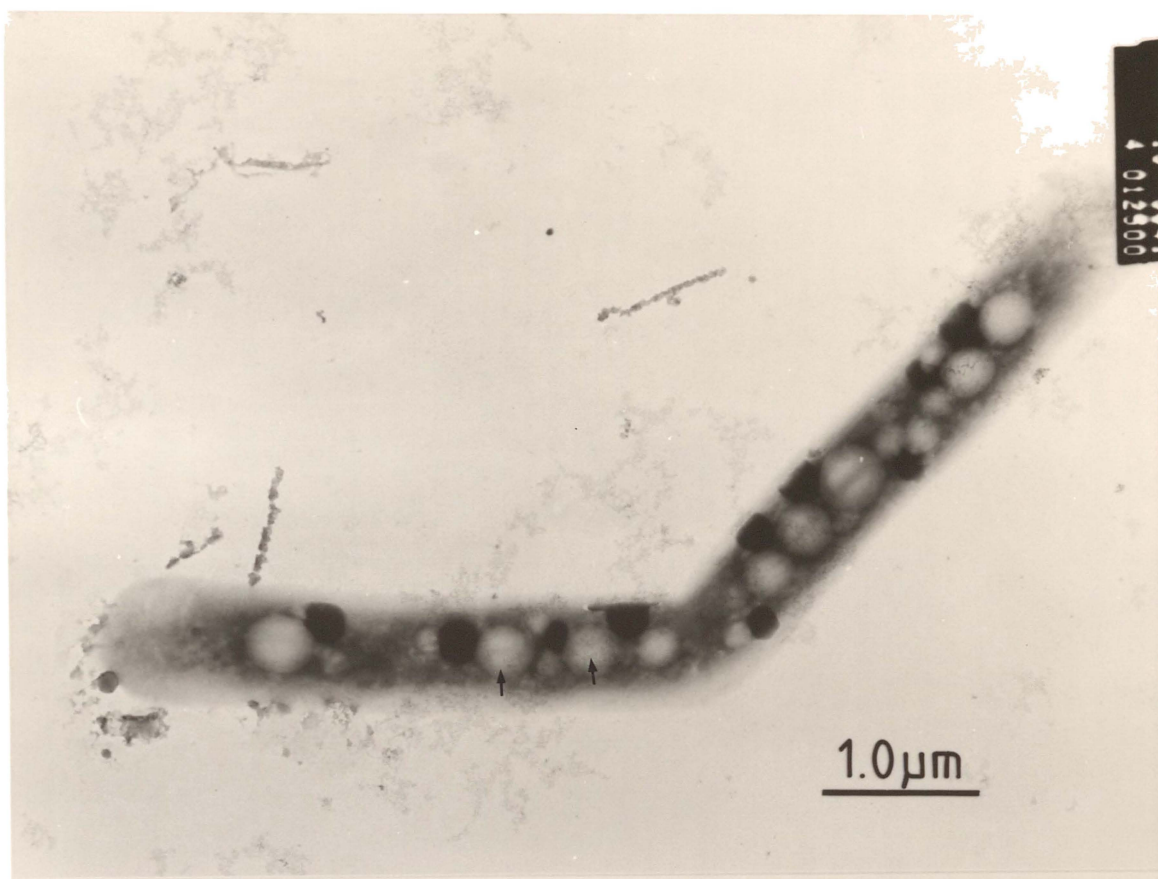


Figure 2.42. An extremely thin rod shaped bacterium present on butvar coated E.M. grids after 1 hour incubation in pool Rt3. Note the presence of slime around the cell, which obscures the definition of the cell envelope.

Figure 2.43. A morphologically distinct rod from the above mentioned pool on butvar coated E.M. grid after 1.5 hours incubation. Note the presence of flagellallum and electron light vesicles.

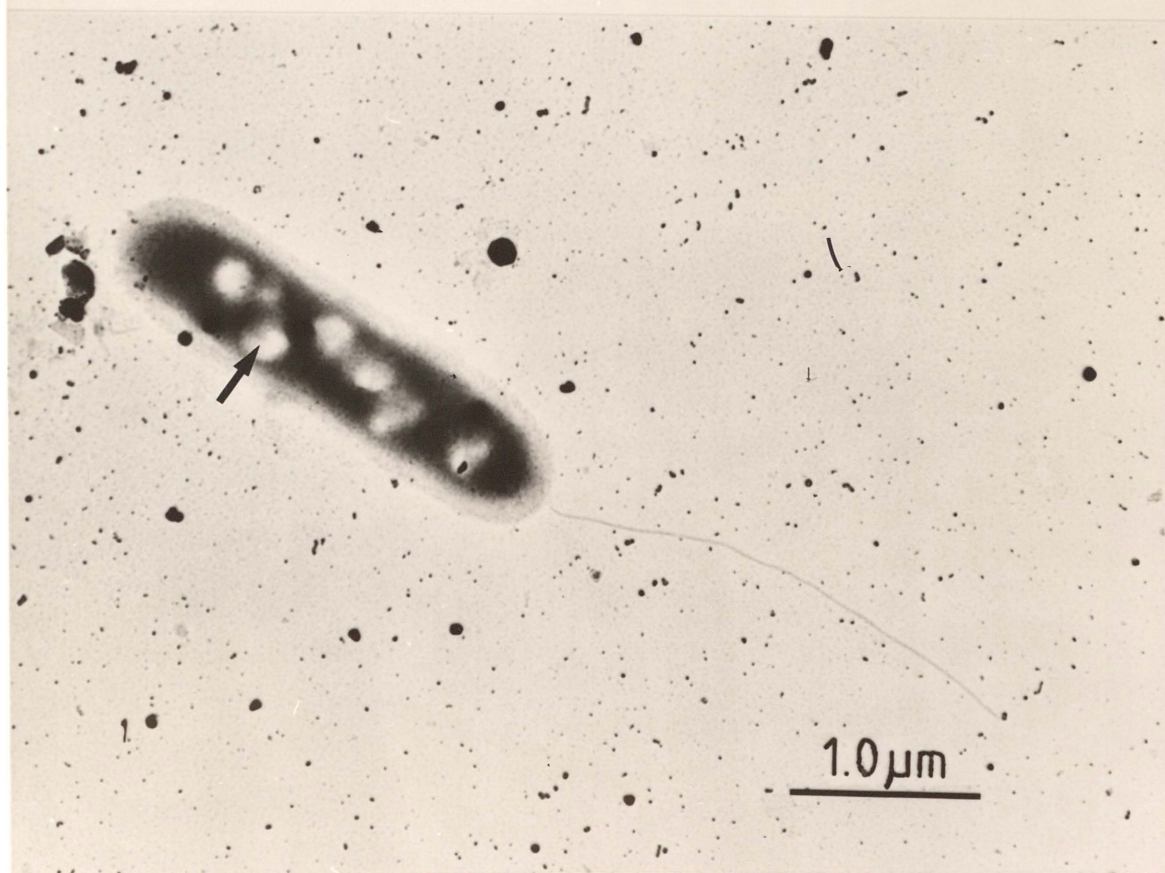


Figure 2.44. Electron micrograph of a polarly flagellated rod from pool WP9 (A). Note the presence of mucilaginous material around the cell (B). Time of incubation of the butvar coated E.M. grid was 1 hour.

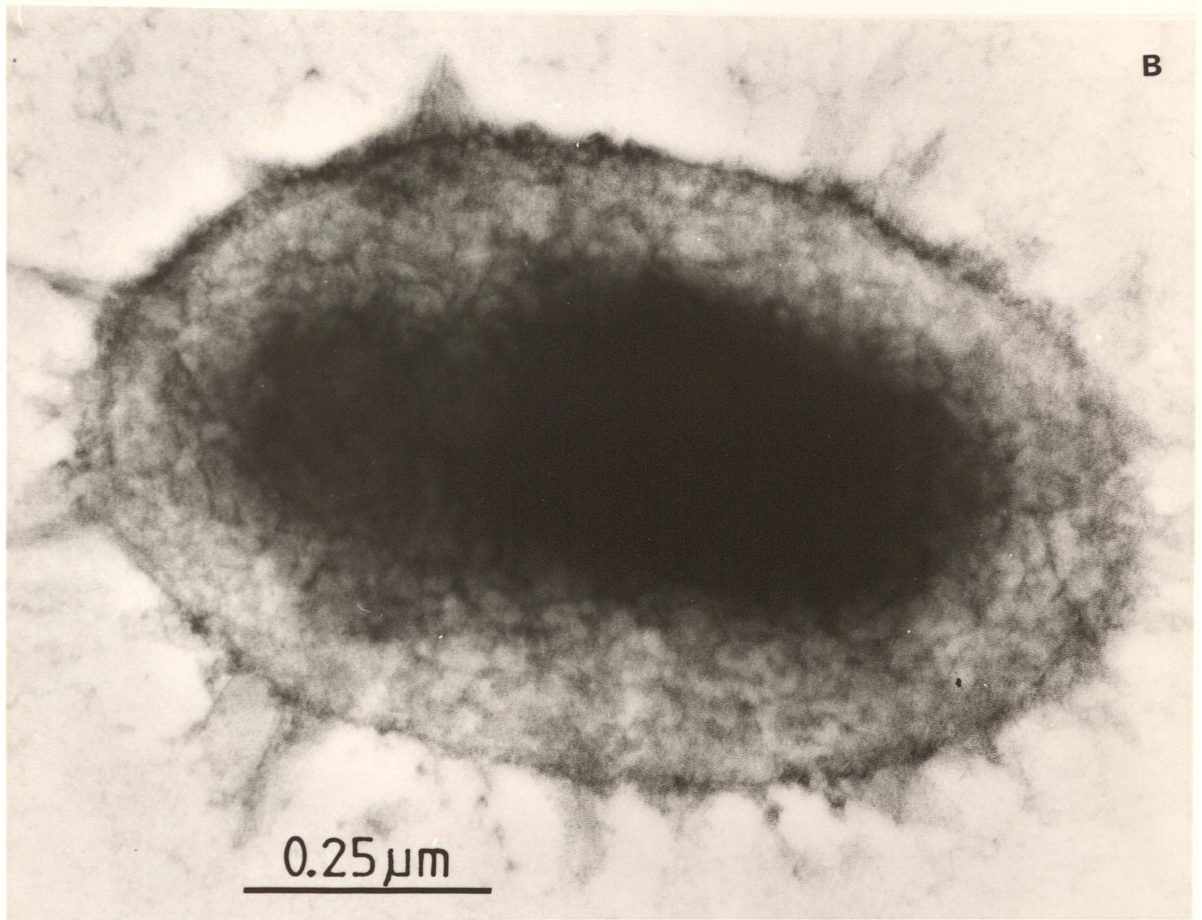
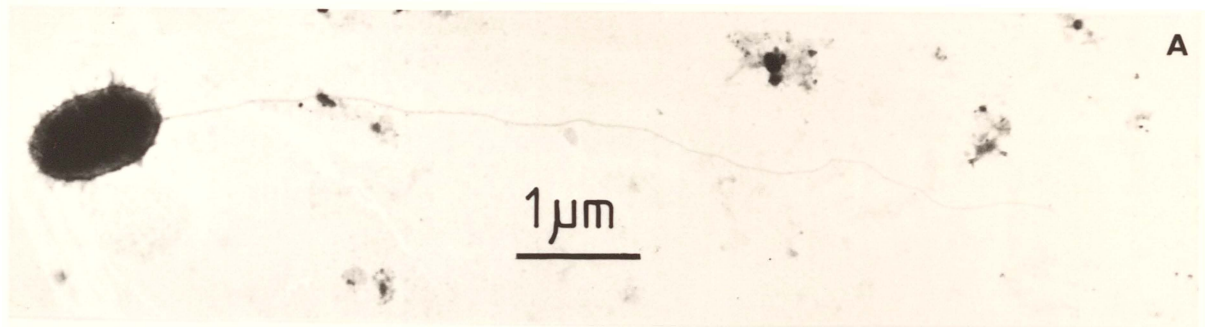


Figure 2.45. A coccus present on butvar coated E.M. grid after 3.5 hours incubation in Wk1. Note the presence of the hexagonal phage-like particle (Arrow).

Figure 2.46. Portion of Figure 2.45 magnified to show the structure of the cell wall of the coccus and the phage-like particle.

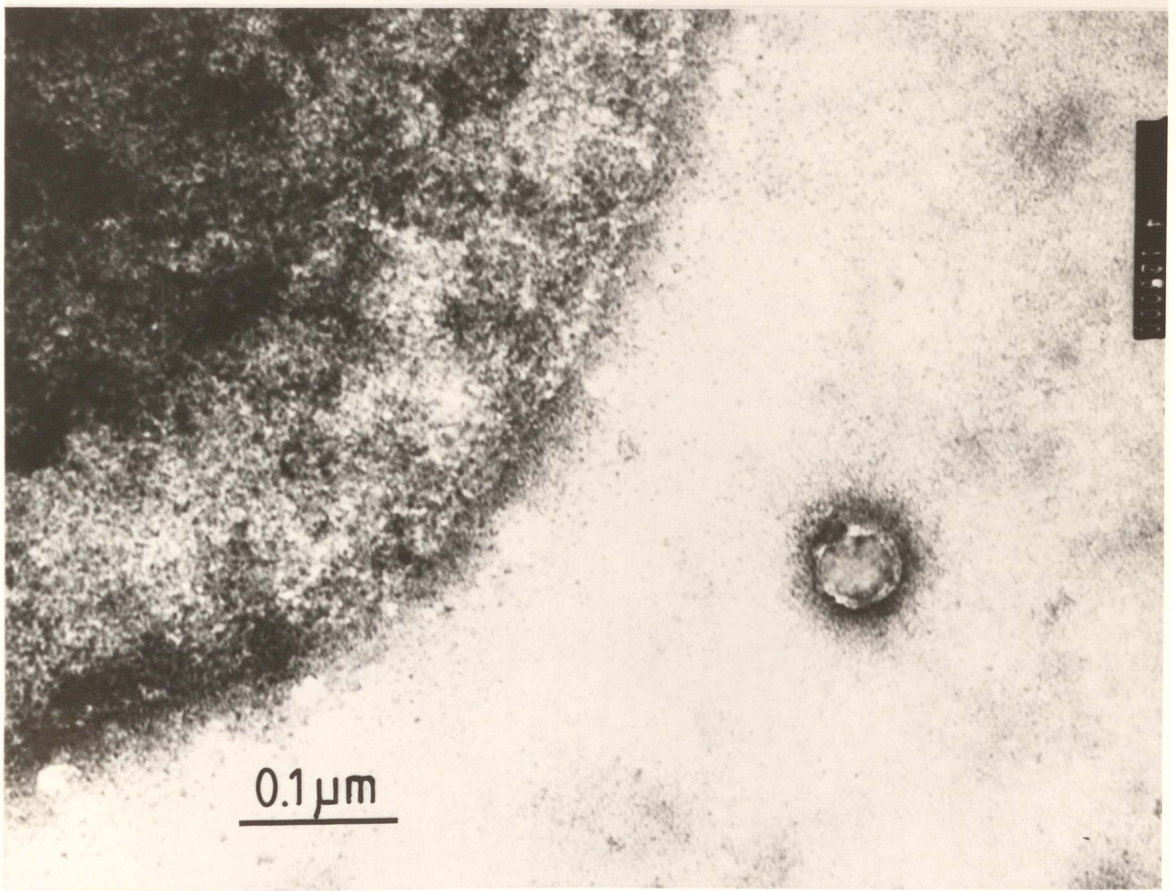
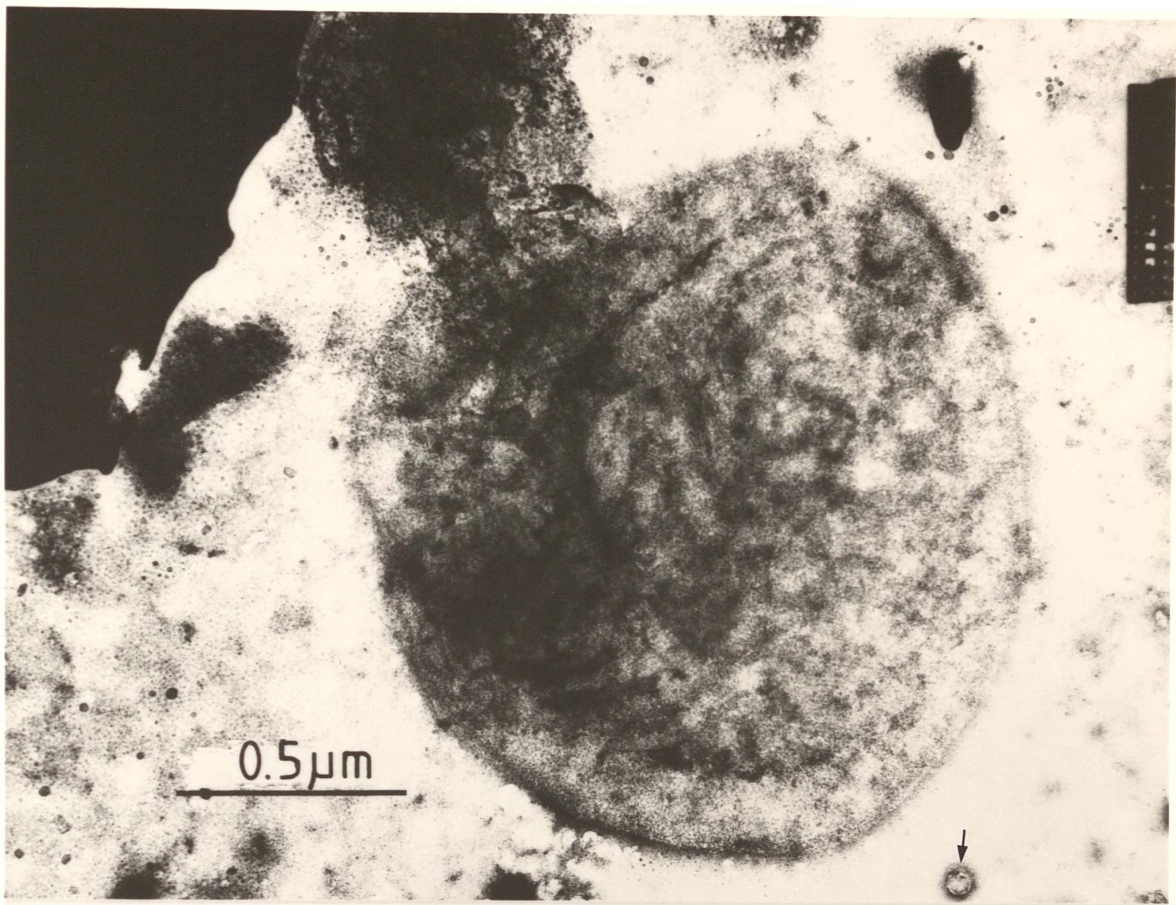


Figure 2.47. An extremely small coccus present in pool Wk1. The butvar coated E.M. grid was incubated for 3.5 hours.

Figure 2.48. A dividing coccus from pool Rt30. The coccus had adsorbed to a butvar coated E.M. grid incubated in the pool for 3.5 hours.

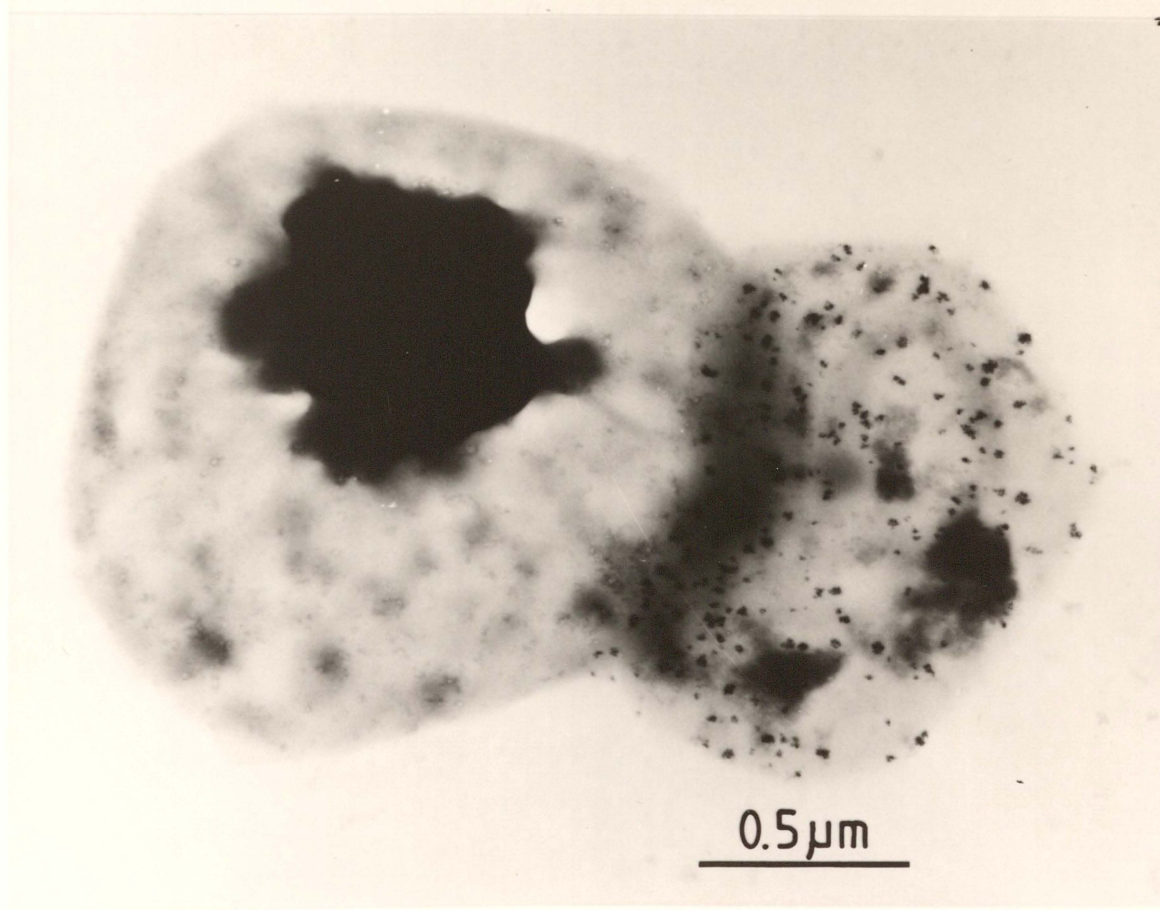
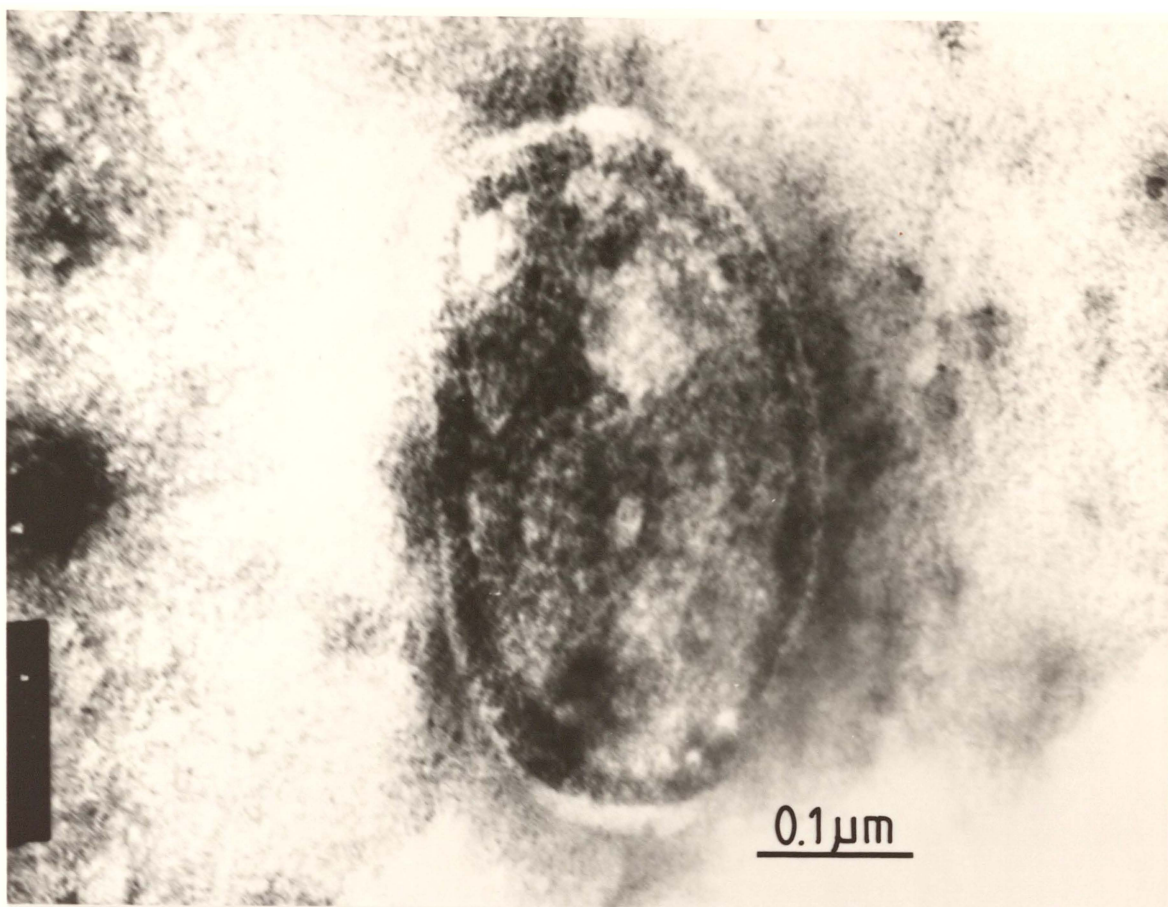


Figure 2.49. A coccus present in pool Wai21 adsorbed to a butvar coated E.M. grid incubated in the pool for 3 hours. Note the presence of appendages of different diameters which may represent flagella and pili.

Figure 2.50. A flagellated coccus present on butvar coated E.M. grid incubated in pool Rt44 for 2 hours. Note the mucilaginous material surrounding the cell.

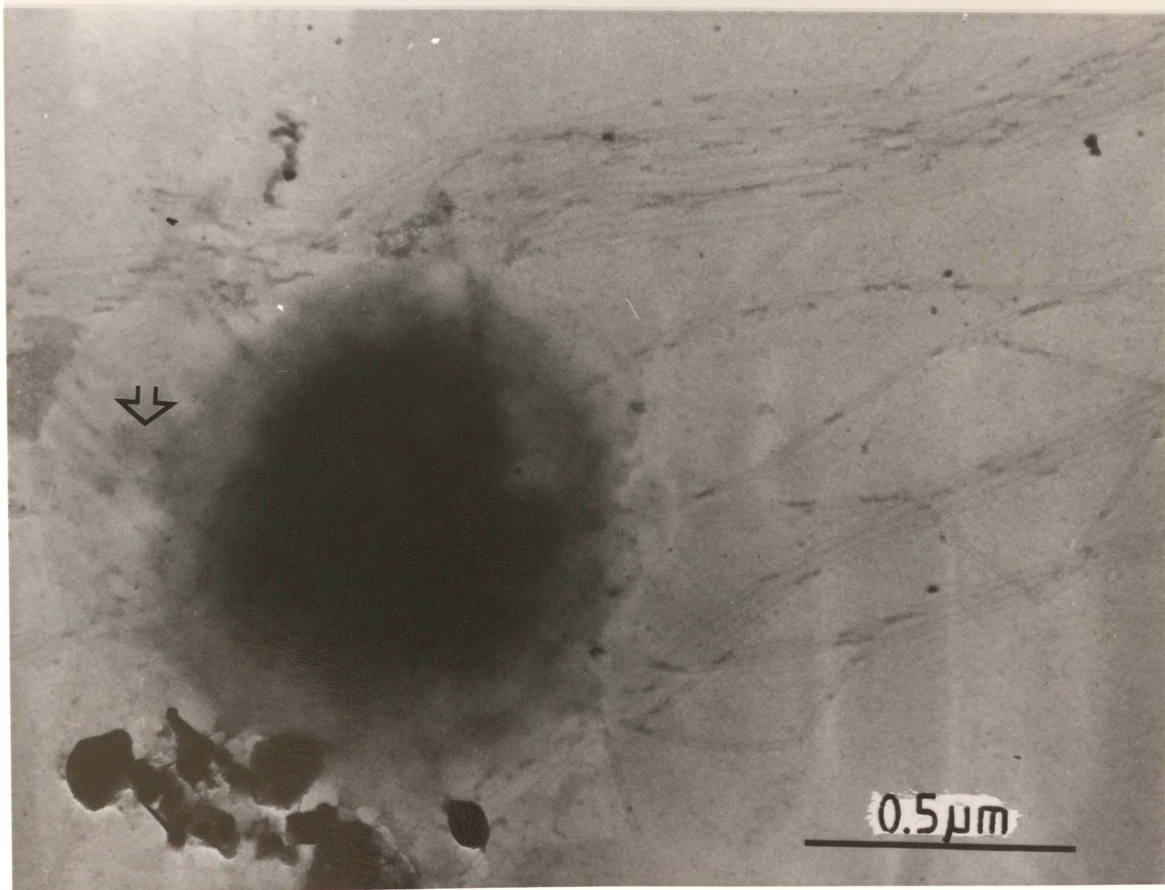
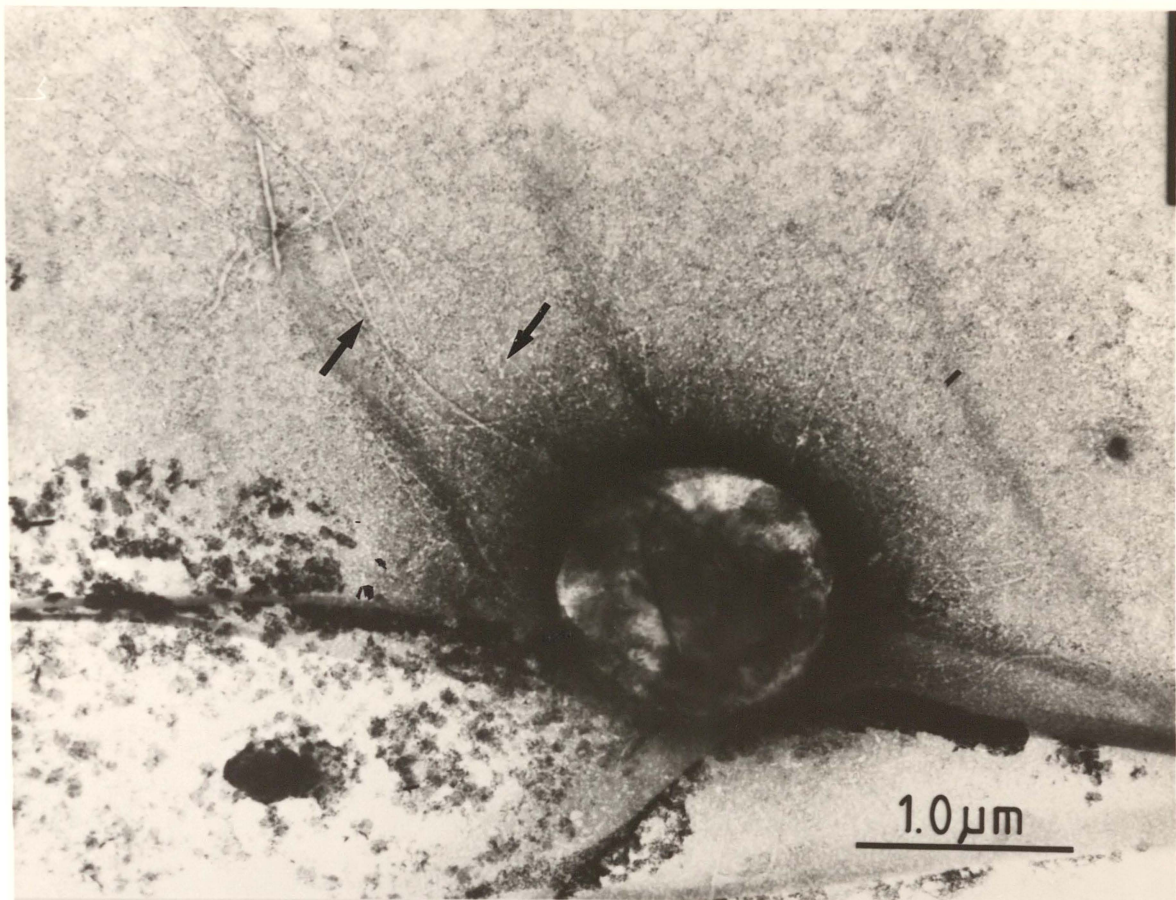
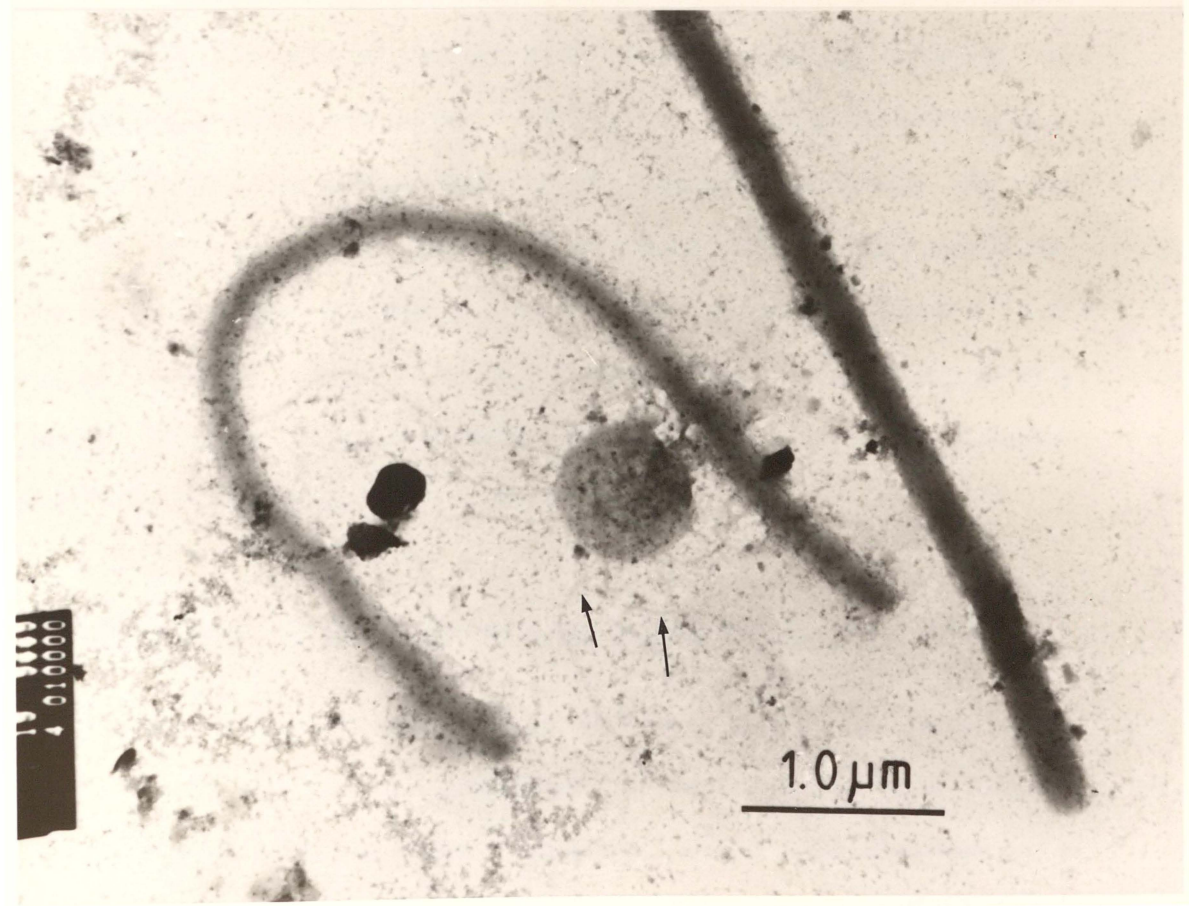
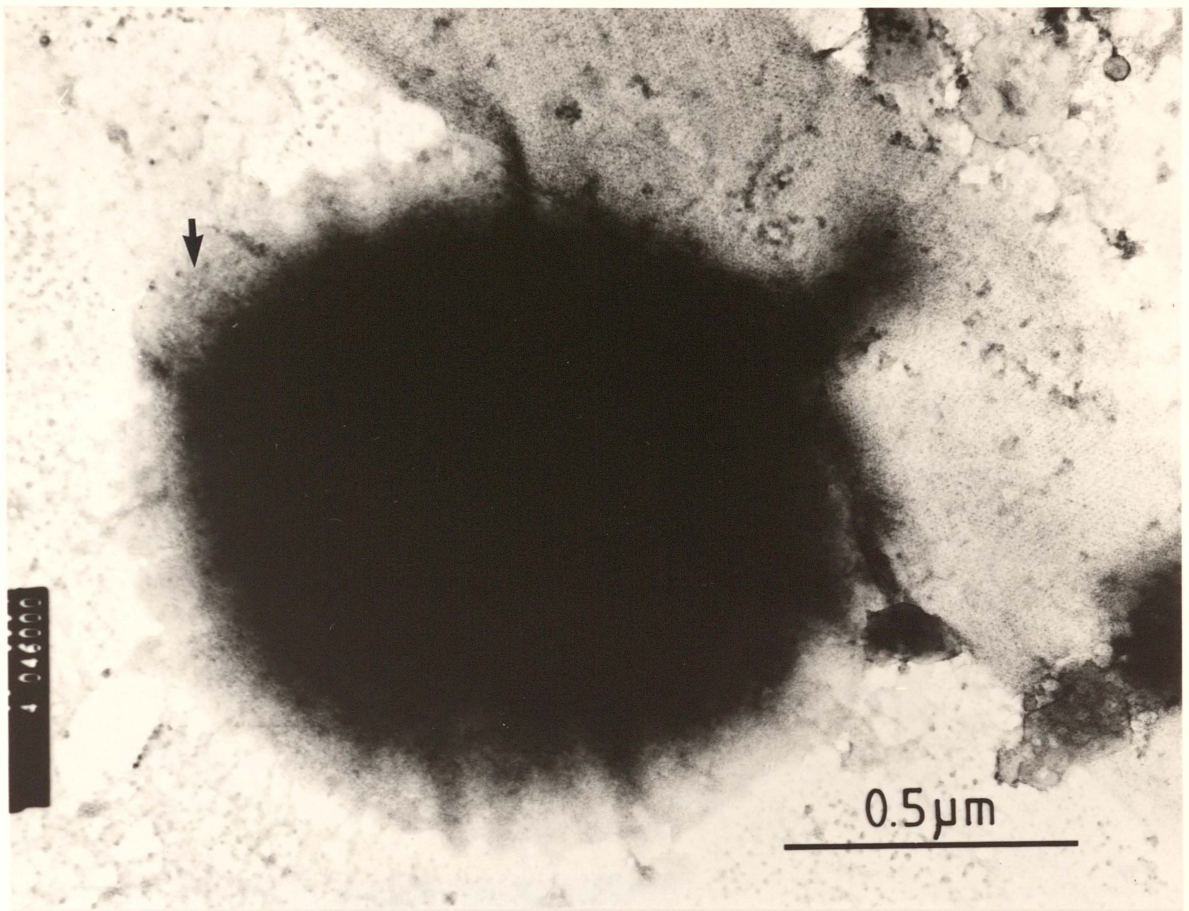


Figure 2.51. Association between a coccus and Thermoproteus type species on a butvar coated E.M. grid incubated in pool Rt51 for 1.5 hours. Note the presence of mucilaginous material surrounding the cell.

Figure 2.52. Association between a coccus and a rod in pool Rt12A. The butvar coated E.M. grid was incubated in pool Rt12A for 1.5 hours. Note the presence of flagella.



cell wall of the former resembled that of Sulfolobus species. Only aerobic Sulfolobus type organisms were enriched (Refer chapter 5, isolation of aerobic archaeobacteria) from this pool and all anaerobic enrichments failed to yield any bacteria (Table 5.3, chapter 5) and hence it is assumed that this organism was a Sulfolobus species. It is interesting to note that numerous hexagonal phage-like particles, either free floating or attached to the cells were observed in the pool (Figures 2.45 and 2.46). A dividing coccus was observed in pool Rt30 (Figure 2.48).

Some of the flagellated and non-flagellated cocci, as mentioned earlier, formed association with rods.

(f) Other morphological types:

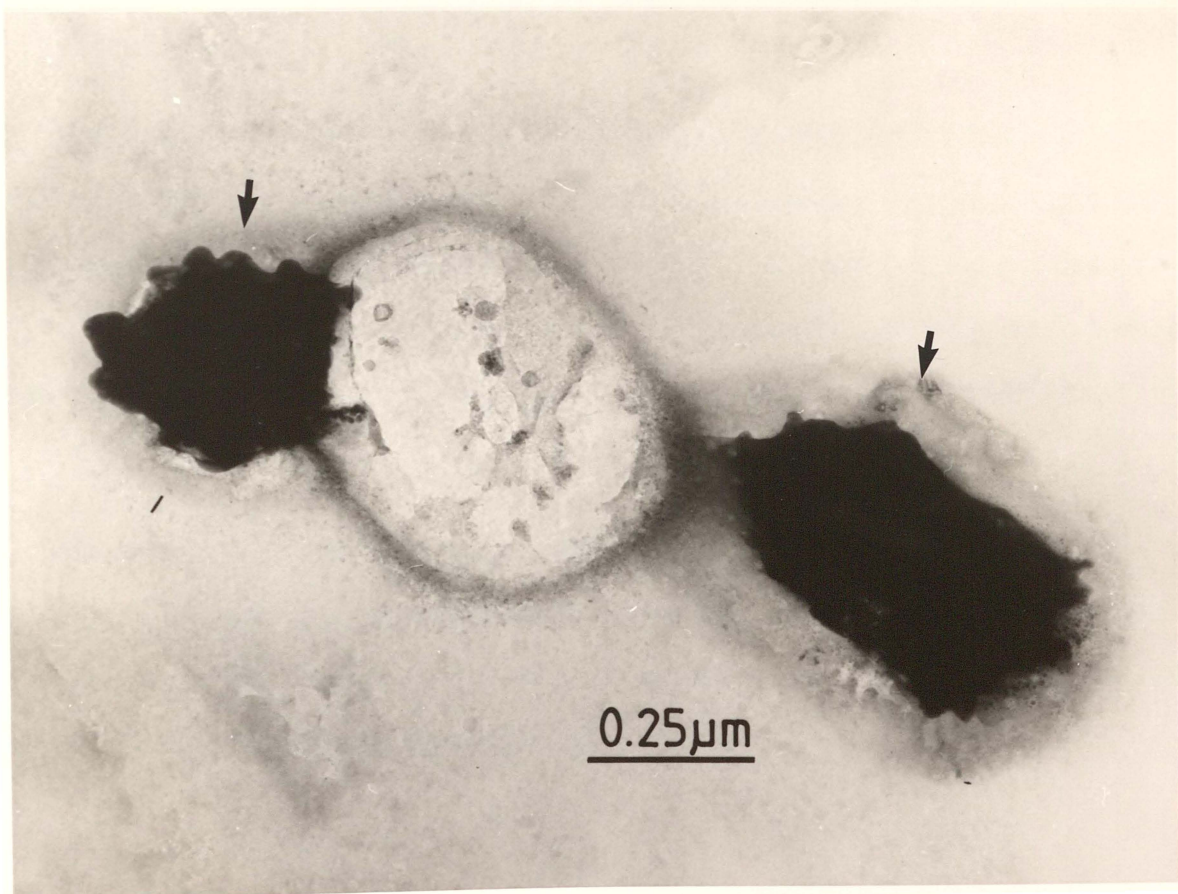
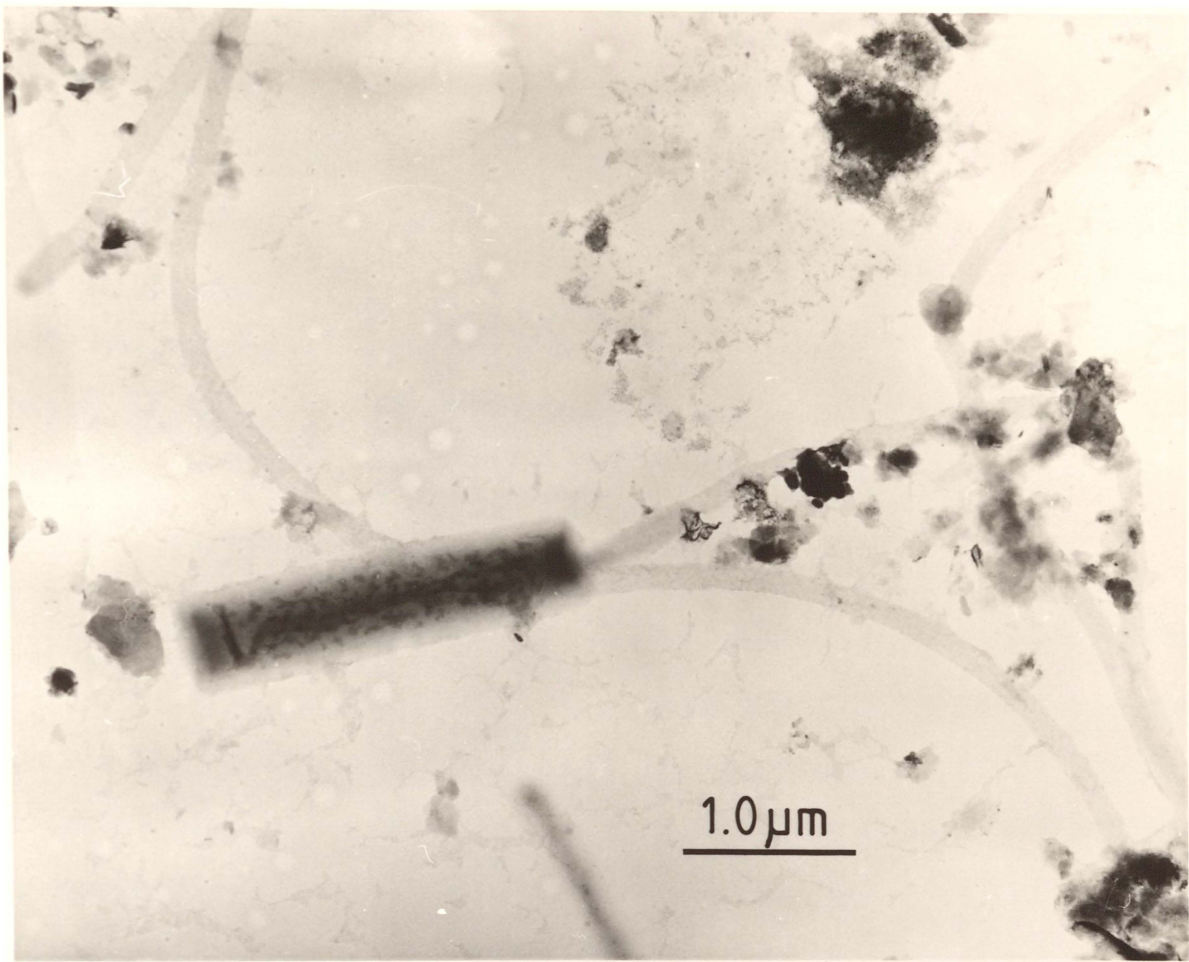
Two unique bacterial morphotypes were observed in a thermal spring of Fiji (Figures 2.53 and 2.54). The spring, designated SSFJ1, was located on the beach and had a high NaCl content because of mixing of sea water with the spring water. The first of these was a square-ended rod shaped bacteria with which filaments were also observed (Figure 2.53). An organism with a pair of electron dense "flap like" structures was also observed (Figure 2.54). A similar organism was later isolated and characterised (Refer chapter 7; Table 7.25)

2.7.2 Studies Of Bacteria Colonising Polycarbonate Membranes

At least three morphologically distinct bacterial morphotypes were present on polycarbonate membranes incubated in six Rotorua pools. The most predominant were rods and filaments. Cocci were observed in only two pools.

Figure 2.53. Filament and a rod shaped bacterium absorbed to butvar coated E.M. grid incubated in pool SSFJ1 for 1 hour. Note the square ends of the rod shaped bacterium.

Figure 2.54. A rod with protruding "flap" like structures The butvar coated E.M. grid was incubated in pool SSFJ1 for 1 hour.



The cell wall ultrastructure of filaments present in pools Rt13, Rt15, Rt44 and Rt56 was very similar. The cell wall was made up of subunits and in a few cells the terminal swellings known as "golf balls" (Zillig et al., 1981a) were also present (Figures 2.55, 2.56, 2.57). These are characteristic features of Thermoproteus and Thermofilum species (Zillig et al., 1981a, 1983a). Morphologically identical bacteria were also observed by Brock et al., (1971) in a spring from Yellowstone National Park, USA.

Pools Rt13, Rt15, Rt44, Rt51, Rt56 and Rt60 had a morphologically similar rod (Figures 2.58, 2.62). The dimension of the cell wall envelope was very similar to the dimension of the cell envelope of the above mentioned filaments. Additionally, in some of the electron micrographs, the presence of subunits in the cell envelope of the rods was clearly discernible (Figure 2.62). Mucilaginous material was also observed surrounding the rods.

Cocci were observed in pools Rt44 (Figure 2.58), Rt51 (Figures 2.59, 2.60) and Rt 56 (Figure 2.61). The cocci present in pool Rt51 were surrounded by a dense network, presumably of mucilaginous material (Figures 2.59, 2.60), whereas cocci in pools Rt44 and Rt56 lacked this feature. Pure cultures of Desulfurococcus species Hvv13 described by Zillig et al., (1982a) is known to possess such a structure.

The presence of mucilaginous material observed in thin sections of bacteria adhering to polycarbonate membrane is in good agreement with other in situ E.M. results, and the advantages of such structures in thermal environments have been discussed earlier.

Figure 2.55. Electron micrograph of a filament from pool Rt15. Note the presence of a "golf ball" present at the end of the cell and the subunit structure of the cell envelope.

Figure 2.56. The cell envelope of the above cell magnified to show the subunit structure

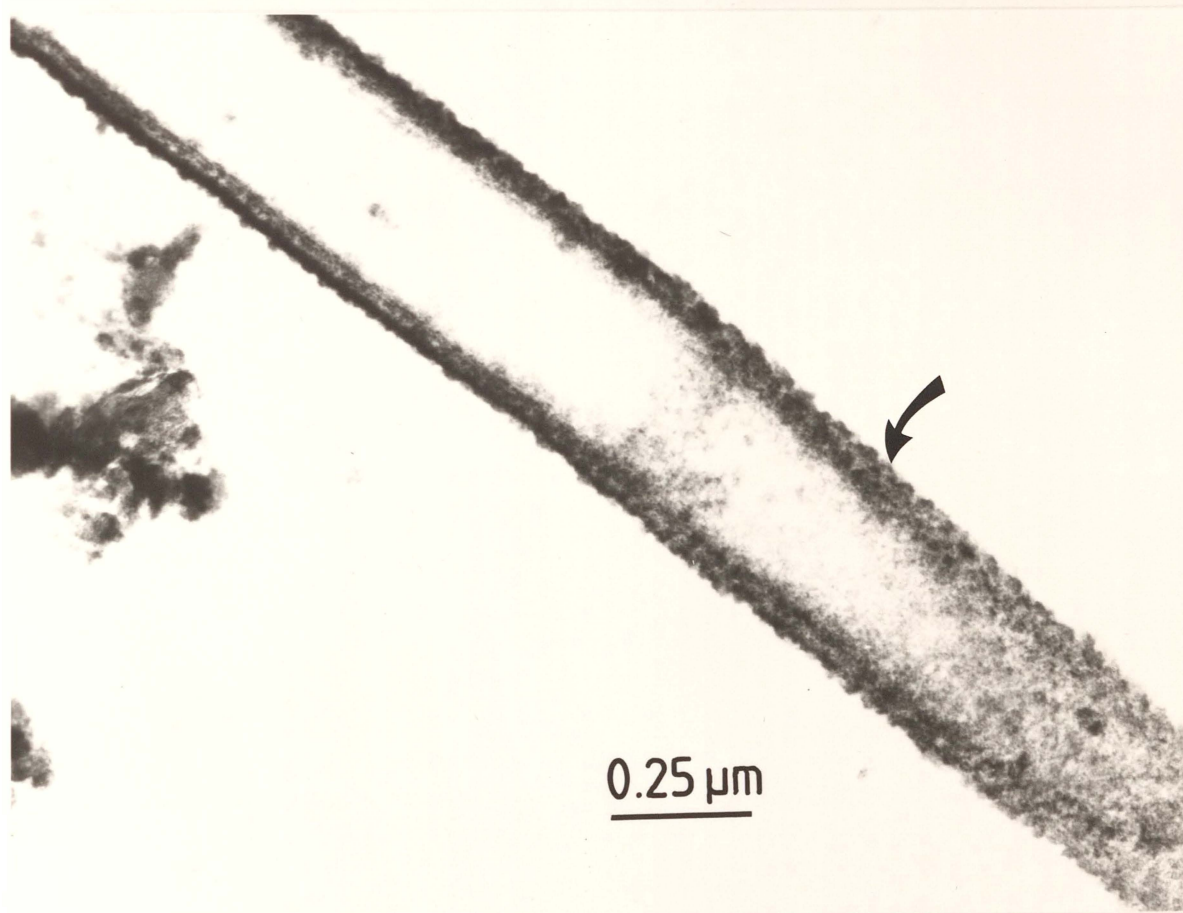
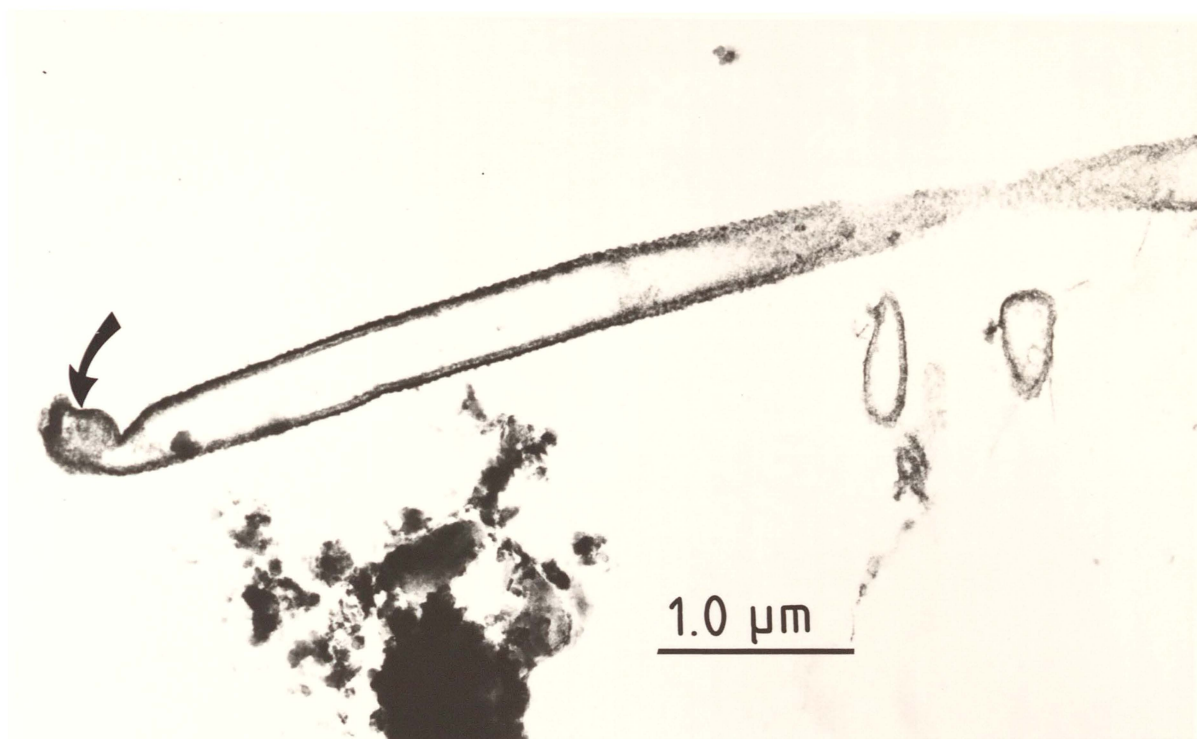


Figure 2.57. A grazing section of bacteria present in pool Rt44. Note the presence of mucilaginous material on the cell presumably used as an adhesion mechanism.

Figure 2.58. Two morphologically distinct cell types from the same pool as above. Note the thick cell envelope of the rod and the much thinner envelope of the coccus. Distinct subunit patterns are present

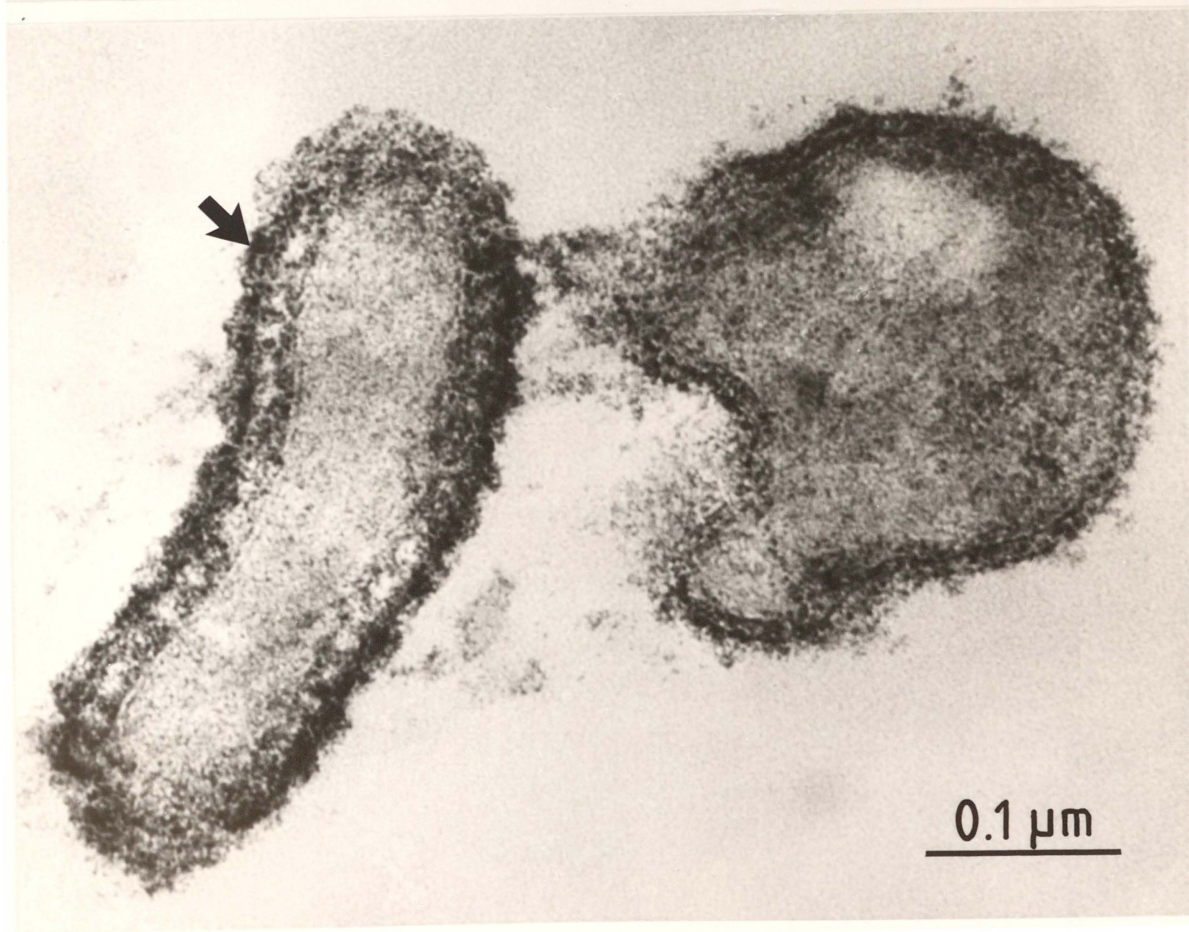


Figure 2.59. A coccus with a dense network of mucilaginous material present in pool Rt51.

Figure 2.60. Magnification of the above cell to show the cell wall structure and the association of the mucilaginous material with the cell envelope.

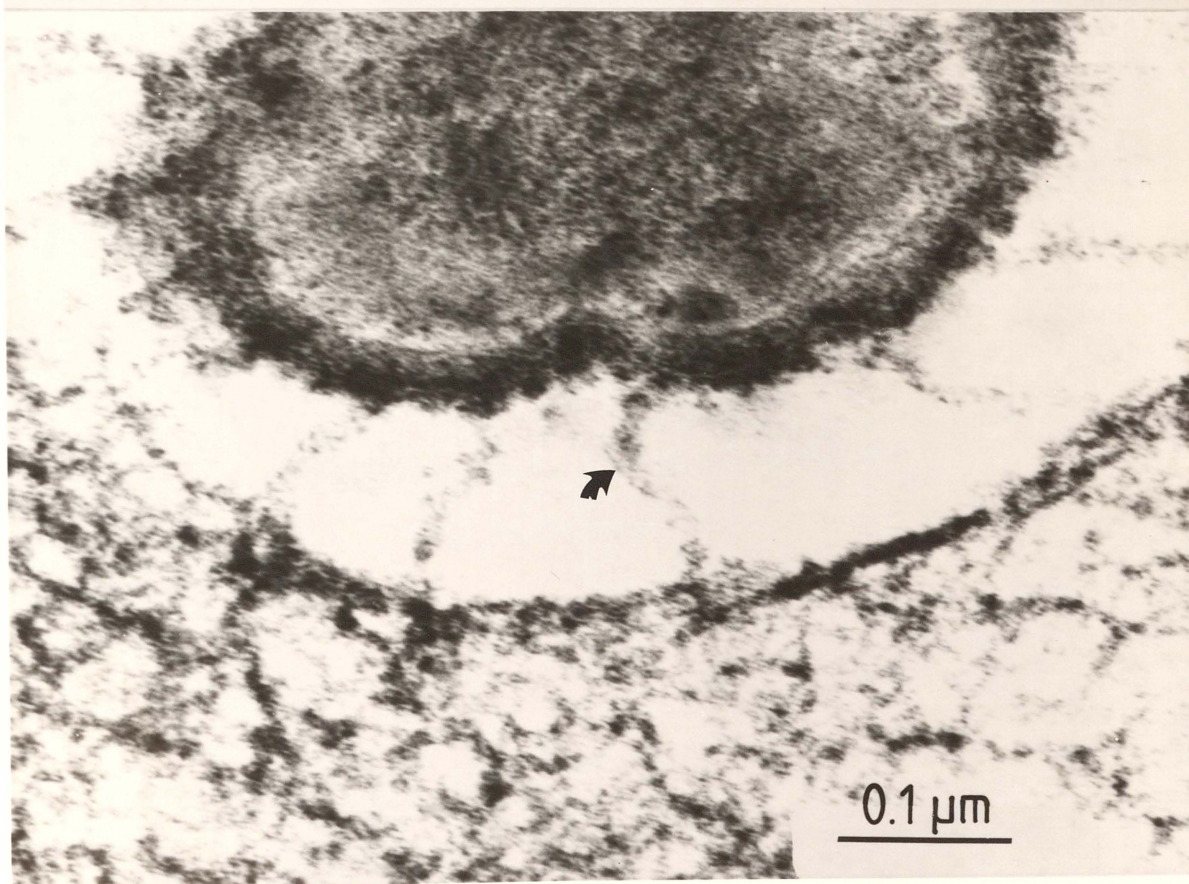
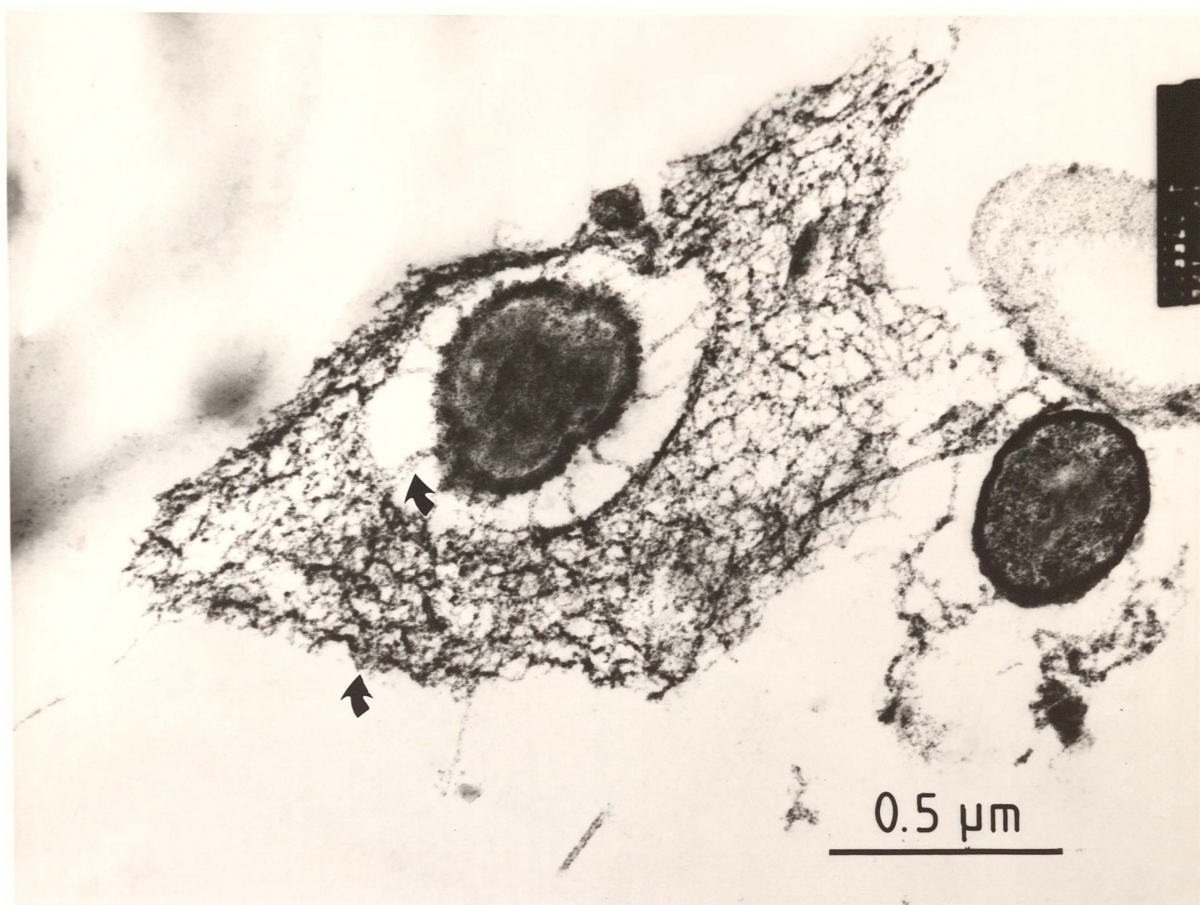
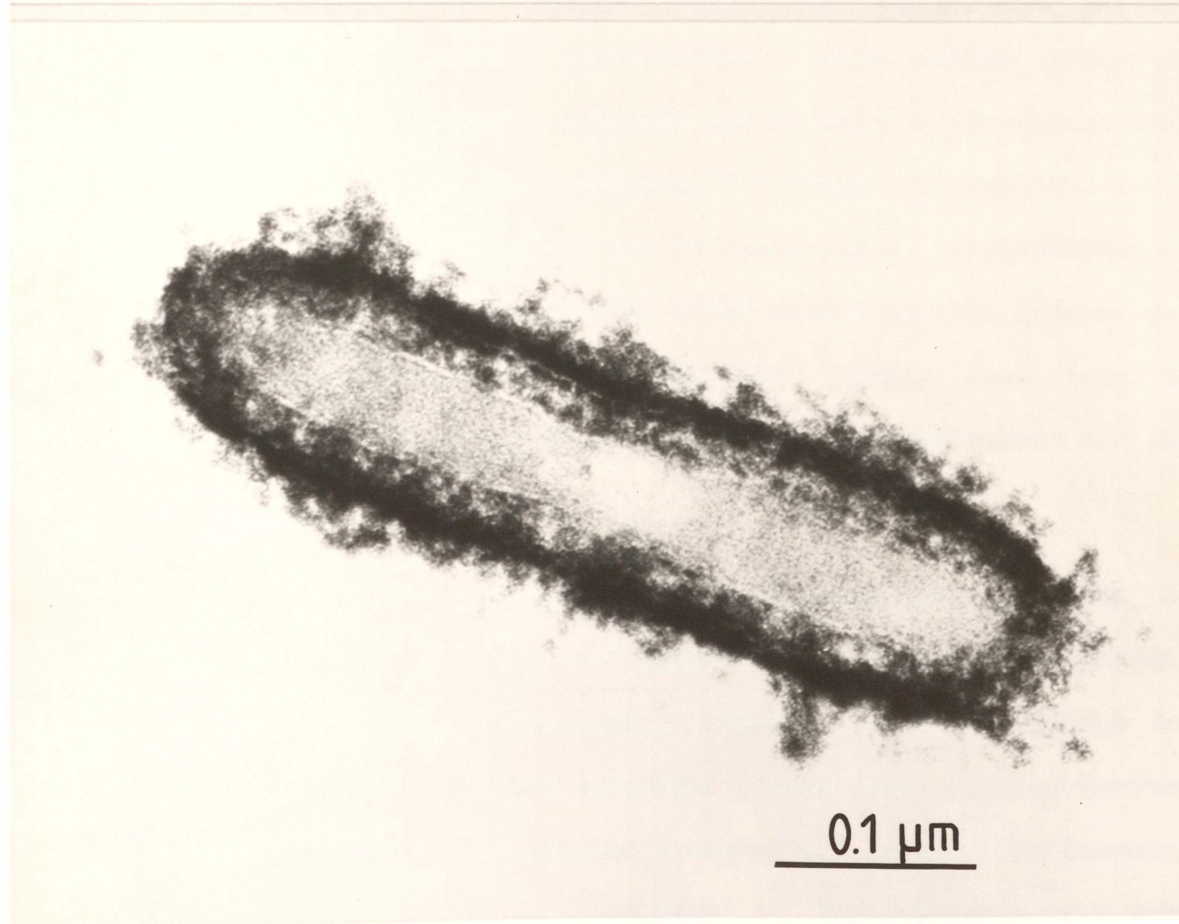
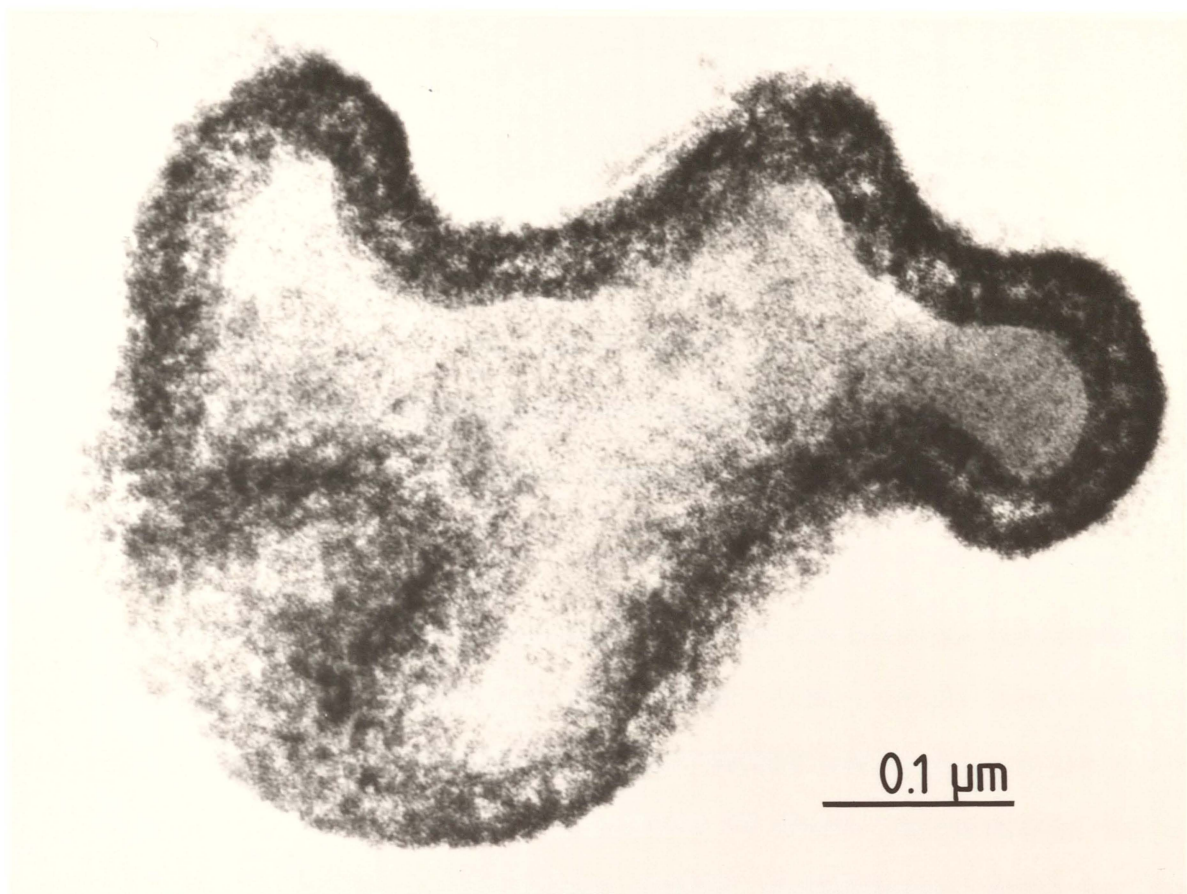


Figure.2.61. A thin section of a coccus present in pool Rt56.
(pleomorphic)

Figure 2.62. A thin section of a rod present in pool Rt60. Note the
subunit structure of the cell envelope and the
presence of mucilaginous material around the cell.



2.8 SUMMARY AND CONCLUSIONS:

The microscopic survey of New Zealand geothermal springs revealed that greater than 90% of the pools harboured bacteria and that the microbial population present was diverse but relatively stable. Rods, cocci and filaments were the major morphotypes present. The rods and cocci were generally distributed throughout the temperature and pH spectrum. However the filaments were more or less restricted to pools with a pH above 6.0.

Further evidence of microbial diversity was revealed by a novel in situ E.M. method in which butvar coated E.M. grids were used as attachment and colonization surfaces. This method was quick, reliable and simple to perform and revealed the presence of phage, spirochetes, spiral bacteria, rods, filaments and other unique morphotypes such as the scutellum bearing filaments. Furthermore, this method aided in identification (e.g. spirochetes, archaebacterial cells with subunit cell wall) and subsequently in the culturing of a number of organisms (e.g. spirochetes). However this procedure was not adequately supplemented by enrichment and isolation of a number of other microorganisms present due to lack of time. Hence the method has been merely used here to demonstrate the amazing microbial diversity present in practically all the springs surveyed.

Electron microscopy of colonized slides indicated the presence of slime and the presence of cell wall subunits on a number of organisms, indicating that these organisms presumably require these structures for attachment. Gram staining of the colonized slides also provided further evidence of the presence of slime around a number of cells. Furthermore Gram staining revealed that the majority of the bacteria were Gram

negative. Gram staining also helped to reveal the presence of scutellum bearing organisms on slides colonized in pool Rt51, and the presence of unique sheathed bacteria from pool Rt8. In both cases, their presence could not be demonstrated using phase contrast microscopy. Similarly, staining improved the chances of observing all the cocci present in slides colonized in pool Rt71, some of which could have been mistaken as particulate material under phase contrast microscopy.

CHAPTER 3

IN SITU MEASUREMENT OF THERMOPHILIC BACTERIAL GROWTH

3.1 INTRODUCTION:

The demonstration of bacterial presence in hot pools, as illustrated in chapter 2 is not unambiguous evidence that such bacteria actually grow in these environments. We might only be capturing the death throes of mesophilic organisms unlucky enough to have fallen into these sites. However, the repeated observation of a particular morphotype from these pools, and the presence of septa in many of the cells can be regarded as good evidence that these bacteria survive and grow in such places. A more direct way of demonstrating that hot pools are habitats for bacteria is to demonstrate bacterial growth in situ. This can be done by microscopic observation, but the method is tedious on the operator, prone to a high degree of error when sediments are involved, and presents logistic difficulties. Initially the measurement of intracellular ATP as an index of active biomass in pool waters was attempted, but extraction problems forced the abandonment of this approach. Subsequently an approach measuring substrate uptake by pool bacteria as an indication of bacterial growth was adopted. ^{14}C or ^3H radioisotope labelled substrates were added to suspensions or films of pool bacteria, and the retention of label used as an index of growth and/or metabolic activity. This is a widely used method in natural ecosystems and has previously been used by Brock et al., (1971) in thermophilic habitats. The results

of uptake experiments on a selected range of pools in the Rotorua area are reported. Pools were selected on the basis of their physical characteristics, especially pH and temperature, and because E.M. observation had indicated bacterial diversity.

3.2 MATERIALS AND METHODS:

3.2.1 Location And Chemical Analysis Of Pools

Eight pools in Whakarewarewa, Kuirau Park and Government Gardens in Rotorua (Table 3-1) were chosen for isotope uptake experiments. Their locations are given in the inside back cover pocket and their chemical analysis, shown in Table 3-1, was done by Ministry of Works (Water and Soil Division, Science Centre, University of Waikato, Hamilton).

3.2.2 Isotope Uptake

In initial experiments only pool water or sediment samples were used. Later, a modification of the method of Brock, et. al., (1971) was used. In this method glass cylinders (20mm in length by 8mm internal diameter and 10mm external diameter) were strung in lots of 50 and were suspended in pools for colonization. After fixed periods of incubations, usually of a week or more, the colonized cylinders were transferred into 7ml bijous bottles or 28ml universal bottles, containing 4ml of spring water, sediment suspension or synthetic media. The bottles were sealed with suba seals, capped and immersed in the spring to equilibrate. After equilibration, isotope was injected into the bijous bottles at a final concentration of 0.1 $\mu\text{Ci/ml}$ for ^{14}C isotopes or 1.0 $\mu\text{Ci/ml}$ for ^3H isotopes. The controls, in addition, were injected with 0.4mls of 40% formalin solution. Bijous bottles were reimmersed into the springs for

Table 3.1: Physical and Chemical Characteristics of Rotorua thermal pools

	Rt3	Rt6	Rt13	Rt14	Rt15	Rt8	Rt56	Rt57
Temp	71°C	70°C	98-100°C	80°C	100°C-102°C	72°C	90.2°C	97°C
pH	7-1	9.4	7.4	7.0	8.9	9.1	7.87	7.2
conductivity (mho)	3030	-	-	-	-	-	-	-
Anions (mM)								
(mM) Cl ⁻	-	-	22.5	23.4	24.1	13.8	-	-
(mM) SO ₄ ⁻⁻	-	-	2.07	1.93	0.97	0.70	-	-
(uM) PO ₄ ⁻⁻⁻	-	-	0.45	1.28	0.54	1.86	-	-
(mM) HCO ₃ ⁻⁻⁻	-	-	1.92	2.46	4.3	8.75	-	-
(uM) NO ₃ ⁻	-	-	-	-	-	0.18	-	-
(mM) SiO ₃ ²⁻	-	-	1.7	1.8	2.26	2.61	-	-
(ug/ml) S ²⁻	3	5	22	4	28	38	16	23
Cations (mM)								
Na ⁺	-	-	26.8	27.8	11.7	25.7	-	-
K ⁺	-	-	1.76	1.76	1.86	0.95	-	-
Mg ⁺⁺	-	-	0.005	0.005	0.004	0.003	-	-
NH ₄ ⁺	-	-	0.03	0.02	44.0	0.007	-	-
Ca ⁺⁺	-	-	0.07	0.07	0.06	0.04	-	-
NH ₄ ⁺ /NO ₃ ⁻	-	-	-	-	-	1.0	-	-
Na ⁺ /K ⁺	-	-	15.2	15.7	6.3	27.0	-	-
Na ⁺ /Ca ⁺	-	-	382	397	195	642	-	-

fixed time intervals. Uptake of isotope by bacteria was terminated by adding 0.4mls of 40% formalin solution. The bijoux bottles were then returned to the laboratory for counting on a scintillation counter.

During the later part of our work, colonized cylinders were transported to the laboratory in thermos flasks containing 80% sediment and 20% pool water to maintain them in conditions similar to those pertaining in the spring for the 3-4 hr time for transport. In the laboratory cylinders were added to bijoux bottles which contained fresh pool water or sediment suspension. The fresh pool water had been collected separately in 500ml bottles and was rendered anaerobic before use by the addition of approx 10ml of 10% $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ per litre. Sediment suspensions were collected in a similar manner but did not require sulphide addition before use, as they were found to have extremely good reducing properties. All transfers to bijoux bottles of cylinders, pool water or sediment were carried out using anaerobic techniques (Appendix 3) and in amounts as described previously. Water baths were used as incubators and temperatures measured by conventional mercury thermometers. Plastic balls or lids were required on water baths at temperatures over 80C.

Isotope uptake by bacteria free in pool water or absorbed to sediments was determined by filtration through a 0.45 μm Millipore filter followed by 3 washes with 3ml of distilled water before filters were counted. Glass cylinders were washed by dipping them into distilled water, and blotting gently on tissue paper to remove excess water before immersing in scintillation cocktail. Filters and cylinders were counted in a scintillation cocktail (Appendix 5) for 10 min on a Packard Tri-Carb3330 scintillation counter with the following window settings:

For ^{14}C :			
Channel	Red	Green	Blue
width	50-1000	50-500	50-300
Gain	23%	23%	23%
For ^3H :			
Channel	Red	Green	Blue
width	50-1000	0	200-1000
Gain	50%	0	10%

3.2.3 Radioisotopes And Chemicals

Sodium acetate- $2\text{-}^{14}\text{C}$, 55mCi/mmol; Sodium pyruvate- $1\text{-}^{14}\text{C}$, 6.6mCi/mmol; D-ribose- $1\text{-}^{14}\text{C}$, 53mCi/mmol; L-leucine- $\text{U-}^{14}\text{C}$, 348mCi/mmol; L-methionine-methyl- ^{14}C , 45.7mCi/mmol; thymidine-methyl- ^3H , 2Ci/mmol and glucose- $\text{U-}^{14}\text{C}$, 5mCi/mmol, were purchased from New England Nuclear, U.S.A.

Ethoxyethanol (scintillation grade), toluene (AR), formaldehyde solution, potassium (+)-tartrate, sodium lauryl sulphate and sodium hydroxide were obtained from BDH, U.K., 1,2 diphenyl oxazole, bovine serum albumin and Folin reagent from Sigma Chemicals, U.S.A; sodium sulphide; $9\text{H}_2\text{O}$ from Ajax Chemicals, Australia, and copper sulphate; $5\text{H}_2\text{O}$ and sodium carbonate from Riedel-De-Haen Ag Seelze, West Germany.

3.2.4 Protein And Sulphide Estimation

Protein was determined using the method of Bott and Brock, (1970). The concentration of the standards used was between 0 to 50 μg and a linear response was obtained for these concentrations. To determine protein concentrations of colonized cylinders or pool water, a set of protein standards was always included in the assay system. Sulphide was determined using the method of Brock et al., (1971).

3.3 RESULTS AND DISCUSSION:

Variability existed between replicates in any individual experiment and between experiments when the level of isotope uptake was compared with samples incubated under apparently identical conditions. To obtain statistically valid results would have required a much greater input of time and resources than was available. However in all the experiments the results clearly indicated ^{14}C -substrate uptake occurred, so that if the method could be improved by reducing variability then valid spring comparisons could be made.

3.3.1 Pools At Temperatures Of Approx 70°C

The pools with temperatures of 70°C (Rt3, Rt6 and Rt8) showed radioactive isotope uptake with pool water, sediment, bacterial mats or colonized cylinders. However, this is not sufficient to allow comparison between pools due to great variability in results as discussed above. Examples of this are discussed below.

(1) Pool Rt 8:

Changes in the use of enclosures for ^{14}C -acetate uptake by bacterial mats in pool Rt8 produced anomalies in results. A much higher and linear response was obtained with ^{14}C -acetate under aerobic conditions when 28 ml universal bottles were used as enclosures (Table 3-2). This is in contrast to extremely low and non-linear response of ^{14}C -acetate uptake response when bijous bottles were used as enclosures (Table 3-3). A plausible explanation is that oxygen may have been a factor limiting ^{14}C -acetate uptake when experiments were conducted in bijous bottles. As can be seen (Table 3-3) no uptake of ^{14}C -acetate occurred under anaerobic conditions. In contrast uptake of ^{14}C -glucose occurred anaerobically but

insignificantly under aerobic condition (Table 3-3).

Table 3-2: Uptake of ^{14}C -acetate by bacterial mats from pool Rt8:

Labelled compound	Time of incubation in hours	counts/min
^{14}C -acetate	Formalin control	159
	1 hr	29,677
	4 hrs	111,727

^{14}C -acetate (0.1 $\mu\text{Ci/ml}$) was added to universal bottles containing 4ml of homogenised bacterial mat. Incubation temperature was 72°C.

Table 3-3: Uptake of ^{14}C -acetate and ^{14}C -glucose by bacterial mats from pool Rt8:

Labelled compound	Time of incubation in hours	counts/min	
		Aerobic	Anaerobic
^{14}C -acetate	Formalin control	573	469
	2 hrs	3040	304
	4 hrs	4075	324
^{14}C -glucose	Formalin control	309	350
	2 hrs	421	759
	4 hrs	453	345

^{14}C -acetate or ^{14}C -glucose (0.1 $\mu\text{Ci/ml}$) was added to 7ml bijoux bottles containing 4ml of homogenised bacterial mat (37 μg of protein). Anaerobic conditions were created by injection of 0.01% Na_2S , 9 H_2O (final concentration) and checked by adding resazurin indicator. Incubation temperature was 72°C.

Considerable variability in isotope uptake was noted for triplicate glass cylinders incubated under presumably identical conditions in Rt8. This variability could be due to differences in degree of colonization of different substrates. Brock et al., (1971) observed similar variability in isotope uptake in replicate colonized coverslips. However, the results here does indicate ^{14}C -uptake with ^{14}C -acetate being taken up more efficiently than glucose under aerobic conditions (Table 3-4).

Table 3-4: Uptake of ^{14}C -acetate and ^{14}C -glucose by colonized glass cylinders in Pool Rt8:

Labelled compound	Time of incubation in hours	counts/min
^{14}C -acetate	Formalin control	53
	2 hrs	281
	4 hrs	616
^{14}C -glucose	Formalin control	56
	2 hrs	107
	4 hrs	133

^{14}C -acetate or ^{14}C -glucose (0.1 $\mu\text{Ci/ml}$) was added to bijou bottles containing colonized glass cylinders. The protein content on each cylinder was 17 μg . The temperature of incubation was 70°C.

At least 8 organisms ranging from strict aerobes to strict anaerobes have been isolated from pool Rt8 which could possibly account for ^{14}C -uptake. For example Thermus (Rt8-AT1) is able to utilize acetate very efficiently under aerobic conditions but not glucose. Bacillus stearothermophilus strain Rt8-AB2 and Thermothrix Rt8-AB1 which are facultative anaerobes can utilize glucose under aerobic or anaerobic conditions. Of the five species of anaerobes present, three species, namely Thermoanaerobium strain 8G4, Clostridium thermohydrosulfuricum strain Rt8-B1 and Fervidobacterium (Chapter 7) can utilize glucose but not acetate anaerobically. The remaining two species namely a thermophilic sulphate-reducing species (Morgan, unpublished results) and a sulphur-respiring rod shaped bacterium utilize neither glucose nor acetate aerobically or anaerobically.

(2) Pool Rt3:

Under aerobic incubation conditions no uptake of ^{14}C -glucose occurred with pool water alone, whereas uptake by pool water organisms was detected when ^{14}C -acetate was used. In contrast, the presence of sediment in a separate series of bijou bottles gave a very pronounced ^{14}C -glucose uptake, with acetate uptake remaining unaltered (Table 3-5). This might suggest the presence of two types of metabolically active organisms in this pool, an aerobe or facultative anaerobe, which can metabolise ^{14}C -acetate, and an obligate anaerobe which can utilize ^{14}C -glucose. The presence of sediment in these experiments being capable of reducing the environment in the bijou bottles to an Eh suitable for anaerobic growth. An alternative suggestion would be that the sediment contains species of bacteria which are not present in overlaying pool water.

Table 3-5: Uptake of ^{14}C -acetate and ^{14}C -glucose by bacteria in pool Rt3:

Labelled compound	Time of incubation in hours	counts/min	
		Pool water (aerobic)	Pool water and sediment (anaerobic)
^{14}C -acetate	Formalin control 0 hrs	701	563
	2 hrs	1505	1854
^{14}C -glucose	Formalin control 0 hrs	1385	1542
	2 hrs	1269	23978

^{14}C -acetate or ^{14}C -glucose (0.1 $\mu\text{Ci/ml}$) was added to bijou bottles containing 4ml of pool water or 3ml of pool water plus 1ml of sediment. The temperature of incubation was 70°C.

Different bacterial morphotypes have been observed in this pool. At least 4 species of bacteria have been isolated from pool Rt3 which may be active in ^{14}C -uptake. These include a strictly aerobic Thermus strain Rt3-AT which grows extremely well on acetate but poorly on glucose, a facultative anaerobe Rt3-AB1 (chapter 8) and two strictly anaerobic glycolytic anaerobes which includes a spirochete isolate and Caldobacterium species (chapter 7).

(3) Pool Rt6:

Limited studies on the brownish bacterial mat with regard to radioactive isotope uptake have been done. ^{14}C -acetate was incorporated by these mats.

At least three types of bacteria have been isolated from this pool; a facultative Thermothrix strain (chapter 8), an aerobic Thermus strain and a strictly anaerobic glycolytic rod shaped bacterium (chapter 7).

The results of ^{14}C -uptake by bacterial population in pools Rt3, Rt6 and Rt8 were further compounded by the fact that considerable fluctuations in these pools occurred during the time of these investigations. For example, pool Rt3 cooled and the level dropped leading to changes in microbial flora. Similarly, pools Rt6 and Rt8 were runoff channels from vents of heat exchangers. Sometimes the vents were shut off and this lead to the drying up of the channels and subsequently the disappearance of bacterial mats (chapter 2).

3.3.2 The Higher Temperature Pools Of 80-100°C

Initial experiments with pools at temperatures of 80°C to 100°C (Rt13, Rt14, Rt15, Rt56 and Rt57) showed no isotope uptake using pool water, sediment slurry or colonized cylinders indicating that the microbial flora was different to that of the pools at temperatures of 70°C. A variety of modifications to the experimental system (described below) failed to demonstrate any radioactive isotope uptake. The following modifications used were used:

(1) 28ml universals bottles in conjunction with precolonized cylinders were used as enclosures. 10ml of aerated pool water and varying amounts of freshly prepared sulphide solution was added so that sulphide could provide an energy source for bacterial growth aerobically. This was found to be requirement for stimulation of isotope uptake as demonstrated by Brock, et al., (1971), but in this case no radioactive uptake occurred.

(2) No uptake occurred when ^{14}C -glucose or ^{14}C -acetate were substituted for other ^{14}C -substrates such as D-ribose, L-methionine, pyruvate or L-leucine.

(3) Temperature variations from 85°C to 100°C or prolonging incubation time from 4 hours to 8 or 16 hours did not demonstrate radioactive isotope uptake.

(4) Varying the amounts of "cold" acetate or glucose did not help in obtaining uptake.

Having repeated some of the experiments several times with no apparent success, it was decided to discontinue this work for sometime, while other methods were used to demonstrate bacterial growth or growth rates at these high temperatures. Two methods were used for this purpose: (1) Enrichments with different growth substrates and (2) Immunofluorescence. Anaerobic enrichments done at 92°C or 98°C were positive from 26 of the 28 pools analysed but none were positive aerobically indicating that strict anaerobic conditions were essential. Methods and results of these two methods will be discussed fully in chapters 5 and 8 respectively.

However, armed with the successful enrichments at 98°C experiments on isotope uptake were reinitiated but strict anaerobic techniques were used as described in Appendix 3. Initial experiments were done on two pools namely Rt 13 and Rt57.

(1) Isotope uptake in pool Rt13:

No isotope uptake was detected when radioactive D-ribose, L-methionine, L-leucine D-glucose and pyruvate were used. The optimum temperature for ^{14}C -acetate uptake was 95°C (Figure 3.1) at an optimum pH of 7.3 (Figure 3.2) but when ^3H -thymidine was used, maximum incorporation occurred at 90°C (Figure 3.1). The presence of air or oxygen in the gas phase of the incubation vessels, in place of nitrogen, resulted in complete inhibition of isotope uptake confirming our enrichment experiment conclusions that only anaerobes were metabolically active in the higher temperature pools.

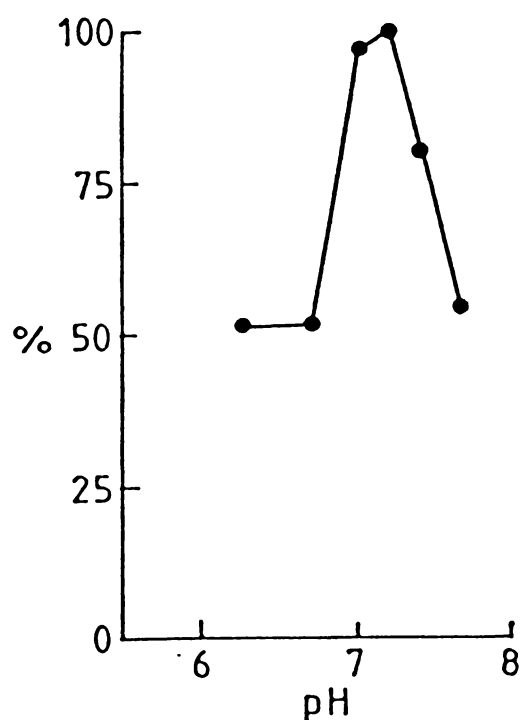
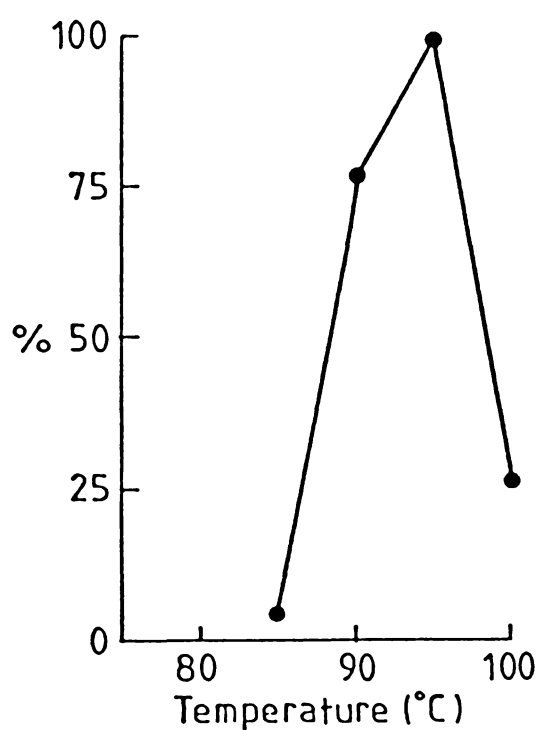


Figure 3.1 Temperature optima for isotope uptake by the anaerobic bacteria from pool Rtl3 using ^{14}C -acetate (top) and ^3H -thymidine (bottom) as labelled substrates. The time of incubation was 4 hours. The 100% values are (in cpm/cylinder) 1786 for ^{14}C -acetate and 610. for ^3H -thymidine.

(Refer errata and addenda on page 11-1)

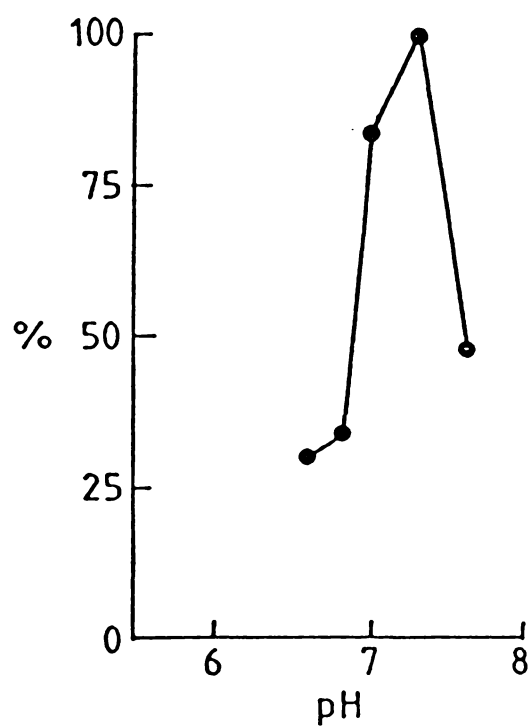


Figure 3.2 pH optimum for isotope uptake by anaerobic bacteria from pool Rt13. ^{14}C -acetate was the labelled substrate used. The time of incubation was 4 hours at 95°C . The pH was adjusted to appropriate values with 0.1M NaOH or 0.1M HCl and were fortified by adding appropriately pH adjusted 0.2M MOPS buffer at a final concentration of 0.005M. 100% value is 268 cpm/cylinder.

(2) Isotope Uptake in Pool Rt57:

Similar results were obtained with isotope experiments performed with anaerobic bacteria from pool Rt57. The temperature optimum for ^{14}C -acetate uptake was 95°C and the pH optimum was 7.2 (Figure 3.3).

3.4 CONCLUSIONS:

Results obtained by isotope uptake experiments indicated that in pools with temperatures below 80°C both aerobes and anaerobes were metabolically active, whereas in pools above 90°C anaerobic bacteria alone were responsible for isotope uptake. This latter finding contrasts with the results obtained by Brock et al., (1971) who reported metabolism in springs at 90°C under aerobic conditions.

The results in this chapter also indicate that microbial activity in most springs resided in sediment bound bacteria rather than those living free in the pool water. Sampling sediments to measure isotope uptake rates was very difficult due to poor reproducibility and non-specific absorption of isotope to sediment particles. To overcome these problems a modification of the approach of Brock et al., (1971), of colonizing glass cylinders of known surface area, was used in pools to simulate a sediment surface area. Great difficulties have been encountered in obtaining repeatable results and the method probably requires further development before reliable conclusions can be drawn.

Future work should ascertain whether isotope uptake is related to colonization rate (as measured by protein content of cylinders). We routinely assayed cylinders after 7-10 days colonization but this may not have been the optimum period. Also, for high temperature pools especially the need for rigorous exclusion of oxygen will probably be

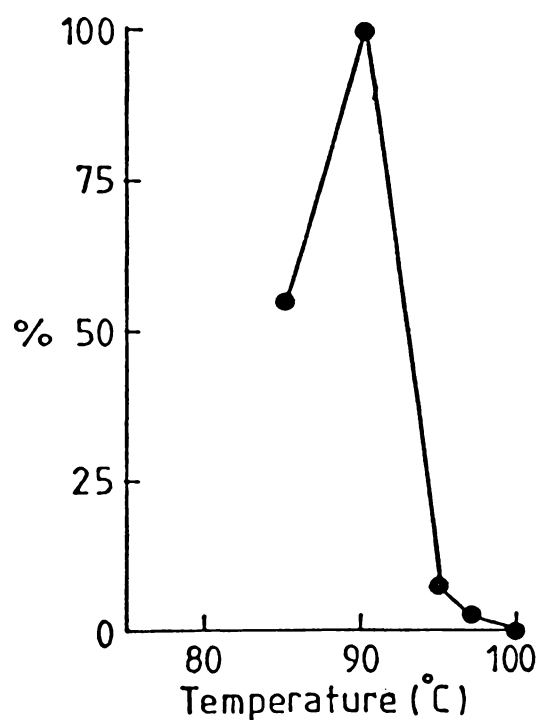
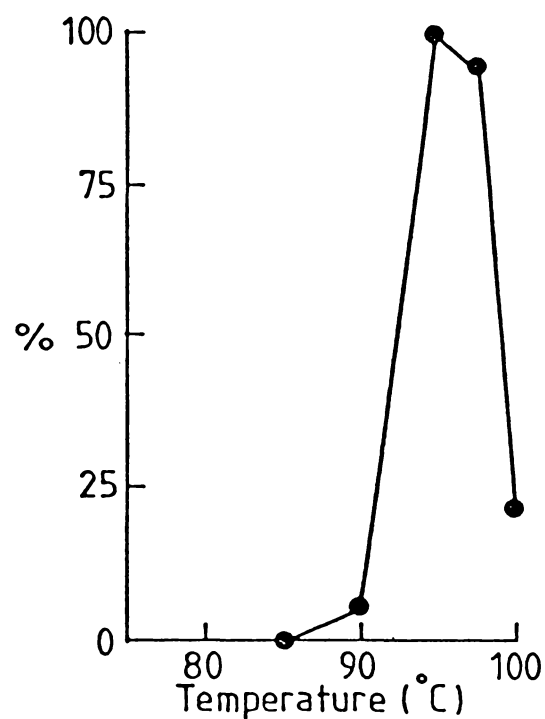


Figure 3.3 Temperature and pH optima of anaerobic bacteria from pool Rt57 (top and bottom respectively). Labelled substrate used was ^{14}C -acetate. Incubation conditions and pH adjustments were as described in figure 25 and figure 26. 100% value for temperature was 696 cpm/cylinder and for pH was 6758 cpm/cylinder.

(Refer errata and addenda on page 11-1)

required to obtain reproducible results. If these problems can be overcome then the isotope uptake method could provide information on the biomass activity in different pools and the relative contributions of aerobes and anaerobes. Together with temperature optima and pH optima curves for isotope uptake in situ comparisons could be made with cultured isolates to identify the dominant species.

CHAPTER 4

IMMUNOFLUORESCENCE

4.1 INTRODUCTION:

The enumeration of bacteria in aquatic and terrestrial habitats is most often accomplished by epifluorescent direct counts with acridine orange stain (Francisco, et al., 1973; Watson, et al., 1977; Hobbie, et al., 1977). This method is based on the interaction of acridine orange with nucleic acids. More recently, a number of fluorochromes with various specificities for biological material have been used for biomass estimation. These include fluorescein diacetate (Jarnagin and Luchsinger, 1980), fluorescein isothiocyanate (Fliermans et al., 1975) and Hoechst dyes 33258 and 33342 (Paul, 1982). However the full extent of their potential in ecological studies is yet to be investigated.

The major disadvantage of using fluorochrome dyes is their lack of specificity, that is the organisms present can be detected and enumerated but not identified.

Methanogenic bacteria possess unique coenzymes which have the ability to fluoresce under UV light (Edwards and McBride, 1975; Mink and Dugan, 1977; Doddema and Vogels, 1978). This feature has been exploited for the simultaneous detection and enumeration of methanogens but is of little value for studies of other bacteria because of the absence of these unique coenzymes.

The fluorescent-antibody (FA), or immunofluorescence (IF) technique provides a highly sensitive method for simultaneous detection and identification of specific micro-organisms directly in the natural sample (Strayer ands Tiedje, 1978; Schmidt, et al., 1968; Hill and Gray, 1967). The technique has been routinely used in clinical laboratories for identification of bacterial (Cherry, et al., 1978) and viral infections (Liu, 1969). It was not until recently that this technique gained recognition in the study of microbial autecology and various applications of its use in this field have been reviewed by Bohlool and Schmidt (1980).

Two methods are presently used for fluorescent antibody staining, the "direct" method and the "indirect" method. For the direct FA technique, the antibody against the microorganism of interest is itself labelled. A number of procedures are available for preparing labelled antibody (Peacock et al., 1971; Rinderknecht, 1962; Calcagno et al., 1973; Reisberg et al., 1970; Lewis et al., 1964). In the indirect or the "sandwich" method, the specific antibody is applied unlabeled and is detected on the surface of the cell by a second antibody which is fluorescent and which is directed against the specific antibody. The indirect FA method was used for studies of hot pool bacteria.

A problem encountered during direct or indirect FA staining of natural samples for bacterial identification and enumeration, was nonspecific staining reactions (Pittman et al., 1967; Schmidt et al., 1968). Bohlool and Schmidt, (1968, 1970) were able to improve this to an acceptable level by the use of alkali hydrolysed gelatin solution added to the sample prior to the addition of the fluorescent antibody.

The only report to date, using the FA staining (direct) technique, in the study of ecology of thermal springs, has been on the growth rates and serology of an extremely thermophilic acidophilic bacterium, Sulfolobus acidocaldarius present in the hot springs of New Zealand (Bohlool, 1975) and U.S.A. (Bohlool and Brock, 1974). Hence, it was of interest to extend this work to include New Zealand isolates other than Sulfolobus acidocaldarius and to determine their growth rates and distribution in New Zealand hot springs.

4.2 MATERIALS AND METHODS

4.2.1 Study Area

The sites for "in situ" studies using immunofluorescence were located in the thermal areas of Rotorua (Whakarewarewa, Kuirau Park and Government Gardens) and Waimangu. The location, temperature and pH of these springs is summarised in the table in the inside back pocket cover.

4.2.2 Source Of Cultures

All the cultures used for preparing antibodies were isolated from New Zealand hot springs. Rt15-AT was a Thermus species (chapter 8), Rt8-AB1 was a Thermothrix species (chapter 8), Wai21-H1 was a hydrogen oxidising Bacillus species (chapter 8), AN1 was an anaerobic coccal shaped archaebacterial species (chapter 5), OK16-A1 was an acidophilic Bacillus acidocaldarius strain (chapter 8) and Tok6-B1 was a glycolytic Thermoanaerobium species (chapter 7). Isolates which were used to check the specificity of the antibodies were also isolated from New Zealand hot springs. Others strains were obtained from the American Type Culture Collection, U.S.A. and represent type cultures of some of the

thermophiles. Growth media and growth conditions used for these organisms are dealt with in the relevant chapters indicated in brackets.

4.2.3 Chemicals

Fluorescein labelled sheep anti-rabbit immunoglobulin was purchased from Wellcome Reagents Ltd, U.K.; acetone from BDH, U.K.; Sephadex G25 from Pharmacia Uppsala, Sweden and Fluorescein isothiocyanate isomer I (10% on celite) from Sigma Chemical Co., U.S.A.

4.2.4 Immunofluorescence Procedures

The above mentioned bacterial strains were grown in 5 litre quantities. After reaching the stationary phase the cells were harvested by centrifugation at $27,000 \times g$ for 30 min. The pellets were washed in saline (0.15 M NaCl), respun at $27,000 \times g$ for 30 min, suspended in 10 ml saline, and frozen at -20 degrees centigrade until required. Antibodies were prepared in young adult New Zealand Albino rabbits with a 2 ml intravenous injection of a 1:1 emulsion of antigen:Freund incomplete adjuvant (Difco Laboratories). Weekly booster doses were injected for a further 4 week period after which the antibody levels were checked by withdrawing 3 to 5 ml of blood from the marginal ear vein. Since the titres obtained were adequate no further booster doses were required and the blood was collected by cardiac puncture. The blood was allowed to coagulate for two hours at room temperature and was stored at 4 degrees centigrade for 8 hours. Supernatant serum was removed by sterile pipetting and the remainder was removed after centrifuging at $1100 \times g$ for 20 min, taking care to avoid haemolysis. Portions of 0.5 to 1 ml were dispensed in vials and heated to 56 degrees centigrade for 20 min and stored at -18 degrees centigrade until required.

Laboratory grown pure cultures or microscope slides which had been incubated in hot pools for colonization by bacteria (as described in chapter 2, section II) for between 3 and 35 days, were used in the following protocol.

(1) Circles were scratched onto slides in triplicates. Normally 6 sets of triplicates were scratched per slide and 6 cultures (or 6 different antibodies) were applied per slide. The grooves made by the scratches allowed the cultures (or the antibodies) to be held within the circle thereby eliminating any chance of cross contamination of cultures (or antibodies) with each other. Colonized slides were treated in the same manner.

(2) Samples of pure cultures were allowed to air dry in these circles and were then fixed with acetone for 10 minutes in slide staining jars. Colonized slides did not require this treatment since they were fixed with formalin after being retrieved from the pools as previously described (Refer chapter 2, section II).

(3) The slides were then removed from the staining jars and allowed to air dry. Appropriate antibody containing sera was then placed in a set of triplicate circles. At this stage different types of antibody containing sera could be used in each set of triplicate circles. The slides were incubated in a humidity chamber at 39 to 40°C for 30 minutes.

(4) After the incubation period, the slides were removed and washed first in standard Phosphate Buffered Saline (PBS) for 10 minutes, and then in distilled water for 5 minutes.

(5) The slides were allowed to air dry and fluorescein labelled (sheep) anti rabbit immunoglobulin was applied. The slides were then returned to the humid chamber for a further incubation of 15 to 30 minutes.

(6) The process, as described in step 4 was repeated, the slides dried and mounted in a drop of glycerol-PBS (pH 9.0) using a cover slip (22mm x

60mm, No. 2)

A Leitz Dialux incidence fluorescence microscope (50W high pressure mercury lamp; blue BG38 filter) was used for observing fluorescence and 200ASA Actachrome film was used for photomicrography.

To control non specific staining of particulate material from slides colonized in hot pools, the method of Bohlool and Schmidt, (1968) was used.

An attempt was also made to conjugate Rt15-AT antibodies with fluorescein isothiocyanate (10% on celite) using the method of Rinderknecht, (1962) for direct FA staining.

4.3 RESULTS:

4.3.1 Potency And Specificity Of Antibodies Of Extreme Thermophiles

The potency and the usable titre of each antibody was tested using the indirect FA test (Table 4.1). The response of antibody production (titre) by all the extreme thermophiles was good and with the exception of the anaerobic cocci, was 1:960. The titre produced by the anaerobic cocci was 1:480. The usable titre for FA tests was between 1:60 to 1:120.

Table 4.1: Antibody potency of some extreme thermophiles:-

Antibody produced against	Titre produced	Usable dilution
<u>Thermus</u> strain Rt15-AT	1:960	1:60 to 1:120
Anaerobic cocci AN1	1:480	1:60
<u>Thermoanaerobium</u> species Tok6-B1	1:960	1:120
<u>Bacillus</u> strain Wai21-H1	1:960	1:60
<u>Bacillus acidocaldarius</u> strain OK16-A1	1:960	1:60
<u>Thermothrix</u> strain Rt8-AB1	1:960	1:50

Most of the studies of FA reactions on pure cultures were conducted with Thermus species Rt15-AT (Table 4.2). FA reactions showed that Rt15-AT antibodies were not strain specific but were species specific as evidenced by different fluorescence intensities obtained with different species or strains used. For example, a +4 reaction was obtained with the Thermus species, Rt6-AT (Figure 4.1) compared to a +1 reaction with the Thermus species, Wai 16-AT (Figure 4.2) or a 0 reaction with Thermus thermophilus.

Similarly, the antibodies were extremely specific at the genus level and did not cross react with a limited number of other thermophiles tested which included aerobic Bacillus species and anaerobic glycolytic bacteria.

Table 4.2. Specificity of antibodies raised against Thermus strain Rt15-AT with other thermophilic isolates:-

Species tested	Reaction (IF)
<u>Thermus</u> strain Rt1-AT	+1
<u>Thermus</u> strain Rt4-AT	+2
<u>Thermus</u> strain Rt6-AT	+4
<u>Thermus</u> strain Rt7-AT	+3
<u>Thermus</u> strain Rt14-AT	+1
<u>Thermus</u> strain Rt15-AT	+4
<u>Thermus</u> strain Tok2-AT	+3
<u>Thermus</u> strain Tok4-AT	+2
<u>Thermus</u> strain Tok5-AT	+4
<u>Thermus</u> strain Tok7-AT	+2
<u>Thermus</u> strain Tok8-AT	+4
<u>Thermus</u> strain TP7-AT	+1
<u>Thermus</u> strain OK7-AT	0
<u>Thermus</u> strain OK8-AT	+2
<u>Thermus</u> strain OK12-AT	+2
<u>Thermus</u> strain OK15-AT	+3
<u>Thermus</u> strain Wai16-AT	+1
<u>Thermus</u> strain Wai19-AT	+2
<u>Thermus</u> strain Wai21-AT	+4
<u>Thermus aquaticus</u> ATCC25104	+4
<u>Thermus thermophilus</u>	0
<u>Bacillus</u> strain TP10-H1	0
<u>Bacillus</u> strain Wai21-H1	0
Anaerobe Rt17-B1	0
<u>Thermoanaerobium brockii</u>	0

The results of antibody specificity of the anaerobic coccus, AN1 is given in Table 4.3. AN1 antibodies were extremely specific and did not cross react with coccal and rod shaped aerobic or anaerobic acidophiles. AN1 type isolates showed differing fluorescence intensities indicating that AN1 antibodies were not strain specific. A typical +4 reaction obtained with Rt12-BC1 is shown in figure 4.3.

Facing Page 4-9

Figure 4.1 FA stained cells of Thermus Rt6-AT showing a strong +4 reaction.

Figure 4.2 FA stained cells of Thermus Wai16-AT showing a very weak +1 reaction.

Figure 4.3 FA stained cells of an archaebacterial coccus Rt12-BC1 showing a strong +4 reaction.

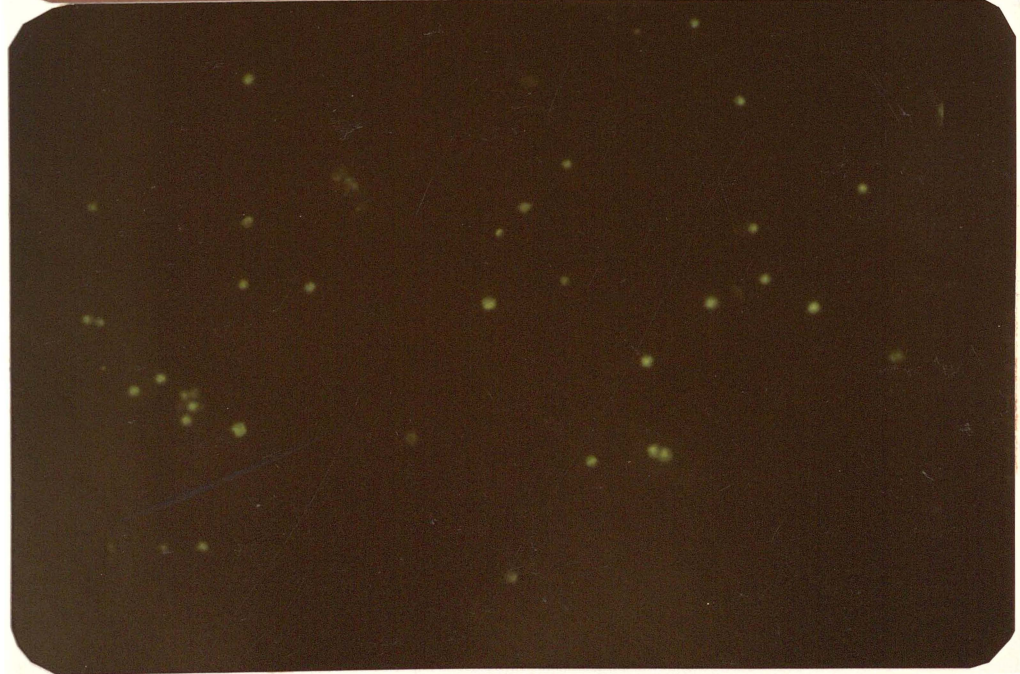
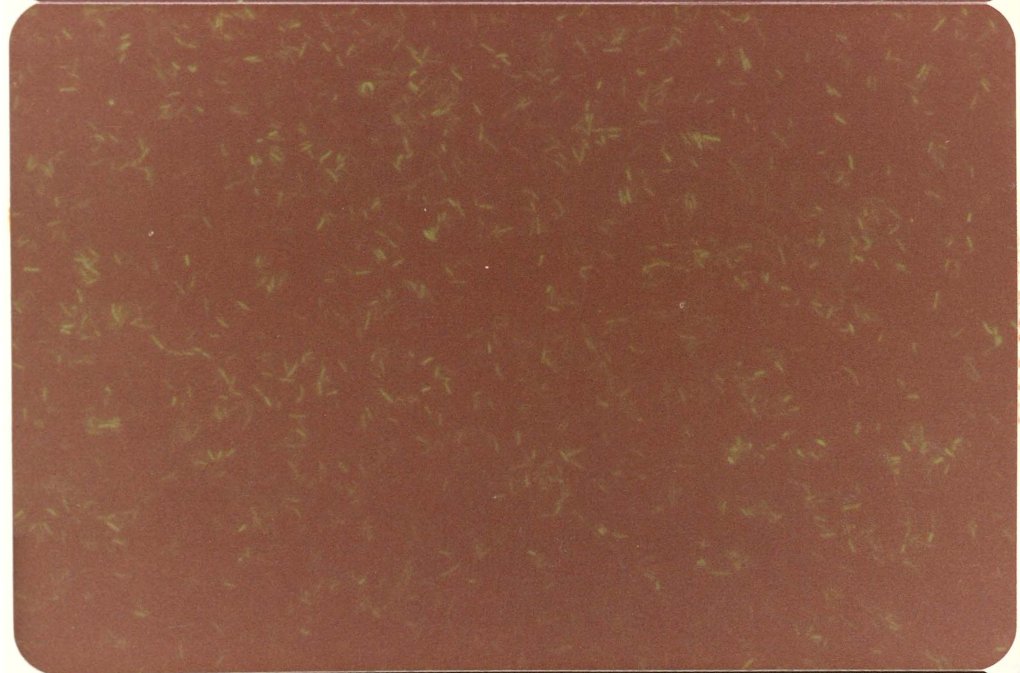
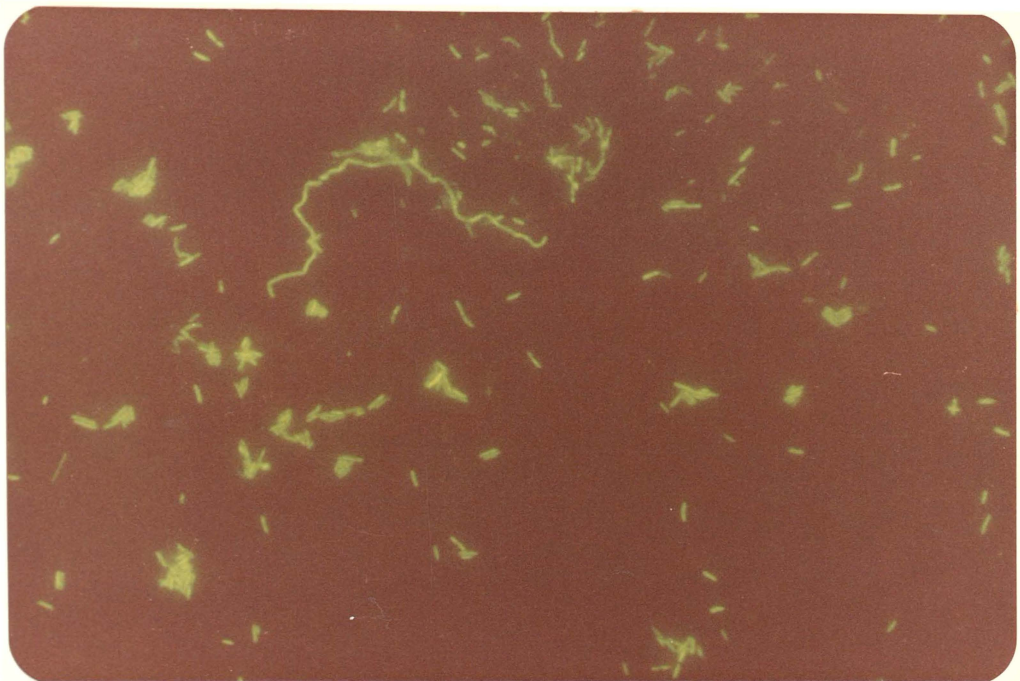


Table 4.3: Specificity of antibodies raised against anaerobic cocci AN1 with other thermophilic isolates:-

Species tested	Reaction (IF)
<u>Strains similar to AN1:</u>	
Rt12-BC1	+4
Rt14-BC1	+1
Rt46-BC1	0 to +1
Rt48-BC1	0
Rt50-BC1	+3
Rt51-BC1	+2
Rt100-BC1	+1 and +2
Tok4-BC1	+2
Tok10-BC1	+3 and +4
Tok11-BC1	+2
Tok14-BC1	+1
WIs4-BC1	+1
<u>Sulfolobus</u> strain Tiki4	0
<u>Bacillus acidocaldarius</u> strain TA6	0
<u>Bacillus acidocaldarius</u> strain WIs11	0
<u>Thermus</u> strain Rt15-AT	0
Acidophilic anaerobic coccus WIs2	0
Acidophilic anaerobic coccus WIs3	0

Thermoanaerobium strain Tok 6-B1 antibodies were observed not to be totally species specific (Table 4.4). The antibodies were found to be strongly FA positive against the glycolytic anaerobe Thermoanaerobacter ethanolicus and weakly FA positive against Clostridium thermohydrosulfuricum strains E100-69 and E2-B1. However, the antibodies were totally non-reactive against another glycolytic anaerobe, Rt17-B1, and against Thermoanaerobium brockii. Similarly, the aerobic extreme thermophiles including Thermus and Bacillus species were found to give a 0 FA reaction.

Table 4.4: Specificity of antibodies raised against Thermoanaerobium strain Tok6-B1 with other thermophilic isolates:-

Species tested	Reaction (IF)
<u>Thermoanaerobium</u> strain Tok6-B1	+4
<u>Thermoanaerobium brockii</u>	0
<u>Thermoanaerobacter ethanolicus</u>	+3
<u>Clostridium thermohydrosulfuricum</u> neotype strain E100-69	+1
<u>Clostridium thermohydrosulfuricum</u> strain E2-B1	+1
Anaerobe Rt17-B1	0
<u>Thermus thermophilus</u>	0
<u>Thermus</u> strain Wai 19-AT	0
<u>Bacillus</u> strain Tp 10-H1	0
<u>Bacillus</u> strain Wai 21-H1	0

Antibodies prepared against Bacillus strain Wai21-H1 were found to be species . specific (Table 4.5). A 0 FA reaction was observed with Thermus species, other Bacillus species, Thermothrix species and Thermoanaerobacter ethanolicus.

Table 4.5: Specificity of antibodies raised against Bacillus strain Wai21-H1 with other thermophilic isolates:-

Species tested	Reaction (IF)
<u>Bacillus</u> strain Wai 21	+4
<u>Bacillus</u> strain TP10-H1	+2
<u>Thermus thermophilus</u>	0
<u>Thermus</u> strain Wai19-AT	0
<u>Thermus aquaticus</u> ATCC25104	0
<u>Thermothrix</u> Rt8-AB1	0
<u>Thermoanaerobacter ethanolicus</u>	0
Anaerobe Rt17-B1	0

The limited number of FA tests carried out with Bacillus acidocaldarius strain OK-16A1 indicated that the antibodies were species specific. No cross reaction was observed with Sulfolobus strain Tiki4 or with Thermus thermophilus and Thermus strain Rt1-AT. However, no tests were performed to determine specificity within Bacillus acidocaldarius strains.

The specificity of Thermothrix strain Rt8-AB1 antibody was not determined.

4.3.2 FA Tests On Natural Populations Of Hot Spring Bacteria

Results obtained from indirect FA tests on slides incubated in pools for varying time intervals are given in table 4.6. All the results obtained were negative or weakly positive.

Table 4.6: Results of indirect FA reactions against natural population of extremely thermophilic bacteria:-

Pool	Incubation of slides (days)	Antisera used*					
		Tok6	Rt15	OK16	RT8	Wai21	AN1
Rt3	3	O	O	O	O	O	NT
Rt6	3	O	+1	O	O	O	NT
Rt8	3	O	+2	O	NT	NT	NT
Rt12	2	NT	NT	NT	NT	NT	O
	3	NT	O	NT	NT	NT	O
Rt13	3	NT	O	NT	NT	O	NT
	5	O	O	NT	NT	O	O
	17	NT	O	NT	NT	O	NT
	35	NT	O	NT	NT	NT	NT
Rt14	1	NT	+1	NT	NT	O	few+1
Rt15	3	NT	O	NT	O	O	O
	5	NT	O	NT	NT	NT	O
	20	NT	O	NT	NT	NT	O
Rt46	2.5	NT	O	NT	O	NT	O
Rt56	2.5	NT	O	NT	O	O	few+2,+1
Rt99	3	NT	O	NT	NT	NT	O

* indicates strains used. Tok6=Thermoanaerobium strain Tok6-B1, Rt15=ThermusRt15-B1, OK16=Bacillus acidocaldarius strain OK16-A1, Rt8=Thermothrix strain Rt8-AB1, Wai21=Bacillus strain Wai21-H1 and AN1=anaerobic archaeobacterial coccus. NT = not tested

4.4 DISCUSSION

A prerequisite for the use of this procedure in autoecological studies is the demonstration that species specific but not strain specific antibodies can be raised against the organism under investigation. This proved to be the case with the six organisms chosen for study. Antibodies of high titre but little cross reactivity between species were obtained with the results for the Thermus Rt15-AT antibody demonstrating the applicability of the antibody to many Thermus isolates (Table 4.2).

However, when these antibodies were used against slides colonized by hot spring bacteria little evidence of immunofluorescent staining was found. This means that the organisms used were either not present in the pools used for slide colonization, that they were there in numbers below the detectable level of the method, or that the method has failed for some reason when used in situ. Colonization of slides was evident under phase contrast microscopy and hence the possibility that the slides were not colonized was ruled out. However, the species against which the antibodies were raised may not have colonized.

Species diversity in thermal environments, or as a matter of fact, in any extreme environment, is much lower than that found in marine or soil environments. Hence, the ecological theory of autogenic succession (Odum, 1969) may not apply to extreme environments. Slides which were colonized for varying time periods (from between 3 to 35 days) and then FA stained were found to give a 0 reaction, ruling out the possibility of autogenic succession. The time period allowed for incubation (35 days) would have allowed bacteria with low generation times to colonize and grow.

In many cases pools were selected from which the test organisms had been originally isolated, and usually on more than one occasion. Because this method had been shown to be successful in New Zealand hot springs for Sulfolobus by Bohlool, the disappointing results obtained were unexpected. Sulfolobus exists in low pH, high temperature pools where species diversity is much lower, which might have avoided problems we have encountered. The pools sampled in this study were generally of lower temperature and greater species diversity (with the exception of pools Rt13 and Rt15). For example more than seven different species have been isolated from pool Rt8, including some of the isolates used to generate antibodies, yet the level of fluorescence detected was low. A possible explanation of this is that culturing the organism under laboratory conditions leads to the expression of different antigenic groups at the cell surface from those normally produced in situ. Thus although good titres are evident with the laboratory reference strain, a much lower titre will exist with the in situ organisms.

The more plausible explanation for results is the choice of strains against which antibodies had been prepared. The immunofluorescence technique was chosen as a tool for ecological investigation in 1981. At this stage very little was known about the species diversity of either New Zealand or overseas thermal spring environments. Organisms isolated from slightly acidic to alkaline hot springs usually turned out to be Thermus species whereas the acidic pools usually harboured Bacillus acidocaldarius and Sulfolobus strains. All of these species were aerobes and it was assumed that these organisms were the predominant microbial flora of the thermal springs. However, this was found not to be the case. Isotope uptake experiments (chapter 3) and the use of a number of enrichment media for extensive cultural studies (chapter 5) conducted

later in the investigations of hot spring bacteria, provided evidence that the microbial flora, especially in the higher temperature pools was made up of archaeobacterial anaerobic species. With the exception of Clostridium thermohydrosulfuricum and Thermoanaerobium brockii strains nothing was known about the ecology of anaerobes in these environments when these experiments on immunofluorescence were initiated. For example, cultural studies have shown that out of the 40 pools screened for the presence of Thermothrix species, only 3 were positive for their presence (chapter 8). Similarly, out of the 266 pools enriched for the presence of Thermoanaerobium strains, only 5 springs yielded such species (Chapter 7). More recently, Kristjansson and Alfredsson, (1983) provided evidence that the incidence of Thermus was greater in springs having temperatures of 55 to 85°C and pH of 6.5 to 10 and their presence in springs at temperatures higher than 85 degrees centigrade was possibly due to their growth in regions of lower temperatures, around the boiling water source and their subsequent leaching into the source, possibly by splashing of the boiling water. The presence of Thermus Rt15-AT could have possibly been occurring on the walls of the spring and hence the negative result obtained with FA staining of colonized slides from pool Rt15 (Table 4.6).

This technique warrants further investigation. With our present knowledge of the springs the method could be checked by using a high temperature spring with only a single dominant organism e.g. spring Rt13 or others listed in Table 4.6, and comparing titres against laboratory and in situ cultures.

PART B

ISOLATION AND CHARACTERIZATION

The only known genera of extreme thermophiles known when this project was initiated, were the aerobic Thermus and Bacillus species and the anaerobic Clostridium and Thermoanaerobium species. All these microorganisms grew optimally at neutral pH and had temperature optima of 65°C to 70°C.

However, in situ EM studies revealed the presence of unidentified bacteria present in the New Zealand thermal springs. Furthermore, the negative results obtained by immunofluorescence procedures on colonized slides raised the possibility that organisms such as Thermus species, Bacillus acidocaldarius and Bacillus stearothermophilus present in the thermal springs were not the dominant microbial flora but were routinely isolated because of the cultural conditions used.

The chapters which follow, describe various procedures used for enrichment of physiologically distinct groups of extreme thermophiles. However, since the physiology and habitat of the aerobic but not the anaerobic microbial flora was thoroughly investigated by Brock, less attention was paid to the former group. Additionally, it was assumed that because of the reducing conditions which existed in thermal springs (due to the presence of H₂S and high temperatures), these would be ideal niches for the growth of anaerobes.

CHAPTER 5

ISOLATION AND CHARACTERIZATION OF ARCHAEBACTERIA CAPABLE OF GROWTH ABOVE 85°C

5.1 INTRODUCTION:

The earth is four and a half billion years old. Evidence from the fossil record has revealed that the first life forms, in the form of bacteria, evolved three and a half billion years ago. (Walter et al., 1980). However, the evolution of more complex life started only 600 million years ago. A great deal of information has been amassed on the process of the latter stage of evolution. Data on the evolution of microorganisms, however, is relatively scarce and most of it has been obtained from fossilized bacterial mats known as stromatolites. These ancient forms are very similar to the present day photosynthetic bacteria. The reason for the paucity of knowledge on evolution of bacteria is that bacterial fossils are not informative structures and the only information obtainable from them is the simple morphology of the cell. The evidence from these types of studies pointed to two fundamental groups of kingdoms of life forms - procaryotes a primitive group and eucaryotes a more advanced group which evolved from the procaryotes. However, this concept of procaryote - eucaryote dichotomy has come under scrutiny recently. This is due to the realisation that (i) to understand the phylogeny of organisms, the elucidation of structure and composition of DNA and RNA is necessary and (ii) that RNA,

especially 16S rRNA, appears to be a more favourable "chronometer" for such studies than those previously used eg. cytochrome c sequencing (Fox et al., 1977; Woese, 1982). This, in conjunction with advances in sequencing techniques of these macromolecules has revolutionised studies on the pylogeny of organisms. Analysis based on 16S ribosomal RNA sequencing has revealed a third line of descent called archaeobacteria.

Initially, methanogens, and two thermophilic bacteria namely Sulfolobus and Thermoplasma, were the first groups of bacteria included in the kingdom Archaeobacteriae (Woese and Fox, 1977) but since then, more bacteria have been included in this group, all of which have been isolated from extreme environments (Oren, 1983; Soliman and Truper, 1982; Rodriguez-Valera, et al., 1983; Stetter, 1982; Zillig et al., 1981a, 1982a, 1983a,b). These findings have made palaeontologists reexamine fossil evidence with more attention and the evidence obtained from such close scrutiny has confirmed the three kingdom concept (Pflug, 1982).

There is increasing evidence for the existence of three primary lines of descent as first proposed by Woese and Fox (1977). However, the relationships of these phylogenetic lines have been unclear. In certain respects, the archaeobacteria resemble eubacteria (e.g. in some aspects of the translation and transcription machinery) whereas in others they resemble eucaryotes (e.g. sequence of 5S rRNA, ribosomal 'A' protein, presence of introns, and histones etc). However, archaeobacteria also contain many unique characters not shared by either eucaryotes or eubacteria (eg. absence of peptidoglycan cell walls, presence of ether-linked lipids in their cell envelope, presence of characteristic tRNAs and rRNAs, unique metabolic pathways and their presence in unusual habitats).

(A) Resemblance of archaeobacteria to Eubacteria:

Eubacteria and archaeobacteria resemble each other in morphological diversity and certain aspects of the translation and transcription machinery. Archaeobacteria and eubacteria both possess similar sized ribosomes (70S), ribosome subunits (50S and 30S) and the three (16S, 23S and 5S) RNA molecules (Woese et al., 1978).

Additionally the coding sequence of 16S-23S-5S in the ribosomes of Halobacterium halobium and Methanococcus vannielli, and the 5S rRNA-protein (2-3 r-proteins bound to 5S rRNA) complex of H. cutirubrum are similar to that of the eubacterial ribosomes (Hofman et al., 1979; Schmid et al., 1982). Furthermore, reconstitution of archaeobacterial 5S rRNA can occur with eubacterial 50s ribosomal component but not with eucaryotic 5S rRNA or 5.8S rRNA, to produce a biologically active subunit (Pieler et al., 1982).

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The genome size of both archaeobacteria and eubacteria is similar (Searcy and Doyle, 1975; Mitchell et al., 1979). Both groups contain plasmids (Weidinger et al., 1979; Thomm et al., 1983), transposable elements (Simsek et al., 1982) and restriction enzymes (McConnell et al., 1978).

(B) Resemblance of archaeobacteria to Eucaryotes:

The secondary structure of 5S rRNA of Halobacterium and Sulfolobus (Hori et al., 1982) Thermoplasma (Luehrsen et al., 1981) and eucaryotic cytoplasmic 5S rRNA are very similar.

Although the ribosomal 'A' proteins of all the three phylogenetic groups are unique (their uniqueness is discussed below) the sequence data of eucaryotic and archaebacterial 'A' protein show close similarities (Matheson and Yaguchi, 1982).

Additionally, electron microscopy studies indicate that the 30S ribosomal subunit of M. thermoautotrophicum and Sulfolobus is structurally similar to that of eucaryotic 40S ribosomal subunit (Lake et al., 1982).

Diphtheria toxin inhibits protein synthesis in eucaryotes and archaebacteria by ADP-ribosylation of the elongation factor (Kessel and Klink, 1980, 1981, 1982). Anisomycin also inhibits protein synthesis in both groups (White and Bayley, 1972).

RNA polymerases of both archaebacteria and eucaryotes are resistant to rifampicin, streptolydigin and α -amanitin (Sturm et al., 1980; Hilpert et al., 1981) and can be stimulated by the plant toxin silybin (Schnabel et al., 1982). The archaebacterial tRNA can be preferentially charged by eucaryotic synthetases but not by eubacterial synthetases.

Recently another significant finding has been the demonstration of introns in archaebacteria (Kaine et al., 1983). Introns are universally present in eucaryotes but have not been found in procaryotes.

(C) Unique Archaebacteria Features:

(i) Cell Envelope:

Murein and diaminopimelic acid are universally present in eubacteria (except Mycoplasmatales). Archaebacteria however lack these constituents (Fox et al., 1977) Gram positive Methanobacteriales, and Halococcus

possess a sacculus which is composed of pseudomurein. Halococcus sacculus contains highly sulphated heteropolysaccharide called glucosaminuronic acid (Schleifer et al., 1982) whereas Methobacteriales sacculus contains talosaminuronic acid and L-amino acids rather than muramic acid and D-amino acids found in eubacteria. All other groups of archaeobacteria (except Thermoplasma, Halococcus and Methanobacteriales) possess cell envelopes containing protein or glycoprotein subunits (Thermococcus celer, Desulfurococcus species, Sulfolobus-Caldariella species and Thermoproteus species) or a fibrillar protein sheath enclosing a short chain of cells (Methanomicrobium). Thermoplasma has glycoproteins in the cytoplasmic membrane.

With the exception of Methanococcus vannielli which is inhibited by D-cycloserine on an as yet unelucidated target site (Jones et al., 1977; Kandler and Konig, 1978), all other archaeobacteria are not inhibited by D-cycloserine or any other antibiotics such as vancomycin, penicillin, nocardicin A and cephalosporin which interfere with eubacterial cell wall formation, such as synthesis of UDP-MurNAC-pentapeptide precursor of murein or with the cross-linking reactions (Hammes et al., 1979; Hilpert et al., 1981).

Though antibiotics which interfere with the lipid cycle of murein biosynthesis in eubacteria also inhibit archaeobacteria, the mode of action in both groups is different. Bacitracin acts on UDP-activated precursors of pseudomurein in the pseudomurein containing methanogens such as Methanobacterium and Methanobrevibacterium (Hammes et al., 1979; Konig et al., 1982). In case of Halococcus and Methanococcus species, bacitracin interferes with biosynthesis of isoprenoid diether lipids (Basinger and Oliver, 1979) and not with the biosynthesis of glycoproteins as believed by Mescher and Strominger, (1975). The

insensitivity of several antibiotics including bacitracin to Methanospirillum and Methanosarcina is due to the antibiotics being impermeable to or inactivated by these organisms rather than to the lack of a suitable target (Hilpert et al., 1981).

(ii) Lipids:

Archaeobacterial cell envelopes possess ether-linked lipids (Tornabene et al., 1978; Langworthy et al., 1974; Kates, 1972) and have been dealt with in Chapter 1.

(iii) tRNA and rRNA:

Extensive sequence studies of 16S rRNA (Magrum et al., 1978; Woese and Fox, 1977; Gupta et al., 1983; Fox et al., 1977), 5S rRNA (Nazar et al., 1978; Hori and Osawa, 1979), ribosomal 'A' proteins (Matheson et al., 1980; Yaguchi et al., 1980), tRNA (Kuchino et al., 1982) and amino-terminal 30S ribosomal proteins (Yaguchi et al., 1982) have revealed that archaeobacteria possess unique sequence which are distinct from eucaryotes or eubacteria. However, as discussed earlier, some of the sequences of the above macromolecules more closely resemble the sequences of macromolecules of eucaryotes than those of eubacteria.

The initiator tRNA of S. acidocaldarius (but not from H. morrhuae and T. acidophilum) resemble yeast initiator tRNA. (Kuchino et al., 1982). Archaeobacteria ribosomes in general, contain significantly more ribosomal proteins (60-65) than have been found in eubacteria (52-55) but still considerably less than those found in eucaryotic ribosomes (70-80). (Schmid et al., 1982).

Archaeobacterial ribosomes lack binding sites for cycloheximide, an inhibitor of protein synthesis of eucaryotic 80S ribosomes and for chloramphenicol, an inhibitor of protein synthesis of eubacterial 70S ribosomes, in spite of some ribosome similarities between eucaryotes and procaryotes. The observed inhibition of methanogens (Hilpert et al., 1981) by chloramphenicol, however, is possibly due to the inhibition of hydrogenase enzyme (Schmid et al., 1982).

The ribosomal and cellular proteins of Halobacteria, Methanobacterium and Thermoplasma are more acidic in nature than eubacterial or eucaryotic ones. The proteins of Sulfolobus however are more basic.

(iv) Unique metabolic pathways:

Cell wall formation and lipid synthesis in archaeobacteria proceeds via novel pathways. Additionally, methanogens exhibit a unique spectrum of coenzymes (Cheeseman et al., 1982; Wolfe, 1979; Vogels et al., 1982; Romesser and Wolfe, 1982).

5.1.1 The Phylogenetic Tree Of Archaeobacteria:

Fox et al., (1980) have constructed a phylogenetic tree obtained from the sequence data of 16S rRNA (Figure 5.1A). Another phylogenetic tree, which included a number of recently isolated thermophiles has been obtained by Tu et al., (1982) and was based on 16S rRNA-DNA hybridisation data (Figure 5.1B). Both the trees revealed almost identical information. The archaeobacteria kingdom could be divided into two main branches. The halophiles and the methanogens formed one branch and the thermophilic bacteria Sulfolobus and Thermoproteus formed the other branch. Data based on the phylogenetic tree of Tu et al., (1982) revealed that

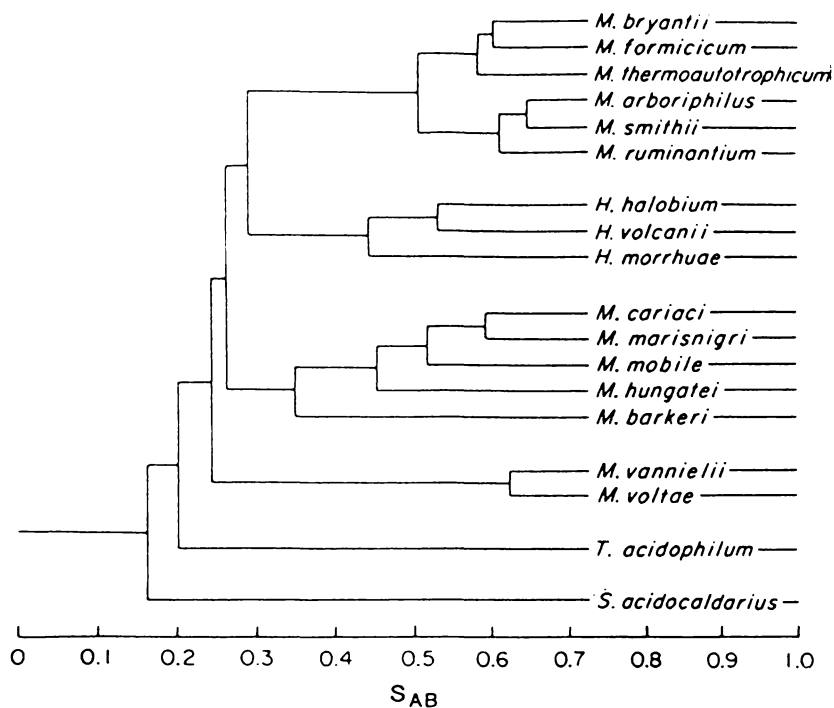


Figure 5.1A The phylogenetic relationships among archaeobacteria based on the sequence data of 16S rRNA by Fox et al., (1980).

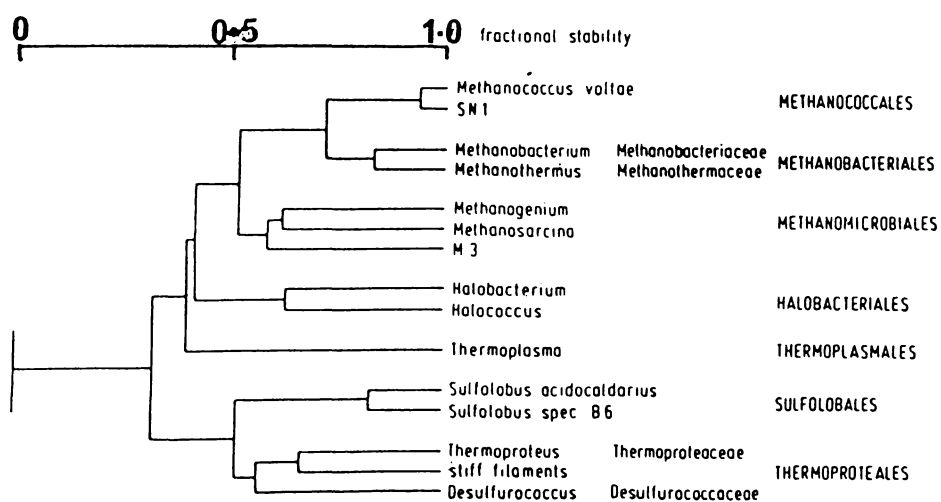


Figure 5.1B Dendrogram of fractional stabilities of hybrids between 16S rRNAs and DNAs of archaeobacteria constructed by Tu et al., (1982.)

the thermophilic methanogens Methanothermus and Methanobacterium were more closely related to each other than to any other archaeobacterium. However, DNA dependent RNA polymerase showed no immunochemical cross reaction with an antibody prepared against the enzyme from Methanobacterium (Zillig et al., 1982b).

Thermophiles in the order Thermoproteales were found to be related to Sulfolobus species. Within the order Thermoproteales, Thermoproteus and Thermofilum (stiff filaments) were more closely related to each other than to Desulfurococcus. The recently isolated organisms Pyrodictium (Stetter, 1982) and Thermodiscus (Stetter et al., 1983) have been placed in the same branch as Thermoproteales (Fischer et al., 1983). It is as yet unclear as to how related Pyrodictium are to Thermoproteales. Fischer et al., (1983) have proposed that the branch containing the thermophilic archaeobacteria presently named thermoacidophilic branch (Tu et al., 1982) be renamed the sulphur metabolising branch. However, methanogens which were not included in this branch have also been shown to metabolise sulphur (Stetter and Gaag, 1983). Hence the name will probably be revised in the near future.

5.1.2 Hypothesis Of Evolution:

Two major findings suggest that eucaryotes did not evolve from procaryotes, as has been proposed by the proponents of the dichotomous origin of life.

Woese and Fox, (1977) obtained evidence from the sequencing data of 16S rRNA that archaeobacteria formed (i) a third line of descent and (ii) they evolved from a common ancestor, called the progenote at approximately the same time as did the eucaryotes and procaryotes.

However, confusion still exists regarding the latter hypothesis of Woese and Fox, (1977). Carlile (1982), Matheson and Yaguchi, (1982) and Hori et al., (1982) have proposed alternative lines or patterns of divergence of archaeobacteria, eubacteria and eucaryotes.

There is ample evidence for the existence of split genes (introns) in eucaryotes (Darnell, 1978; Breathnach and Chambon, 1981). Doolittle (1978), has suggested that split genes were relics of primitive genetic organisation in eucaryotic cells and had not been acquired by these cells during evolution. In Tetrahymena thermophila the splicing mechanism of rRNA was found to be catalysed by the RNA itself and enzymes or ATP were not involved in the reaction. It has been assumed that this mechanism of splicing of rRNA was a primitive one (Abelson, 1982). However, this is not true for all splicing mechanisms; in the case of yeast tRNA for instance the reaction was mediated enzymatically. Since introns are thought to be primitive it is a belief that they serve no function. Recently, it was shown that the intron in yeast tyrosine tRNA was necessary for the correct modification of its tRNA product. (Johnson and Abelson, 1983).

Archaeobacteria are thought to be primitive microorganisms and in support of this it is not surprising that introns have been found in two tRNAs of the thermoacidophilic species, Sulfolobus solfataricus (Kaine et al., 1983).

The study of archaeobacteria is still in its infancy. Further studies, particularly on the thermophilic anaerobic archaeobacteria, might reveal more information on evolution, since thermal environment in which these organisms exist are presumably the reminiscent of the hot and reducing primitive world that existed billions of years ago.

5.1.3 The Species Of Extremely Thermophilic Archaeobacteria:

Extremely thermophilic archaeobacteria have been isolated from a number of thermal regions of the world. The organisms range from those in the proposed order Sulfolobales which are aerobic sulphur oxidising acidophiles to the Thermoproteales which are sulphur reducing anaerobes, and a small group of methanogens. The the first two groups namely the members of the order Sulfolobales and order Thermoproteales are dealt with in this chapter and the latter in the following chapter.

ORDER Sulfolobales:

The aerobic acidophilic archaeobacteria Sulfolobus acidocaldarius are small, spherical cells, with a primitive sub-microscopic morphology, lacking true cell walls and being surrounded by a very fine extracellular protein coat; some strains show intracellular bodies of an unidentified nature (Brock et al., 1972; Brierley and Brierley, 1973; Millonig et al., 1975). Pili are apparently involved in the attachment of the bacteria to substrates such as sulphur (Weiss, 1973b).

Sulfolobus species were first isolated from the acid thermal springs in Yellowstone National Park, USA (Brock et al., 1972; Brierley and Brierley, 1973; Weiss, 1973a) and from hot sulphur rich, acidic soils of this area (Fliermans and Brock, 1972). Sulfolobus like organisms have been isolated from an Italian thermal spring (De Rosa et al., 1975), from New Zealand hot springs (Bohlool, 1975) and Japan (Furuya et al., 1977, 1980). Sulfolobus has only been isolated from thermal environments with a pH between 2 and 3 and temperatures above 55°C where an oxidizable energy source of either sulphur or ferrous iron is present. These organisms have not yet been reported to exist in metal leaching environments; but another extreme thermophile Thermoplasma acidophila,

has been isolated from a coal refuse pile (Darland et al., 1970).

The optimum temperature for growth of nearly all the Sulfolobus strains is between 70-87°C at pH 2-3 although some strains are able to oxidise sulphur at 84°C and probably at 90°C and pH 1.0 (Shivvers and Brock, 1973). A number of serotypes exist within Sulfolobus (Bohlool and Brock, 1974; Bohlool, 1975). However at present, only three species of Sulfolobus have been described, based on physiological and morphological characteristics, G+C content and RNA dependent DNA polymerases. Table 5.1 presents the salient features of these three Sulfolobus species.

Sulfolobus strains as already described in chapter 1 have been exploited for their capability of metal leaching. They can leach copper from chalcocite (Brierley, 1977), molybdenum from molybdenite (Brierley, 1974) and can grow when the concentration of molybdenum is 7.82mM, which far exceeds the molybdenum tolerance of other metal leaching organisms.

ORDER Thermoproteales:

The order Thermoproteales includes morphological heterogeneous anaerobic bacteria ranging from rods, filaments and cocci to disc shaped organisms. The following genera are grouped into this order. Thermoproteus, Thermofilum, Thermococcus, Desulfurococcus, Thermodiscus and Pyrodictium

Thermoproteus tenax was the first extremely thermophilic anaerobe isolated which was capable of growth optimally at 85°C (Zillig et al. (1981a). Since then, several organisms have been isolated which have growth optima between 85°C and 105°C (Stetter, 1982; Stetter et al., 1983; Baross et al., 1982; Zillig et al., 1981a, 1982a, 1983a,b). Table 5.2 summarises some of the properties of the species of these

Table 5.1 Salient features of *Sulfolobus* species:^(a)

	<i>Sulfolobus acidocaldarius</i> ^(b)	<i>Sulfolobus brierleyi</i> ^(c)	<i>Sulfolobus solfataricus</i> ^(a)
Diameter (μm)	0.8 - 1.0	1 - 1.2	0.8 - 2μm
GC content (%)	37	31	36
Temp. growth range (°C)	55 - 80	45 - 75	50 - 89
Optimum growth temp. (°C)	70 - 75	70	87
pH growth range	1 - 5.9	-	1.5 - 5.0
Optimum pH	2 - 3	1.5 - 2.0	3.5 - 5.0
Autotrophy	-	+	-
ID reaction to <i>S. acidocaldarius</i> purified RNA polymerase antibodies(a)	+	Incomplete	Incomplete

(a) Zillig et al., 1980
(c) Brierley and Brierley, 1973

(b) Brock et al., 1972

ID = Immuno diffusion.

anaerobic archaeobacteria. It is likely that variants with different growth conditions may exist within each species. For instance Thermoproteus strain H3 had a temperature optimum of 90°C.

Since the members of this group have been isolated very recently, very little is known about their metabolism in the thermal pools apart from a few laboratory studies.

The main feature of all of the organisms belonging to order Sulfolobales and Thermoproteales was their capability to grow chemolithotrophically and/or heterotrophically (Tables 5.1, 5.2). The ability to grow chemolithotrophically was not surprising since gasses like CO₂, H₂, CO and H₂S are often abundant in thermal water with far lower concentrations of other elements being present primarily due to silicification. The lack of biomass due to the absence of phototrophs leads to inefficient carbon cycling and this must also mitigate against the presence of heterotrophs. Not surprisingly, higher concentrations of carbon compounds are often toxic to a number of the heterotrophic organisms isolated from thermal areas

Energy for chemolithotrophic growth by Sulfolobus species is obtained via oxidation of sulphur, ferrous iron or metal sulphides (Brock et al., 1972; Brierley and Brierley, 1973; Brierley, 1978) and some strains are autotrophic, obtaining all their organic carbon from carbon dioxide fixation (Brock et al., 1972). Most strains are capable of heterotrophic growth on yeast extract and other organic substrates (Brock et al., 1972; De Rosa et al., 1975, Brierley, 1978). Some strains showed enhancement of growth on sulphur or iron in the presence of yeast extract (Brierley and Brierley, 1973), while others showed decreased sulphur oxidation although they gave abundant growth (De Rosa et al.,

Table 5.2 Properties of extremely thermophilic, chemolithotrophic sulphur metabolizing archaeobacteria:

Species	Morphology	Diameter (µm)	Length (µm)	Temp. optima (°C)	pH optima	Doubling time (hrs)	Autotrophy	Presence of subunits in cell wall
<i>Thermoproteus tenax</i> (a)	Rod to filament	~0.4	1-100	88°C	5.5	1.7	Facultative	+
<i>Thermoproteus neutrophilus</i> (b)	Rod to filament	?	?	85°C	6.8	4 to 8	Obligate	+
<i>Thermofilum pendens</i> (c)	Rod to filament	~0.15	1-100	88°C	5.2	10	?	+
<i>Desulfurococcus mucosus</i> (d)	Cocci	~1.0	-	85°C	6.0	4	Facultative	?
<i>Desulfurococcus mobilis</i> (d)	Cocci	~1.0	-	85°C	6.0	3	Facultative	+
<i>Thermococcus celer</i> (e)	Cocci	~1.0	-	88°C	5.5	0.9	Facultative	+
AN1 (f)	Cocci	~1.0	-		7.3	0.83	?	?
<i>Pyrodicticum occultum</i> (g,h)	Discs with hyphae	0.3 to 2.5	-	105°C	6.5	2	Obligate	+
<i>Thermodiscus maritimus</i> (b)	Discs	?	-	85°C	6.5	?	Obligate	?

(a) Zillig et al., 1981
(c) Zillig et al., 1983a
(e) Zillig et al., 1983b
(g) Stetter, 1982

(b) Fischer et al., 1983)
(d) Zillig et al., 1982)
(f) Morgan, (1979)
(h) Stetter et al., (1983)

1975). Such results indicate that varying degrees of mixotrophic capabilities exist: those strains showing decreased inorganic substrate oxidation in the presence of organic supplements being the ones capable of ready heterotrophic growth and presumably capable of greater repression of the chemolithotrophic and autotrophic metabolic mechanisms.

All the anaerobic Thermoproteales species require sulphur as a terminal electron acceptor. Some of them are strict autotrophs whereas others are facultative chemolithotrophs and yet others are mixotrophs (Table 5.2). In the case of Thermoproteus tenax, sulphur requirement is stringent and only replaceable by malate. It is a facultative chemolithotroph capable of growth either on a limited number of organic compounds or on CO₂ as a sole carbon source (Zillig et al., 1981a). Thermoproteus neutrophilus, Thermodiscus marmitus (Fischer et al., 1983) and Pyrodictium occultum (Stetter 1982; Stetter et al., 1983) on the other hand are strict autotrophs capable of growth in the presence of CO₂, H₂ and sulphur only. A number of Thermoproteus species isolated from New Zealand thermal areas were found to have a similar metabolism. Additionally, sulphur was replaceable by cystine in the New Zealand isolates (Jasperse, P., M.Phil., unpublished results). Desulfurococcus mobilis, Desulfurococcus mucosus (Zillig et al., 1982a) and Thermococcus celer (Zillig et al., 1983b) are mixotrophs in which sulphur is not essential but stimulates growth.

Recently, evidence of chemolithotrophic microbial communities capable of growing at 250°C has been presented by Baross and Deming, (1983). These communities produced CO, CH₄ and H₂ indicating that these gasses when found in thermal waters could be produced in part by microbial activity. (Baross et al., 1982, Baross and Deming, 1983). Doubt on the validity of organisms growing at 250°C has recently been

provided by Trent et al., (1984) who provided evidence that apparent growth at such high temperatures could have been due to artifacts produced in the medium and to contaminants introduced during sample processing. The existence of life forms at temperatures greater than 100°C must therefore still be in doubt. There is however no dissension that thermophiles grow at temperatures up to 105°C.

Evidence is presented in this chapter that bacteria exist in New Zealand hot springs up to temperatures of 102°C and that the organisms present are aerobic and anaerobic archaeobacteria.

5.2 MATERIALS AND METHODS:

5.2.1 Inocula Sources

Pools covering the temperature ranges from 64°C to 103°C and pH range from 2.1 to 9.1 were sampled from a number of thermal regions of New Zealand and Fiji. Procedures for sampling, pH and temperature measurement were performed as previously described. The sites and description of pools sampled are provided in the back cover pocket.

5.2.2 Media And Cultivation Conditions

<1> Four different media were used for enrichment and/or isolation of anaerobic archaeobacteria. These were:

(1) Initial enrichments were performed in a pool enrichment medium (PEM). PEM consisted of pool water as a basal medium to which nutrient sources were added. An inoculum source of 9 ml pool water and 1 ml sediment slurry was added to Hungate tubes containing 0.1ml of 1% trypticase peptone-yeast extract mixture, 20mg sulphur and 100µl of 10%

Na_2S , $9\text{H}_2\text{O}$. Control tubes were prepared similarly and in addition contained 0.4mls 40% formalin. These acted as controls for determination of initial cell counts present before enrichments.

Enrichments were incubated under aerobic and anaerobic conditions. Anaerobic conditions were achieved by flushing tubes with oxygen free nitrogen (OFN) prior to sealing. Replicate enrichments from each site were incubated at 85°C, 92°C and 98°C.

(2) Acidified Allens medium was prepared essentially as described by Allen, (1959) except that the carbon content was reduced to 0.05% yeast extract and trypticase peptone was omitted, the pH was adjusted to 3.5 and was rendered oxygen free as described in appendix 3. A hydrogen/carbon dioxide gas mixture (80:20) was used in the gas phase instead of N_2 .

(3) CM medium was used for enrichment, isolation and characterization studies of some cocci. CM medium contained 0.1% NaCl and also contained in addition to sulphur, nitrate as an electron acceptor (Appendix 4).

(4) Modified Allens medium (pH 6.8) was used for two purposes. (a) To initiate enrichments and (b) for subsequent transfers from initial enrichments from PEM or CM medium. Modified Allen's medium was essentially as described by Zillig et al (1981a) except that the pH of the medium was adjusted to 6.8 and anaerobic conditions were achieved as described in Appendix 3.

most probable modified
The numbers of organisms present in pools were estimated in Allen's medium or CM medium, using sediment slurry or pool water as an inoculum source. The preparation of the sediment slurry for enumeration was as

follows: Sediment as well as pool water was collected and the sediment allowed to settle out for six hours, after which the supernatant was siphoned out and a 1:1 dilution of the sediment was prepared using the appropriate medium. A twenty fold dilution was made^{then} by adding 1ml of the sediment slurry to 9mls of medium, from which serial dilutions were prepared. When pool water was used, serial dilutions were prepared directly without the twenty fold dilution step. Triplicate tubes were inoculated from each dilution and incubated at 82°C and 90°C.

<2> For enrichment and isolation of aerobic Sulfolobus species, the media of Brock et al., 1972 was used with one minor modification. Enrichments were initiated at 83°C. This temperature was chosen since Brock, (1978) had observed the existence of high temperature strains within the group. This meant that Sulfolobus species with higher temperature optima could be possibly isolated if higher temperatures for initial enrichments were used.

5.2.3 Growth Characterization

Electron microscopy and phase contrast microscopy were performed as described previously.

Cells were counted using a Petroff-Hausser cell counting chamber (C.A.Hausser and Son, U.S.A) as described by the manufacturer.

For nutrient utilization tests of anaerobic Thermoproteus species, the appropriate carbon source at a concentration of 0.1%, was used in basal modified Allens medium with sulphur at a concentration of 0.2%. Occasionally, 0.2% cystine was used as an electron acceptor in place of sulphur. Sulphur was not sterilized prior to use and enrichments set up with sulphur as an inoculum source were observed to be negative.

5.3 RESULTS AND DISCUSSION:

<1> ENRICHMENT OF ANAEROBIC ARCHAEOBACTERIA

In general enrichments initiated in the four different media gave rise to a mixed bacterial population, including rods, filaments and cocci when incubated in PEM, or a single morphotype when CM medium or acidified Allens medium was used.

5.3.1 Enrichment Of Rods:-

Within 36 to 48 hours, 29 out of the 37 enrichments initiated anaerobically in PEM at 85°C, 92°C and 98° became positive as evidenced by an increase in cell numbers and sulphur "corrosion". The change in colour of sulphur from bright yellow to greenish yellow and the subsequent crumbling of sulphur from a coarse to a fine powdery texture was indicative of corrosion. Rod shaped to filamentous cells were the only morphological types present at 98°C with the exception of culture Rt42, which had cocci as well. The morphological types of cells present in enrichments at 92°C are given in Table 5.3. A similar result was obtained at an incubation temperature of 85°C.

Table 5.3. Results of enrichment of bacteria in PEM at an incubation temperature of 92°C:

Pool No.	Temp. (°C)	pH	Bacterial Morphotype	
			Rods/Filaments	Cocci
<u>ROTORUA:</u>				
Rt3*	70	7.2	-	-
Rt8*	64	9.1	-	-
Rt13*	97.5	6.9	+	-
Rt14*	82	7.2	+	+
Rt15*	96-102	8.7	+	+
Rt30*	97	3.69	-	-
Rt41	100	7.4	+	-
Rt42*	97	7.3	+	+
Rt51*	82	7.3	+	+
Rt56*	90	7.9	+	+
Rt57*	97	7.2	+	+
Rt58	103	8.1	+	-
Rt59	103	7.2	+	+
Rt60	97.7	7.2	+	+
Rt61	94.5	8.1	+	-
Rt68	95	7.5	+	+
Rt70	98.2	3.4	-	-
Rt71	98.5	3.3	+	+
Rt72	97	6.6	+	+
Rt74	NA	NA	+	+
Rt80	NA	NA	+	+
Rt82	91	6.76	+	+
Rt84	NA	NA	+	+
Rt87	94	7.8	+	+
Rt88	95	8.3	+	+
Rt89	94	8.2	+	+
Rt90	98	7.3	+	+
Rt91	96	7.6	+	+
Rt93	96	6.8	+	+
Rt98	100	8.5	+	+
Rt99	98	4.8	+	-
<u>WAIMANGU:</u>				
Wai8	85	3.03	-	-
Wai9	88	3.1	-	+
Wai11	96	8.5	+	+
Wai17	80	8.5	+	-
Wai18	81	7.34	-	+
Wai19	79	7.7	-	-
Wai21	67	6.5	-	-
<u>WAIRAKEI:</u>				
Wk1	97	3.15	-	-

NA = Not analysed. Media used was as described in Materials and Methods. An increase in cell numbers and sulphur corrosion was used as an index of growth. * indicates that enrichments were performed at least twice at different times and identical results were obtained.

However enrichments initiated in modified Allens medium at 75°C, yielded rods only (Table 5.4).

Table 5.4: Results of enrichment of bacteria in modified Allens medium at 75°C:

Pool No.	Temperature (°C)	pH	Bacterial Morphotype	
			Rods	Cocci
Rt3	70	7.2	-	-
Rt8	64	9.1	-	-
Rt12	66	6.9	+	-
Rt14	80	7.0	+	-
Rt34	82	6.4	-	-
Rt51	82	8.1	+	-
Rt56	90	7.9	+	-
Rt57	97	7.2	-	-

All enrichments were repeated at least twice.

Comparing results from PEM enrichments (Table 5.3) and enrichments from modified Allens medium (Table 5.4), two observations were evident. (1) Cocci could be enriched in PEM but not in modified Allen's medium. Refer to cocci of group 2 for more details. (2) Results of enrichments in both PEM (Table 5.3) and modified Allen's medium (Table 5.4) were similar with the exception of the negative result obtained for Rt57 when enriched in modified Allen's medium. Subsequent experiments, based on a positive enrichment of Rt57 sample in Allen's medium at 85°C (and its inability to grow at 75°C) indicated that the organisms present in the pool were capable of growth at 85°C but not at 75°C. Cultures from Rt51 and Rt56 enriched in modified Allen's medium at 75°C and Rt51, Rt56 and Rt57 grown at 85°C had a characteristic cell wall structure which was similar to that described for *T. tenax* (Zillig *et al.*, 1981a). This indicated that at least two groups of *Thermoproteus* species existed. One group which had a minimum temperature for growth higher than 75°C, and perhaps existed in boiling pools, and another group which could grow at 75°C, and perhaps existed in the lower temperature pools.

Since the rods but not the cocci (with the exception of the cocci from pool Rt42, Table 5.3) were capable of growth at 98°C, this in conjunction with the end product dilution technique proved to be a convenient and easy method of separating the rods from the cocci. An anaerobic rod shaped organism was isolated in pure culture in Allen's medium adjusted to pH 6.5 from pool Rt13 and designated Rt13-BS1. The cell-wall had a characteristic appearance similar to that of T.tenax (Figure 5.2) and until more is known it is assumed the New Zealand isolate Rt13-BS1 is a Thermoproteus species. Isolate Rt13-BS1 was able to grow on 0.1% glutamate or yeast extract and trypticase peptone as carbon sources with cystine or sulphur as electron acceptor. Thiotone, glucose, ribose and pyruvate could not serve as carbon source or as electron donors with or without sulphur or cystine as electron acceptors. Polysulphide did not stimulate growth or replace sulphur as electron acceptor. Its inability to grow on glucose differentiates it from T.tenax isolated by Zillig et al., 1981a. Most of the other substrates tested with isolate Rt13-BS1 however, have not been tested with T.tenax. Four cultures Rt13, Rt51, Rt56 and Rt57 enriched in Allen's media comprised of rod shaped bacteria only and electron microscopy revealed that all the cultures had a cell-wall morphology similar to that of Rt13-BS1 as exemplified in Figure 5.2.

Table 5.5 presents data on the estimation of numbers of organisms present in pool Rt13 (sediment or pool water) at incubation temperatures of 88°C and 95°C in Allen's medium.

Figure 5.2 Electron Micrograph of a pure culture of rod shaped bacterium from pool Rt13 exhibiting a cell-wall characteristic of the archaebacterial Thermoproteus species.

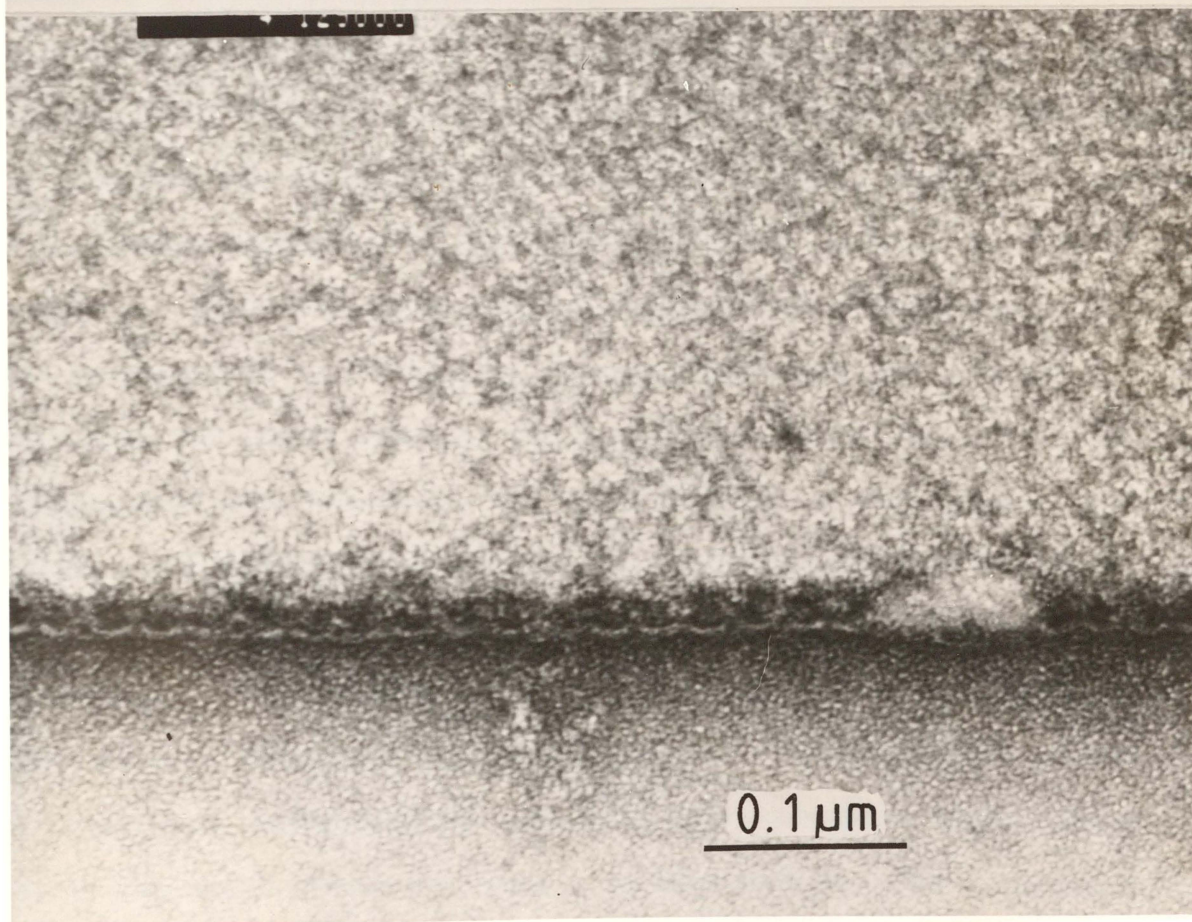
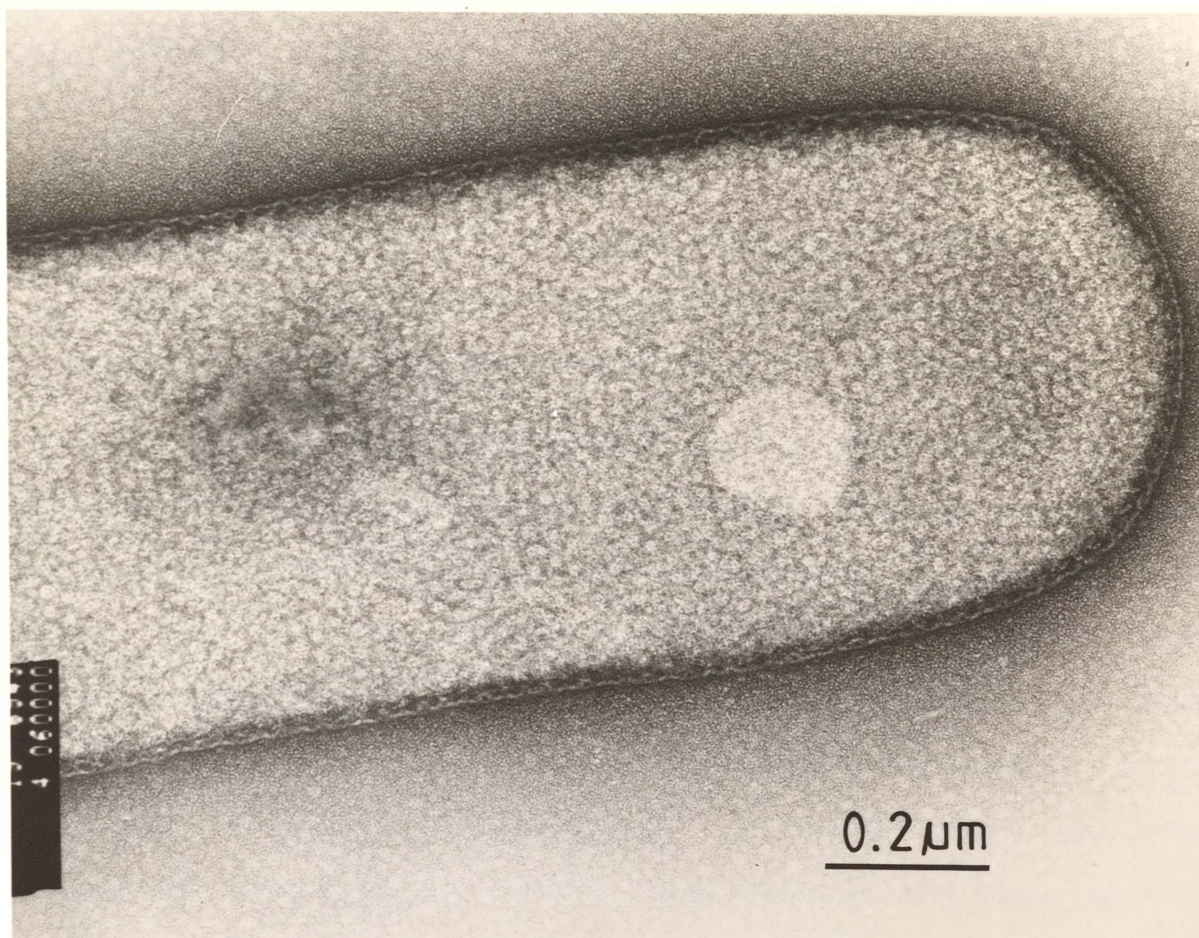


Table 5.5: Most probable numbers of Thermoproteus type cells in pool Rt13:

Inoculum source	No. of cells/at	
	88°C	95°C
Pool water	$1.1 \times 10^3/\text{ml}$	$4.6 \times 10^2/\text{ml}$
Sediment slurry	$4.3 \times 10^4/\text{ml}$	$9.3 \times 10^3/\text{ml}$

Slightly higher values were obtained at the 88°C incubations for both water and sediment estimations than at 95°C. This was in accordance with the known optimum temperature for growth of both T. tenax (85°C) and for pure cultures from New Zealand thermal pools (88-90°C). Pool Rt13 had a temperature of 97°C, the MPN results indicate that the pure culture obtained from this pool probably represents the dominant species in the pool, at least on the media used. If higher values had been obtained at 95°C compared to 88°C, then organisms with higher optimum temperatures would be suspected. This was not the case, therefore growth of organisms in the pool occurred suboptimally. However, an optimum temperature of 97°C for isotope uptake (close to the pool temperature) was obtained for the organisms from pool Rt13 (refer chapter 3). Uptake of substrates need not show the same temperature optimum as the cell, especially over the relatively short incubation periods used, and care must be exercised in correlating optimum temperature for isotope uptake with that of cell growth. The possibility exists that either another population of rods exists in the pool with a higher growth optimum (closer to that of pool temperature) or that the cultures isolated have a different optimum temperature when in the pool environment. The possibility of thermostabilizing factors existing in the thermal water which was used in isotope uptake experiments, but presumably absent in the synthetic media used for culturing these organisms under laboratory conditions, would affect the results of both studies. For example, the

thermostability of thermophilic phage particles increased if the pool water from which they had been isolated, was added to the broth (Sakaki and Oshima, 1975). Obviously a number of other factors could also determine the optimum conditions for growth. For example, the type of carbon source used determined the temperature optimum for bacteria in a thermal spring of USA (Brock and Brock, 1971b).

The greater number of cells found in pool sediments might indicate a lower E_h and/or higher substrate concentration in this environment. If the rods were adsorbed to sediment particles then the MPN approach might significantly underestimate the number of cells in the sediment, because the number of cells per particle could be high. Adsorption to EM grids was observed and colonization of glass slides and glass cylinders occurred rapidly. Although the high pool temperature 95-97°C would tend to minimise the amount of oxygen dissolved, the turbulent nature of the pool would counteract this. When resazurin was added directly to pool water it was not completely reduced indicating that at least traces of oxygen were present. Whether cells in pool water merely survived or were actively growing under such conditions was not ascertained.

Growth did occur in enrichments from several of the pools set up at 98°C aerobically but only after 15 days incubation, compared to approximately 36-48 hours anaerobically. This could be attributable to two causes, (1) Growth occurred under facultative anaerobic conditions, and sulphide was produced from sulphur slowly until the medium was reduced, the whole process taking a longer period of time, or (2) the temperature of 98°C rendered the medium anaerobic after a period of time after which the organisms started growing. More work is required to prove any of these hypotheses. The oxygen sensitivity of these organisms is largely unknown. For instance, the behaviour of resting cultures of

Pyrodictium occultum (growing at 105°C) in the presence of oxygen was found to be unpredictable, in that, in some cases the cultures were not revivable but in other cases they were, indicating that there might be a protective mechanism against oxygen (Stetter et al., 1983). It has also been observed that trace amounts of oxygen increased the rate of methane production in a mixed culture of algae and a methanogen (Pirt and Lee, 1983), again an indication that some anaerobic archaeobacteria might be able to tolerate and in some cases be stimulated by the presence of traces of oxygen.

Thermoproteus species are most definitely ubiquitous in thermal environments and have previously been isolated from the hot springs of Japan, Italy, the USA and Iceland. The Thermoproteus species found in New Zealand hot springs are different from T.tenax described by Zillig et al (1981a) since they were able to grow optimally at neutral pH. This was evident from enrichment results initiated in PEM medium (Table 5.3) and from other pure culture studies. For example, Thermoproteus species Tok12 was found to have a pH optimum of 6.5 (Jasperse, P., unpublished M.Phil., thesis), a pH optimum very similar to that of the recently described T. neutrophilus (Fischer et al., 1983). However, the former was a strictly heterotrophic species whereas the latter is a strict autotroph. Some of the New Zealand Thermoproteus isolates also have a slightly higher temperature optima. Isolate Tok12-S2 and KT10-S1 have a temperature optima of 90°C (Jasperse, P., M.Phil., thesis) compared to 88°C for T.neutrophilus and 85°C for T.tenax.

5.3.2 Enrichment Of Cocci:-

At least four morphological types of cocci were observed in the 3 types of enrichment media used.

Group 1 cocci:

The coccus from pool Rt42 belonged to group 1 cocci and as already indicated, this was the only coccus capable of growth at 98°C. Such cocci were motile and possessed a cell-wall structure very similar to that of a rod present in the pool and with which it was frequently found in association (Fig. 5.3). The subunit structure of the cell wall of the coccus from pool Rt42 differentiated it from other New Zealand hot spring isolates and Desulfurococcus mucosus and Desulfurococcus mobilis (Zillig et al., 1982a) but resembled that of Thermococcus celer (Zillig et al., 1983b). However, T. celer required 4% NaCl for growth whereas the coccus from Rt42 did not as indicated by growth in modified Allens media which contained no NaCl at all. No attempts were made to isolate this coccus in pure culture and the organism was not studied further.

Group 2 cocci:

Cocci but not rods were enriched from 7 out of the 50 pools sampled at 75°C in CM medium (Table 5.6). Although enrichments were successful at 75°C, all the seven coccal isolates had growth temperature maxima between 85°C and 90°C. However, when enrichments were carried out in modified Allen's medium at 75°C (Table 5.4) or 85°C, rods but not cocci were evident from pools Rt12, Rt14, Rt51 and Rt56. Similarly, when coccal enrichments achieved in CM medium were transferred to modified Allen's medium, no growth occurred. The most probable reason for the lack of growth of the cocci in Allen's medium was their requirement for

Figure 5.3 An enrichment culture of pool Rt42 grown at 98°C, showing association between a flagellated coccus (A, B) and a Thermoproteus type species (A). Magnification of the cocci (C) and rod (D) to show the sub unit cell wall pattern.

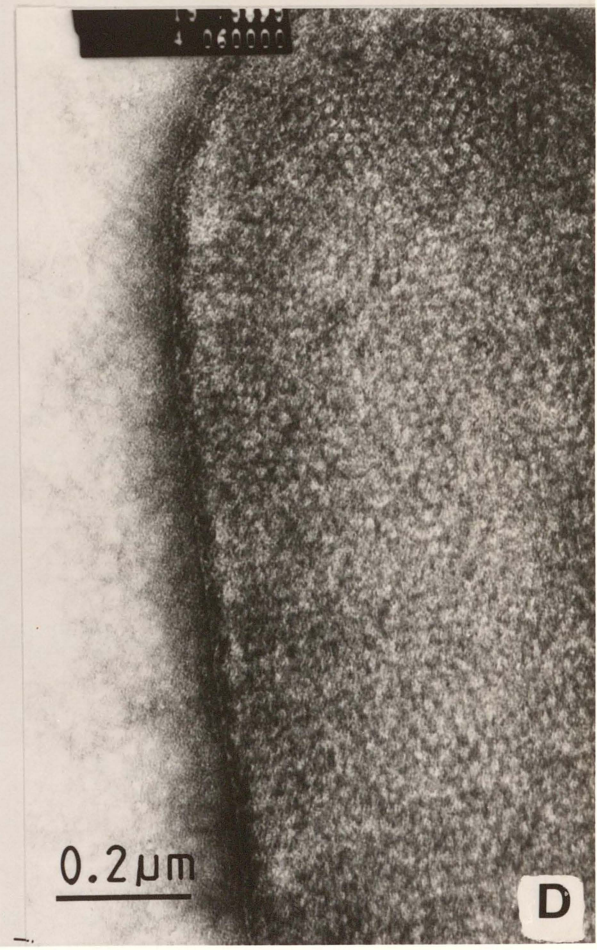
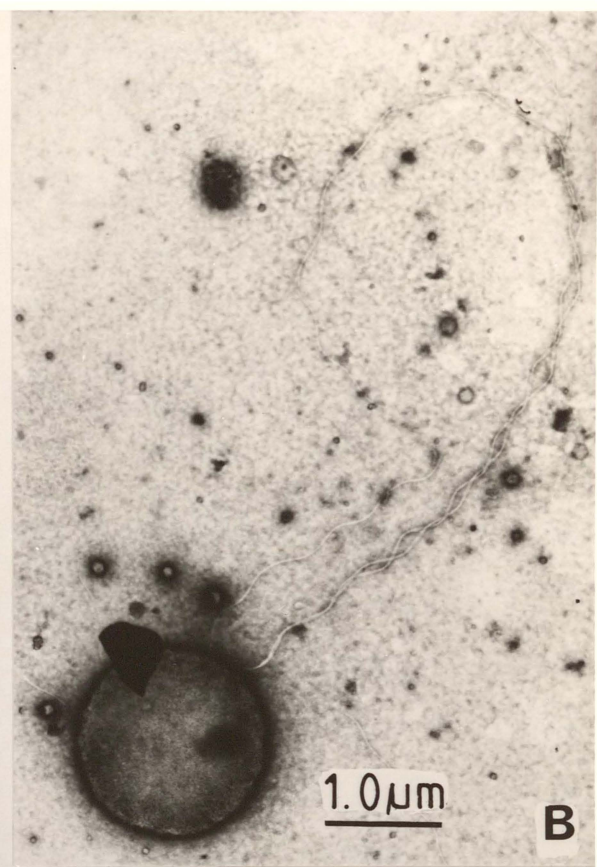


Table 5.6. Enrichment of cocci in CM medium at 75°C:

Pool No.	pH	Temperature (°C)	Presence of Cocci
<u>WHITE ISLANDS:</u>			
WIs1*	2.5	52.8	-
WIs2*	3.1	95.2	-
WIs3*	2.9	83.7	-
WIs4*	5.0	90-96	+
WIs5*	3.5	64.5	-
WIs6*	2.8	75	-
WIs7*	3.2	75	-
WIs8*	ND	+105	-
WIs9*	2.6	89.7	-
WIs10*	2.4	64	-
WIs11*	3.8	56.2	-
WIs12*	2.9	91.4	-
WIs13*	3.0	65	-
<u>ROTORUA:</u>			
Rt3	7.5	63	-
Rt7	9.1	91-98	-
Rt9	8.4	84	-
Rt10	7.9	94	-
Rt12*	6.9	66	+
Rt13*	7.4	96	-
Rt14*	7.0	80	+
Rt15	8.7	91	-
Rt16*	5.5	67	-
Rt34	6.4	82	-
Rt39	3.3	80.5	-
Rt51*	8.1	82	+
Rt56*	7.7	90.2	+
Rt61	8.1	94.5	-
Rt97	7.2	78	-
Rt99	4.8	95	-
Rt100	7.3	81	-
Rt101*	2.9	66	-
<u>WAIMANGU:</u>			
Wa19	3.1	88	-
<u>FIJI:</u>			
FJ1*	ND	ND	-
FJ2*	ND	ND	-
FJ3*	ND	ND	-
FJ4*	ND	ND	-
FJ5*	ND	ND	-
<u>KETATAHI:</u>			
KT1	ND	ND	-
KT2	ND	ND	-
KT3	ND	ND	-
<u>WAIOTAPU:</u>			
WT1	2.1	88	-
WT3	4.0	76.8	-
WT4	-	73.6	-
WT5	5.5	69.8	-
WTB	7.0	96	-
WTC	3.8	78	-
WTD	4.4	84.4	-
WTE	2.9	82	-
<u>TOKAANU:</u>			
Tok4	5.7	92	+
Tok10	6.6	75	+

Enrichments were done as described in Materials and Methods. In addition, 0.2%, 2% and 4% NaCl in CM medium was used to enrich from the Fijian pools (since they were collected from thermal areas on the beach). Indicates that enrichments were initiated at least more than twice and similar results were obtained on each occasion.

NaCl. CM medium contains 0.1% NaCl which supported growth of the cocci but rods were inhibited by NaCl even at such a low concentration. Allen's media on the other hand does not contain NaCl and hence was incapable of supporting the growth of cocci but was able to support the growth of rods. It is possible that other components of CM medium were responsible for inhibiting the growth of rods eg the higher organic content, but these were not investigated.

In pure cultures the cocci had faster growth rates than that of rods, for example AN1 has a generation time of 50min (Morgan, H.W., unpublished results) compared to 330min for Thermoproteus Rt13-S1 (Jasperse, P., M.Phil. thesis) and yet their presence in pools was difficult to demonstrate as indicated by their infrequent occurrence on colonized glass slides, EM grids and polycarbonate membranes. A number of reasons are possible. (i) Their inability to attach to glass slides as efficiently as rods. (ii) Requirement of NaCl, sulphide etc. present in the pool which are needed as growth stimulating factors but are present only in suboptimal amounts (iii) Purely physical stresses, such as temperature and pH which lower growth rates.

A search of the literature for the chemical composition of New Zealand thermal springs revealed that sodium and chloride levels in the Whakarewarewa thermal area of Rotorua, Waimangu and Tokaanu were generally higher than the springs in Taupo, Tikiteri, Waiotapu, Orakei Korako and with a few exceptions all springs in Kuirau Park thermal area of Rotorua. The chemical composition of a number of springs were analysed by the Water and Soil Division of Ministry of Works, Hamilton and the data on sodium and chloride content of some springs is provided in Table 5.7.

Table 5.7. Sodium, chloride and sulphide contents of some New Zealand thermal springs:

Pool No.	pH	Temp. (°C)	mg/litre			Presence of Cocci
			Sodium	Chloride	Sulphide	
ROTORUA:						
Rt3	7.5	65	370	335	3	-
Rt10	7.9	94	637	571	50	-
Rt12	6.9	66	606	803	ND	+
Rt13	7.4	98	617	797	22	-
Rt14	7.0	80	647	842	4	+
Rt15	8.7	96.4	639	821	28	-
Rt30	3.7	97	398	495	6	-
Rt34	6.5	75.7	411	482	ND	-
Rt42	7.3	97	630	731	21	-
Rt44	7.8	62	636	896	ND	+
Rt51	7.3	82	628	842	19	+
Rt56	7.9	90.2	857	528	16	+
Rt61	8.1	94.5	751	806	ND	-
TOKAANU:						
Tok21	5.6	89	1392	1915	ND	+

The data suggests that the distribution of the group 2 cocci is controlled by temperature and/or sodium chloride content of the pool. The cocci were not found in pools above 90°C regardless of NaCl concentration. In pools below 90°C only those with greater than 600 mg/litre sodium contain such organism. Sulphide content of the pools does not appear to influence the distribution of the cocci. Analysis of a far greater number of pools needs to be carried out to substantiate these claims.

The group 2 cocci such as Rt14-BS1 were present in quite high numbers in the pools from which they originated. For instance, pools Rt56 and Rt51 were estimated to have populations of greater than 10^4 cells per ml of these organisms as determined by most probable numbers when incubated in CM medium at their respective pool temperatures (82°C for Rt51, and 90°C for Rt56) for 24 hours. Initial experiments had shown that the cocci from Rt51 grew poorly at 90°C. This indicated that the

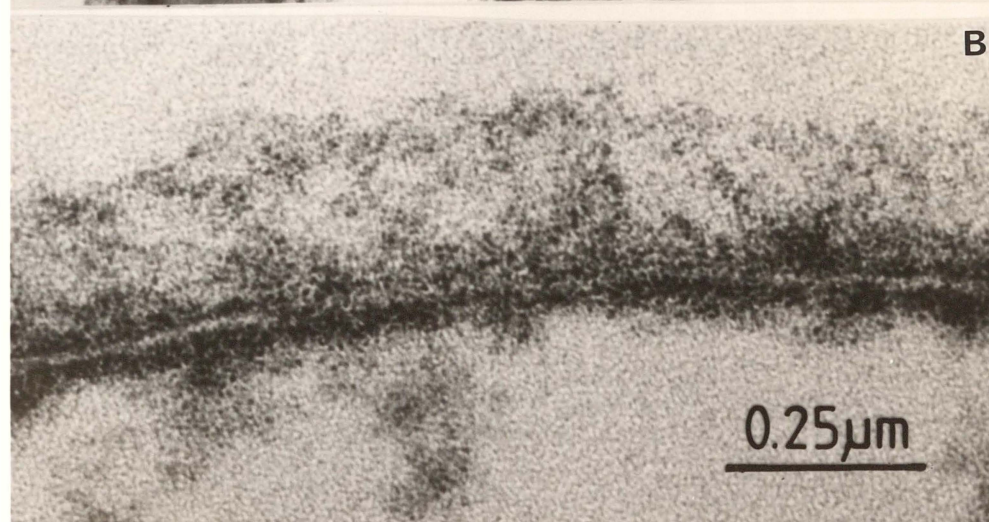
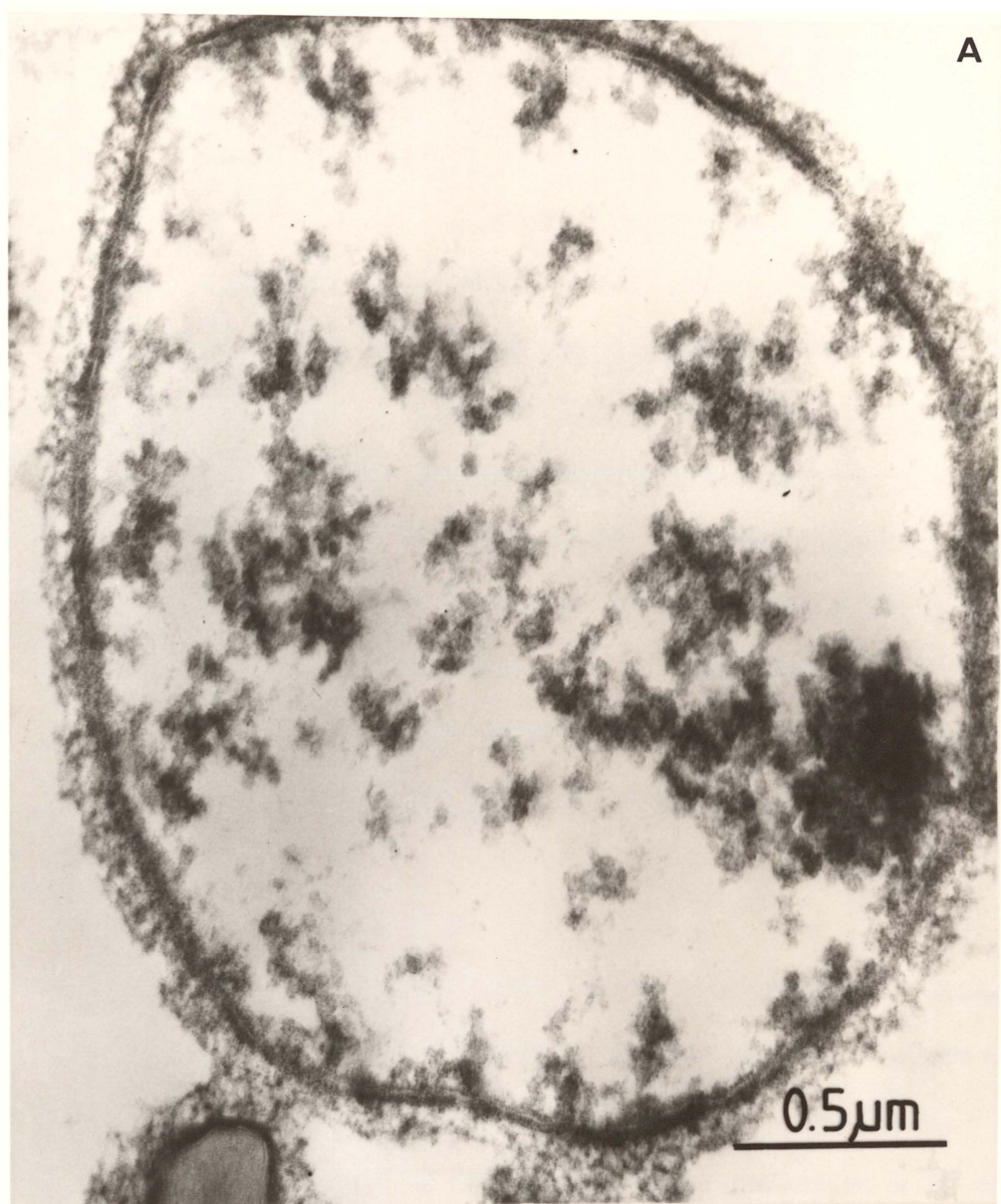
cocci from Rt56 had a higher temperature optima than those from pool Rt51.

Pure cultures of the cocci present in pools Rt12 and Rt14 were isolated by serial dilution and investigated further. Both isolates were motile as observed under a phase contrast microscope. Culture Rt12-BS1 from pool Rt12 required sulphide for growth whereas culture Rt14-BS1 from pool Rt14 was not affected by the presence or absence of sulphide in CM medium.

Fluorescence antibody (FA) staining of antibodies prepared against AN1 when cross reacted with other isolates of this group of NaCl requiring strains, gave differing fluorescent intensities indicating that the group was composed of heterogeneous and distinct serotypes. The FA staining results have been discussed earlier in chapter 4, Table 4.3. Results from Pyrolysis-mass spectroscopy of a number of strains from this group indicates that the strains could be divided into at least two broad groups (Martin, A.M., unpublished Ph.D. thesis).

The relationship of the cocci in this group (Table 5.6) including AN1 (a culture of similar type previously isolated by Morgan, 1979) with cultures of Desulfurococcus species and Thermococcus celer is of interest. Thin sections of the cell wall of Rt14-BS1 (Fig. 5.4) revealed the presence a 25nm thick cell envelope and the presence of electron dense cytoplasmic material attached to the cell membrane, features which were similar in both AN1 and Desulfurococcus mobilis (Zillig et al., 1982a) but quite different from Thermococcus celer which possessed a cell wall composed of a distinct subunit layer. However, Rt14-BS1 and AN1 required NaCl but not sulphide for growth, which differentiated them from D. mobilis and D. mucosus T. celer also

Figure 5.4 Electron Micrograph a thin section of a AN1 type isolate-
Rt14-BS1.
(A) Whole cell.
(B) Cell envelope detail.



required NaCl for growth but the amount required (4%) and the cell wall structure clearly differentiated it from Rt14-BS1.

The geographic distribution of these archaebacterial cocci was also interesting. T. celer has only been isolated from submarine springs of Italy (Zillig et al., 1983b), D.mucosus and D.mobilis from Icelandic hot springs but not from those of Japan or Italy (Zillig et al., 1982a) and Rt14-BS1 type isolates from New Zealand thermal areas but not from Fiji. Even within the New Zealand thermal region, Rt14-BS1 type isolates are restricted to a few places. They have been isolated from some pools in Rotorua and Tokaanu and only one hot pool of White Island but not from Ketatahi or Waiotapu. A possible explanation lies with sodium and chloride content of the springs and has been discussed earlier.

Group 3 cocci:

In addition to the above mentioned cocci, acidophilic anaerobic cocci were isolated from a number of acidic thermal springs including all White Island sources with the exception of pool WIs 4 (Table 5.8). These acidophilic cocci were isolated in modified Allen's medium at a pH of 3.5 and had a morphology similar to that shown in Figure 2.48.

The optimum temperature of growth of the cocci in acidified Allens medium was approximately 70°C with no growth occurring at 80°C. No growth occurred above pH 6.0 or below pH 2.0.

Table 5.8. pH and temperature of pools yielding anaerobic cocci at an incubation temperature of 70°C in acidified Allen's medium

Pool No.		pH	Temperature (°C)	Presence of Cocci
WIs	1	2.5	52.8	+
WIs	2*	3.1	95.2	+
WIs	3	2.9	83.7	+
WIs	4*	5.0	90-96	-
WIs	5	3.5	64.5	+
WIs	6	2.8	75	+
WIs	7	3.2	75	+
WIs	9	2.6	89.7	+
WIs	10	2.4	64	+
WIs	11	3.8	56.2	+
WIs	12*	2.9	91.4	+
WIs	13	3.0	65	+
Wai	9	3.1	88	+
WP	1	2.87	89.8	-
WP	2	2.9	89.9	+
WP	3	2.76	81	-
WP	5	ND	98.5	-
WP	8	6.8	83.6	-

* indicates that at least two attempts at enrichments were made and in case similar results were obtained.

The cultures usually lacked turbidity when grown in deoxygenated acidified Allens media but bacterial presence could be demonstrated by phase contrast microscopy. However, the addition of 0.1% polysulphide or 0.05% sodium sulphide stimulated growth to an extent that visual turbidity was observed. This was probably due to the lowering of the Eh of the deoxygenated media by sulphide. The cocci required CO₂ and H₂ for growth and no growth occurred if the gas mixture was replaced by N₂. Methane was not produced from CO₂ and H₂ and therefore the isolates were not methanogens. Sulphur was routinely added to acidified Allen's medium but its requirement by this group was not investigated further.

One of the cultures was isolated and purified from pool WIs2 and designated WIs2-BAC1. WIs2-BAC1 was not inhibited by 100µg/ml vancomycin and it was also susceptible to complete lysis with 10% SDS indicating

that it did not possess a eubacterial wall structure and was possibly an archaeobacterial coccus. It did not cross react with immunofluorescent antibody prepared against AN1, an anaerobic coccus growing at neutral pH at 75°C indicating that the acidophilic cocci were substantially different from the neutral pH organisms.

Group 4 cocci:

A 4th group of cocci could be identified based on a number of factors such as their inability to grow at 98°C, inability to grow optimally at a pH of 3.5 and the non requirement of NaCl for growth. The cocci in this group (group 4) appear to be similar to Desulfurococcus species.

This distinct group of cocci were capable of growth up to temperatures of 98°C (Table 5.3). The coccus in pool Rt15 which was incapable of growth in CM medium, at a temperature of 98°C and incapable of growth at a pH of 3.5 is an example of a coccus from this group. However, further studies on this isolate were not done. Other strains falling in this group have been purified by Jasperse, P. (M.Phil thesis). This includes some of the cocci which have been listed in Table 5.3.

<2> ENRICHMENT OF AEROBIC SULFOLOBUS-TYPE ARCHAEBACTERIA

A very limited number of pools (listed in Table 5.3) were screened for the presence of Sulfolobus strains since a lot of information of their distribution was known. The main aim of this survey was to demonstrate their presence in very high temperature pools (95°C to 99°C) since at such high temperatures with low pH, bacterial life is thought to

be inhibited.

Positive enrichments of coccal shaped organisms were obtained from pools having temperatures greater than 95°C, namely Rt30, Rt70, Rt71 and Wk1. The enrichment obtained from Rt30 was purified by the end point dilution method and designated Rt30-A1.

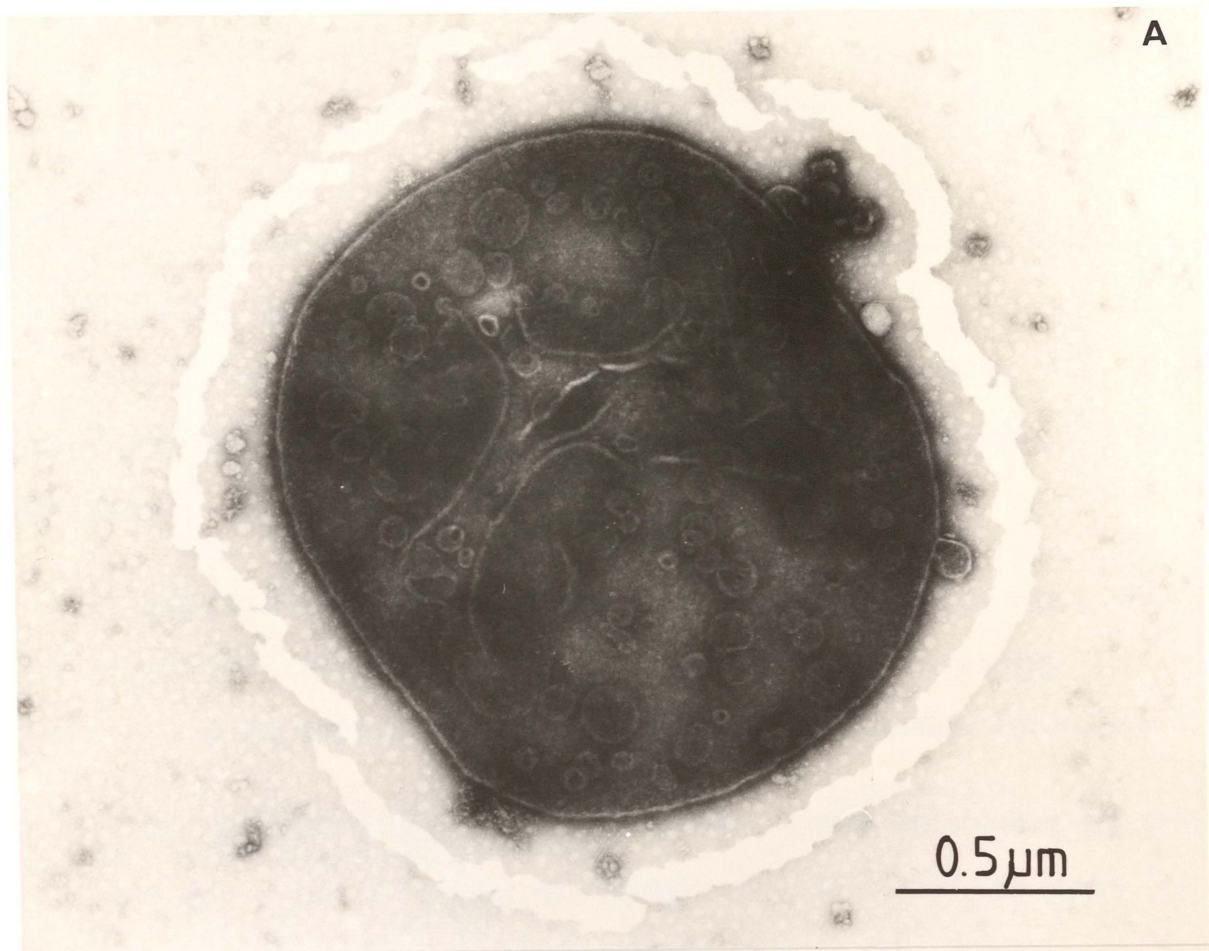
Cells of Rt30-A1 were 1-2µm in diameter and were spherical to spherical lobes (Fig. 5.5). The maximum temperature for growth was below 90°C, and no growth occurred at pH 6.0. The optimum temperature for growth was approximately 80°C. Rt30-A1 was routinely grown at a pH of 3.0.

Rt30-A1 was not inhibited by 100µm/ml penicillin. It lysed immediately in the presence of 5% SDS. Lysis also occurred in the anaerobic modified Allen's medium described earlier. This obviously differentiates these aerobic organisms from the anaerobic group 3 cocci.

5.4 SUMMARY AND CONCLUSIONS:

Results of enrichment cultures initiated in the four types of media used (PEM, CM and modified Allens medium) indicated that at least 4 distinct physiologically types of cocci and two distinct morphological types of rods were present in New Zealand hot springs. A mixed microbial flora comprising rods, filaments and cocci was observed in PEM medium at 85°C and 92°C whereas only cocci were observed in CM and acidified Allens medium at 75°C and 70°C respectively. The limited number of pool samples enriched in modified Allens medium yielded rods but no cocci. Isotope uptake data (presented in chapter 2) and evidence of enrichments in this chapter indicates that anaerobic archaeobacteria were probably the dominant microbial flora in springs exceeding temperatures of 80°C.

Figure 5.5 Electron micrograph of a pure culture of Sulfolobus species RT30-A1, showing a typical spherical morphology (A) and a lobe-shaped morphology (B). Note the presence of a distinct outer cell envelope.



Springs differed in their archaebacterial composition, some springs maintaining a mixed flora whilst others appear to possess only a single isolate. For example, only Thermoproteus species was isolated from pool Rt13. Whether this species is able to grow chemolithotrophically has not been determined, but if it does then it is not surprising that very low counts ($4.3 \times 10^4/\text{ml}$) were obtained, since growth rates under chemolithoautotrophic conditions are normally lower than heterotrophic conditions.

At least 4 physiologically distinct groups of anaerobic cocci exist in New Zealand thermal environments. Cocci belonging to groups 1, 2 and 3 have at present been observed only in New Zealand thermal environments.

On the other hand, Thermoproteus species is more ubiquitous and have been isolated from a number of thermal environments of the world. At least two different groups of Thermoproteus species are present in New Zealand thermal springs. These two groups can be differentiated on the basis of minimum temperature required for growth.

The absence of Pyrodictium species from New Zealand thermal areas is perhaps a reflection of the different type of niche this organism inhabits or that the growth conditions employed were not suitable for the growth of these organisms.

Repeated sampling of a number of pools for enrichment, yielded the same microbial flora indicating that the organisms inhabiting these pools were a stable structure of the thermal environment. However, a number of these springs themselves were physically unstable which in turn could possibly lead to destabilization of the chemical constituent of the springs. For instance, springs Rt51, Rt56 and Rt44 fluctuated in temperature and water level. These types of fluctuations occurred within

a span of a few days to a few weeks. The reversal of these fluctuations were as quick to occur and this possibly made colonization of such pools by other bacteria impossible. The reestablishment of the original conditions in the pools could however allow the same microbial flora to recolonize. Drastic changes in pool characteristics over a long period of time could change the microbial flora significantly. This has already been discussed in relation to the spirochete populations in pool Rt3 (chapter 2).

Sulfolobus species can be isolated from extremely hot and acidic New Zealand thermal pools and appear to be similar to Sulfolobus strains isolated elsewhere. However an observation made earlier on (chapter 2) is worth mentioning here. The coccal cells from pools Rt30, Rt70 and Rt71 were never observed to form microcolonies (Table 2.1) in slides colonized in situ. Furthermore, the organisms could have been passed off as debris had the slides not been Gram stained. Weiss, (1973a) had observed that Sulfolobus strains produced two types of colonies (compact and dispersed) on slides immersed "in situ". He also observed that Sulfolobus strains in pools with temperatures below 89°C were normal in appearance compared to the ones present in pools with temperatures of 93°C. Brock, (1978) provided evidence that Sulfolobus present in acid pools with boiling temperatures (93°C) in Yellowstone National Park USA were less metabolically active than their counterparts in acid pools having much lower temperatures (80°C). It is possible that Sulfolobus present in boiling acid springs do not grow optimally at their environment temperatures.

Abundant information still lies in the pools regarding the types of archaeobacteria present in these environments and obviously requires more screening, enrichment and isolation work to characterise these organisms. This will possibly provide an answer to the question of phylogeny and distribution of these organisms.

CHAPTER 6

ISOLATION AND CHARACTERIZATION OF THERMOPHILIC METHANOGENS

6.1 INTRODUCTION:

A number of thermophilic methanogens have been isolated from anaerobic digestors, the first being Methanobacterium thermoautotrophicum (Zeikus and Wolfe, 1972). Since then strains of this species have been isolated from environments such as sediments of polluted rivers (Ronnow and Gunnarsson, 1981), sewage digestors (Fuchs et al., 1978) and geothermal sites (Sandbeck and Ward, 1982; Zeikus et al., 1980). Considerable strain variability has been noted within M. thermoautotrophicum. For instance, a geothermal strain grew optimally at a temperature of 58°C compared to a temperature optimum of 65°C to 70°C exhibited by all other strains. A vitamin B₁₂ requiring strain was also isolated from the same thermal region. None of the other M. thermoautotrophicum strains are known to require such growth factors (Sandbeck and Ward, 1982). Additionally, M. thermoautotrophicum strain YT1 isolated from a thermal spring was found to be immunologically distinct from its counterpart isolated from a sewage digester (Zeikus et al., 1980).

Table 6.1 Properties of thermophilic and extremely thermophilic methanogens:

Species	Isolation source	Morphology	Dimensions	Gram reaction	pH optima	Temp. optima(°C)	Doubling time (hrs)	Growth on			Sulphide dependence	NaCl requirements	mol. % G+C
								H ₂ CO ₂	Acetate	Formate			
<i>Methanococcus</i> (a) <i>thermolithotrophicus</i>	Thermal sea sediment	Cocci	1.5µm	-ve	6.5-7.5	65°	0.9	+	-	+	?	+	31.3
<i>Methanococcus jannaschii</i> (b)	Thermal sea vent	Cocci	1.5µm	?	6.0	85°	0.43	+	-	-	+	+	31
<i>Methanosarcina</i> sp. (c)	Sludge digester	Cocci	?	?	6.0	50°	12.0	-	+	-	?	?	?
<i>Methanomicrobium</i> sp. (d)	Kelp sludge	oval to coccoid	0.7-1.8µm	Too fragile for analysis	7.2	55-60°	1.8	+	-	+	-	-	59.69
<i>Methanobaeterium thermoautotrophicum</i> (e)	Sewage sludge	Rods to filaments	0.35-6µm 3-100µm	+ve	7.2-7.6	65-70°	5.0	+	+	-	+	+	52
<i>Methanogenium thermophilicum</i> (f)	Thermal effluent	Cocci	1-1.3µm	-ve	7.0	55°	2.5	+	-	+	-	+	59
<i>Methanothermus frevidus</i> (g)	Geothermal spring	Rods	0.3-0.4µm 1-3µm	+ve	6.3	83°	2.8	+	-	-	+	?	33

(a) Huber et al., 1982

(c) Zinder and Mah, 1979

(e) Zeikus and Wolfe, 1972

(g) Stetter et al., 1981

(b) Jones et al., 1983

(d) Ferguson and Mah, 1983

(f) Rivard and Smith, 1982

More recent isolates of thermophilic methanogens include species belonging to the family Methanomicrobiaceae (Ferguson and Mah, 1983), Methanococcus thermolithotrophicus (Huber et al., 1982), Methanogenium thermophilicum (Rivard and Smith, 1982) and Methanosarcina species (Zinder and Mah, 1979). The habitats and distinguishing features of these thermophilic methanogens are summarised in Table 6.1.

The upper temperature limit for methanogenesis in thermal environment was thought to be less than 80°C (Zeikus et al., 1980; Ward, 1978). However, the recent isolation of Methanothermus fervidus (Stetter et al., 1981) and Methanococcus jannaschii (Jones et al., 1983) and the observation of methane production from mixed microbial communities at 100°C and 250°C (Baross et al., 1982; Baross and Deming, 1982), indicates that as yet undescribed species of methanogens may exist in the thermal environments. These methanogens and in particular Mt. fervidus appear to be much rarer in nature and possibly inhabit very specialized niches within the geothermal environments. For example, out of the 137 samples enriched for methanogens at 88°C, only 2 samples were positive and both yielded Methanothermus fervidus strains (Stetter et al., 1981). Mt. fervidus is unique among methanogens since it grows at temperatures where biological production of CO₂ and H₂ is absent and presumably grows by using non-biologically derived CO₂ and H₂ present in the geothermal waters.

This study was undertaken to determine the presence and distribution of known species of methanogens including M. thermoautotrophicum and Mt. fervidus in New Zealand hot springs. Attempts were also made to culture and study new isolates from these environments.

6.2 MATERIALS AND METHODS:

6.2.1 Chemicals

Hydrogen/carbon dioxide gas mixtures (80/20) were obtained from New Zealand Industrial Gases Limited.

6.2.2 Field Locations And Sampling Procedure

The sampling procedure was described in chapter 2. The location of sampling sites are in the pocket in the back cover.

6.2.3 Enrichment And Isolation Procedure

Three different media used to enrich methanogens are described below.

LPBM (described in chapter 7) was a synthetic medium designed to enrich autotrophic methanogens such as M. thermoautotrophicum whereas medium 3 (Balch et al., 1979) was designed to enrich more complex microbial consortia. Enrichments were initiated by adding 0.1 to 0.2ml sediment slurry into Hungate tubes containing 10ml of medium 3 or the defined mineral medium, LPBM. The procedure for preparing and dispensing anaerobic media has been described in chapter 5 and appendix 3. The gas phase used for enrichments was either N₂ or an 80:20 mixture of hydrogen:carbon dioxide (80:20). All incubations were at 65°C for a period of up to 8 days, during which time enrichments under the hydrogen:carbon dioxide gas phase were flushed with a fresh gas mixture daily. Pure cultures from positive enrichments were obtained by end point dilution in the defined medium (LPBM) or in medium 3 containing penicillin at a final concentration of 0.1mg/ml. Pure cultures of

methanogens were tested for the presence of heterotrophic contaminating organisms by inoculating loopfuls into TYEG medium. Growth in TYEG medium after 2-4 days at 65-70°C was evidence for heterotrophic contamination. TYEG was prepared as described in chapter 7. In other studies which were aimed at detecting the presence of biologically derived CO₂ and H₂, glycolytic anaerobes were enriched directly from pool samples using the media and techniques described in chapter 7.

Because of the success achieved in obtaining enrichments of sulphur respiring archaebacteria (chapter 5) by using pool water as a basal medium, limited enrichments were initiated using the same technique to enrich for methanogens. 10ml of pool water was collected from a depth of 15-20cm by means of sterile syringes and injected into sealed Hungate tubes which had been flushed and filled with a mixture of hydrogen and carbon dioxide (80:20). Coenzyme M (MENSA) at a final concentration of 1µg/ml and/or acetate at a final concentration of 10mg/ml was injected into the enrichment tubes. Coenzyme M was used since it was found to be stimulatory to Mt. fervidus (Stetter et al., 1981). Tubes containing pool water only were used as controls. All incubations were at a temperature of 88°C for a period of 6 to 7 days.

Since most of the methanogens reported in the literature (except Mt. fervidus and Mc. jannaschii) were capable of optimal growth between 65-70°C, it seemed more likely that a much wider range of methanogens could be isolated at such temperatures. Hence an incubation temperature of 65°C was chosen. For Mt. fervidus and Mc. jannaschii type isolates a temperature of 88°C was chosen.

6.2.4 Characterization Tests

A GLC equipped with a thermal conductivity detector was used to detect methane. The conditions used for such analysis have been described in chapter 7. Microscopic observations were made as described elsewhere.

6.3 RESULTS AND DISCUSSION:

(1) Enrichment of methanogens in a complex heterotrophic medium:

Bacteria were present in 9 enrichment samples incubated in medium 3 under a H_2/CO_2 gas phase but only 8 samples were positive when incubated under a N_2 gas phase (Table 6.2). Filaments and rods were present in all the pools and in addition Wai19 (Figure 6.1) had a coccal population.

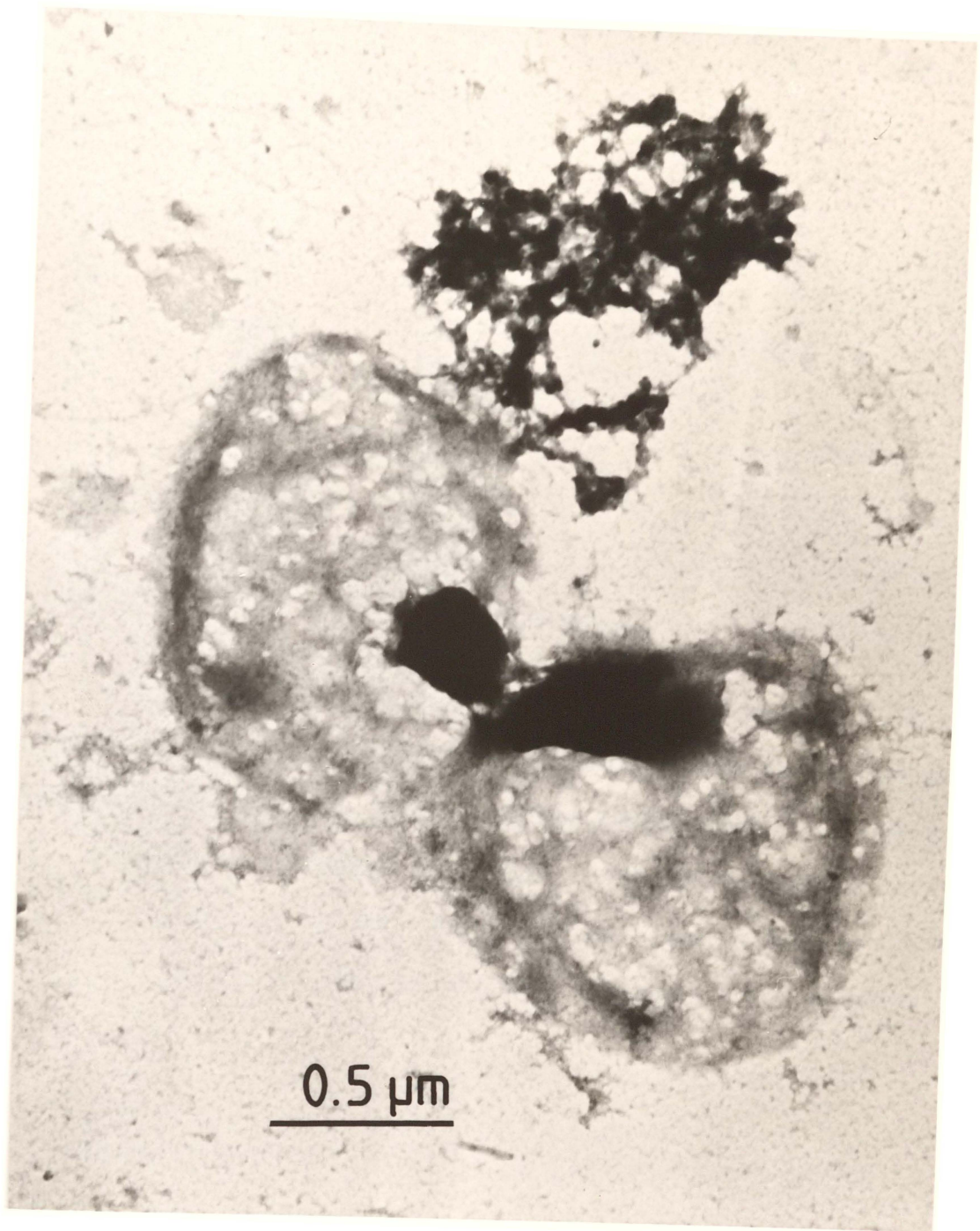
The enriched cultures were subcultured several times in medium 3. Methane was detected in only three samples, all of which had been enriched under CO_2/H_2 . These were Rt3, Rt100 and Wai19.

All the positive enrichments in medium 3 when subcultured in TYEG medium gave rise to turbidity, indicating once again that the enrichments were not methanogens. Enrichments from pools Rt3, Rt100 and Wai19, which were methane positive, also gave rise to bacterial cells in TYEG medium indicating the presence of mixed cultures.

Pure cultures of Rt3 and Rt100 were obtained by serial transfers in medium 3 which contained penicillin (0.1mg/ml). The absence of growth in TYEG medium and the presence of methane indicated that pure cultures of methanogens had been isolated. These organisms did not grow above 75°C and were morphologically similar to M. thermoautotrophicum. The cultures required a H_2/CO_2 atmosphere for growth and did not grow in the

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Figure 6.1 EM of a coccal-shaped methanogen present in pool Wai19 enrichment culture.



presence of acetate under a N_2 gas phase.

Table 6.2: Results of enrichment cultures grown in medium 3 in the presence of H_2/CO_2 and N_2 at $65^\circ C$:

Pool No.	Temp. ($^\circ C$)	pH	Growth in presence of	
			H_2/CO_2	N_2
Rt3	70.0	7.2	+	+
Rt34	75.7	6.53	+	+
Rt92	84.1	8.6	-	-
Rt97	78.0	7.14	+	+
Rt100	81.0	7.33	+	-
TK4a	90.1	5.8	+	+
TK5	61.0	5.6	+	+
TK6	84.0	4.7	-	-
TK12	78.0	6.94	-	-
OK2	57.0	7.6	-	-
OK4	60.0	6.9	-	-
OK9	83.0	6.8	+	+
WP8	83.6	6.8	-	-
WP14	76.2	5.74	-	-
WP20	74.8	5.55	-	-
WP21	67.7	6.58	-	-
WTB	96.0	6.98	+	+
Wai19	79.0	7.7	+	+

The coccus from Wai19 was difficult to purify since the rods which grew readily in TYEG were found to be resistant to penicillin even at concentrations of 0.2mg/ml. Increasing the concentration of acetate or H_2/CO_2 increased the number of rods. The cocci but not rods were susceptible to lysis by 5% SDS. The cocci was flagellated as observed under the electron microscope but motility was never observed under phase contrast microscopy.

(2) Enrichment of methanogens in the defined LPBM mineral medium:

12 of the 35 enrichments were positive for the presence of bacteria in the defined LPBM mineral media incubated under H_2/CO_2 (Table 6.3). After several transfers in the defined media they were checked for contaminating organisms. None of the enrichments were capable of growth

in TYEG medium and all were positive for methane production. Morphologically they were Gram positive rods and filaments and resembled M. thermoautotrophicum.

The absence of glycolytic anaerobes in the autotrophic methanogenic cultures was surprising since it has been observed that M. thermoautotrophicus cultures under laboratory conditions were often contaminated by Cl. thermohydrosulfuricum strains. The clostridial strains have been presumed to grow on the the lysed components of M. thermoautotrophicum. This indicates that the glycolytic anaerobes present in the thermal springs of New Zealand (Table 6.3) may not be Clostridium thermohydrosulfuricum strains or alternatively these strains were incapable of growth in autotrophic M. thermoautotrophicum cultures. Whether extremely thermophilic glycolytic anaerobes other than Cl. thermohydrosulfuricum are capable of growth on the lysed products of M. thermoautotrophicum is yet to be investigated.

Cultures Rt1-M1, Rt3-M1 and Rt8-M1 were purified by the end point dilution method. All three were incapable of growth above 75°C.

The optimum temperature of growth for Rt8-M1 was between 65 to 70°C as evidenced by visual turbidity at pH 7.0. Sulphide was always required for growth and no growth occurred in its absence even in a medium which was totally deoxygenated as indicated by complete reduction of resazurin. The optical density (660nm) was never greater than 0.12 when incubated at 65°C or 70°C. Such poor optical densities have been obtained for M. thermoautotrophicum in batch experiments (Zeikus and Wolfe, 1972; Zeikus et al., 1980). However, much higher values (O.D. of 8.0 at 660nm) were obtained under continuous culture (Zeikus and Wolfe, 1972).

Rt1-M1 and Rt3-M1 had a similarity coefficient of greater than 90% when analysed by Py-Mass Spectroscopy (Martin, A.M., Ph.D. thesis unpublished results).

Table 6.3: Distribution of methanogens and glycolytic anaerobes in some New Zealand hot springs:

Pool No.	Temp.(°C)	pH	Enrichment of	
			Methanogens in LPBM.	Glycolytic anaerobes in TYEG
Rt1	84.0	7.0	+	NT
Rt3	63.0	7.0	+	+
Rt6	64.0	9.4	+	+
Rt7	91.0	9.1	-	+
Rt8	64.0	9.1	+	+
Rt10	90.0	7.9	-	+
Rt12	66.0	6.9	-	+
Rt13	98.0	7.4	-	-
Rt14	80.0	7.0	-	+
Rt16	67.0	5.5	-	+
Rt17	96.0	5.2	-	+
TP 1	95.0	ND	-	-
TP 2	95.0	ND	+	-
TP 3	95.0	ND	+	-
TP 4	95.0	ND	+	-
TP 5	89.0	6.47	-	-
TP 6	78.0	7.28	+	+
TP 7	72.0	ND	-	-
TP 8	48.0	ND	+	+
TP 9	92.0	7.14	-	-
TP 10	72.0	6.84	-	+
TP 11	96.0	6.6	-	-
TP 12	ND	ND	+	-
Tok 1	96.0	7.86	-	-
Tok 2	81.0	7.13	-	-
Tok 3	76.0	6.93	-	-
Tok 4	89.0	5.63	-	-
Tok 5	52.0	5.93	-	+
Tok 6	97.0	7.0	+	+
Tok 7	53.0	6.04	-	+
Tok 8	75-80	5.59	+	+
Tok 9	86.0	7.0	-	-
Tok 10	75.0	6.5	-	-
Tok 11	98.0	4.0	-	-
Tok 12	97.0	7.0	-	-

Enrichment experiments were performed separately for glycolytic anaerobes and for methanogens at 6 5°C. TP1, TP2 and TP3 were wood samples taken from a fumarole. TP4 was also a wood sample but was taken from another fumarole. Tok6 a wood sample which was immersed in a pool.

(3) Enrichment of methanogens at 88°C:

Enrichments cultures which had been initiated at 88°C failed to yield methanogens as indicated by the absence of methane, in spite of bacterial presence (Table 6.4). However, the presence of ethane was observed from Wai9 enrichment culture.

Table 6.4: Results of enrichment cultures from Waimangu thermal area grown in enriched pool water at 88°C with CO₂/H₂

Pool No.	Temp.(°C)	pH	Bacterial morphology in pool water enriched with	
			MENSA	Acetate+MENSA
Wai8	85.0	3.03	-	-
Wai9	88.0	3.1	Cocci	Cocci
Wai11	96.0	8.5	Motile rods	-
Wai17	80.0	8.5	-	-
Wai18	81.6	7.34	Few rods	Few rods
Wai19	79.0	7.7	-	-
Wai21	67.0	6.5	Dividing and single rods	-

6.4 CONCLUSION:

From the limited number of screenings attempted (Table 6.3), it can be concluded that the occurrence of M. thermoautotrophicus in New Zealand thermal environments is widespread. The use of a defined medium and incubation under autotrophic condition is an ideal method of enriching and isolating M.thermoautotrophicus. 12 out of the 35 enrichment cultures gave rise to methanogens resembling M. thermoautotrophicus (Table 6.3). Microbial methanogenesis has been generally associated with the anaerobic decomposition of organic matter

in the absence of high amounts of certain electron acceptors (e.g. O_2 , NO_2^- , SO_4^{2-}). The presence of methanogens in wood samples in the thermal springs without the presence of combined trophic groups of bacteria such as glycolytic bacteria (Table 6.3) or sulphate reducing bacteria (chapter 7) which bring about effective mineralization of the organic matter, indicates that the methanogens may be capable of chemolithotrophic methanogenesis via bacterial reduction of CO_2 with geothermal hydrogen.

The use of medium 3 as an enrichment media on the other hand, gave rise to mixed cultures of methanogens (Table 6.2) and glycolytic anaerobes. The purification of methanogens from such mixed cultures has been achieved by using antibiotics which were inhibitory to the growth of eubacteria but not for the growth of archaeobacteria. Pure cultures of M. thermoautotrophicus were isolated from such mixed cultures by using penicillin. However, this method failed to yield a pure culture of the coccoid methanogen present in an enrichment from pool Wai 19 due to an antibiotic resistant or insensitive strain of a glycolytic anaerobe.

Growth was evident in enrichment cultures incubated at $88^\circ C$. However, methanogens (eg Mt. fervidus) were absent as indicated by the absence of methane production. This indicated that bacteria capable of growth either autotrophically or heterotrophically, were enriched. The presence of motility and the presence of binary fission in enrichment culture from pool Wai11 and Wai18 respectively indicated that the organisms did not belong to the Thermoproteales group of archaeobacteria.

The presence of ethane in enrichment cultures from pool Wai9 (Table 6.4) was interesting since none of the other enrichments or controls were positive for its presence.

CHAPTER 7

ISOLATION AND CHARACTERIZATION OF EUBACTERIAL ANAEROBES

7.1 INTRODUCTION:

A number of sporulating and non-sporulating thermophilic and extremely thermophilic anaerobes have been isolated from both thermal and non-thermal environments (Table 7.1). It is interesting to note that the geothermal niche has been the source for isolation of both the sporulating and non-sporulating thermophilic and extremely thermophilic glycolytic anaerobes whereas with the exception of Acetogenium kivui (Leigh et al., 1981) all isolates from non-geothermal environments are sporulating organisms (Table 7.1). Does this mean that only geothermal habitats are ideal places for non-sporulating thermophilic glycolytic anaerobes or is this merely a reflection on inadequate isolation procedures being used or our failure to search for them in non-geothermal environments. Recently, Varel (1984) has provided evidence of the presence of non-sporulating extremely thermophilic glycolytic anaerobes in a beef waste fermentor. The incidence of these organisms in this niche was surprisingly high and was 87% of the total isolates.

The organisms (Table 7.1) show great morphological, physiological and metabolic diversity. Morphologically all the organisms are rods or filaments of varying diameters and can exist singly, in pairs and/or in chains. In addition, coccal cells were frequently observed in

Table 7.1 Properties of thermophilic and extremely thermophilic glycolytic anaerobes:

Species	Habitats	Morphology	Dimensions (μm)	Gram reaction	Sporulation	Flagellation	Temp. growth range ($^{\circ}\text{C}$)	Temp. optima ($^{\circ}\text{C}$)	pH growth range	pH optima	Generation time (mins)	Autotrophy	Reductant requirement	G+C content (%)	Fermentation end product
<u>Acetogenium kivui</u> (a)	Lake sediment	Rods single, pairs or chains	0.7-2.0 x 7.5	-ve	-ve	-ve	50-72	66	5.3-7.3	6.4	120	Facultative	+	38	Acetate
<u>Clostridium thermoautotrophicum</u>	Thermal and non-thermal	Rods	0.8-1.0 x 3-6	V	oval (D)	Peritrichous	36-70	56-60	4.7-7.5	5.7	120	Facultative	?	53-55	Acetate
<u>Clostridium thermoaceticum</u> (c)	Horse manure	Rods	0.4x2.8	+ve	Spherical (ND)	Peritrichous	45-65	55-60	?	?	?	-ve	-	54	Acetate
<u>Clostridium</u> (d) <u>thermosaccharolyticum</u>	Spoiled canned food	Rods single pairs	0.5x 2.4-5.6	-ve	Spherical (D)	Peritrichous	28-67	55-60	?	?	?	-ve	?	29-32	Butyrate, lactate, acetate, CO_2 , H_2
<u>Clostridium</u> (e) <u>thermohydrosulfuricum</u>	Thermal & non-thermal environmts	Rods,	0.4-0.5 x 1.8-8.0	-ve	Spherical (D)	Peritrichous	40-78	68	5 - 9	5.9-7.5	72	-ve	-ve*	34.1	Ethanol, acetate lactate, CO_2 , H_2
<u>Clostridium thermosulfurogenes</u> (f)	Thermal springs	Rods	0.5-72.0	-ve	Spherical (D)	Peritrichous	>35- <75	60	4.0-7.6	5.5-6.5	114	-ve	?	32.6	Ethanol, CO_2 , H_2 , acetate, lactate
<u>Clostridium thermocellum</u> (g)	Thermal & non-thermal environmts	Rods	0.5-0.6 x 4-5	-ve	Oval (D)	Peritrichous	37- <70	50-64	?	?	68	-ve	?	88-39	Ethanol, acetate lactate, CO_2 , H_2 (Butyrate traces)
<u>Thermoanaerobium brockii</u> (h)	Thermal springs	Rods & filaments pairs or chains	1.0 x 2-20	+ve	-ve	Peritrichous*	40-80	65-70	5.5-9.5	7.5	60	-ve	-ve*	30-31.4	Lactate, ethanol, acetate, CO_2 , H_2
<u>Thermoanaerobacter ethanolicus</u> (i)	Thermal springs	Pleomorphic	0.5-0.8 x 4-100	V	-ve	Peritrichous	37-78	69	4.4-9.8	5.8-8.5	40	-ve	-ve*	32	Ethanol, CO_2 , acetate, H_2 , Lactate
<u>Thermobacteroides acetoehtylicus</u> (j)	Thermal springs	Singly or in pairs	0.6 x 1.5-2.5	-ve	-ve	Peritrichous	40-80	65	5.5-8.5	7.0	98	-ve	+ve	31	Ethanol, acetate, butyrate, isobutyrate CO_2 , H_2

- (a) Leigh et al., 1981
 (b) Wiegel et al., 1981
 (c) Fontaine et al., 1942; Gottschalk et al., 1981
 (d) Sjolander, 1937; McClung, 1934; Resting cultures produce ethanol
 (e) Wiegel et al., 1979; Hollaus and Klaushofer, 1973a, b.
 (f) Schink and Zeikus, 1983a
 (g) Mg et al., 1977; Zeikus et al., 1981
 (h) Zeikus et al., 1979
 (i) Wiegel and Ljungdahl, 1981
 (j) Ben-Bassat and Zeikus, 1981; Zeikus et al., 1980

V Indicates Gram +ve initial growth phase but Gram -ve in latter stage of growth.
 * Indicates unpublished results
 D Indicates spores

Thermoanaerobacter ethanolicus cultures (Wiegel and Ljungdahl, 1981). With the exception of Cl. thermoaceticum and Ta. brockii, all are Gram negative organisms. Sporulation is a common feature of the Clostridium species but spore production is very difficult to demonstrate with a number of these species; for example with Cl. thermohydrosulfuricum (Wiegel et al., 1979) and Cl. thermosaccharolyticum (Hsu and Ordal, 1969a) special media and/or conditions are required to demonstrate their presence (Wiegel et al., 1979; Hsu and Ordal, 1969b).

All the glycolytic anaerobes listed in table 7.1 are capable of heterotrophic growth on glucose. In addition, Acetogenium kivui (Leigh et al., 1981) and Cl. thermoautotrophicum (Wiegel et al., 1981) are capable of autotrophic growth on CO₂ and H₂. Cl. thermocellum (Ng et al., 1977) on the other hand until recently was the only thermophilic bacterium capable of growth on cellulose. However, more recently another two thermophilic cellulolytic bacteria, Caldocellum species (Wiegel, 1982) and Clostridium stercorarium (Madden, 1983). Other thermophilic bacteria with the ability to degrade polymers such as xylan have also been isolated and initial investigations have revealed that they are taxonomically new species (Weimer et al., 1984; Biomass Digest, 1982).

Acetogenium kivui (Leigh et al., 1981), Cl. thermoautotrophicum (Wiegel et al., 1981) and Cl. thermoaceticum (Fontaine et al., 1942) are homofermentative fermentors and produce acetate as the only fatty acid during fermentation on glucose. Additionally, acetate is the only product formed by Acetogenium kivui and Cl. thermoautotrophicum during autotrophic growth on CO₂ and H₂. Cl. thermosaccharolyticum is the only thermophilic glycolytic anaerobe known to produce substantial amounts of butyric acid and no ethanol during growth on glucose (Gottschalk et al., 1981). However ethanol is produced from glucose in resting cultures (Lee

and Ordal, 1967). Thermoanaerobacter ethanolicus, Thermoanaerobium brockii, Clostridium thermohydrosulfuricum and Clostridium thermocellum produce almost the same end products but in different quantities. This is because of subtle differences in the enzymatic pathway producing these products (Zeikus et al., 1981, Zeikus, 1980, 1983; Lamed and Zeikus, 1980; Morris, 1983). Clostridium thermosulfurogenes (Schink and Zeikus, 1983a) is the only known organism listed in table 7.1 which produces sulphur from thiosulphate. Thermobacteroides acetoethylicus produces equal amounts of acetate and ethanol and no lactate (Ben-Bassat and Zeikus, 1981).

A number of thermophilic and extremely thermophilic sulphate-reducing bacteria have also been isolated and studied. Desulfovibrio thermophilus (Rozanova and Khudyakova, 1974) a non-sporulating species was isolated from a deep-sea thermal petroleum stratum. The optimum temperature for growth of this organism was 65°C and the maximum 85°C and it required NaCl for growth. Furthermore, the cells divided unequally. Thermodesulfobacterium commune was isolated from geothermal springs (Zeikus et al., 1983). This organism grew optimally at 70°C and was distinctly different from Desulfovibrio thermophilus in its morphology and physiology. A surprising feature of this eubacterial organism was the presence of ether linked lipids which had only been previously reported in archaebacterial cells (Chapters 5 and 6). Desulfotomaculum nigrificans is a sporulating thermophilic sulphate-reducer with an optimum temperature for growth at 55°C and a maximum temperature for growth at 70°C (Campbell and Postgate, 1965).

Almost all the eubacterial thermophilic organisms which have been isolated from geothermal environments have been associated with algal-bacterial mat ecosystems, and little attention has been paid to their presence in thermal springs with little or no biomass production or springs of extreme pH and temperature.

Keeping these factors in mind, studies were initiated to identify the presence of ecological niches for extremely thermophilic eubacterial anaerobes in New Zealand hot springs. A number of species, some of which were similar to strains described in the literature and some novel, were isolated. Their documentation and the isolation and characterization of some of these species is presented in this chapter.

7.2 MATERIALS AND METHODS:

7.2.1 Chemicals

All chemicals were reagent grade. Antibiotics were purchased from Sigma Chemical Company and oxygen free nitrogen (OFN) was obtained from New Zealand Industrial Gas Ltd.

7.2.2 Sampling Procedure

The sampling procedure has been described fully in chapter 2.

7.2.3 Media And Cultivation Condition

A number of different media were used to enrich for anaerobes. These included a complex medium trypticase-yeast extract-glucose (TYEG) medium described by Zeikus et al., (1979) for enrichment of glycolytic anaerobes. Other enrichment media such as MMB, BM3A and medium 77 were

also used.

(a) TYEG (Zeikus et al., 1979) consisted of basal mineral media containing per litre of distilled water NH_4Cl , 0.9g; MgCl_2 , 0.2g; KH_2PO_4 , 0.75g; K_2HPO_4 , 1.5g; trace mineral solution, 9ml; 10% FeSO_4 , 0.03ml; 0.2% resazurin, 1ml; 5 ml of vitamin solution (Wolin et al., 1963); yeast extract, 3g; trypticase peptone, 10g and glucose 5g. Trace mineral solution contained per litre distilled water: 12.5g nitrilotriacetic acid neutralised to pH 6.5 with KOH, $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$, 0.2g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.017g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1g; ZnCl_2 , 0.1g; CuCl , 0.02g; H_3BO_3 , 0.01g; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.01g; NaCl , 1.0g and Na_2SeO_3 , 0.02g.

(b) MMB was essentially as described by Leigh et al., (1981) but was fortified with 10g/litre of 3-(N-morpholino)propanesulfonic acid (MOPS) and 2-(N-morpholino)ethanesulfonic acid (MES) and 1g/litre of trypticase peptone and casamino acids. The pH was adjusted to 5.6.

(c) Balch's medium 3 (Balch et al., 1979) without acetate and designated BM3A was used in initiating enrichments from the Fijian hot spring samples. BM3A was used since out of the 27 samples analysed, only 2 were positively enriched in TYEG medium. Again BM3A contained more chloride and sulphate ions to more closely reflect the composition of Fijian spring water which contains high concentrations of these elements due to mixing with seawater.

(d) Sulphate-reducing thermophiles were enriched medium 77 as described by Zeikus et al., (1983). Further characterization of the enrichments was not done due to lack of time.

The Hungate technique (Hungate, 1950) as modified by Latham and Wolin, 1978) with minor changes was used to prepare anaerobic media. Media was autoclaved in an Oxford dispensing jar (model S-A) for 1 minute which was sufficient to reduce the medium as indicated by resazurin or methylene reduction. A steady stream of O_2 -free nitrogen gas was allowed to flow through the inlet of the dispenser, via a bent syringe needle as the media cooled. 10ml of 10% $Na_2S \cdot 9H_2O$ was used as a reducing agent in all media except for medium 77 in which it was replaced by sodium thioglycollate (1 gm/litre). Unless indicated 10 ml of media was dispensed into tubes under O_2 -free nitrogen. For enrichments, medium walled pyrex tubes (size 12 x 152mm) fitted with '0' number stoppers and secured in a press before autoclaving were used. For all other studies, such as, nutritional, growth and antibiotic inhibition studies 23 ml anaerobic culture tubes (Bellco) fitted with butyl rubber septums and caps, were used.

Primary enrichments were initiated by adding 0.1 to 0.2 ml of sediment slurry to the appropriate pre-reduced medium. All enrichments initiated in TYEG medium, BM3A and medium 77 were incubated at 70°C under a N_2 atmosphere whereas enrichments initiated in MMB medium were incubated at 62°C under a $H_2:CO_2$ (70:30) atmosphere. After 1 to 3 days, tubes were examined both visually and microscopically for growth. All positive cultures were transferred and incubated at least three transfers in the same media after which they were purified using the end point dilution method in the appropriate liquid medium. However, anaerobes enriched in TYEG medium were further purified in TYEG agar deeps. (2% w/v of agar). Pure cultures were then stored at -20°C in a 50:50 glycerol-medium mixture.

unless stated otherwise,
For studies on nutrient utilization by glycolytic anaerobes, yeast extract (YE) medium was used. YE medium was TYEG medium minus trypticase peptone and glucose. Additions of nutrients were made to YE medium from 10% sterile stock solutions to a final concentration of 0.5%.

7.2.4 Growth And Metabolic Characterization

Growth was determined by measuring increase in turbidity at 600nm by inserting Bellco tubes directly into a spectronic 20 (Bausch and Lomb, N.Y.). All growth experiments were performed in duplicate.

Fermentation end products were analysed by gas chromatography. Metabolic gases were analysed in a Pye PU4500 gas chromatograph equipped with a thermal conductivity detector. A stainless steel column (2.7m by 3mm outer diameter), packed with Carbosieve B, 120/140 mesh (Supelco Inc., Bellefonte, Pa.) was used for analysis of H₂ and CO₂ at an oven temperature of 180°C detector temperature of 76°C and an injector temperature of 200°C. O₂-free ^{N₂} was used as a carrier gas at a flow rate of 8 ml/min. For analysis, 0.4ml from the headspace of the culture tube was withdrawn with a 0.5ml gas tight syringe (Dynatec Precision Sampling Corporation, Louisiana) and injected into the gas chromatogram. Concentration of gasses dissolved in the medium were obtained by calculation (Stephen and Stephen, 1963). Volatile fatty acids, ethanol and lactate were determined in a Pye G.C.D. gas chromatograph equipped with a flame ionization detector. Chromosorb 101, 100-120 mesh (Supelco Inc., Bellefonte, Pa.)) was packed in a glass column (1.8m by 4.5mm) and the oven was run isothermally at 160°C with a detector temperature of 225°C and an injector temperature of 215°C for determination of ethanol or volatile fatty acids. O₂-free nitrogen was used as a carrier gas at a flow rate of 60 ml/min. Lactic acid was determined by the same method,

but the column temperature was 200°C and the flow rate of nitrogen carrier gas was 52 ml/min. Propionic acid at a final concentration of 10mM was used as an internal standard for determination of volatile fatty acids and ethanol.

Peak areas of all analysis were automatically integrated on a Hewlett Packard 3390A integrator.

Glucose was determined by Glucose GOD-Perid method. (Boehringer Mannheim GmbH, West Germany).

7.2.5 Cellular Characterization

An Olympus BH phase contrast microscope was used for direct examination and cell size determinations. Thin sections of cells were prepared for electron microscopy by fixing an exponential culture grown in TYEG medium by the method of Sleytr and Glauert (1976). Fixed cells were dehydrated in a graded series of ethanol, embedded in Spurr's resin (Spurr, 1969) and sectioned with glass knives on a Reichert ultramicrotome. Sections were placed on 400 mesh grids, stained with 1% uranyl acetate (pH 4.0) and lead citrate. Negative staining was accomplished by floating 200 mesh butvar coated grids on a drop of bacterial culture for one minute. Excess culture was drawn off the grid with filter paper and the grid stained by applying a drop of 1% uranyl acetate (pH 4.0) for 30 seconds removing excess stain and the grids (both negatively stained and thin sections) examined under a Phillips EM 400 electron microscope at an accelerating voltage of 80kV.

Inhibition studies by antibacterial agents were performed in Hungate tubes which contained 5 ml of TYEG medium to which the appropriate antibacterial agents from filter sterilized concentrated stock solutions were added, so as to give the appropriate final concentration. Incubations were done at 65°C for an appropriate time (depending on the growth rate of the organism under study). Calculations on percentage inhibition were done as explained earlier (Patel et al., 1985a).

7.2.6 G+C Content

Cells were lysed using the method of Owen and Hill, (1979) and DNA recovered to the first precipitation stage as described by Marmur (1961). Further purification of the DNA was achieved on a Sepharose 4B column (Gibson and Ogden, 1979). The T_m of the DNA was measured in a Pye SP8-400 spectrophotometer with stepwise heated polyethylene glycol 4000 circulating through the heating block. The temperature of the DNA solution was measured directly in the cuvette with a YSI 427 thermistor probe (Yellow Springs Instrument Co., Ohio) inserted through a stopper and read directly on a Digisense thermometer (Cole Palmer Inst. Co., Illinois). *E. coli* DNA (Sigma Chemicals) was used as a standard. The %G+C of *E. coli* was 51.2.

7.3 RESULTS AND DISCUSSION:

7.3.1 PART I. STUDIES ON CALDOACTIVE GLYCOLYTIC ANAEROBES

7.3.1.1 General Features:-

(1) Distribution

At an incubation temperature of 70°C, 51 of the 266 pools screened gave positive enrichments, as indicated by turbidity and microscopic examination, for extremely thermophilic glycolytic anaerobes (Table 7.2). The pH and temperature of the pools screened (Fig. 7.1A) and those which yielded caldoactive glycolytic anaerobes is represented graphically in Figure 7.1B. Since full data on 68 pools was not available, they have not been represented.

Table 7.2 Temp. and pH of thermal springs yielding thermophilic anaerobic glycolytic bacteria:

Pool No	Temp (°C)	pH	Bacterial morphology
<u>Rotorua</u>			
Rt3*	70	7.2	Two types of rods
Rt6*	64	9.4	Rods, filaments and cocci
Rt7	91	7.3	Rods, diameter > 1.2µm
Rt8*	70	9.0	Rods, single in twos & filaments
Rt10	94	7.9	NA
Rt12	66	6.9	Rods with spheroids, single or in twos
Rt14	80	7.0	Rods with spheroids, single or in twos
Rt16	69	5.5	Rods with spheroids, single, in twos or in clumps
Rt17	96	5.2	Rods with spheroids, single or in twos
Rt28	95	6.0	Rods with spheroids, single or in twos
Rt44*	62	7.81	Rods and filaments.
Rt46	90	3.6	Filaments > 10µm
Rt66*	78	6.12	Sporulating rods, filaments, rods single or twos
Rt67	80	6.6	Rods 1.2µm in diameter
Rt68	95	7.52	NA
Rt97	78	7.14	Rods with spheroids.
<u>Waimangu</u>			
Wai4*	56	4.9	Rods single, in twos with or without spheroids
Wai5*	54	4.0	Rods and filaments (2-10µm x 0.8µm)
Wai6	75	5.3	NA
Wai7	82	6.3	NA
Wai10	91	7.4	Rods
Wai14	70	7.7	NA
Wai16	70	-	Rods, single or in twos and filaments
Wai19	79	7.7	NA
Wai21*	67	6.25	Rods
<u>Tokaanu:</u>			
Tok5*	52	5.93	Motile rods, single in twos or in chains. Could not be revived from glycerol preserved enrichment cultures
Tok6	97	7.0	Rods single in twos or filaments
Tok7	53	6.04	As in Tok6
Tok8*	77	5.6	As in Tok6
<u>Taupo:</u>			
TP6	78	7.3	As in Tok6
TP8	48	ND	NA
TP10*	72	6.84	Motile rods and filaments
<u>Tikiteri:</u>			
TK4a	90.1	5.8	Rods single or in twos and filaments
TK4x	93.5	6.5	As in TK4a
TK15	84.5	5.33	As in TK4a
TK19	63	6.1	NA
TK20	75	4.9	NA

Table 7.2 (Cont'd) Temp. and pH of thermal springs yielding thermophilic anaerobic glycolytic bacteria:

Pool No	Temp (°C)	pH	Bacterial morphology
TK21	73	6.0	Rods could not be revived from glycerol preserved enrichment cultures.
TK22	62	3.5	As in Tk49. Thin sporulating rods
TK26	75	5.5	NA
TK28	75	6.25	NA
TK29	68	6.5	NA
<u>Orakei Korako:</u>			
OK2*	51	7.6	NA
OK3	81	8.6	Rods single or in twos
OK4*	60	6.9	Rods single or in twos, lysis after prolonged incubation
OK5*	66.5	8.58	Rods single, in twos or in clumps
OK8	83.5	8.5	Coccobacillary type of rods, single or in twos
OK9	83	6.8	As in OK4
OK10	74	7.86	As in OK4
OK12	80	7.4	As in OK4
<u>Fiji Islands:</u>			
Wg7B*	69	8.01	Filaments > 10µm
Wg14*	67.2	6.80	Rods, single in twos or in chains
<u>Mt Erebus:</u>			
E2	62	ND	Rods and filaments
Garden compost	60	ND	Rods single, in twos and filaments
Uni. Lake 15 (site 3)		ND	Filaments, some with terminal spores

Enrichment medium was TYEG broth. Incubations were inspected for turbidity for periods up to 72 hours. Tubes not showing turbidity within this time were regarded as negative and discarded.

The Fijian springs were also set up for enrichments in TYEG containing 2%, 4% and 8% NaCl. With 2%, the pools indicated in this table, were positive. However, no organisms were obtained with 4% or 8% NaCl. All strains were grown in TYEG medium for up to 72 hours at 70°C. Enrichments were considered negative after this time period and discarded.

NA = not analysed by phase contrast microscopy.

ND = not determined.

* = indicates presence of algal and/or bacterial mats.

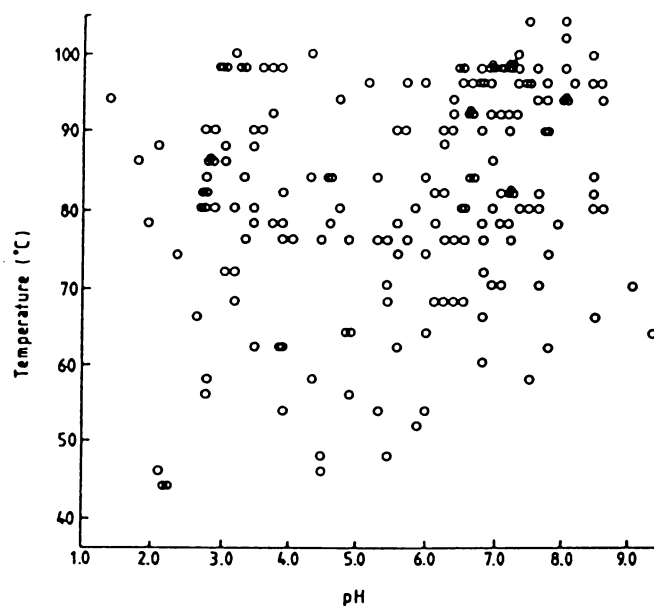


Figure 7.1A Graphical representation of temeprature and pH of the pools screened for caldoactive glycolytic anaerobes.

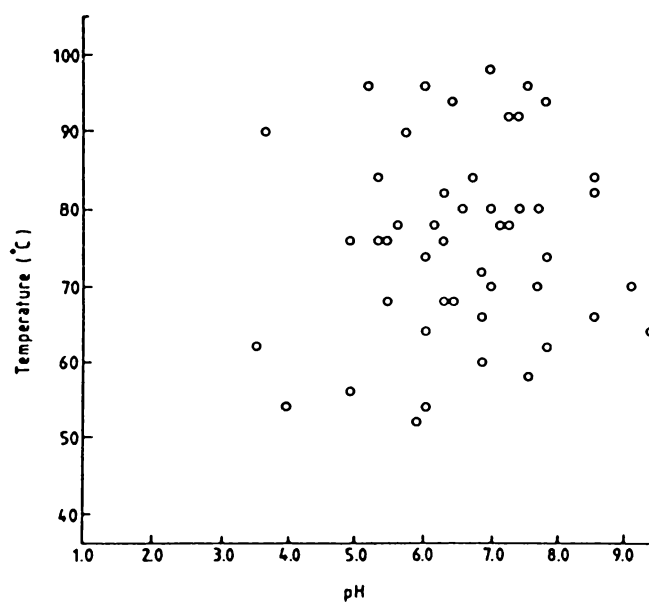


Figure 7.1B Temperature and pH of pools' yeilding caldoactive glycolytic anaerobes.

The chances of obtaining extremely thermophilic glycolytic anaerobes decreased with temperatures above 75°C with none being enriched from pools above 97.5°C (Figure 7.2B). Similarly, pools below pH 3.6 did not yield glycolytic anaerobes (Figure 7.2A). It is interesting to note that the pH profile of Figure 7.2A represents the pH growth range of a number of pure cultures of caldoactive glycolytic anaerobes.

However, all 266 samples when incubated at 82°C were found to be negative. It is possible that the enrichment conditions used had selected for a restricted physiological type of caldoactive glycolytic anaerobe, that is organisms which grow at neutral pH and a temperature optimum of around 70°C on glucose as the energy source. Moreover, a number of acidic pool samples enriched at pH 3.5 were negative. Such screenings were however, limited. Before a firm conclusion can be drawn on temperature and pH limitation on distribution of caldoactive glycolytic anaerobes, a much more thorough investigation is required. Such an investigation will need to use parameters which mimic as closely as possible the conditions of the pool environment. For example, Ljungdahl et al., (1981) were able to enrich a number of anaerobic cultures from Icelandic hot springs by simply using temperatures and pHs similar to that of the source pools.

(2) Most Probable numbers

Wiegel et al., (1979) have demonstrated that the numbers of thermophilic glycolytic anaerobes present in hot springs were between 500-5000 times greater than those obtained from mesophilic environments. High counts were obtained in MPN estimation of caldoactive glycolytic anaerobes from 3 New Zealand thermal springs (Table 7.3).

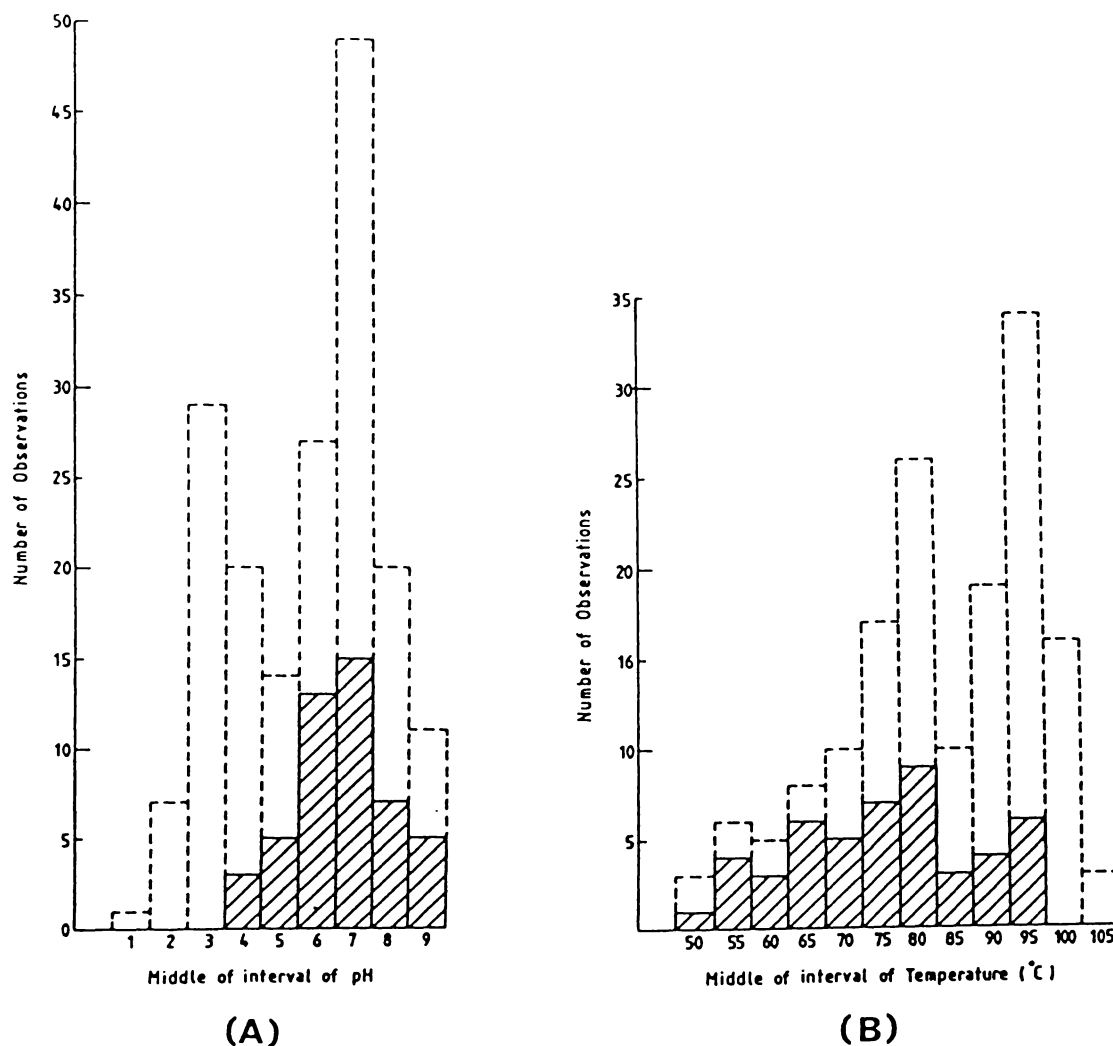


Figure 7.2A Histogram of pools yielding caldoactive glycolytic anaerobes at various pHs. Pools having temperatures above 97.5°C were deleted from the histogram plot since no caldoactive glycolytic anaerobes were isolated from such pools.

Figure 7.2B Histogram of pools yielding caldoactive glycolytic anaerobes at various temperatures. Pools having pHs below 3.6 were deleted from the histogram plot, since no caldoactive glycolytic anaerobes could be isolated from such pools.

(Refer errata and addenda on page]]-])

The numbers of caldoactive glycolytic anaerobes decreased with an increase in temperature with low densities present at temperatures above 80°C. Pools Rt3 and Rt8 had similar temperatures but markedly different population densities. This might reflect the presence of algal-bacterial mats in Rt8 providing a richer organic medium than Rt3, or may be a reflection of the nutrient composition of the spring water.

Table 7.3 MPN of caldoactive glycolytic anaerobes present New Zealand hot springs.

Pool No.	Temperature (°C)	pH	MPN/100ml of pool water
Rt3	70	7.2	2,400
Rt8	70	9.1	>24,000
Rt13	98	7.4	0
Rt14	80	7.0	230
Rt15	96.4	8.7	0

MPN was performed essentially as described on page 5-19 except that TYEG was the medium used at 70°C.

(3) Morphology and fermentation end products

In enrichment cultures, the most common bacterial morphologies present were rods and filaments with cocci being rare (Table 7.2). Rods and filaments varied in morphology and size. Unique spheroid producing bacteria were observed in a number of springs from Rotorua and from a single Waimangu thermal spring. Some bacteria were actively motile whereas most enrichment cultures contained non-motile microorganisms.

All the positive enrichments were subcultured up to three times in TYEG medium and stored in a 50:50 glycerol-TYEG mixture. A number of these bacteria were purified for further studies on their fermentation end products from glucose, since end products have been useful in classification of anaerobes. The strains could be grouped, into 3

categories based on the type of major end product formed, namely ethanol (Table 7.4), acetate (Table 7.5) and lactate (Table 7.6). However, it should be noted that not all the isolates conformed to the production of a single dominant end product, and the conditions of culture could vary the ratio of end products. For example the production of ethanol or lactic acid in Cl. thermohydrosulfuricum was dependent on the initial culture pH (Wiegel et al., 1979). An initial pH of 7.5 favoured ethanol production whereas a pH below 6.5 favoured lactate formation. Similarly T. ethanolicus produced significantly more lactate than ethanol or as an yet unknown reason (Wiegel et al., 1979). Indeed, a Cl. thermohydrosulfuricum strain isolated from compost (Table 7.6) was found to have lactate as a major end product whereas a number of other Cl. thermohydrosulfuricum isolates from thermal springs produced ethanol as a major end product (Table 7.4) in spite of their growth under identical conditions. Hence it was not possible to classify these organisms solely on the basis of end products formed. Further studies on the morphology, Gram reaction, sporulation and cell wall ultrastructure of the organisms in each group were required to differentiate the dominant organisms, and all discussions presented in this chapter have been done on this basis.

7.3.1.2 Isolation And Characterization Of Thermoanaerobacter Type - Isolates:-

A caldoactive glycolytic anaerobe, very similar to T. ethanolicus was isolated from Taupo (Table 7.4) and designated TP6-B1. The organism could not grow on TYEG agar plates if exposed to air. Similarly, they failed to grow on plates even when all procedures were performed in an anaerobic glove box, hence pure cultures were obtained by using an end point dilution method in TYEG agar shakes. Creamish colonies, 1mm to 2mm

Table 7.4 Fermentation end product and properties of some caldoactive glycolytic anaerobes producing ethanol as the major end product:

Properties	<u>Ta. brockii</u> type		<u>Cl. thermohydrosulfuricum</u> type				<u>T. ethanolicus</u> type		Others	
	<u>Ta. brockii</u>	Tok6-B1	<u>Cl. thermohydrosulfuricum</u> E100-69	Rt8-B1	Tk5-B1	E2-B1	<u>T. ethanolicus</u>	TP6-B1	Wai16-B1	OK4-BA1 ^a
(1) Fermentation end products (mM):										
Ethanol	15.3	30	30	30	27	26.7	38	15	14.2	40
Acetate	5.1	22	5	6	9	2.5	3	3	9.1	Tr
Lactate	12.5	2.2	16	2	NA	14.3	2.5	10	5.1	NA
(2) Properties:										
Gram reaction	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Morphology	Rods to filaments mini cells		Rods single, in pairs or in chains & filaments	ibid	ibid	ibid	Rods to filaments blebs & minicells	Rods, fila- ments & mini cells	Rods single in pairs & filaments	Rods single or in pairs
Sporulation	-ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
pH optima	7.5	6.5	6.9-7.5	~7.0	7.0	7.0	5.8 to 8.5	7.5	6.5-7.5	~7.0
Temp optima(°C)	70	70	69	70	65-70	65-70	69	70	65-70	~70
Doubling time (minutes)	70	60	70	NA	NA	NA	40	90	NA	NA
Flagellation	+	+	+	+	NA	+	+	+	-	-

All organisms were grown in 10mls TYEG and incubated at 70°C overnight.

^a = obtained from other enrichment procedures described in latter section.

Tk8-B1 and Tk9-B1 were sporulating organisms but end products were not analysed and hence were excluded from this table.

Tr = trace amounts

NA = not analysed

Table 7.5 Fermentation end product and properties of some caldoactive glycolytic anaerobes producing acetate as the major end product:

Properties	<u>Thermobacteroides acetoethylicus</u> type									
	<u>Tb. acetoethylicus</u>	Wa15-B1	Wa110-B1	Rt46-B1	TP10-B1	Tk20-B1	OK3-B1	OK4-B1	OK10-B1	OK12-B1
(1) Fermentation end products (mM):										
Ethanol	9	minor	minor	0.3	4.0	3.2	2.3	1.6	2.7	3.0
Acetate	15	major	major	6.4	10.0	22.3	14.6	17.9	12.5	14.2
Iso-butyrate	2	Tr	Tr	-	0.1	-	Tr	Tr	Tr	Tr
N-butyrate	9	Tr	Tr	-	0.1	-	-	Tr	Tr	Tr
Iso-valerate	-	Tr	-	-	0.3	-	Tr	Tr	Tr	Tr
N-valerate	Tr	-	-	-	-	-	-	-	-	-
Lactate	- ve to Tr	-	-	1.0	3.0	5.0	3.0	6.07	< 3	< 3
(2) Properties:										
Gram reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	NA
Morphology	Rods single or in pairs	Dumbell shaped rods, single or pairs	ibid.	Fila-ments	Filaments and rods					
Sporulation	-	-	-	-	-	-	-	NA	-	-
pH optima	~7.0	7.0 - 7.8	NA	7.0 - 7.5	7.0	7.5	NA	NA	NA	NA
Temp. optima (°C)	65°	65-70°	65-70°	65-70°	65-70°	65-70°	NA	NA	NA	NA
Doubling time(min)	30	NA	NA	NA	NA	NA	NA	NA	NA	NA
Flagellation	+	+	+	-	NA	NA	NA	NA	NA	NA

All organisms were grown in 10ml TYEG and incubated at 70°C overnight.

Table 7.6 Fermentation end product and properties of some caldoactive glycolytic anaerobes producing lactate as the major end product:

Properties	OK5-B1	OK8-B1	OK9-B1	<u>Clostridium thermohydrosulfuricum</u> from compost ^a	<u>Fervidobacterium nodosum</u> type Rt17-B1 ^b	<u>Wa14-B1</u>
(1) Fermentation end products (mM)						
Ethanol	0.9	1.0	1.24	2	1.14	1.8
Acetate	4.6	10.0	7.9	12	6.6	5.7
Iso-butyrate	Tr	-	-	-	-	-
n-butyrate	-	-	-	Tr	-	-
Iso-valerate	-	-	-	-	-	-
n-valerate	Tr	-	-	-	-	-
Lactate	46.3	42.7	35.85	30	9.8	8.2
(2) Properties:						
Gram reaction	NA	NA	NA	-ve	-ve	-ve
Morphology	Single or paired rods	Small rods single or in pairs	Rods single or in pairs	Rods to filaments	Rods single, in pairs or chains produce spheroids	
Sporulation	-	-	-	+	-	-
pH optima	NA	NA	NA	NA	7.0	7.0
Temp. optima (°C)	NA	NA	NA	NA	70°	65°
Doubling time(min)	NA	NA	NA	NA	105	NA
Flagellation	NA	NA	NA	NA	+	NA

All organisms were grown in 10ml TYEG and incubated at 70°C overnight.

a = A sporulating Clostridium thermohydrosulfuricum type organism and hence been discussed with Clostridium tnermohydrosulfuricum type isolates.

b = Initially produced acetate as the major end product but subsequently produced lactate as the major end product.

NA = not analysed.

* Refer paper (Patel et al., 1985a-back pocket cover

in diameter were obtained in TYEG agar shakes after 48 hours incubation at 65°C.

Cultures grown in TYEG lost viability if stored at room temperature for longer than 2 weeks and hence during all growth experiments, this organism was subcultured at weekly intervals. Stock cultures were maintained at -20°C in a 50:50 glycerol-TYEG medium mixture.

(1) Morphology

Both T. ethanolicus and TP6-B1 were Gram negative non-sporulating flagellated rods, 0.7 to 0.8µm x 5-7µm in dimensions. Minicells and blebs (Fig.7.3) were present in both cultures. Minicells were produced due to unequal cell division and were also protruberances of the cell and were distinguishable from spores and prespores or from spheroids produced by Caldobacterium nodosum.

(2) Growth Optima

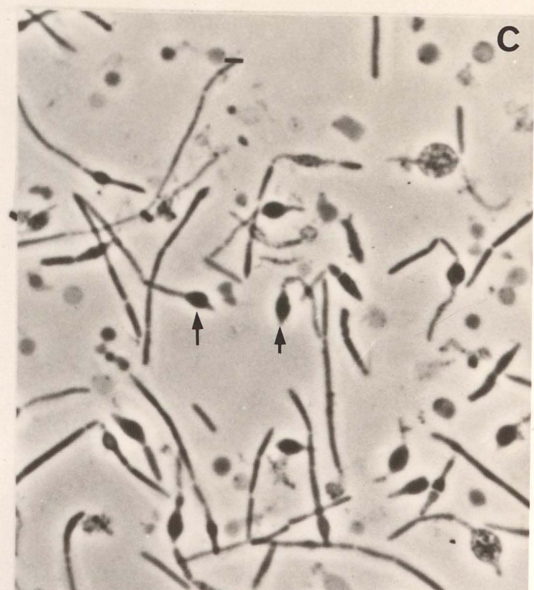
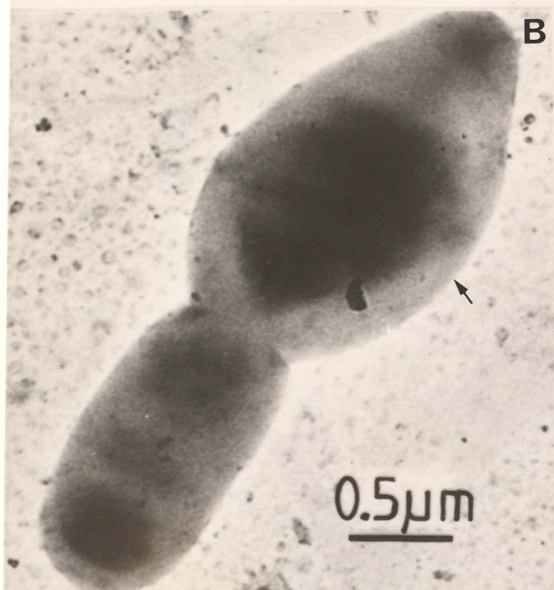
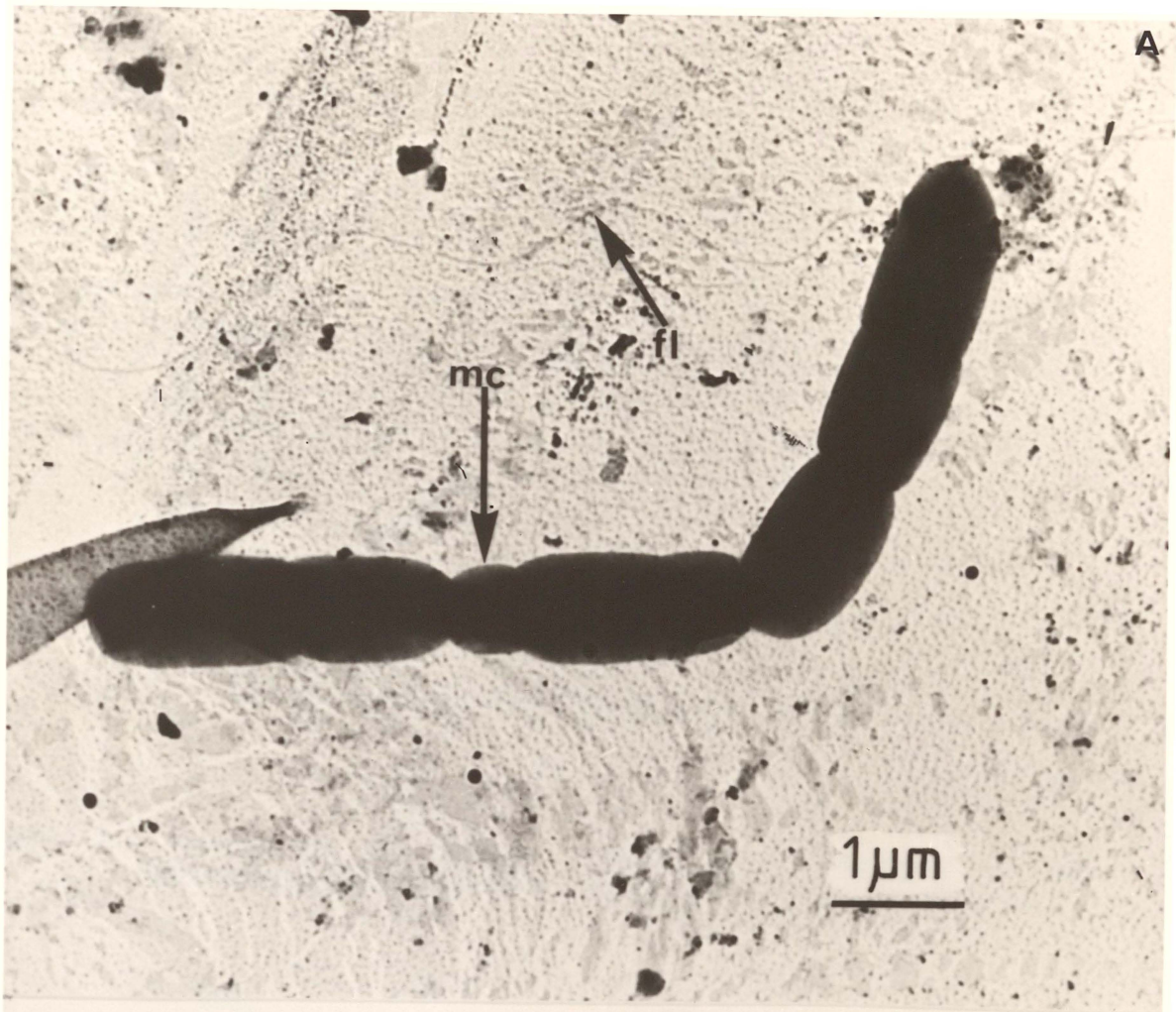
TP6-B1 was capable of growth from pH 5.0 to 9.0 with an optimum pH of 7.5 (Fig 7.4). T. ethanolicus had a similar pH range for growth, however the optimum pH was much broader and was between 5.8 to 8.5 (Wiegel and Ljungdahl, 1981). The minimum temperature for growth of TP6-B1 was above 45°C but that of T. ethanolicus was 37°C. Both anaerobes had a temperature maxima of below 80°. The temperature optima of both organisms was 70°C. The temperature-growth relationship of TP6-B1 is shown in Fig 7.5.

Figure 7.3 Light Microscopic and electron microscopic observations of isolate TP6-B1.

(A) A filamentous cell has divided to form cells of unequal length and mini-cell (MC).

(B) Cell showing bleb formation

(C) Bleb formation after the culture has reached stationary phase of growth. (Light Microscopy). x4000



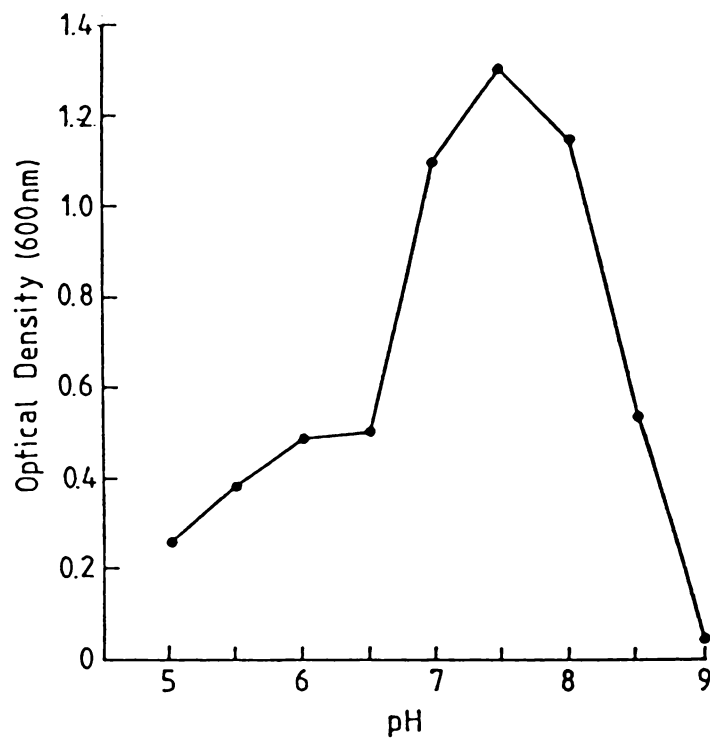


Figure 7.4 Growth of NZ isolate TP6-B1 as a function of pH. Absorbance was measured at 600nm after 14 hours of growth.

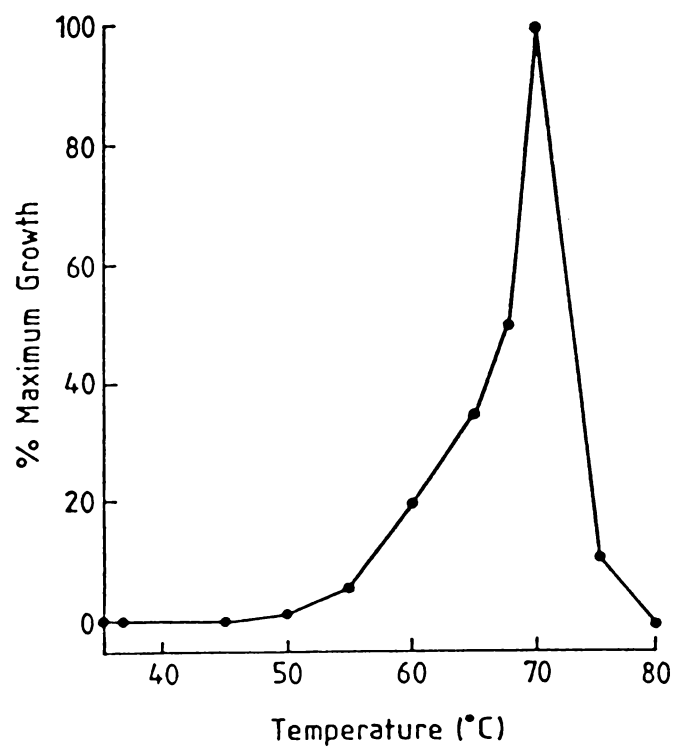


Figure 7.5 Growth of TP6-B1 as a function of temperature. After 14 hours of growth in TYEG medium at various temperatures, absorbance was read at 550nm. An absorbance of 0.8 is equal to 100% growth.

(3) Cell Wall Ultrastructure

Electron microscope observations of thin sections of TP6-B1 (Fig 7.6) and T. ethanolicus (Fig. 7.7) showed a typical Gram negative cell wall ultrastructure in that a very thin and dense, presumably a peptidoglycan layer was surrounded on either side by electron light cell wall layers. These two cell wall layers were discernible as containing subunits in case of T. ethanolicus but were diffuse in case of TP6-B1.

(4) Antibiotic Sensitivity

The effect of antibacterial agents on growth of TP6-B1 and T. ethanolicus is presented in Table 7.7.

Table 7.7 Effect of antibiotics and inhibitors on growth of TP6-B1 and T. ethanolicus:

Antibacterial agent	Amount	Strains (% inhibition)	
		TP6-B1	<u>T. ethanolicus</u>
Neomycin	10µg/ml	55%	49%
	100µg/ml	87%	97%
D-cycloserine	10µg/ml	0	0
	100µg/ml	94%	56%
Tetracycline	10µg/ml	94%	94%
	100µg/ml	100%	98%
Streptomycin	10µg/ml	80%	94%
	100µg/ml	94%	??
Penicillin G	10µg/ml	94%	99%
	100µg/ml	98%	100%
Chloramphenicol	10µg/ml	94%	0%
	100µg/ml	98%	100%
Sodium azide	250µg/ml	92%	70%
	500µg/ml	94%	94%
Sodium chloride	1%	1%	ND
	2%	64%	ND

Figure 7.6 Electron Micrograph of thin section of isolate TP6-B1

(A) Whole cell.

(B) Cell-wall detail showing three layered structure
membrane, D=dense layer, O=outer layer, I=inner layer,
M=mesosomes.

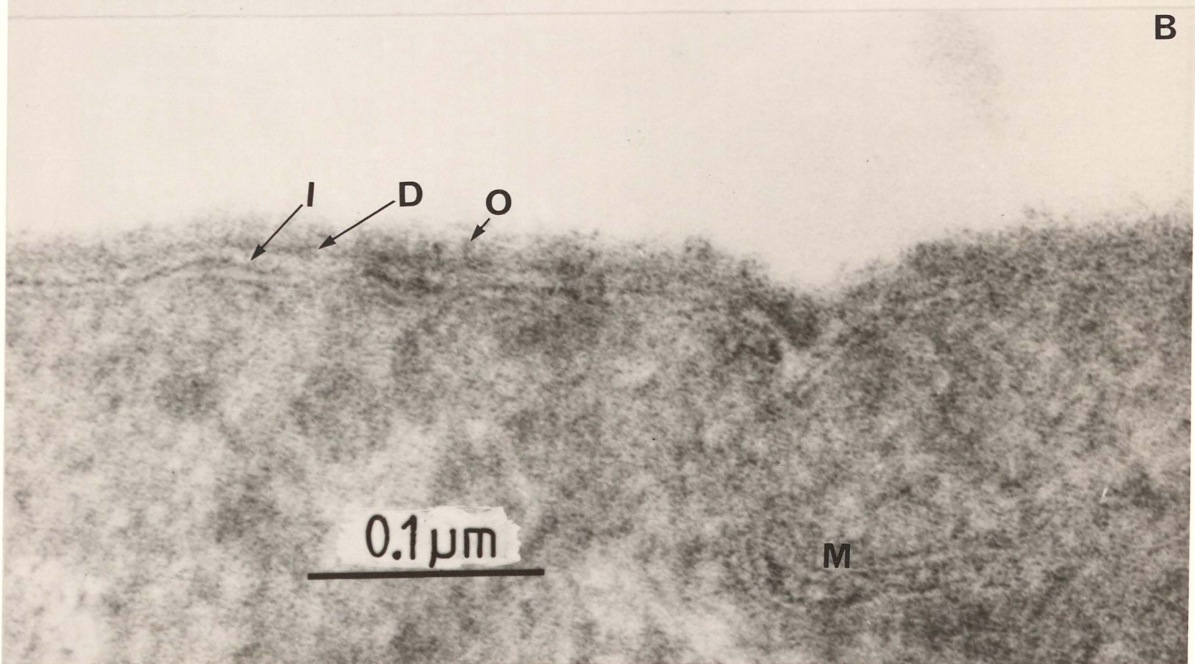
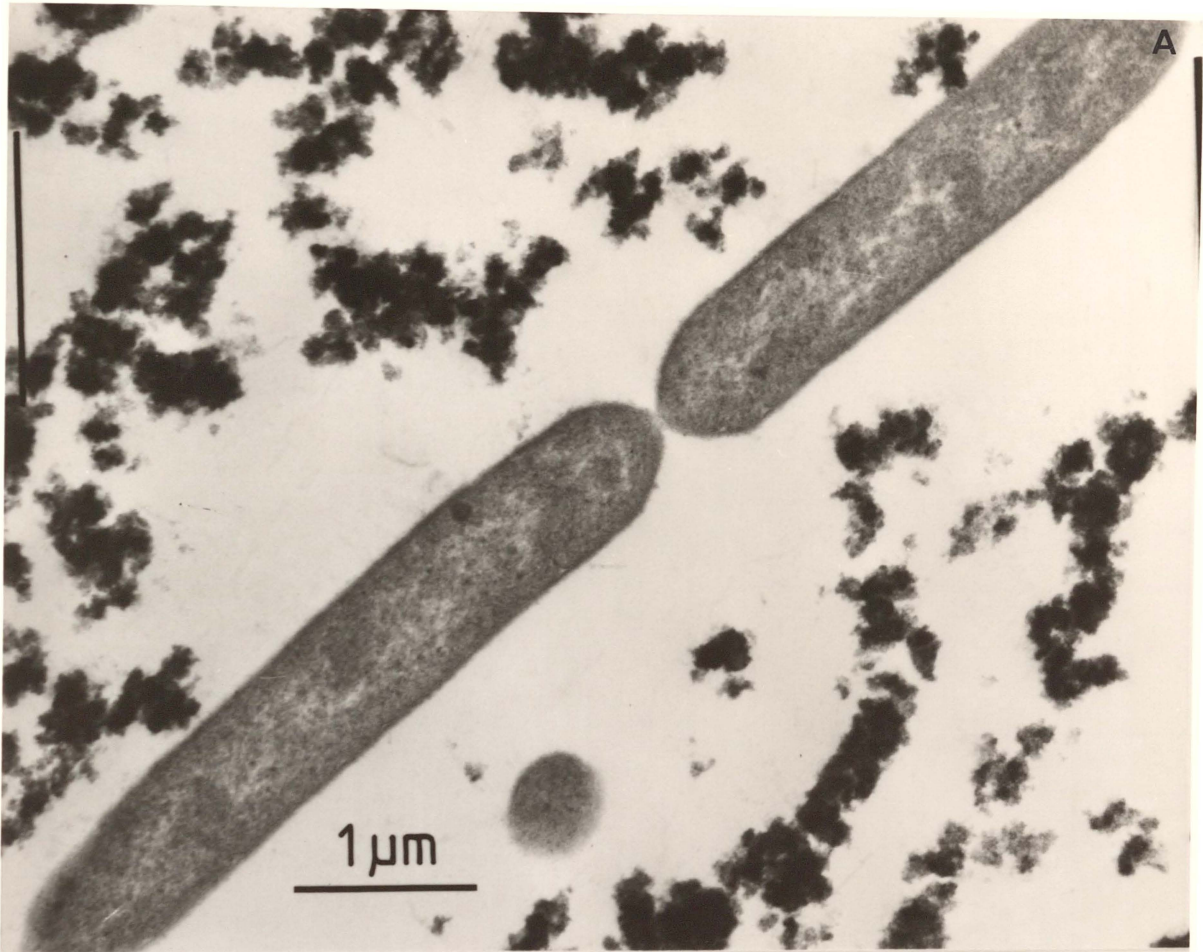
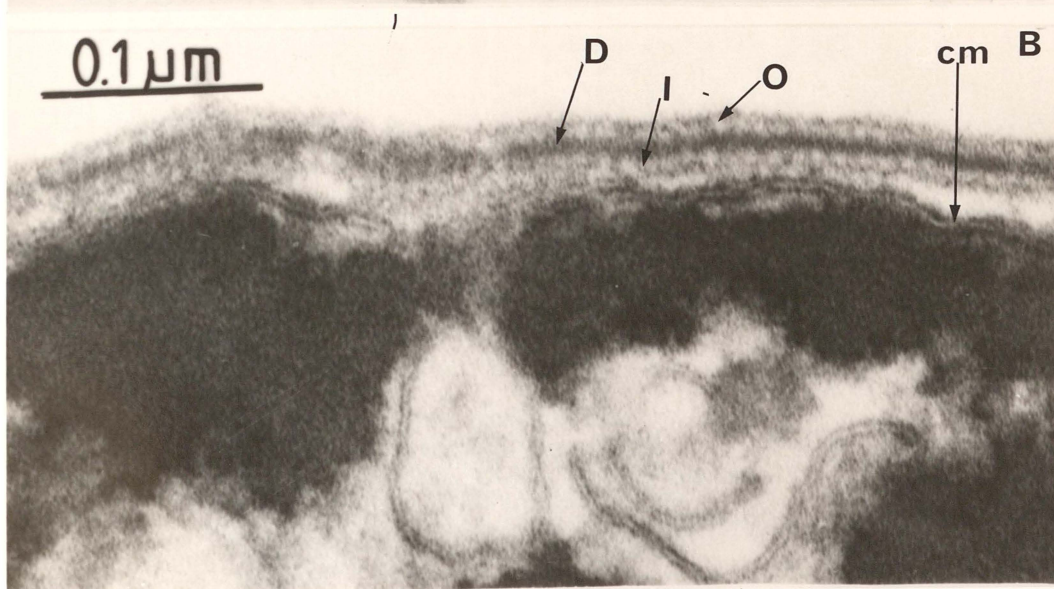


Figure 7.7 Electron Micrograph of a thin section of Thermoanaerobacter ethanolicus JW200 (ATCC 31550)
(A) Whole cell.
(B) Cell-wall magnified to show the three layered structure of the cell-envelope. CM=cytoplasmic membrane, D=Dense layer, O=outer layer, I=inner layer.



The inhibition spectrum of antibacterial agents on TP6-B1 was very similar to that of T. ethanolicus. Neomycin, D-cycloserine, tetracycline, streptomycin, penicillin and chloramphenicol at a concentration of 100µg/ml totally inhibited both strains. Both strains were in general more sensitive to the antibacterial agents compared to Tok6-B1 and Ta. Brockii.

(5) Nutritional Characterization

TP6-B1 required yeast extract or trypticase peptone when growing on a fermentable substrate. Beef extract could replace yeast extract or trypticase peptone when glucose was used as a nutrient. Limited growth occurred in the absence of glucose on trypticase peptone or yeast extract, but not on beef extract. Table 7.8 provides the nutritional spectrum of TP6-B1. TP6-B1 in general was more nutritionally versatile than T. ethanolicus.

Table 7.8 Nutritional spectrum of TP6-B1:

Substrate Tested	Species	
	TP6-B1	<u>T. ethanolicus</u> (a)
Adonitol	+	NA
Sorbitol	+	-
m-inositol	+	-
Mannitol	+	-
Galactose	+	+
Fucose	+	-
Raffinose	+	-
Rhamnose	+	-
Arabinose	+	NA
Pectin	+	NA
Chitin	+	NA
Lactose	+	NA
Xylose	+	+
Sucrose	+	+
Maltose	+	NA
Amylopectin	+	NA
Glycogen	+	NA
Maize starch	+	+
Acacia	+	NA
Cellobiose	+	+

Substrate (0.5%) was added to LPBM medium that contained 0.2% yeast extract. Growth was determined after 36 hours at 65°C. No growth occurred when acetate, lactate, succinate, gelysate or beef extract were used as substrates. (a) Data obtained from Wiegel and Ljungdahl, (1981).

(6) Growth rates and end product formation

Growth of TP6-B1 in TYEG is shown in Figure 7.8. Ethanol was the major end product formed together with minute amounts of lactate. Acetate, H₂ and CO₂ were also formed. The generation time was 90 minutes.

Table 7.9 presents fermentation balances obtained with TP6-B1 and T. ethanolicus with glucose as the growth substrate.

Table 7.9 Fermentation products from glucose by TP6-B1 and T. ethanolicus

Products formed	Amount produced (total μmol)	
	TP6-B6	<u>T. ethanolicus</u>
Ethanol	280.1	170.0
Acetate	132.3	38.6
Lactate	34.7	280.0
CO ₂	339.4	184.0
H ₂	107.5	11.5
Glucose utilized	196.0	197.0
Carbon recovery	1.07	1.21
C ₁ /C ₂ ratio	1.05	0.88
O/R ratio	0.96	1.05

TP6-B1 and T. ethanolicus were grown in TYEG medium at 65°C for 18-20 hours. Results are given as micromoles of product formed or glucose consumed per tube.

Ethanol and acetate were also produced from a number of other carbohydrates fermented (Table 7.10). A ethanol/acetate ratio of approximately 1 was obtained with saccharides whereas an average value of 2.0 was obtained with polysaccharides. Much higher values were obtained with monosaccharides e.g. glucose. In case of glucose fermentation (the other substrates were not tested), ethanol yields could vary from 1.0 moles/mole to 1.7 moles/mole of glucose fermented. Such variation in ethanol yields obtained from glucose fermentation has also been noted in T. ethanolicus (Wiegel et al., 1981).

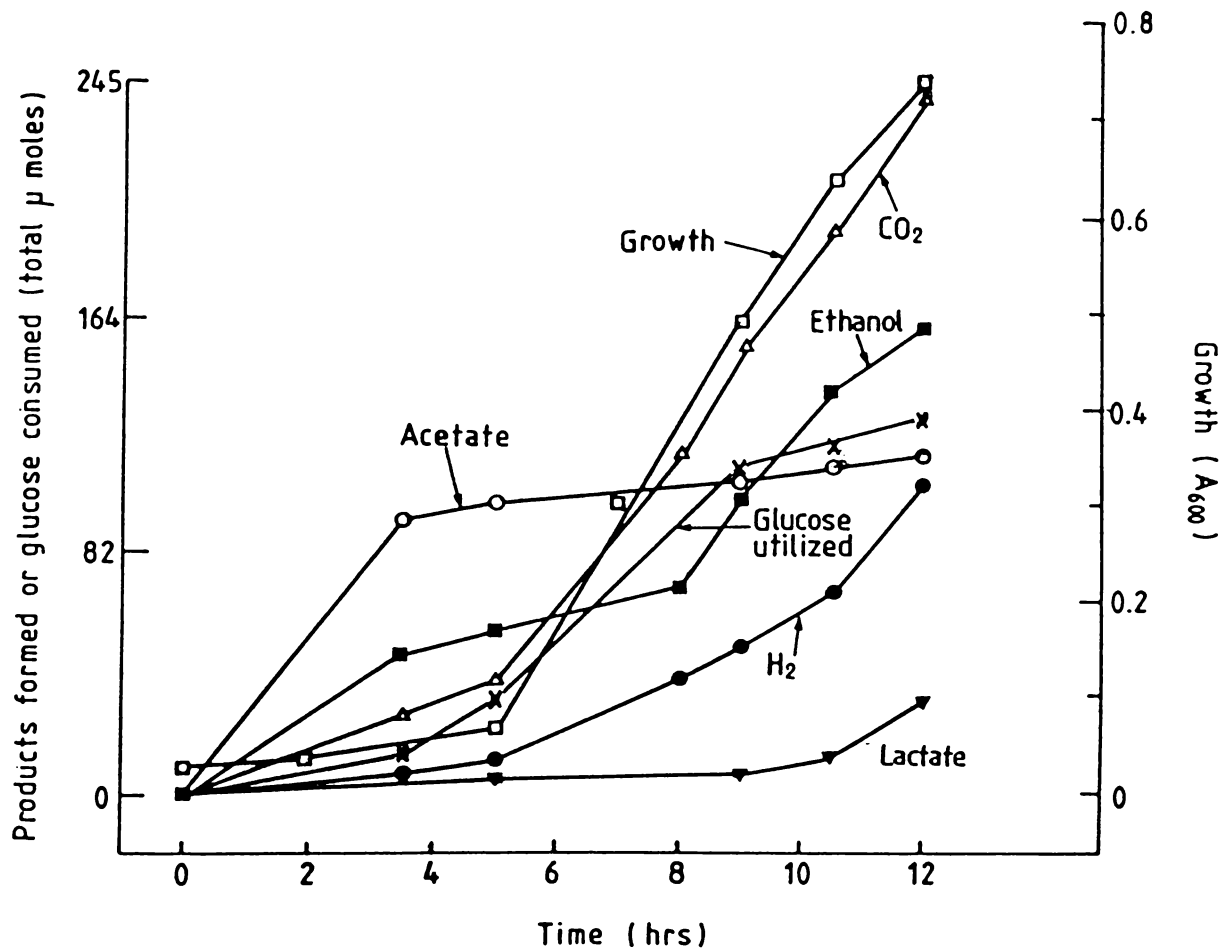


Figure 7.8 The relationship of end-product formation to growth of isolate TP6-B1 in TYEG medium at pH 7.5. Results are given as total micromoles formed per tube at the indicated times.

Table 7.10 Relationship of ethanol and acetate ratios to substrate utilized by TP6-B1

Substrate utilized	OD (660)	Products formed(Total μmol)		Ethanol/Acetate ratio
		Ethanol	Acetate	
Glucose	-	162	1	162
Sucrose	0.11	18	18	1.00
Maltose	0.29	26	25	1.04
Lactose	0.16	23	21	1.09
Amylopectin	0.37	38	14	1.8
Glycogen	0.36	38	20	1.9
Starch	0.38	40	24.6	1.6
A cacia	0.39	38	16.0	2.4

LPBM (10ml) contained 0.3% yeast extract plus 0.5% substrate and was incubated at 70°C for 36 hours. Values are given as total μmoles per tube.

(7) Effect of Hydrogen

Hydrogen inhibited growth of TP6-B1 and T. ethanolicus to the same extent and is shown in table 7.11. However the ratios of end products formed were affected.

Table 7.11 Effect of hydrogen on growth and fermentation of TP6-B1 and T. ethanolicus

Product formed (total μmoles)	TP6-B1		<u>T. ethanolicus</u>	
	N2	H2	N2	H2
Ethanol	76	53	98.8	36.2
Acetate	64	28	31.0	1.0
Lactate	40	53	232.8	165.0
Ratios:				
Ethanol/acetate	1.2	1.9	3.1	36.2
Ethanol/lactate	1.9	1.0	0.4	0.2
OD(660nm)	0.5	0.37	0.45	0.34
% inhibition	-	26%	-	29%

7.3.1.3 Isolation And Characterization Of Thermoanaerobium Type Isolates

(1) Ecology

Ta. brockii was isolated from the thermal springs of Yellowstone National Park, USA, which had temperatures between 65°C to 80°C and where actively decomposing photosynthetic biomass occurred (Zeikus et al., 1979). However, Ta. brockii type isolates were enriched from pools with and without algal mats (Morgan, et al., 1981). The temperature of these pools ranged from 52°C to 97°C and the pH from 5.6 to 9.0 (Table 7.12).

Table 7.12 Features of New Zealand thermal springs yielding Thermoanaerobium brockii isolates

Pool No.	Temp(°C)	pH	Strain designation
Tok5	52°C	5.9	Not studied further since culture had lysed.
Tok6	97°C	7.0	Tok6-B1.
Tok7	53°C	7.4	Not studied further.
Tok8	77°C	5.6	Not studied further.
Rt8*	64°C	9.0	Rt8G4.

*Isolated and presently studied by Dr K.B. Bertram.

All the strains indicated in Table 7.12 had a similar morphology and were non motile Gram positive rods. Further studies were limited to Tok6-B1 which was compared to Ta. brockii HTD4. Tok6-B1 formed creamish colonies, 2mm in diameter on TYEG agar plates after 48 hours incubation at 70°C under anaerobic conditions.

(2) Cellular Features

Both Tok6-B1 and Ta. brockii were similar in morphology and existed as rods, singly, in pairs or in chains and as filaments. Minicells (spherical cells) were often observed with filaments and paired rods. Paired rods sometime occurred in unequal lengths presumably due to unequal cell division. The cells were 0.8 μ m-1.0 μ m in width and varied in length from 2 μ m to 20 μ m.

In stationary phase cultures of Ta. brockii, cellular granulation, elongation and swelling followed by lysis occurred. This was in contrast to Tok6-B1 in which stationary phase lysis was absent.

Spores were not observed in either Tok6-B1 or Ta. brockii when cultured in GC medium and xylose soil extract agar (Hollaus and Sleytr, 1972) or in 2% (Wt/Vol) agar roll-tubes containing 0.5% glucose and incubated at 60°C for 5 days (Wiegel, et al., 1979). Spores were also not observed in any liquid medium used.

EM observation of thin sections of the cell wall of these cultures revealed the presence of a monolayer in Tok6-B1, Ta. brockii and 8G4. Zeikus et al., (1979) have provided evidence that the monolayer of Ta. brockii was diffuse. However, in our hands a monolayer composed of distinct sub-units in all the three bacterial strains was observed.

The G+C content of DNA of Tok6-B1 and Ta. brockii were similar. Tok6-B1 had a G+C content of 29.5% whereas strains of Ta. brockii gave values between 30-34% (Zeikus et al., 1979).

(3) Temperature and pH optima

(fig 7.9)

Tok6-B1 and Ta. Brockii had temperature optima of 70°C and a maximum growth temperature below 80°C. However, a significant difference existed between the two isolates with respect to pH (Figure 7.10). Tok6-B1 had an optimum pH for growth of 6.5 whereas Ta. Brockii had an optimum of 7.5. Both organisms had a wide pH range over which growth occurred (from pH 4.5 to 9.0).

(4) Effects of antibacterial agents

The effect of antibiotics on the growth of Tok6-B1 and Ta. Brockii is presented in Table 7.13.

Table 7.13 Effect of antibiotics and inhibitors on growth of Tok6-B1 and Ta. Brockii

Antibacterial agent	Amount	Strain (% inhibition)	
		Tok6-B1	<u>Ta. Brockii</u>
D-cycloserine	10ug/ml	0%	20%
	100ug/ml	100%	100%
Tetracycline	10ug/ml	100%	100%
Streptomycin	10ug/ml	17%	20%
	100ug/ml	100%	100%
Penicillin G	10ug/ml	100%	100%
Chloramphenicol	10ug/ml	100%	100%
Sodium azide	250ug/ml	48%	31%
	500ug/ml	50%	36%
Sodium chloride	1%	0%	85%
	2%	96%	100%

The inhibition spectrum of antibacterial agents of Tok6-B1 was very similar to that of Ta. Brockii. 10µg/ml of tetracycline, penicillin and chloramphenicol and 100µg/ml of D-cycloserine and streptomycin

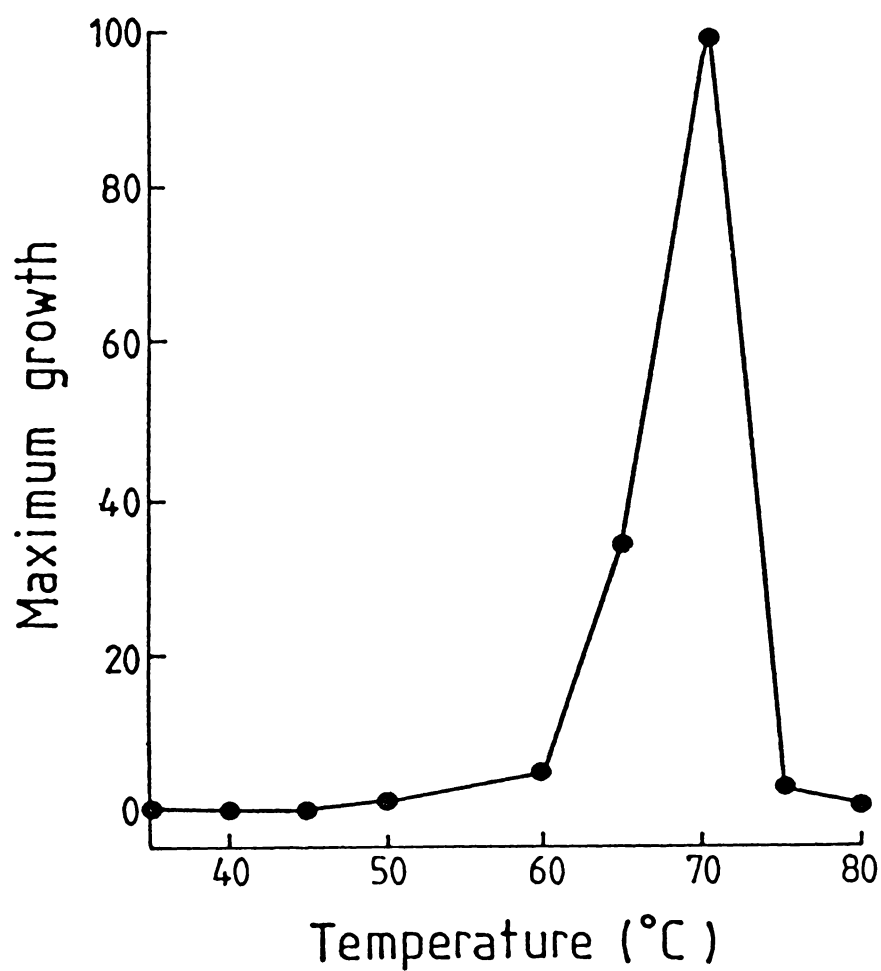


Figure 7.9 Growth of Tok6-B1 as a function of temperature. After 10 hours incubation in TYEG medium at various temperatures, absorbance was read at 540nm. An absorbance of 0.78 is equal to 100% growth.

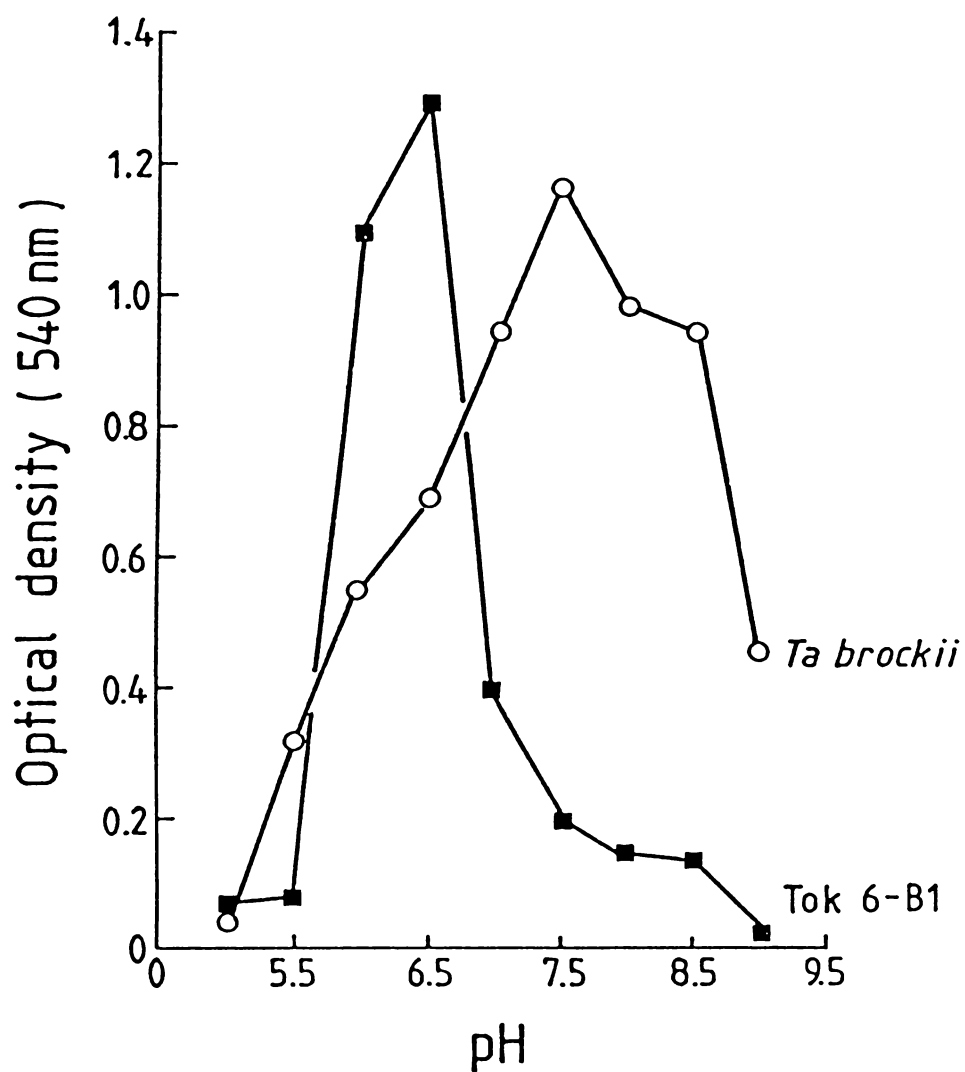


Figure 7.10 Growth of Tok6-B1 and *Ta. Brockii* in TYEG medium as a function of pH. Absorbance was measured after 10 hours for isolate *Ta. Brockii* and after 16 hours for Tok6-B1.

totally inhibited both strains. Tok6-B1 was more resistant to moderate concentrations of sodium chloride in the medium.

(5) Nutritional Requirements

Tok6-B1 and Ta. brockii both required yeast extract for growth. This was an obligate requirement for Ta. brockii but could be replaced by trypticase peptone for Tok6-B1. Neither organism could grow on carbohydrates alone. Attempts to devise a minimal medium for Tok6-B1 by substituting, yeast extract and trypticase peptone by amino acid and vitamin equivalents, failed. Similarly, a minimal medium devised for growing Cl. thermocellum (Johnson et al., 1981) did not sustain growth of Tok6-B1.

(6) Substrate Utilization and Fermentation Products

Tok6-B1 was able to grow on a wide range of carbohydrates (Table 7.14).

Table 7.14 Nutritional spectrum of Tok6-B1

Substrate	Growth (A _{600nm})
Amylopectin	0.34
Trehalose	0.18
Soluble starch	0.35
Maize starch	0.49
Dextrin	0.15
Fructose	0.34
Xylose	0.24
Mannose	0.52
Ribose	0.10
Chitin	0.51
Pectin	0.10
Cellobiose	0.11
Pyruvate	0.10
Sucrose	0.08
Glucose	0.45
Inositol	0.10
Glycogen	0.30
Lactose	+
Sorbitol	+
Raffinose	+
Maltose	+
Mannitol	+
Galactose	+
Arabinose	+
Arbutin	+

Substrates were added at a concentration of 0.5% (from 10% sterile stock solutions) to basal medium containing 0.3% Yeast extract. Growth was determined after 20 hours at 70°C. No growth occurred on lactate, acetate, fumarate, succinate, xylitol, inulin, theonine, fucose, casamino acids, formate, oxalate, glycollate, serine and melizitose.

+ = turbidity but not measurable

Tok6-B1 was able to ferment xylose, pectin, mannose and arabinose which were not utilised by Ta. Brockii. The ability to ferment pentose sugars such as xylose is regarded as an important attribute of the fermentation of biomass to alcohol. Ethanol was the major end product formed during fermentation of xylose, starch, glucose, lactose and cellobiose by Tok6-B1.

Although, Tok6-B1 and Ta. Brockii grew on glucose and produced ethanol as the major end product, they showed substantial differences in the ancillary products formed. Tok6-B1 produced substantial amounts of acetate and small amounts of lactate. Ta. Brockii produced significantly less acetate and more lactate (Lamed and Zeikus, 1980). Ta. Brockii but not Tok6-B1 produced traces of n-butyrate. Table 7.15 presents fermentation balances obtained with Tok6-B1 and Ta. Brockii with glucose as the substrate.

Table 7.15 Fermentation products from glucose by Tok6-B1 and Ta. Brockii

Products	Amounts produced (total umoles)	
	Tok6-B1	<u>Ta. Brockii</u>
Ethanol	230.67	174.5
Acetate	127.8	31.9
Lactate	22.04	118.3
CO ₂	315.0	195.0
H ₂	146.4	12.9
Glucose utilized	166.32	154
Carbon recovery	1.1	1.05
C ₁ /C ₂ ratio	1.04	0.95
O/R ratio	0.89	1.08

Tok6-B1 and Ta. Brockii were grown in TYEG medium at 65°C for 15 hours. Results are given as micromoles of product formed or glucose consumed per tube.

Typical end product formation kinetics during glucose fermentation by Tok6-B1 are shown in Figure 7.11. Ethanol, lactate, acetate, hydrogen and carbon dioxide production paralleled growth and glucose consumption.

Ethanol and acetate were also produced from a number of other carbohydrates utilized by Tok6-B1 (Table 7.16).

Table 7.16 Relationship of ethanol and acetate ratios to substrate utilized by Tok6-B1

Substrate Utilized	OD (660nm)	Products formed (mM)		Ethanol/Acetate ratio (mole/mole)
		Ethanol	Acetate	
Glucose	0.69	198.8	100.9	1.97
Xylose	0.30	58.8	40.5	1.45
Lactose	0.21	25.0	25.0	1.0
Cellobiose	0.24	24.1	23.9	1.0
Starch	0.24	33.4	33.4	1.0

LPBM (10mls) contained 0.1% yeast extract plus 0.5% substrate and incubated at 70°C for 15 hours. Values are given as total μ moles per tube.

Acetate/ethanol yields were much higher for xylose and glucose compared to polysaccharides such as cellobiose or starch and disaccharides such as lactose.

(7) Effect of Hydrogen

Hydrogen inhibition was more marked in Ta. brockii (46% inhibition) compared to Tok6-B1 (7% inhibition). Similarly changes in the ratios of the end products formed occurred during hydrogen inhibition. Table 7.17 presents data of effect of hydrogen on Tok6-B1 and Ta. brockii.

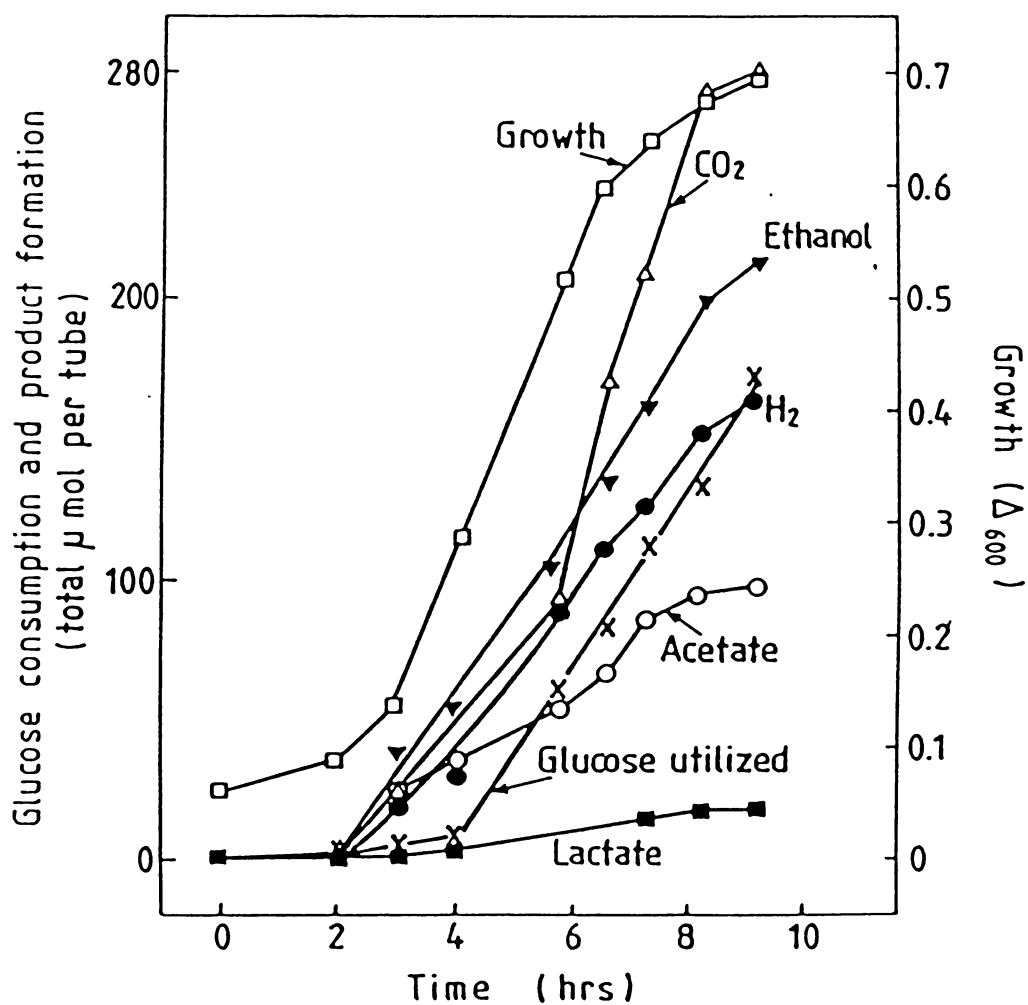


Figure 7.11 The relationship of end-product formation to growth of NZ isolate Tok6-B1 in 0.1% MOPS-Buffered LPBM medium supplemented with 0.2% yeast extract and 0.5% glucose. Results are given as total micromoles products formed or glucose utilized per tube at indicated times.

Table 7.17 Effect of hydrogen on Tok6-B1 and Ta. Brockii

Product formed (total μ moles)	Tok6-B1		<u>Ta. Brockii</u>	
	N ₂	H ₂	N ₂	H ₂
Ethanol	161.9	120	267	158.6
Acetate	126.8	122	58.2	24.6
Lactate	14.9	108.3	164.7	169.1
Ratios of				
Ethanol/Acetate	1.35	1.0	4.6	6.6
Ethanol/Lactate	10.9	1.1	1.6	0.9
OD at 600nm	1.13	1.06	1.07	0.56
% inhibition		7%		48%

(8) Immunodiffusion with Tok6-B1 Antibodies

Two precipitating bands were obtained when disrupted Tok6-B1 cells was reacted with Tok6-B1 antibodies. No reaction occurred with disrupted Wai16-B1 or Ta. Brockii cells. However, a strong precipitating band was obtained with disrupted T. ethanolicus and TP6-B1 cells with a fainter precipitating line occurring with disrupted Cl. thermohydrosulfuricum strains E100-69 and E2-B1 (Figures 7.12A, 7.12B).

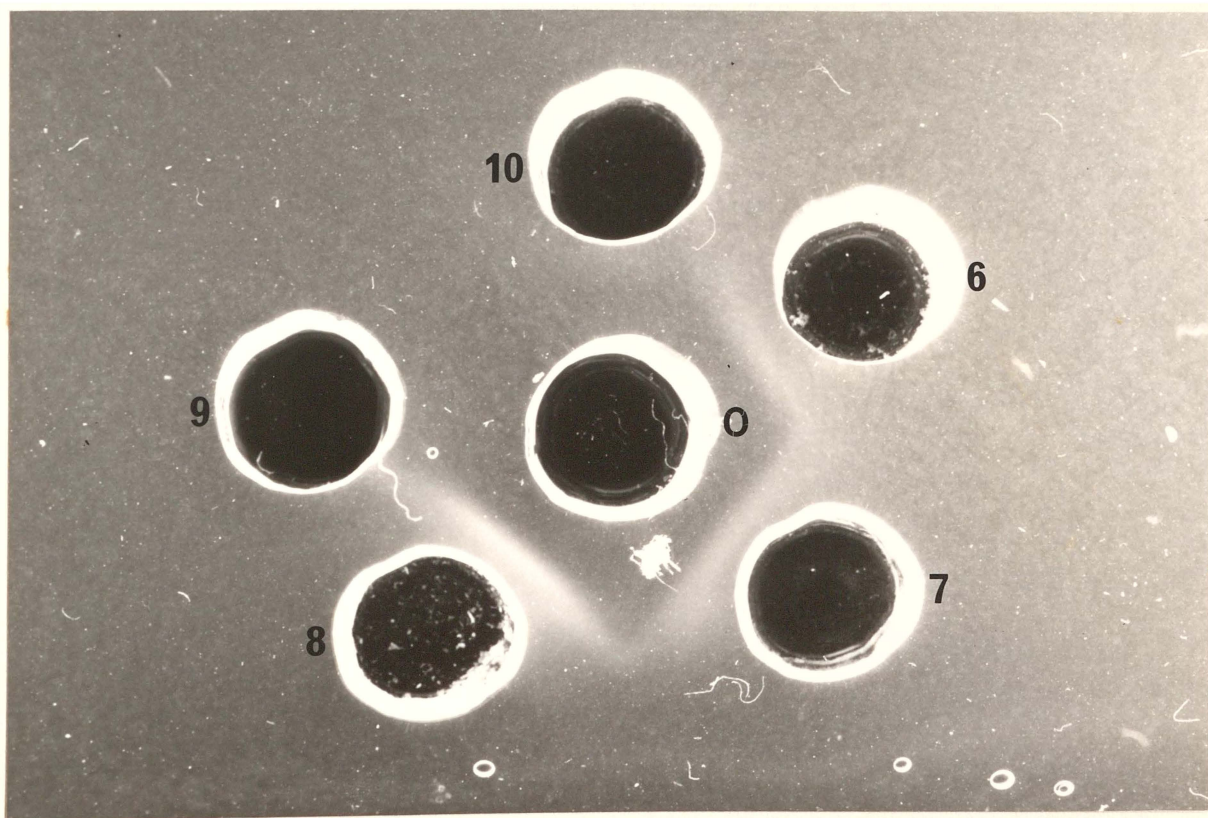
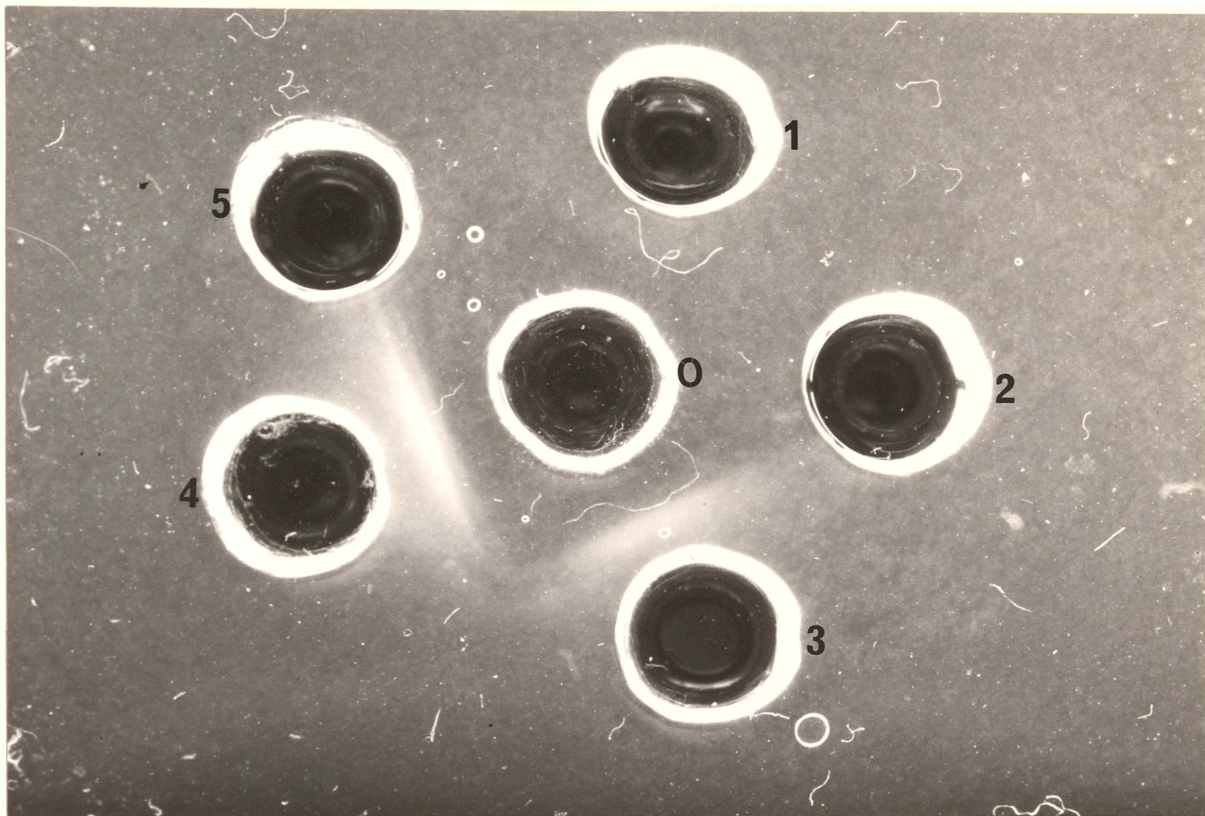
The fluorescent antibody (FA) reaction of Tok6-B1 antibodies to a few caldoactive glycolytic anaerobes was presented in Table 4.4 of Chapter 4 which confirmed results obtained by immunodiffusion reaction. Tok6-B1 antibodies cross reacted as evidenced by FA reaction with T. ethanolicus, TP6-B1, Cl. thermohydrosulfuricum E100-69 but not with Ta. Brockii.

Results of immunodiffusion and FA tests indicate that partial common antigenic properties amongst Cl. thermohydrosulfuricum, TP6-B1 and T. ethanolicus exists and that Ta. Brockii has antigens totally unrelated to Tok6-B1.

Figure 7.12A Immunodiffusion precipitation reaction of (0) Tok6-B1 antibodies against (1) Ta. Brockii, (2) distilled water control, (3) T. ethanolicus, (4) Tok6-B1 and (5) TYEG medium control.

Immunodiffussion precipitation test was performed using standard slide Outerlony method. Each slide was prepared by pipepting 8 ml of molten 1.5% Agar containing 0.8% NaCl. The agar was allowed to solidify and wells bored with the aid of an appropriate sized cork borer. The centre well was filled with Tok6-B1 antibodies and the surrounding wells with antigens or appropriate controls. Cell extracts (prepared from sonicated cells) were used as antigens.

Figure 7.12B Immunodiffussion precipitation reaction of (0) Tok6-B1 antibodies against (6) Cl. thermohydrisulfuricum E100-69, (7) CL. thermohydrosulfuricum E2-B1, (8) TP6-B1, (9) Rt17-B1 and (10) Wai16-B1.



7.3.1.4 Isolation And Characterization Of Clostridium Type Isolates -

(1) Ecology

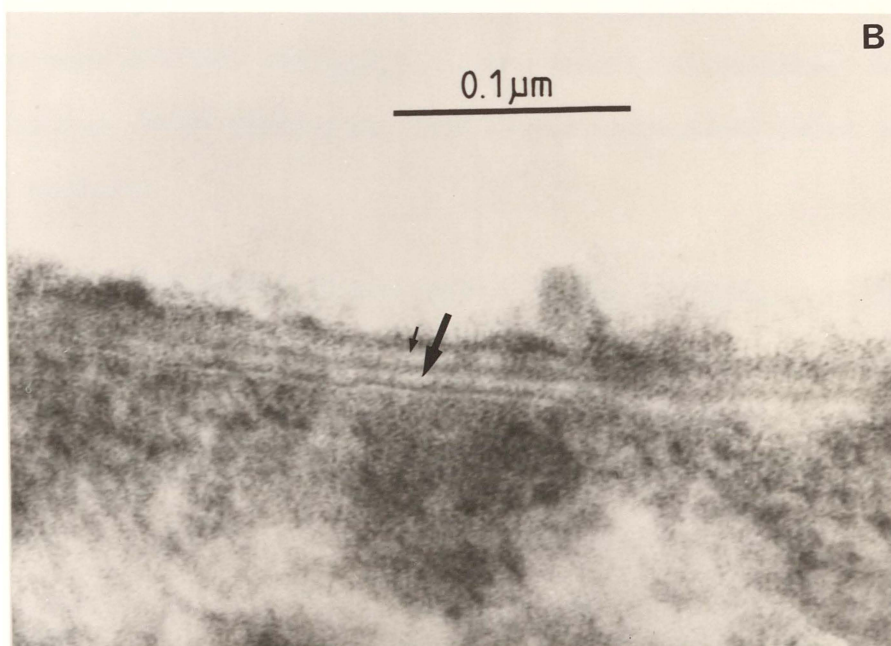
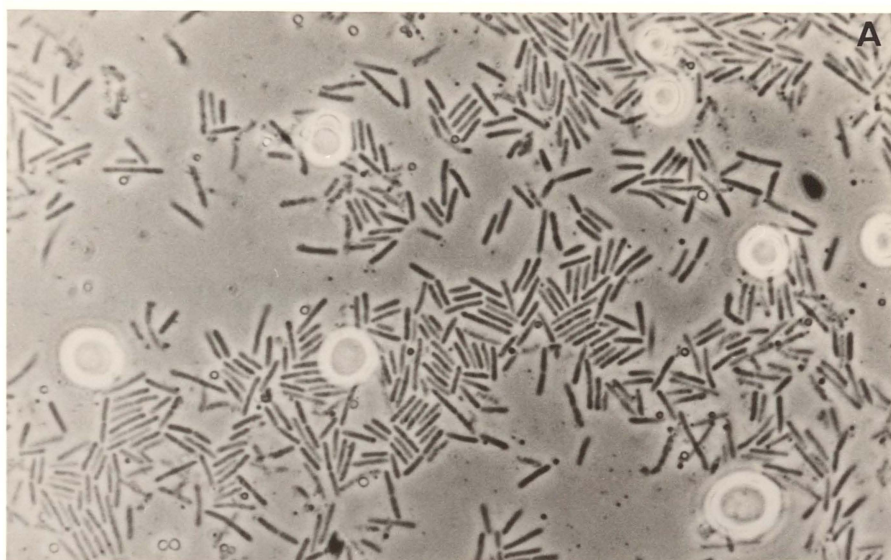
Since not all the 51 caldoactive glycolytic enrichments were purified for studies on sporulation, the distribution of sporulating Clostridium type organisms especially Cl. thermohydrosulfuricum type isolates in New Zealand hot springs could not be determined. Only 5 morphologically similar sporulating rods resembling Clostridium thermohydrosulfuricum were isolated using end point dilution in TYEG agar shakes from thermal and non-thermal environments and designated Rt8-B1, E2-B1, Tk5-B1, Tk20-B1 and Com-B1. Rt8-B1, Tk5-B1 and Tk20-B1 were isolated from New Zealand thermal springs, E2-B1 from a volcanic area of Mt. Erebus and Com-B1 from a garden compost heap. Cl. thermohydrosulfuricum E100-69, the type culture was obtained from Dr. Hollaus for comparative work with the above mentioned isolates.

It is not surprising to find Cl. thermohydrosulfuricum in such varied and different environments. Klaushofer and Parkkinen (1965) and Hollaus and Klaushofer (1973) isolated Cl. thermohydrosulfuricum strains from an Austrian sugar factory whereas Zeikus et al., (1980) isolated them from thermal areas of Yellowstone National Park, USA. Wiegel et al., (1979) on the other hand isolated Cl. thermohydrosulfuricum from thermal sources such as compost and hot springs and non-thermal sources such as digestors and mud samples.

(2) Morphology

All 5 isolates were Gram negative, sporulating, sluggishly motile rods which occurred singly, in pairs or in chains. Figure 7.13 is a phase photomicrograph of Cl. thermohydrosulfuricum E100-69 which was

Figure 7.13 Micrographs of Cl. thermohydrosulfuricum E100-69. (A)
A phase coontrast micrograph showing the morphology and (B)
An EM of a thin section showing the double layered cell
wall ultra-structure.



typical of the morphology of all 5 isolates. Figure 7.13 also shows the ultrastructure of the cell wall of Cl. thermohydrosulfuricum E100-69. Note that the cell wall of this organism is dissimilar to that of other caldoactive glycolytic anaerobes. E2-B1 and Rt8-B1 were peritrichously flagellated similar to that of the neotype strain, E100-69. The other 3 strains were not examined for flagellation.

(3) Nutritional Requirements:

All five strains required growth promoting factor(s) from yeast extract. Yeast extract could in some cases be replaced by a number of other biological media such as gelysate, trypticase peptone and/or beef extract (Table 7. 18). Growth on trypticase peptone, yeast extract, gelysate and beef extract was fair in the respective cultures that were able to utilise them indicating that these substrates could not act as sole carbon sources.

Table 7.18 Nutritional requirements of Cl. thermohydrosulfuricum type isolates

Media	Strains				
	Rt8-B1	E2-B1	Tk5-B1	Tk20-B1	E100-69
Yeast Extract	++	++	++	++	++
Gelysate	-	-	-	+	-
Trypticase peptone	+	+	++	+	+
Beef Extract	-	-	++	+	-

- indicates no growth, + indicates fair growth, ++ indicates good growth.

(4) Temperature and pH optima

No strains were capable of growth above 78°C, with an optimum temperature for growth between 65°C to 70°C.

All strains were able to grow between pH 5.0 to 8.5 with an optimum between pH 7-7.5.

(5) Antibiotic Inhibition:

The effect of antibacterial agents on Rt8-B1 is presented in Table 7.19. Other strains were not tested.

Table 7.19 Effect of antibacterial agents on growth of Rt8-B1:

Antibacterial agents	Amount	% inhibition
Neomycin	10µg/ml	8%
	100µg/ml	100%
D-cycloserine	10µg/ml	0%
	100µg/ml	0%
Tetracycline	10µg/ml	100%
Streptomycin	10µg/ml	0%
	100µg/ml	100%
Chloramphenicol	10µg/ml	100%
Penicillin G	10µg/ml	100%
Sodium azide	250µg/ml	24%
	500µg/ml	23%
Sodium chloride	1%	0%
	2%	100%

Rt8-B1 was extremely sensitive to 10µg/ml of penicillin G, tetracycline and chloramphenicol but was resistant to neomycin and streptomycin at similar concentrations. Rt8-B1 was totally resistant to D-cycloserine and partially resistant to sodium azide.

(6) Substrate Utilization

All New Zealand isolates were able to utilize a wide variety of carbohydrates including glucose, mannose, raffinose, arabinose, accacia, cellobiose, chitin, pectin, glycogen, sucrose, amylopection and maltose (Table 7.20). The New Zealand isolates in general conformed to the substrate spectrum of known Cl. thermohydrosulfuricum strains described by Wiegel et al., (1979) and Hollaus and Klaushofer (1973). However a few differences were observed. Strains Rt8-B1, E2-B1 and Tk20-B1 were able to grow on rhamnose and strains Rt8-B1 Tk5-B and Tk20-B1 were able to grow on m-inositol whereas none of the Cl. thermohydrosulfuricum isolated so far have been able to do so (Wiegel et al., 1979; Hollaus and Klaushofer, 1973). Similarly indole was produced from trypticase peptone by strains Rt8-B1 and E2-B1 but not by the strains described by Hollaus and Klaushofer, (1973). Hydrogen sulphide was produced from sodium sulphite and sodium thiosulphate by all New Zealand isolates, a feature common to other Cl. thermohydrosulfuricum strains (Hollaus and Klaushofer, 1973).

Table 7.20 Substrate utilization by Cl. thermohydrosulfuricum E100-69(a) and Cl. thermohydrosulfuricum type isolates

Substrates tested.	Isolates				
	E100-69	Rt8-B1	E2-B1	Tk5-B1	Tk20-B1
Glucose	+	+	+	+	+
Rhamnose	-	+(slow)	+	-	+
Mannose	+	+	+	+	+
Raffinose	V	+	+	+	+
Fructose	+	+	+	NA	NA
Fucose	-(b)	+	-	NA	NA
Galactose	+(b)	+	+	NA	NA
Adonitol	NA	+	NA	NA	+
Sorbitol	V	+	+	+(slow)	+
M-inositol	V	+	NA	+	NA
Arabinose	V(b)	+	+	+	+
Ribose	+(b)	+	+	NA	NA
Xylose	+	+	-	NA	NA
Accacia	NA	+	+	+	+
Cellobiose	+	+	+	+	+
Chitin	NA	+	+	+	+
Pectin	+	+	+	+	+
Potato starch	V	+	+	-	+
Glycogen	V	+	+	+	+
Sucrose	+	+	+	+	+
Lactose	V	+	NA	+	+
Amylopectin	NA	+	+	+	+
Maltose	+	+	+	+	+

(a) Data obtained from Hollaus and Klaushofer, 1973.

(b) Data obtained from Wiegel et al., 1979.

NA= Data not analysed.

(7) Fermentation End Product

Ethanol, acetate, lactate, CO₂ and H₂ were the products formed from glucose fermentation. The fermentation balances obtained from glucose fermentation by Cl. thermohydrosulfuricum E100-69 are compared with New Zealand isolates in Table 7.21. Considerable variation in end products especially in lactate and ethanol concentrations, can be observed if results of the same end products from the same organisms shown in Tables 7.4 and 7.6 are compared.

Table 7.21 Fermentation end products of Cl. thermohydrosulfuricum type isolates

Fermentation products (total umol)	Strains					
	E100.69		Rt8-B1	E2-B1	Tk4-B1	Tk20-B1
	(A)	(B)	(A)	(A)	(A)	(A)
Ethanol	165.0	108.0	168	267.4	1.64	22
Acetate	88.0	51.7	84	25.0	184.8	81
Lactate	281.6	225.0	18.1	142.8	100.0	+
CO ₂	239.0	150.0	284	+	+	+
H ₂	107.5	93.0	165	+	+	+
Glucose utilized	220.8	179.4	150	NA	NA	NA
Carbon recovery	118%	108%	102%	NA	NA	NA
C1/C2 ratio	0.95	0.94	1.13	NA	NA	NA
O/R index	1.09	0.97	1.13	NA	NA	NA

+ indicates detected but not quantitated.

NA = not analysed

All strains were grown at 70°C for 15-18 hours in medium (A) or medium (B). Medium A= TYEG medium. Medium B= MOPS buffered LPBM+0.2% yeast extract + 4% glucose. Results are given as micromoles of product formed or glucose consumed per tube.

The ethanol to acetate ratios of a number of carbohydrates fermented by Tk20-B1 is given in Table 7.22. A ratio of approximately 1 was obtained irrespective of the type of carbohydrate utilized ie whether disaccharide, polysaccharide or monosaccharide.

Table 7.22 Relationship of ethanol and acetate ratios to substrate utilized by Tk20-B1

Substrate Utilized	OD (660nm)	Products formed (total μ m)		Ethanol/Acetate ratio
		Ethanol	Acetate	
Glucose	0.14	27.0	39.0	0.7
Lactose	0.26	21.7	29.0	0.75
Sucrose	0.23	34.8	29.8	1.16
Maltose	0.33	40.0	25.0	1.60
Cellobiose	0.10	26.0	20.0	1.30
Amylopectin	0.30	36.8	29.4	1.25
A. cacia	0.17	15.0	21.0	0.71

LPBM (10ml) contained 0.3% yeast extract plus 0.5% substrate and incubated at 70°C for 24 hours. Values are given as total μ moles per tube.

7.3.1.5 Isolation And Characterization Of Isolate Wai16-B1 -

An extremely thermophilic glycolytic anaerobe which produced ethanol as the major end product was isolated from pool Wai16 and designated Wai16-B1. Wai16-B1 did not fit into any of the existing known species (Table 7.4). Wai16-B1 was Gram negative and thus could be differentiated from Tok6-B1 and Ta. brockii. The cells did not sporulate under the conditions successfully used for sporulating Clostridium thermohydrosulfuricum strains. Additionally, Wai16-B1 together with Rt17-B1 and Ta. brockii did not react with Tok6-B1 antibodies as evidenced in immunodiffusion reaction (Figure 7.12). Furthermore the cells were rod shaped organisms which existed singly, in pairs or as filaments and were 4-15 μ m by 1 μ m. Wai16-B1 cells were peritrichously flagellated and were sluggishly motile when observed as wet mounts under a phase contrast microscope.

(1) Growth optima

Wai16-B1 grew optimally at 65-70°C with no growth occurring at 80°C. Growth occurred from pH6 to pH9.0 with an optimum between 6.5 to 7.5.

(2) Nutritional characterization

Wai16-B1 required yeast extract for growth. Growth on 0.8% yeast extract was very poor (OD₆₆₀ = 0.02) indicating that it alone could not support growth. A number of carbohydrates tested were able to support growth in the presence of Yeast extract (Table 7.23).

Table 7.23 Nutritional spectrum of isolate Wai16-B1

Substrates tested	Utilization	pH of media after growth	Production of acid and/or gas
Sorbitol	+	6.3	AG
Mannitol	+	6.4	AG
m-inositol	-	7.2	-
Galactose	+	6.1	AG
Raffinose	-	7.3	-
Rhamnose	p	6.9	A
Arabinose	p	6.7	A
Mannose	+	6.1	AG
Fructose	+	5.9	AG
Cellulose	-	7.2	-
Pectin	+	6.5	A
Starch	+	6.2	AG
Sucrose	-	7.2	-
Maltose	+	6.1	AG
Lactose	+	6.2	AG
Xylose	+	6.0	A
Glycerol	-	7.2	-
Pyruvate	+	6.4	AG
Nitrate broth	+	?	G

A= acid produced; G= gas produced; p= poor growth
 LPBM (10ml) containing 0.3% Yeast extract and 0.5% carbohydrate.
 Incubations were done at 70°C for 15-18 hours, after which gas production was recorded from durham tubes and pH using a pH electrode.

Gas was produced from nitrate broth anaerobically and Wai16-B1 was the only thermophilic glycolytic anaerobe with the exception of Rt17-B1 to grow and produce gas from nitrate broth.

7.3.1.6 Isolation And Characterization Of Thermobacteroides Type Isolates -

Two strains designated Wai5-B1 and Wai10-B1 were isolated from pools Wai5 and Wai 10 respectively. These two isolates shared a number of features in common with Thermobacteroides acetoetylicus and included morphological similarities (short coccobacillary Gram negative, non-sporulating motile rods) and their inability to produce lactic acid during glucose fermentation. Hence these two New Zealand isolates were provisionally designated as Thermobacteroides species. Time did not permit further examination of these strains.

7.3.1.7 Filamentous Isolates Rt46-B1 And TP10-B1 -

(1) Morphology

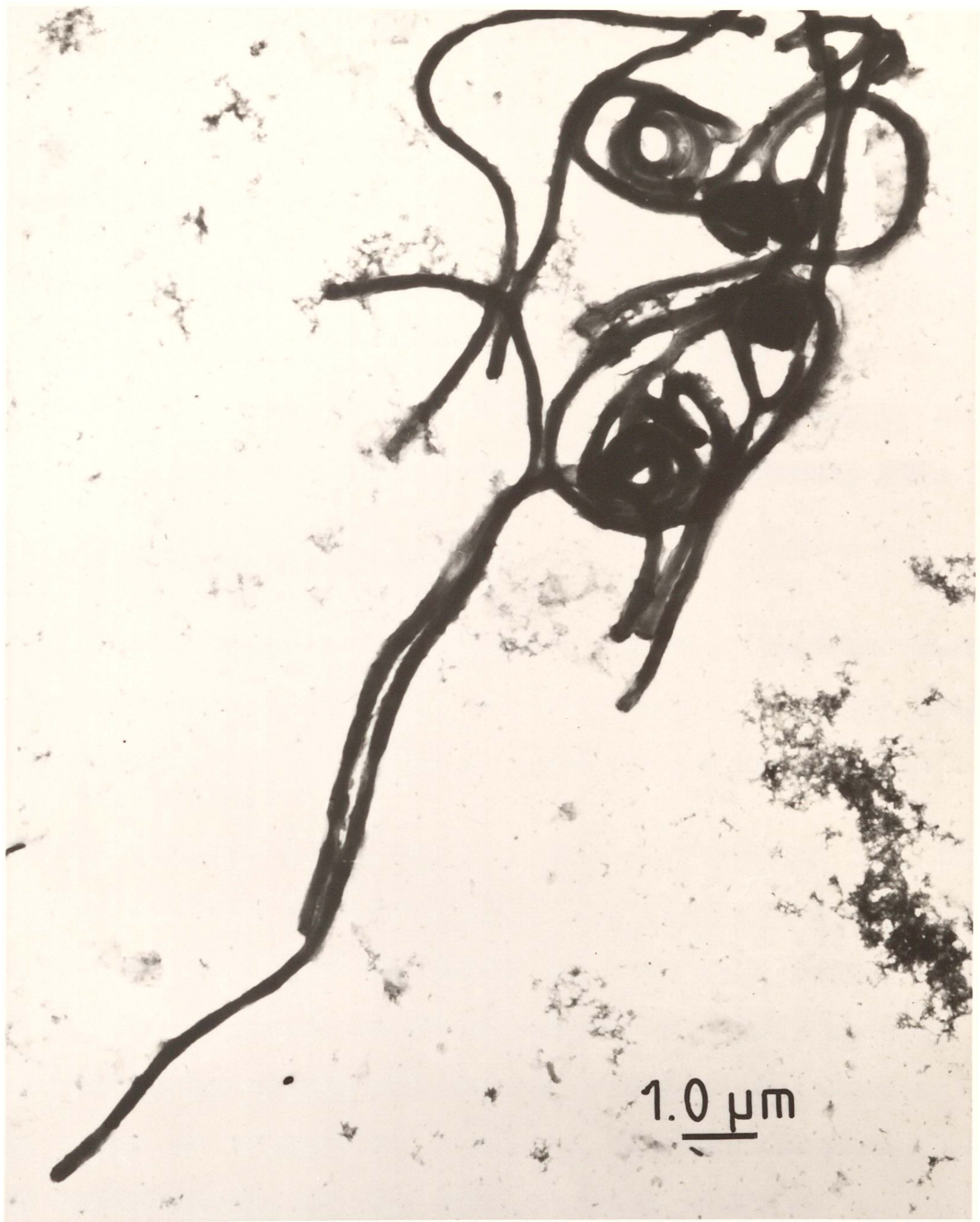
TP10-B1 and Rt46-B1 were both Gram negative filaments. However, TP10-B1 filaments divided to form different sized chain of cells and sometimes as many as 35 cells to a chain were observed some of the cells were motile. Rt46-B1 filaments on the other hand did not divide as chains of cells (Figure 7.14). Under certain conditions of growth, for example in TYEG containing sodium sulphite, Rt46-B1 coiled spring like filaments.

(2) Temperature and pH optima

The temperature maximum for growth of TP10-B1 and Rt46-B1 was below 78°C with an optima between 65-70°C. The pH range for growth was much more restrictive for Rt46-B1 than TP10-B1 or any other caldoactive glycolytic anaerobes studied so far and was between 6.5 to 8.0 with an optimum of 7.0 to 7.5. The pH range for growth of TP10-B1 was between

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Figure 7.14 An EM of anaerobe Rt46-B1 showing the filamentous nature of the cell.



5.0 to 8.5 with an optimum around pH7.0.

(3) Nutritional Requirements

Both required yeast extract for growth. Much better growth was achieved with 1% yeast extract than with 0.3% yeast extract. Growth of Rt46-B1 was much better on TYEG containing 25mM sodium sulphite than on TYEG containing sodium thiosulphate.

Both strains were incapable of growth aerobically in Thermothrix media (Caldwell et al., 1976), Castenholz media (Brock and Freeze, 1969) and nitrate broth hence differentiating it from Thermothrix species (Caldwell et al., 1976) or Thermus species (Brock and Freeze, 1969).

(4) Fermentation End Products

TP10-B1 utilized glucose and produced H₂, CO₂, acetate, lactate, ethanol, iso-valerate, butyrate and iso-butyrate. Rt46-B1 produced acetate, lactate, ethanol, CO₂ and H₂ during glucose fermentation.

Optical densities higher than 0.15 were never obtained for TP10-B1 and Rt46-B1 and hence less attention was paid to these organisms because of the need to use other procedures such as cell counts, protein estimation during growth or ATP for evaluating growth accurately. The few observations and studies done were to justify earlier comments that diverse types of caldactive glycolytic anaerobes are present in New Zealand thermal springs and that some of these organisms may not be widely distributed in nature. In this case filamentous caldactive glycolytic anaerobes were only cultured from pools Rt46 and TP10. However both these microorganisms were physiologically distinct.

7.3.2

PART II OTHER ANAEROBIC ENRICHMENT MEDIA:Enrichment in MMB medium (pH 5.6) under H₂:CO₂ (70:30) gas phase

Out of the 27 cultures initiated for enrichments at 62°C, only 7 were positive after 5 days incubation. Filaments, rods and vibriods were the only morphologies present. Motile rods were present in 4 enrichments (OK2, OK4, OK10, COM) and all appeared morphologically similar (Table 7.24).

Table 7.24 Enrichment cultures obtained under CO₂ and H₂ gas phase in MMB medium (pH 5.6) incubated at 62°C

Pool	Temp (°C)	pH	Morphology
OK2	57	7.6	Motile rods (2-5mm by 1mm) and non-motile shorter rods
OK4	60	6.9	Motile rods (2-5µm by 1µm) and non-motile shorter rods.
OK5	62.5	8.58	Filaments (15µm by 1µm) and chains of rods.
OK8	83.5	8.5	Vibriods only (4-5µm by 1µm)
OK10	74	7.9	Motile rods (2-5µm by 1µm)
WP5*	98.5	ND	Rods (5-12µm by 0.7µm). Some with terminal, spherical spores.
COM*	98.0	ND	Few motile rods (2-5µm by 1µm)

* indicates fumarole samples.

The samples which were negative for growth were OK1, OK6, OK7, OK9, OK11, OK12, OK13, OK14, OK15, OK17, OK18, OK9, WP1, WP3, WP4, WTA, WTB, WTC, WTD, and WTE.

ND =Not Determined.

Whether all the organisms present in the enrichments were capable of growth on CO₂ and H₂ or whether they utilized substrates from trypticase peptone and/or yeast extract was not tested. However, a depletion in the gas phase did occur in all enrichments, and since methane was not produced, it is assumed that some of the organisms could utilize CO₂ and

H₂ for growth. Such a feat is not surprising since geothermal environments contain gasses like CO₂ and H₂, which are known to be utilized by chemolithotrophs. Interestingly enough the two fumarole samples tested were also positive. Microscopic examination of 11 fumarole samples (Chapter 2) had revealed no bacterial presence. Additionally, enrichments initiated in TYEG medium (Part 12 of this Chapter) were negative for bacterial growth.

Subsequent transfers of OK2, OK4 and OK10 performed in MMB (pH5.6) under CO₂ and H₂ at 75°C were positive. Transfer of OK4 enrichment into TYEG at 70°C was also positive for growth with glucose fermentation and production of 40mM ethanol, and traces of acetate and lactate. In addition electron micrographs of enrichment cultures of OK4 incubated at 62°C in MMB medium under H₂ and CO₂ phase and OK4 cultures show morphological similarities (Figure 7.15). Both had striations in the middle of the cells, and both had similar dimensions and hence it is assumed that both are similar cultures. However, enrichments from MMB incubated cells were flagellated and those grown in TYEG were non-flagellated. Additionally, TYEG grown cells were coated with slime. This could be just an effect of growth under different conditions. It is known, at least in case of T. ethanolicus that the production of flagella is growth phase dependent.

Enrichment in BM3A

7 pools from Fiji were positive for the presence of organisms out of the 27 set up for enrichments in BM3A. The morphological features of some these organisms was unique (Table 7.25).

Figure 7.15A An impure culture of OK4 grown on CO_2/H_2 . Note the "striations" on some cells, and flagella

Figure 7.15B A pure culture of OK4-B1 grown in TYEG. Note that all cells have "striations."

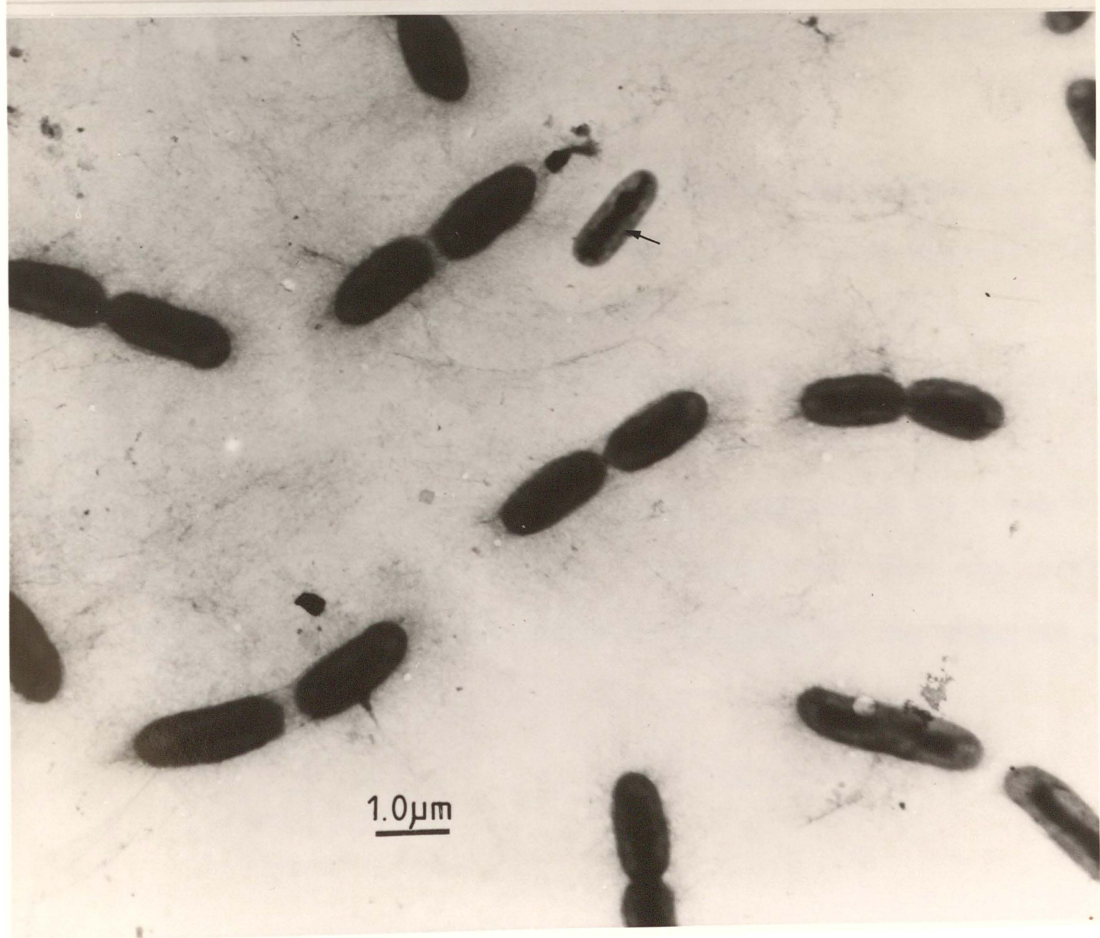


Table 7.25 Thermal features of Fijian geothermal springs yielding enrichments initiated in BM3A:

Pool	Temp (°C)	pH	Morphology
Ta4	62.8	8.71	Filaments with internal granular material
Na2	99.7	7.86	Rods single, twos or threes with internal granular material
Na3	98.7	8.1	Motile rods and cocci
Na4	95.5	7.57	Phase dense spheres with protruding phase light arms (2 per cell)
Na8	95.8	8.08	Filaments with internal granular material; Rods with terminal, spherical spores.
SS1	99.0	7.6	Filaments with internal granular material
SS3	85.4	7.52	Filaments; a phase dense sphere with protruding phase light arms (2 per cell)

Other springs which gave negative results were Ta1, Ta2, Ta2, Na1, Na5 Na6, Na7, SS4 and SS5.

All incubations were done at 70°C.

One of the cultures Na4 was studied further. The unusual feature of this organism was discussed earlier in Chapter 2 (Fig. 2.54) and was first observed when "in situ" electron microscopy method was used to study bacterial morphology in hot pools.

Na4 appeared pure but was purified further by end point dilution in BM3A medium at 70°C, and designated NA4-B1. The optical density (660nm) of Na4-B1 was approximately 0.1. Addition of glucose (0.1% or 0.5%), acetate (0.1%), trypticase peptone-casamino mixture (0.2%) and incubation under H₂:CO₂ (80:20) gas phase did not increase turbidity. It lysed immediately with 10% SDS and was totally inhibited by 10µg/ml of vancomycin, and 150µg/ml of penicillin. Tetracycline did not inhibit growth at 100µg/ml, however, the growth of the flaps of the organism were inhibited.

7.3.3 PART III ENRICHMENT OF THERMOPHILIC SULPHATE-REDUCERS

Isolation and Characterisation of Thermophilic Sulphate-reducers.

From the 43 enrichments initiated in medium 77 for sulphate-reducers, 16 were observed to be positive after 3-5 days incubation at 70°C as evidenced by blackening of the medium. Microscopic examination of these enrichment cultures indicated diversity within the rod shaped bacteria; for example some were thin and long rods (0.5µm by 8 to 10µm) whereas others were short and fat rods (0.7-0.9µm by 1-1.5µm). The types of bacteria present in initial enrichment cultures persisted for another two successive transfers. The morphological types of bacteria present in enrichments is given in Table 7.26.

Table 7.26 Morphology of bacteria present in sulphate-reducing enrichments initiated in medium 77 and incubated at 70°C for 3-5 days.

Pool	Temp. (°C)	pH	Morphology
ROTORUA			
Rt3	70	7.2	Rods
Rt8	70	9.1	Rods, some motile
Rt12	66	6.9	Small motile rods and larger granulated rods
Rt34	75.7	6.53	Thin rods
Rt44	62	7.81	Motile rods, single or in pairs
Rt66	78	6.12	Small and larger motile rods; single or in pairs.
FIJI			
Na2	99.7	7.86	Short fat rods and thinner rods
Na7	99.3	8.18	Motile rods and thinner rods
Na8	95.8	8.08	Short fat rods and thinner rods
Ta1	83	8.76	Small rods and thinner rods
Ta3	67.8	8.74	Rods
Wg7B	69.7	8.01	Thin rods
Wg7C	89.7	8.3	Rods
Wg14	67.2	6.86	Small motile rods and thinner rods
WAIMANGU			
Wai20	90	6.85	Fat rods and thinner rods
Wai21	67	6.25	Rods

Pools from which sulphate-reducers could not be isolated were Rt2, Rt13, Rt14, Rt15, Rt16, Rt42, Rt51, Rt56, Rt57, Rt58, Rt60, Rt61, Rt67, Rt68, Rt72, Rt74, Rt80, Rt82, Rt84, Na1, Ta2, WgA, Wg9B, Wg10, Wg11C, Wg12A and Wg12B.

Only one of the enrichment cultures, namely Na8 was investigated further. As indicated in Table 7.26 it was a mixed culture consisting of thin rods and shorter, thicker rods which usually existed singly or in pairs. The thin rods were capable of growth on TYEG indicating it was a fermentative anaerobe. Both organisms sporulated. The thin rod sporulated after 2 days incubation and the spores were oval and located terminally. The spores of the short rod were sub-terminal and the morphology and sporulation of this organism was similar to that of Desulfotomaculum nigrificans. However, Desulfotomaculum nigrificans has a temperature maximum for growth at 65°C compared to 78°C for the short rod. The short rod could be a temperature variant of Desulfotomaculum nigrificans. Additional studies with the mixed culture indicated that the short rod could not grow on pyruvate or ethanol with or without sulphate. Though growth occurred in an acetate plus sulphate medium best growth was achieved in a lactate plus sulphate medium.

Unfortunately time did not permit purification of these organisms for any further studies. However, it does appear from this small survey that sulphate-reducers are present in New Zealand thermal springs. By using only one enrichment medium a number of morphologically distinct bacteria were observed. It is unlikely that of these morphotypes all were sulphate-reducers, some could be fermentative organisms presumably capable of growing on the yeast extract present in the medium; for example, as already described in the mixed culture obtained from pool Na8. Further work has been undertaken by Graham Attwood M.Sc. thesis). It should be added that the diversity of the sulfate-reducers in thermal springs will only be realised fully if a wide range of substrates are used as carbon sources for enrichments. The isolation of Thermodesulfobacterium commune (Zeikus et al., 1983) has already proved

to be of interest because of the presence of ether-linked lipids -An archaeobacterial feature in a eubacterium!

7.4 SUMMARY AND CONCLUSIONS:

Due to the lower oxygen solubility with increasing temperature, a predominance of anaerobes might have been expected in thermal springs. Only approximately 20% of the pools surveyed gave positive results for the presence of glycolytic anaerobes, which does not bear out the expectation. The 20% value might well be an underestimate of the total anaerobic population in that the conditions of temperature, pH and substrate concentration used for isolation may have been restrictive. Pools giving negative results were not subjected to further isolation attempts so that pools with low population densities of such organisms could well have been missed. Many of the pools sampled had pH/temp regimes well outside the isolation parameters so that isolation attempts under conditions of the pool environment might be more productive.

A surprising feature of the isolates obtained was the metabolic diversity exhibited under the standard isolation conditions used. Broadly three groups of organisms were defined according to the major fermentation end product. Although only selected isolates were investigated in greater detail, we have shown that one of these, Rt17-B1,* was a unique isolate in several respects and differed significantly from reported type species. Tok6-B1 although having many similarities to Ta. brockii was also sufficiently different in its metabolism to warrant calling it a new species and TP6-B1 was closely related but not identical with T. ethanolicus. The relationship of many of the remaining isolates both with the New Zealand isolates referred to, and the type species warrants further investigation. Undoubtedly some will

* Refer paper (Patel et al., 1985a-back pocketcover

be identical to or very similar to the isolates described, but even on the meagre evidence of end product analysis isolates such as OK4-B1, Rt46-B1 and the three lactate producing strains (Group 3 Table 7.6) appear unique.

With an increasing interest in the technological applications of thermophiles and the anaerobic fermenters in general a programme to identify and characterize some of the previously unreported organisms identified in this survey could be of importance.

CHAPTER 8

ISOLATION AND CHARACTERIZATION OF AEROBIC AND FACULTATIVELY ANAEROBIC EXTREME THERMOPHILES

8.1 INTRODUCTION:

Extremely thermophilic aerobes are in many respects the most easily and widely studied group of thermophiles. Not surprisingly, the number of species isolated to date is quite large and diverse. An extensive systematic study of the group as a whole has not been carried out, so that the relationship between many of the isolates is not clearly defined. Table 8.1 lists the species that have been isolated and described.

Of the spore formers, Thiobacillus thermophilica has not been reported from as wide a geographic distribution as the more ubiquitous Bacillus species. Although the species has a maximum growth temperature of 80°C, its optimum is generally at 55-60°C. Growth was inhibited by organic compounds, the organism survives autotrophically by oxidation of sulphide, thiosulphate and sulphite to sulphate.

The sporeforming Bacillus species are widespread and have been isolated from most of the world's thermal regions. Most of the species can be classified by the scheme devised by Walker and Wolf (1971) which is based on phenotypic characters. Some of the properties of the neutral Bacillus species are listed in table 8.2. The group which causes most

Table 8.1: List of extremely thermophilic aerobes (eubacteria) described to date:

Species	Isolation source	Reference
(A) <u>Spore formers</u>		
<u>Neutral Bacillus group:</u>		
<i>B. thermocatenulatus</i>	Geothermal spring, Russia	Golovacheva et al., 1975
<i>B. caldolyticus</i>	Geothermal spring, USA	Heinen and Heinen, 1972
<i>B. caldovelox</i>	Geothermal spring, USA	Heinen and Heinen, 1972
<i>B. caldotenax</i>	Geothermal spring, USA	Heinen and Heinen, 1972
<i>B. flavothermus</i>	Geothermal spring, NZ	Heinen et al., 1982
<i>B. stearothermophilus</i>	Thermal springs	Marsh and Larsen, 1953; Golovacheva et al., 1965
	Water	Miquel, 1888; McBee and McBee, 1956; Harris and Fields, 1972
	Snow	Golikova, 1926
	Soil	McBee and McBee, 1956; Harris and Fields, 1972
	Compost	Epstein and Grossowicz, 1969
	Ocean sediment	Bartholomew and Rittenberg, 1949; Bartholomew and Paik, 1966
	Mineral wax	Bairiev and Mamedov, 1963
<i>B. schlegelii</i>	Eutropic lake sediment	Aragno, 1978; Schenk and Aragno, 1979
<u>Acidic Bacillus group:</u>		
<i>B. acidocaldarius</i>	Geothermal spring, Russia	Khraptsova and Loginova, 1981
	Geothermal spring, USA	Darland and Brock, 1971
	Geothermal spring, Japan	Uchino and Doi, 1967; Oshima et al., 1977; Uchino, 1982
	Geothermal spring, Italy	Buonocore et al., 1976; De Rosa et al., 1973
<i>B. acidocaldarius</i>	Soil	Hippchen et al., 1981; Poralla and Koning, 1983
<u>Other groups:</u>		
<i>Thiobacillus thermophilica</i>	Geothermal spring, Russia	Egorova and Deryugina, 1963
Isolate PS3	Geothermal spring, Japan	Kagawa, 1976
(B) <u>Non sporeformers</u>		
<i>Thermus</i> species	Iceland	Kristjansson and Alfredsson, 1983; Cometta et al., 1982b; Pask-Hughes and Williams, 1977
<i>Thermus</i> species	Thermally polluted river, Belgium	Degryse et al., 1978
<i>Thermus</i> species	Geothermal spring, Russia	Egorova and Loginova, 1974; Andreev et al., 1983
<i>Thermus ruber</i>	Geothermal spring, Russia	Loginova and Egorova, 1975
<i>Thermus</i> X-1	Thermally polluted river, USA	Ramaley and Hixson, 1970
<i>Thermus aquaticus</i>	Geothermal spring, USA	Brock and Freeze, 1969
<i>Thermus</i> species	Geothermal spring, USA	Ramaley and Bitzinger, 1975; Ulrich et al., 1972
<i>Thermus</i> NH	Hot water heater, UK	Pask-Hughes and Williams, 1975
<i>Thermus thermophilus</i>	Geothermal spring, Japan	Oshima and Imahori, 1974
<i>Thermus flavus</i>	Geothermal spring, Japan	Saiki et al., 1972
<i>Thermus caldophilus</i>	Geothermal spring, Japan	Taguchi et al., 1982; 1983
<i>Thermus</i> T351	Geothermal spring, NZ	Hickey and Daniel, 1979
<i>Thermomicrobium roseum</i>	Geothermal spring, USA	Jackson et al., 1973
<i>Thermomicrobium fosteri</i>	Lake sediment	Phillips and Perry, 1976
<i>Thermothrix thioparus</i>	Geothermal spring, Mexico	Caldwell et al., 1976; Brannan and Caldwell, 1980
<i>Thermoleophilum album</i>	Geothermal springs and non-thermal environments, USA	Zarilla and Perry, 1984; Merkel et al., 1978
Isolate Wai33 A1	Geothermal spring, NZ	Hudson et al., 1984
Isolate V2	Geothermal spring, Japan	Kagawa, 1976; Hasegawa et al., 1976
Isolate K2	Geothermal spring, USA	Ramaley et al., 1975
Isolate Tk6	Geothermal spring, Japan	Kawasumi et al., 1980

Table 8.2: Properties of Bacillus species:

	<u>B. stearothermophilus</u>	<u>B. caldodenax</u>	<u>B. caldolyticus</u>	<u>B. caldovelox</u>	<u>B. thermocatenulatus</u>	<u>B. thermoflavus</u>	<u>B. schlegelii</u>	<u>B. acidocaldarius</u>
Gram reaction	variable	variable	variable	variable	+ve	+ve	+ve	variable
Optimum temperature	60-65°C	80°C	72°C	60-70°C	65-70°C on solid media 55-60°C in liquid media	60°C (aerobic) and 65°C (anaerobic)	70°C	60-65°C
Optimum pH	6.5-7.5	7.5-8.5	6-8	6.3-8.5	ND	6-9		3.5
Temperature maximum	65-75°C	85°C	82°C	76°C	78°C	<75°C	< 80°C	70-75°C
Spore shape	oval or cylindrical	oval	cylindrical	oval	cylindrical	NA	spherical	oval to cylindrical
Autotrophy	-	-	-	-	-	-	+	-
Colony pigmentation	cream	cream	cream	cream	yellow	dark yellow	cream	cream
Anaerobic growth	variable	W	W	W	+	+	-	-
G+C content (%)	51-65	64.8	52.3	65.1	NA	61	67.7	45-50

(compiled from references on page 8-2)

confusion this system of classification is B. stearothermophilus. Firstly, the minimum, maximum or optimum temperatures can change depending on media composition or the age of the inoculum (Gordon and Smith, 1949; Long and Williams, 1959; Heinen and Lauwers, 1981) and secondly, some strains are prototrophic (Epstein and Grossowicz, 1969; Atkinson et al., 1975; Lee et al., 1982) while others require growth factors, eg. methionine and biotin for growth at high temperature (Heinen, 1971; Kuhn et al., 1979). Additionally, there has been confusion over the substrate range of some isolates, eg. growth on n-alkanes as sole carbon sources (Mateles et al., 1967) being refuted by Kachholz and Rehm, (1980).

Thermus species were first isolated from the thermal springs of Yellowstone National Park, USA (Brock and Freeze, 1969) and have subsequently been isolated from most thermal regions (Table 8.1). The organisms are generally present in springs with temperatures between 55-85°C and neutral to alkaline pH (Kristjansson and Alfredsson, 1983). They are also present in many man made thermal environments including thermally polluted rivers (Ramaley and Bitzinger, 1975; Zeikus and Brock, 1972; Degryse et al., 1978) and cooling towers and laundry heaters (Pask-Hughes and Williams, 1975; Brock and Boylen, 1973).

All Thermus species are Gram negative, non sporulating, heterotrophic rods to filaments. Under favourable conditions, eg. media containing high organic compounds, all Thermus species form multicellular spherical bodies called "rotund bodies" (Degryse et al., 1978). Other common features of Thermus species are the presence of a characteristic annelid cell wall structure and the inability to oxidise carbohydrates in spite of their ability to grow on them. Difference in pigments, growth optima, physiology and G+C content are known to occur within Thermus

species. Table 8.3 shows the salient features of some Thermus species.

Cometta et al., (1982a, b) from a study of the distribution of Thermus strains along a natural temperature gradient and in thermal runoff, and from the behaviour of Thermus aquaticus in continuous culture have suggested that the species are genetically unstable. For example, pigmentation was rapidly and irreversibly lost on culture. It has been suggested that pigments might serve a photoprotective function in the spring habitat and that strains growing under lower light intensities, eg. below algal mats or enclosed water heaters would not require pigments. A similar degree of physiological variability has also been noted. Cometta et al., (1982b) studied a number of strains of Thermus species and observed that all were physiologically diverse in the spectrum of nutrients utilized, even though they were all isolated from the same habitat. They have speculated that such genetic instability might be a common feature of all extreme thermophiles and was presumably necessary for these organisms to survive in a continuously changing geothermal environment.

Table 8.1 also contains features of other nonsporulating aerobes which are clearly different from the Thermus species. Thermomicrobium roseum can be differentiated from Thermus species by its pleomorphic morphology, pink pigmented colonies, and absence of rotund bodies (Jackson et al., 1973) cell wall ultrastructure (Merkel et al., 1980). Thermophile V2 was isolated from a Japanese hot spring (Kagawa, 1976; Hasegawa et al., 1976). It was Gram negative and motile but had a temperature optimum for growth of only 60°C and a maximum at 70°C The G+C content of the organism was only 52%. Another unique extreme thermophile was isolated from the Japanese hot springs and designated TK-6 (Kawasumi et al., 1980). Strain TK-6 was a Gram negative obligate chemolithotroph

Table 8.3: Properties of Thermus and Thermomicrobium species:

	<u>Thermus aquaticus</u>	<u>Thermus flavus</u> (a)	<u>Thermus thermophilus</u> (b)	<u>Thermus caldophilus</u>	<u>Thermus ruber</u>	<u>Thermus X-1</u>	<u>Thermomicrobium roseum</u>
Reference cited	Brock & Freeze(1969)	Saiki et al., (1972)	Oshima & Imahori (1974)	Taguchi et al. (1983)	Loginova and Egorova, (1975)	Ramaley & Hixson,(1970) Ramaley et al., (1975)	Jackson et al., (1973)
Habitat	Hot spring USA	Hot spring, Japan	Hot spring, Japan	Hot spring Japan	Hot spring, Russia	Polluted river, USA	Hot spring, USA
Colony colour	Yellow Orange	Yellow-brown	Yellow-brown	NA	Red	Cream	Pink
Optimum temperature(°C)	70-72	70-75	65-72	70-75	60 on solid medium 50-55 in liquid medium	70	70-75
Optimum pH	7.5 - 7.8	7.0 - 7.5	7.0	7.0	8.0	NA	8.2 - 8.5
pH range for	6.0 - 9.5	6.0 - 9.0	5.0 - 9.6	ND	ND	7.5 - 10.0	6.0 - 9.4
Generation time (min.)	60 - 70	42 - 45	18 - 20	ND	60	50 - 60	300
Growth in rich medium; nutrient broth, boullion	No	Yes	Yes	ND	Yes	No	No
Presence of slime	Yes	ND.	Yes	ND	ND	No	No
G+C ratio(%)	67	69 - 71	69	ND	ND	64	64.3

(a) Requires several amino acids and vitamins for growth. Saiki et al., 1972

(b) Requires lysine for growth. Degryse et al., 1978.

with an optimal temperature between 70-75°C and a G+C content of 43.7%. Strain TK-6 is unique since it is the only strict autotrophic hydrogen oxidising bacterium known to exist within the hydrogen-oxidising group of bacteria. Isolate K-2 was isolated by Ramaley et al., (1975) from thermal springs of USA. The colonies were pink in colour and the organism was capable of growth up to 80°C with an optimum at 60°C. The pH range for growth was 6.0-9.4 and the optimum for growth was between 7.6 and 7.8. These Gram negative cells had a G+C content of 64.4%. Wai33 AT and Thermus species both possessed yellow pigmented colonies but were differentiated on the basis of morphology and cell wall ultrastructure. Wai33 AT was an extremely filamentous bacterium (Hudson et al., 1984) and possessed a Gram negative cell wall structure distinct from Thermus species (Hudson, unpublished observation). The recently described Thermoleophilum album is also unique since it is the only known thermophile which has an obligate requirement of alkanes for growth. Thermothrix thioparus is different to all other thermophiles in its ability to produce sulphur from thiosulphate.

A number of moderate thermophiles (temperature optimum of 45-50°C) have also been isolated from geothermal environments and include methane oxidising bacteria (Shen et al., 1982), hydrogen oxidising bacteria (McGee et al., 1967; Goto et al., 1977, 1978), Thiobacillus species (Williams and Hoare, 1972), budding bacteria (Kahan, 1961) and thermophilic Pseudomonas species (Egorova and Bogdanova, 1974). However, these organisms will not be discussed further since it was not the object of this thesis to look at organisms with such moderate temperature optima for growth. The aim of the study was (i) to find out whether the extremely thermophilic aerobic microflora of New Zealand thermal was similar to that observed and (2) to isolate and identify any new

species of aerobic extreme thermophiles present in New Zealand hot springs.

8.2 MATERIALS AND METHODS:

Conventional methods for enrichment, isolation and characterization of aerobic thermophilic bacteria were used.

For studies on Thermus species, the media described by Brock and Freeze (1969), Degryse et al., (1978) or Oshima and Imahori (1974) was used. Hydrogen oxidising bacteria were enriched as described by Schenk and Aragno (1979). For isolation of the hydrogen oxidising bacteria capable of growth heterotrophically, the medium of Brock and Freeze (1969) referred to as Allen's medium was used.

Acidophilic Bacillus acidocaldarius was isolated and characterized as described by Darland and Brock (1971) or Oshima et al., (1977).

Thermothrix species were isolated and characterized as described by Brannan and Caldwell (1980). Thiobacillus species were enriched on media described by Le Roux et al (1977).

Attempts were also made to isolate Thermomicrobium species using the method described by Jackson et al., (1973).

Conventional microbiological media such as Trypticase Soy Broth, Nutrient Broth, Nitrate Broth, Peptone Broth etc were used either at normal strength or were diluted as required. Similarly, for pH studies, diluted Trypticase Soy Broth was used but adjusted to the required pH values.

8.3 RESULTS AND DISCUSSION:

8.3.1 Isolation And Characterization Of Thermus Species

(1) Distribution

Table 8.4 presents data on the pools in which Thermus-like isolates were found to be present. Their identification as Thermus species was based on cell morphology and cell dimensions, and typical yellow colonies together with random Gram negative staining. It was observed previously that at lower incubation temperatures (55°C), the microbial diversity was higher than when enrichments were incubated at 70°C. At 70°C, Thermus species usually predominated. However, as can be seen in Table 8.4, other morphological types of bacteria were also observed but these were not studied further. Results of data from Table 8.4 are presented graphically in Figure 8.1.

Thermus species were present in springs with temperatures between 45°C to 95°C and pH between 5.5 to 9.4. However, the temperatures and pHs of Icelandic hot springs from which Thermus species could be isolated was between 55°C to 85°C and 6.5 to 10 respectively (Kristjansson and Alfredsson, 1983).

The isolation of Thermus species from New Zealand thermal springs having high temperatures (95°C or higher) and/or lower pH (5.5 or lower) was inconsistent. Such inconsistencies in the isolation frequency was also observed by Kristjansson and Alfredsson (1983) in their studies of Thermus species along a thermal gradient in a runoff channel. They attributed the presence of Thermus species in pools with temperatures above 85°C (especially in boiling springs) to be the result of splash water washing in cells from adjoining rocks. They also speculated that

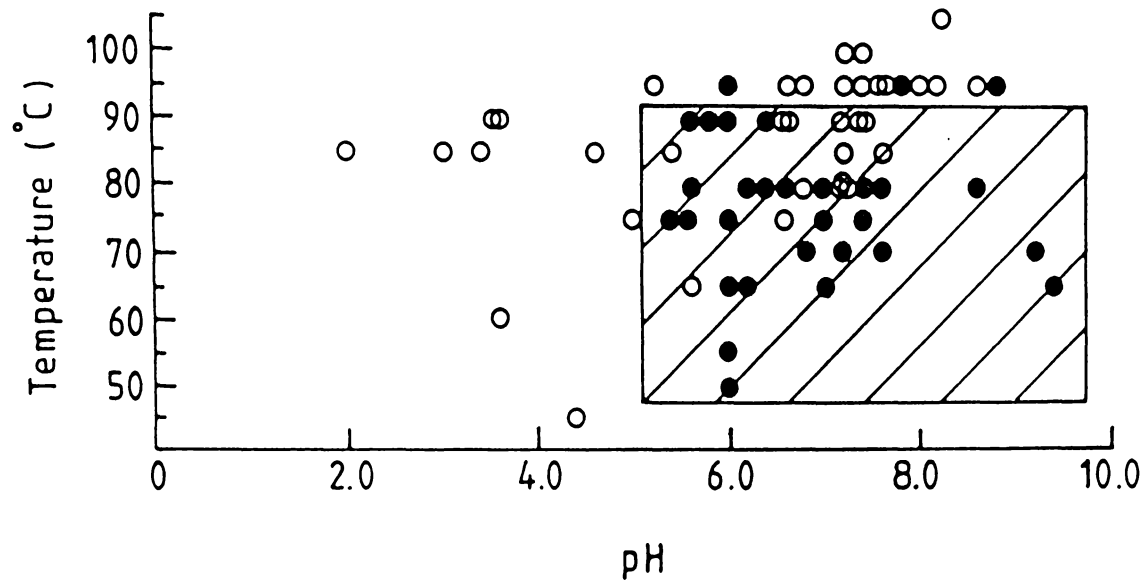


Figure 8.1 Graphic representation of data from table 8.4 depicting the presence of *Thermus* species in New Zealand thermal springs. Only 67 out of the 80 pools have been plotted on the graph because data on the remaining 13 pools was incomplete.
(Refer errata and addenda on page]]-])

Table 8.4. Distribution of Thermus in New Zealand hot springs

Pool No	Temperature(°C)	pH	Bacterial Morphology
<u>Rotorua:</u>			
Rt1	85	ND	<u>Thermus</u> like
Rt2	82.5	7.66	<u>Thermus</u> and <u>Bacillus</u> like
Rt3	70	7.2	<u>Thermus</u> like
Rt4	87	7.2	<u>Thermus</u> like
Rt6	64	9.4	<u>Thermus</u> like
Rt7	91	7.3	<u>Thermus</u> like
Rt8	70	9.1	<u>Thermus</u> and <u>Bacillus</u> like
Rt10	94	7.9	-
Rt12	66	6.9	<u>Thermus</u> like
Rt13	98	7.4	-
Rt14	80	7.0	<u>Thermus</u> like
Rt15	96.4	8.7	<u>Thermus</u> like
Rt16	67	5.5	-
Rt17	96	5.2	-
Rt25	ND	2.1	-
Rt26	85	3.0	-
Rt27	ND	ND	-
Rt28	95	6.0	<u>Thermus</u> like
Rt34	75.7	6.53	-
Rt35	80	6.56	-
Rt42	97	7.3	-
Rt46	90	3.6	-
Rt51	82	7.34	-
Rt57	91	7.2	-
Rt58	103	8.12	-
Rt60	97.7	7.16	-
Rt61	94.5	8.1	-
Rt66	78.0	6.12	<u>Thermus</u> like
Rt67	80.0	6.6	<u>Thermus</u> like
Rt68	95	7.52	Sporulating bacilli, motile rods
Rt72	97	6.64	-
Rt74	ND	ND	-
Rt80	ND	ND	-
Rt82	91	6.69	-
Rt84	ND	ND	-
Rt86	92	6.69	-
Rt97	78	7.14	-
<u>Waimangu:</u>			
Wai2	89	3.5	-
Wai6	75	5.3	<u>Thermus</u> like
Wai7	82	6.3	<u>Thermus</u> like
Wai10	91	7.4	<u>Thermus</u> like
Wai11	96	8.5	-
Wai14	70	7.7	Rods, filaments and <u>Thermus</u>
Wai16	70	ND	<u>Thermus</u> , filaments
Wai17	80	8.5	<u>Thermus</u> like

Table 8.4(cont) Distribution of Thermus in New Zealand hot springs

Pool No	Temperature(°C)	pH	Bacterial Morphology
Wai19	79	7.7	<u>Thermus</u> and <u>Bacillus</u> like
Wai20	96	6.85	Sporulating bacilli & filaments - putrid smell
Wai21	67	6.25	Motile bacilli & <u>Thermus</u> like
Wai22	97	7.7	-
<u>Tikiteri:</u>			
Tk4a	90.1	5.8	<u>Thermus</u> like
Tk4x	90.1	6.1	<u>Thermus</u> like
Tk10	75.3	7.3	<u>Thermus</u> like
Tk11	45.3	4.49	-
Tk15	84.5	5.33	-
Tk18	85.0	1.9	-
Tk19	63	6.1	<u>Thermus</u> like
Tk20	75	4.9	-
Tk21	73	6.1	<u>Thermus</u> like
Tk22	62	3.5	-
Tk24	84	3.4	-
Tk25	84	4.7	-
Tk26	75	5.5	<u>Thermus</u> like
<u>Taupo:</u>			
TP1	95	ND	-
TP2	95	ND	-
TP3	95	ND	-
TP4	95	ND	-
TP5	89	6.47	<u>Thermus</u> like
TP6	78	7.28	<u>Thermus</u> like
TP7	72	ND	<u>Thermus</u> like
TP8	48	ND	<u>Bacillus</u> like
TP9	92	7.14	
TP10	72	6.84	<u>Thermus</u> and
			<u>Bacillus</u> like
<u>Tokaanu:</u>			
Tok1	96	7.86	<u>Thermus</u> like
Tok2	81	7.13	<u>Thermus</u> like
Tok3	76	6.93	<u>Thermus</u> like
Tok4	89	5.63	<u>Thermus</u> like
Tok5	52	5.93	<u>Thermus</u> like
Tok6	97	7.00	
Tok7	53	6.04	<u>Thermus</u> and <u>Bacillus</u> like
Tok8	77.5	5.59	Rods single or in twos and <u>Thermus</u> like

the detection and isolation of Thermus species at pH lower than 6.0 was possible in certain springs where effluents of an alkaline and an acidic spring came together. However, the number of springs with pH between 4.0 and 6.0 is low in Iceland, hence their conclusion that Thermus species can only exist in springs with pH above 6.0 is still open to question.

Several pools within the apparent pH and temperature range for Thermus gave negative enrichments on several occasions indicating that other pool properties are important. Ramaley and Bitzinger (1975) provided evidence that Thermus species could not be isolated from high sulphide containing (40mg/litre) hot springs in Colorado. It is possible that a similar situation occurs in New Zealand thermal springs since some New Zealand hot springs are very rich in sulphide and could vary from as low as 1 to 2mg/litre to as high as 45mg/litre. Similarly, the presence of other inhibitory substances (e.g. arsenic, mercury) in hot springs might be influential in the distribution of these organisms.

Samples of soil around fumaroles (Taupo region) did not yield Thermus species. This is consistent with the understanding that Thermus species exist below 90°C and they cannot be isolated from soil; that is their habitat is essentially aquatic.

(2) Isolation and Characterisation

A number of pure cultures were obtained for further studies (Table 8.5). Pure cultures were obtained by picking pigmented colonies from primary enrichments and re-streaking.

All isolates had a similar cell morphology, were Gram negative nonsporulating rods or short filaments (Fig. 8.2A) with maximum growth temperature below 80°C. Electron micrographs of thin sections of Rt8-AT,

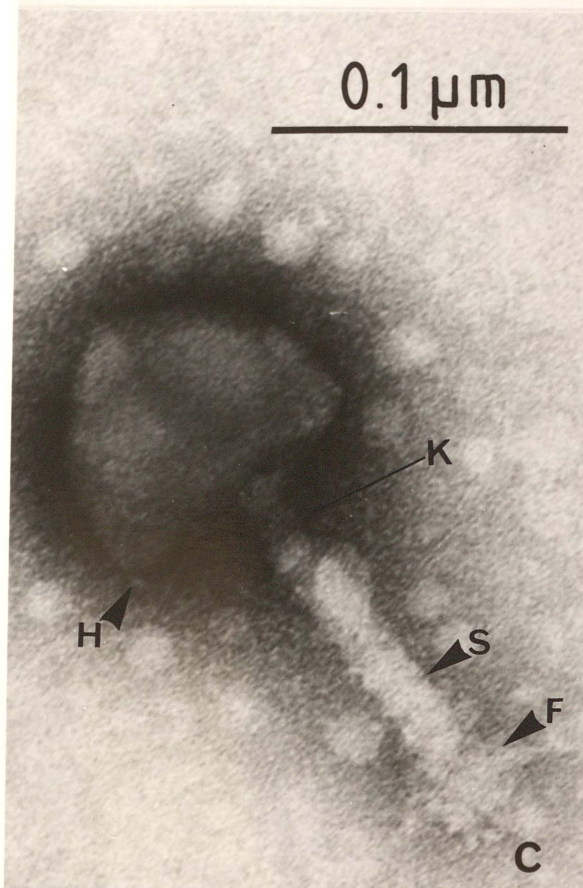
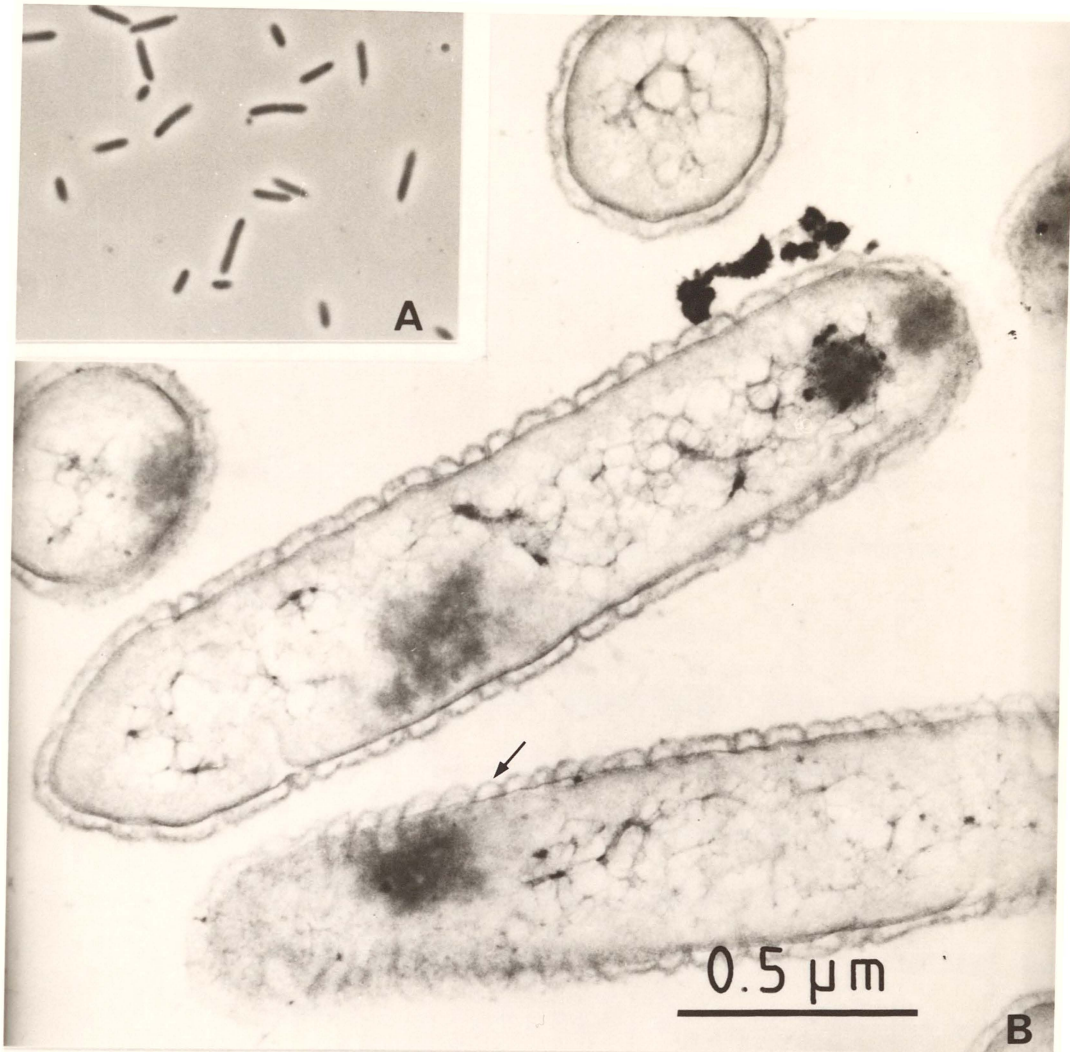
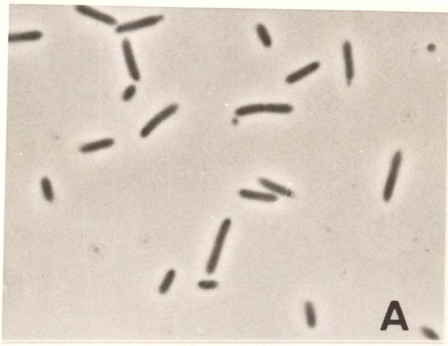
Table 8.5 Growth of *Thermus* species in different media and colony colour:

	<u>T. aquaticus</u>		<u>T. thermophilus</u>	Rt15-AT	Rt3-AT	Rt4-1A	Rt8-AT	Pool Wai19	
	25104	25105						Wai19-AT1	Wai19-AT2
Allen's medium	++++	++++	++++'	++++	++++	++++	++++	++++	++++
Castenholz's medium	++++	++++	++++	++	+	++++	++++	++++	++++
Oshima's medium	-	-	++++	-	++++	++++	-	++++	-
Nitrate Broth	-	+	+	-	++++	++++	++++	-	-
BHI Broth	+	-	-	-	-	NT	NT	-	-
TSB pH 7.0	+	+	+	+	-	+	+	+	+
TSB pH 8.0	+	+	+	+	-	+	+	+	+
TSB pH 9.0	+	+	+	-	-	+	+	+	+
TSB pH 10.0	+	+	+	-	-	-	+	+	+
TSB pH 11.0	-	-	-	-	-	-	-	-	-
Colony Colour	Yellow-brown	Yellow-orange	Yellow-brown	Yellow-brown	Pink	Yellow-brown	Yellow-orange	Yellow-brown	Yellow-brown

TSB = Trypticase Soy Broth, growth recorded as positive (+) or negative (-) ++++ excellent growth
 - no growth

All strains were proteolytic as evidenced by precipitation zones forming in casein agar plates. None were lipolytic (tributyryl agar plates) and except for Rt3-AT none were amylolytic (starch agar plates).

Figure 8.2 (A) Phase contrast photomicrograph of Thermus Wai19-AT2 showing typical rod-shaped and filamentous morphology <Mag. X 3700>. (B) EM of a thin section of Thermus Wai19-AT2 showing a typical annelid cell wall structure. (C-D) Negatively stained preparation of phage infectious to Thermus Wai19-AT2. (C) A whole virus particle showing the presence of a head (H), collar (K), sheath (S) and tail fibres (F) and (D) defective phage particles showing the unassembled phage components. Note the presence of tail fibres.



Wai19-AT and Rt15-AT revealed an annelid cell wall structure typical of Thermus species (Fig. 8.2B).

The Thermus species isolated from New Zealand hot springs, namely Rt3-AT, Rt8-AT, Rt4-1A and Wai19-AT2 were inhibited by penicillin, streptomycin, chloramphenicol, neomycin and novobiocin at 10µg/ml. D-cycloserine was inhibitory at a concentration of 100µg/ml and sodium azide at 500µg/ml. The antibiotic sensitivity of these New Zealand isolates is similar to that of T. aquaticus (Brock and Freeze, 1969) and other Thermus species (Degryse et al., 1978).

Differences in pigmentation and physiology were evident in the cultures isolated (Table 8.5). Colonies were usually pale yellow to yellowish brown. Rt8-AT was bright yellow-orange and resembled T. aquaticus. With the exception of Rt3-AT, pigments extracted from the New Zealand isolates had an absorption spectrum (Fig. 8.3) similar to T. aquaticus (maximum 450nm). Rt3-AT was pink in colour with a distinct pigment absorption spectrum with a peak at 480nm (Fig. 8.3), and thus resembles isolate K2 of Ramaley and Bitzinger (1975) and T. ruber (Loginova and Egorova, 1975).

Rt3-AT also differed physiologically in being the only isolate to show no growth in trypticase soy broth (TSB) and poor growth in Castenholz medium. Physiological differences were evident in all the strains particularly with respect to rich media e.g. Oshima's.

Two Thermus strains were isolated from Pool Wai19. Wai19-AT1 but not Wai19-AT2 was able to grow in a rich medium. A phage isolated from the same pool was able to infect and lyse Wai19-AT2 but not W19-AT1. This phage had a very restrictive host range (Table 8.6). The morphology was similar to that of Thermus thermophilus phage, ϕ YS40 (Fig. 8.2C).

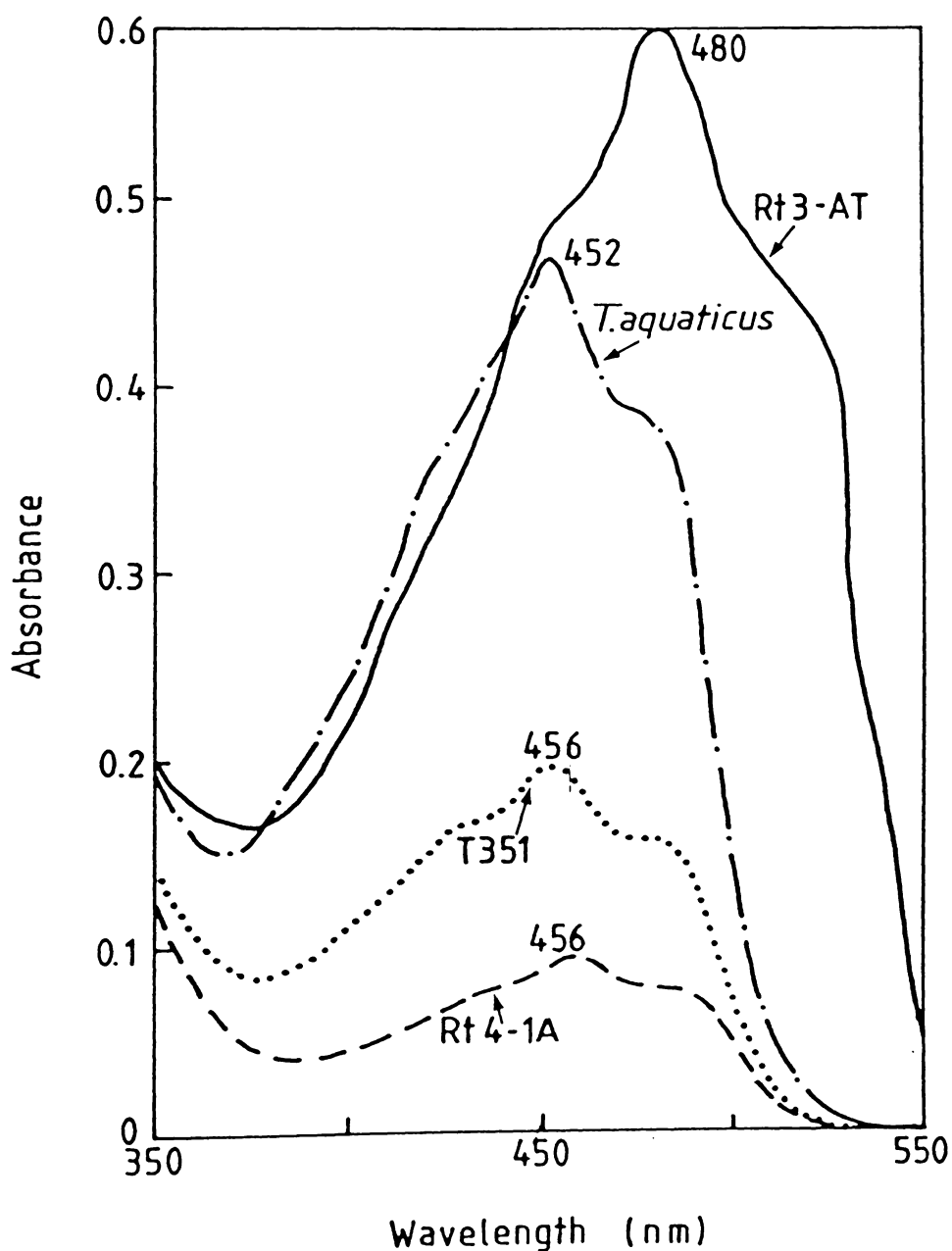


Figure 8.3 Absorption spectra of pigments produced by *Thermus* isolates- *Thermus aquaticus*, Rt3-AT, RT15-AT (T351), and Rt4-1A. Cells were grown in Allen's medium, washed with 0.8% NaCl solution, suspended in 90% acetone (Vol/vol), centrifuged and the spectra of the acetone extract determined in a Beckman spectrophotometer.

Table 8.6: Host range of phage Wai19-AT2 and ϕ YS40:

Bacterial strains	Phages	
	Wai19-AT2	ϕ YS40
<u>Thermus</u> Wai19-AT1	-	-
<u>Thermus</u> Wai19-AT2	+	-
<u>T. aquaticus</u> (25104)	-	-
<u>T. thermophilus</u> HB8	-	+
<u>Thermus</u> 7-AT	-	-
<u>Thermus</u> T351	-	NT
<u>Thermus</u> TOK 3	-	-
<u>Thermus</u> L2	-	NT
<u>Thermus</u> OK6	-	NT
<u>Thermus</u> Rt6	-	NT
<u>Thermus</u> 1-AT	-	-

A common feature of Wai19-AT1 phage and ϕ YS40 was their ability to form defective viral particles in their respective hosts (Fig 8.2D). The morphology of Wai19-AT1 phage (Fig 8.2E) is clearly different from that of the spirochete phage (Refer to paper on spirochetes).

8.3.2 Isolation Of Hydrogen Oxidising Bacteria:

(1) Distribution

Seventeen of the 31 samples were positive for hydrogen oxidising bacteria. At least three morphological types of rods were recognizable; small rods, large rods and thin rods. The thin rods had terminal, spherical spores (Table 8.7).

After three passages in autotrophic medium, some of the positive cultures were transferred into Allen's (heterotrophic) medium. This enabled the selection of autotrophs capable of heterotrophic growth. Further purification was done on Allen's medium solidified with 1.8% Agar at 70°C. Two distinct groups of bacteria were observed based on colony size and colony type. Cultures of organisms belonging to Group I, produced white colonies, which were 0.5mm in diameter after 40 hours of

Table 8.7 Morphology of Hydrogen Oxidising Bacteria

Site/Pool	Optical Density (660 nm)	Morphology
<u>Rotorua:</u>		
Rt1	0.21	Small and large rods
Rt1A	0.001	Small rods
Rt3	0.21	Small and large rods
Rt6	0.001	Rods
Rt7	0.0	-ve
Rt8	0.02	Rods
Rt9	0.01	Rods single or in pairs
Rt10	0.0	-ve
Rt12	0.0	-ve
Rt14	0.012	Rods
Rt15	0.0	-ve
<u>Tokanuu:</u>		
ToK4	ND	Rods, single, pairs or in chains
ToK5	ND	Rods
ToK6A	ND	Small and large rods, thin rods with terminal spherical spores
Tok7	ND	Small and large rods, thin rods with terminal spherical spores
ToK8	ND	Rods
<u>Taupo:</u>		
TP1	ND	-ve
TP2	ND	-ve
TP3	ND	-ve
TP4	ND	-ve
TP5	ND	-ve
TP6	ND	Rods single, in pairs or in chains
TP7	ND	Rods single, in pairs or in chains
TP8	ND	Rods single, in pairs or in chains
TP9	ND	-ve
TP10	ND	Rods single, in pairs or in chains
<u>Waimangu:</u>		
Wai4	ND	-ve
Wai5	ND	-ve
Wai10	ND	-ve
Wai11	ND	-ve
Wai16	ND	-ve
Wai17	ND	-ve
Wai21	ND	Rods
<u>Mount Erebus:</u>		
E4 ₄	ND	-ve
<u>Waikato University Pond:</u>		
3WU	ND	Rods
6WU	ND	-ve
<u>Lake:</u>		
L1	ND	-ve

ND indicates not determined;

growth. Two springs contained cells of this type and the pure cultures isolated were designated Rt1-AH1 and Rt3-AH1. Pure cultures obtained from pools 3WU, Tok4, Tok5, Tok6A, Tok7, Tok8, TP6, TP7, TP8, TP10 and Wai21 were designated by suffix -H1 and belonged to Group II. All the members of Group II produced slimy and creamish-grey colonies. The 3WU-H1 colonies were 1-2mm in diameter whereas colonies of the remaining isolates were 5mm in diameter after 30 hours of growth.

(2) Characterization Studies

(a) Group I hydrogen oxidising isolates (Rt1-AH1 and Rt3-AH1):

Both Rt1-AH1 and Rt3-AH1 formed extremely small (0.5mm) white colonies on Allen's medium after hours growth at 70°C. The cells were 1-2µm by 0.8-1.0µm, occurred singly or in pairs, did not sporulate and were Gram negative. Comparative properties of these organisms are given in Table 8.8. Both strains appeared very similar with respect to a number of tests performed. However, Rt3-AH1 grew very poorly in rich medium (Oshima's medium, Nutrient broth), did not utilise lactose and utilized maltose very slowly. Rt1-AH1 on the other hand, grew very well on rich medium and on lactose but did not utilize maltose.

Table 8.8 Properties of Rt1-AH1 and Rt3-AH1

Property	Rt1-AH1	Rt3-AH1
pH Growth range (1% TSB)	7.0 to 8.5	7.0 to 9.1
Temperature maximum (°C)	78	78
Substrate utilization:(a)		
Lactose	+	-
Maltose	-	+(slow)
Glucose	+	+
Galactose	+	+
Arabinose	+	+
Growth on rich (Oshima's) medium	+	+
Enzyme production:		
Amylase	-	-
Lipase	+	-
Protease	+	+

TSB = Trypticase Soy Broth

(a) = A number of other substrates tested were not utilised; and include erythritol, adonitol, glycogen, starch, m-inositol, dextrin, glycerol and rhamnose.

Based on the colony and bacterial morphology and ability to proliferate as facultative autotrophs these organisms can be differentiated from the strict heterotrophs such as Thermus spp., Thermomicrobium spp., Bacillus spp., isolate V-2, isolate K-2 etc. Isolates Rt1-AH1 and Rt3-AH1 are also distinct from the Japanese isolate TK-6 because the latter is a strict autotroph (Kawasumi et al., 1980) while the former are facultative autotrophs. Both New Zealand isolates also differ from the isolates of Goto et al., (1977, 1978) in their ability to grow at much higher temperatures, their ability to utilize some carbohydrates, and their colony colour. Hence isolates Rt1-AH1 and Rt3-AH1 have presently been regarded as new species of extremely thermophilic hydrogen oxidising bacteria. Except for minor differences, Rt1-AH1 and Rt3-AH1 are very similar to each other and are possibly strains of the same species.

(b) Studies on organisms belonging to Group II:

All the strains belonging to Group II were Gram variable sporulating rods, 3-7 μ m by 1 μ m when grown on solid media. The spores were cylindrical, were produced terminally and distended the vegetative cell in which they were borne. Sporulation depended on the carbon source and the type of strain used. For example, TP10-H1 but not Tok4-H1 formed spores on casein agar plates within 12 hours incubation at 70°C. Similarly, 3WU-H1 but not TP6-H1 or Wai21-H1 formed spores on 2% trypticase soy broth at 65°C within 12 hours incubation. These organisms can be classified as Bacillus species based on their morphology, colony characteristics, Gram reaction, sporulation, and their inability to ferment glucose anaerobically.

The physiology of hydrogen oxidising Bacillus strains is different from other B. stearothermophilus in that the former is a facultative autotroph whereas the latter a heterotroph. Similarly all the hydrogen oxidising Bacillus strains are prototrophs in that they can utilize a wide range of substrates as sole carbon sources. B. stearothermophilus 136 (Epstein and Grossowicz, 1969) is the only known B. stearothermophilus strain capable of prototrophic growth. B. caldotenax B. caldolyticus B. caldotenax and all other B. stearothermophilus strains require growth factors.

However, the hydrogen oxidising Bacillus strains of group II resemble B. stearothermophilus and related species with respect to the spectrum of carbohydrates utilized and enzymes produced (Table 8.9).

A wide variation in the type of substrate utilized by different strains within the Bacillus isolates of Group II hydrogen oxidisers was obtained (Table 8.9). However a very similar antibiotic inhibition spectrum was obtained indicating that all were possibly strains of the same species. The inhibition spectrum, measured as zones of inhibition (mm) were as follows for all the isolates of group II hydrogen oxidisers: Nalidixic acid (30µg), 0; Ampicillin (2µg), 21; Tetracycline (30µg), 17; Erythromycin (15µg), 10; Kanamycin (30µg), 12; Chloramphenicol (30µg), 9; Bacitracin (10units), 11; Polymyxin B (300units), 8; Streptomycin (10units), 12; Vancomycin (30µg), 12; Griseofulvin (30µg), 0.

One of the isolates TP10-H1 was studied further as the type strain. TP10-H1 grew optimally between 65 and 70°C with an optimum pH between 7 and 8. The morphology of the organism on agar is the same as described earlier. However, in a liquid medium (TSB) extremely filamentous cells

Table 8.9 Properties of Bacillus strains:

	TP7-H1	TP8-H1	TP10-H1	TOK4-H1	TOK5-H1	TOK7-H1	<u>B. caldotenax(a)</u>	<u>B. caldotenax(a)</u>	<u>B. caldolyticus(a)</u>	<u>B. stearothermophilus(a)</u> 136 262	
Maximum growth temperature (°C)	78	78	75	78	78	78	85	76	82	65	78
pH growth range	5-8.5	5-8.0	5-8.5	5-8.5	6-8.0	5-8.5	6-9.5	6-9.5	NT	NT	NT
Glycogen	-	-	+	-	-	-	-	-	+	-	+
Starch	W	+	+	+	+	+	-	-	+	-	W
Dextrin	-	+	+	-	-	-	+	+	+	+	+
Lactose	-	-	+	+	W	W	-	-	-	-	-
Maltose	NT	+	+	+	-	-	+	W	W	+	-
Glycerol	+	+	+	+	-	+	+	+	W	W	-
Glucose	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	+	-	W	W	-	-	-
Galactose	+	+	+	+	+	+	-	-	-	-	-
m-Inositol	+	+	+	-	-	-	-	-	-	W	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	+	-
Arabinose	-	-	-	+	+	+	-	-	-	-	-
Gas from NO ₃ under anaerobic conditions	-	+	-	-	+	+	-	-	-	-	-
Growth in anaerobic dextrose broth	-	-	-	-	-	-	W	W	W	-	T
Growth under autotrophic conditions	+	+	+	+	+	+	-	-	-	NT	NT
Starch digestion ^(b)	+	+	R	+	+	+	R	R	+	-	R
Casein hydrolysis ^(b)	+	R	+	+	+	+	+	+	+	-	+
Tributylin hydrolysis ^(b)	R	+	+	+	+	+	+	+	NT	NT	NT

(a) Results of type cultures except for pH range obtained from Wolf and Sharp (1981). B. stearothermophilus 136 was a prototrophic strain (Epstein and Grossowicz, 1969) and B. stearothermophilus 262 was a strain isolated from Icelandic hot springs.

(b) Digestion recorded as + for large zones of hydrolysis, and R for a restricted zone.

W = Weak growth NT = Not Tested.

were formed together with extensive slime production. Higher concentrations of yeast extract (0.5%) but not trypticase peptone (1%) were found to be inhibitory. Thin sections of TP10-H1 revealed the presence of a Gram positive type cell wall. The API-ZYM test strip showed high levels of extracellular enzyme production by TP10-H1 (Table 8.10).

Table 8.10 Constitutive enzyme produced by TP10-H1 and detected using the API-ZYM system.

Enzyme	Reaction
Alkaline phosphatase	2
Esterase	3
Esterase lipase	4
Lipase	1
Leucine arylamidase	4
Valine arylamidase	2
Cystine arylamidase	2
Trypsin	2
Chymotrypsin	4
Acid phosphatase	5
Phosphoamidase	4
α -galactosidase	5
β -galactosidase	2
β -glucuronidase	0
α -glucosidase	2
β -glucosidase	1
N-acetyl- β -glucosaminidase	2
α -mannosidase	0
α -fucosidase	0

TP10-H1 was grown on Casein agar. The cells were resuspended in distilled water ($A_{660} = 1.3$) and 60 μ l was added to each reaction cupule. The strips were incubated at 60°C for 10 hours before results were read. Enzyme production was assessed qualitatively on a scale of 0 to 5, 0 indicating no enzyme production and 5 high enzyme production.

8.3.3 Isolation And Characterization Of Bacillus Acidocaldarius

(1) Distribution

Only 2 of the 24 samples (Rt1 to Rt19, Rt22 to Rt26) set up for enrichment of acidophilic, aerobic extreme thermophiles, were positive at 70°C. The organisms present were tentatively identified as B. acidocaldarius strains and designated Rt17-A1 and Rt26-A1, after the pool numbers from which they isolated. A B. acidocaldarius strain was also isolated from a spring in Orakei Korako thermal region and designated OK16-A1 (A.M. Martin, Ph.D. thesis). Another 4 of the 11 thermal soil samples obtained from Mt. Erebus (E4₁ to E4₄) were positive for bacterial presence at 66°C, but not at 70°C. All four isolates were Gram variable, sporulating rods with no growth occurring in Allen's medium, pH 7.0 incubated either at 66°C or 70°C. This ruled out the possibility of them being B. stearothermophilus. The morphology, growth in acidic medium but not in neutral medium and Gram reaction indicated that these four strains were B. coagulans. B. coagulans have been isolated previously from geothermal springs (Belly and Brock, 1974). However, these four strains were not investigated any further.

(2) Characterization

Colonies of Rt17-A1 and Rt26-A1 were similar, and were 1-2mm after 36 hours growth at 60°C. The cells were rods to filaments 2-5 µm by 0.5-1.0µm and stained Gram positive. Both strains had a sluggish motility and possessed peritrichous flagella. The temperature maxima for growth was below 80°C with no growth occurring at 37°C. Best growth was obtained between 60 and 65°C. No growth occurred at pH 7.0. Both strains grew well at pH 3.0. Spores were formed on starch agar. The spores were terminal and cylindrical in shape.

The results of substrate utilization by Rt17-A1 and Rt26-A1 are given in Table 8.11.

Table 8.11. Substrate spectrum of B. acidocaldarius strains Rt17-A1 and Rt26-A1.

	Rt17-A1	Rt26-A1
Starch	+	+
Glycerol	+	+
Sucrose	+	+
Lactose	W	+
Glucose	+	+
Galactose	+	+
Rhamnose	-	+
Mannose	+	+
Fructose	+	+
Mannitol	+	+
m-inositol	-	+
Sorbitol	-	-
Xylose	+	+
Ribose	+	+
Pyruvate	+	+
Citrate	-	-
Acetate	-	-
Succinate	W	W
Ethanol	-	-
Casamino acids	+	+

W = weak

The New Zealand B. acidocaldarius strains Rt17-A1 and Rt26-A1 have a substrate spectrum similar to those isolated in Japan (Oshima et al., 1977) and USA (Darland and Brock, 1971). However, minor variations were observed. The New Zealand isolates could utilize pyruvate whereas the Japanese isolates could not.

B. acidocaldarius strains appear to be widely distributed, though (according to the literature). not as widespread as their neutral counterpart B. stearrowthermophilus. B. acidocaldarius can be isolated from acidic geothermal springs. However, not all geothermal springs with acidic pH and temperatures close to 80 to 90°C are favourable for growth of B. acidocaldarius. In this survey, two pools, namely Rt16 and Rt25 were ideal pools for growth of B.

acidocaldarius, but this species was found to be absent as indicated by negative enrichments. The culturing technique might not have been adequate; however similar negative results were obtained on several occasions. More recently, strains resembling B. acidocaldarius were isolated from neutral pH mesophilic soils (Poralla and Konig, 1983; Hippchen et al., 1981). The significance of this finding is yet to be investigated further.

8.3.4 Isolation And Characterisation Of Facultative Anaerobes

During the course of investigations on the aerobic flora, the presence of facultative anaerobes was encountered. No attempt was made to comprehensively survey springs but some of the organisms were readily isolated and cultures purified are briefly described in this section.

Three different types of facultative anaerobes were isolated. These were:

Type 1 - motile, Gram negative nonsporulating rods capable of producing sulphur from thiosulphate.

Type 2 - motile Gram negative nonsporulating rods unable to grow at temperatures above 65°C.

Type 3 - Gram variable sporulating rods tentatively identified as Bacillus stearothermophilus.

(a) Type 1 facultative anaerobes:

Such organisms were demonstrated in pools Rt6, Rt8 and Wai14, but not from pools Rt13, Rt14, Rt34 and Rt35. Organisms were demonstrated by enrichment on Beijernick's autotrophic thiosulphate medium as described by Egorova and Deryugina, (1963) incubated at 70°C. Most studies were restricted to an isolate from pool Rt8 designated Rt8-AB1. Following

enrichment on Beijernick's medium samples were inoculated into nitrate agar where growth was much faster. Well isolated colonies were transferred to nitrate broth and after incubation were again streaked on nitrate agar. This process was repeated three times to ensure cultures were pure. Colonies on nitrate agar were yellow and 1-2mm in diameter after 48 hours at 60°C.

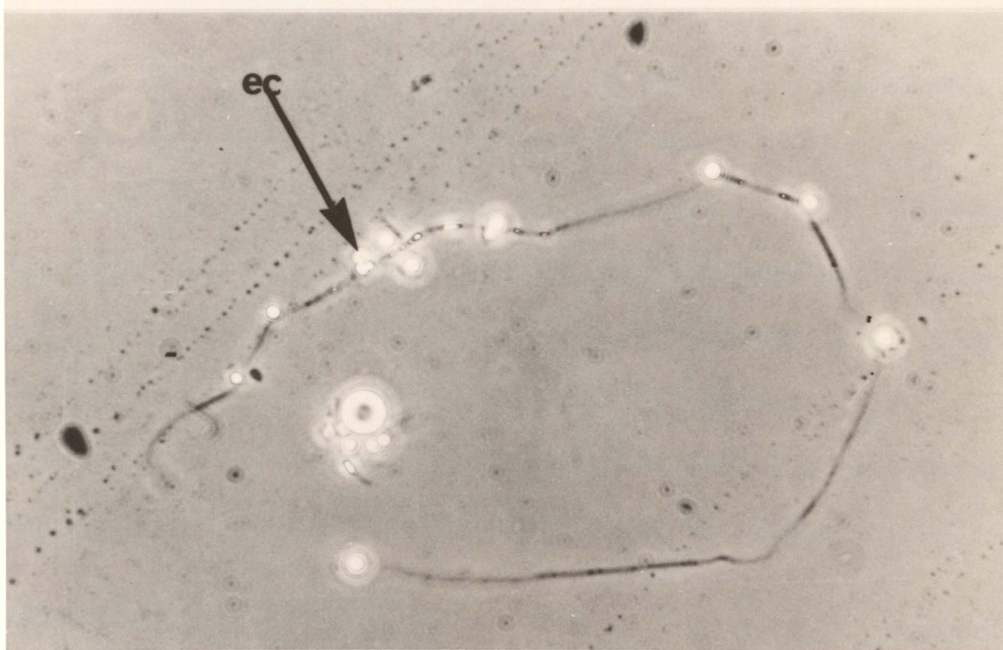
Under heterotrophic growth on nitrate broth and nitrate agar, cells formed long chains, often consisting of 20 to 30 cells. The culture retained the ability to grow on Beijernick's medium autotrophically and cells had the same morphology on both media. They were motile, Gram negative, nonsporulating rods, which on aging became non-motile and filamentous, during which time intracellular and extracellular sulphur was produced (Fig 8.4). Under autotrophic conditions, thiosulphate could be replaced by sulphide but not by sulphite or metabisulphite. Optimal growth occurred with 0.3% sulphide with no growth occurring with 0.1% or 0.5% sulphide.

Growth occurred optimally between 65-70°C in nitrate broth or in autotrophic Beijernick's thiosulphate medium. The maximum temperature for growth in both media was greater than 75°C but less than 80°C.

Rt8-AB1 was capable of growth in anaerobic nitrate broth and could utilize glucose anaerobically if nitrate was present.

The ability to grow on thiosulphate autotrophically is a trait similar to that of thermophilic Thiobacillus species (Egorova and Deryugina, 1963; Le Roux et al., 1977; Williams and Hoare, 1972) and Thermothrix thioparus (Caldwell et al., 1976) isolated from geothermal springs.

Figure 8.4 Phase contrast micrograph of Rt8-AB1 filaments depositing sulfur from thiosulphate, either intracellularly (ic) or extracellularly (ec)



However, Rt8-AB1 bears little resemblance to thermophilic Thiobacillus species. Thiobacillus thermophila (Egorova ad Deryugina, 1963) is a strictly autotrophic sporulating strain; Thiobacillus isolate 58 (Le Roux et al., 1977) is also a strict autotrophic non-motile rod or filament and Thiobacillus strains of Williams and Hoare, (1972) are incapable of growth above 60°C. Based on the observed variability in morphological characteristics in different media, the production of sulphur from thiosulphate and aerobic and anaerobic growth in nitrate broth, Rt8-AB1 was classified as a Thermothrix species. This is the first report of their isolation from New Zealand thermal springs.

(b) Type 2 facultative anaerobes:

Organisms of this type were observed in enrichment in nitrate broth at 55°C and were easily identified by their darting motility. Positive enrichments were obtained from pools Rt3, Rt8, Rt12, Wai27 and Fj3 but not from Rt6. Subsequently organisms with darting motility were also observed in anaerobic spirochete enrichments from pool Rt3. They were the only morphological type present in the enrichment culture containing 30µg/ml of rifampicin. Hence it might be possible to use rifampicin as a selective agent for the isolation of these type of organisms.

Enrichments at 70°C in nitrate broth (or Allen's medium) from positive springs were negative for such bacteria, instead Thermus species were enriched. Two cultures were purified for further studies, Rt3-AB1 and FJ3-AB1, of which Rt3-AB1 is regarded as a type species.

Isolate FJ3-AB1 was significantly different from Rt3-AB1 in its requirement for sodium chloride. Isolate Fj3-AB1 could grow aerobically in nutrient broth supplemented with 0 to 4% NaCl, the optimum concentration being 1%. It could grow anaerobically in TYEG medium

supplemented with 0.8% NaCl and was totally inhibited by 10µg/ml of penicillin. Motility stopped if SDS was added, and cell granulation was followed by cell lysis.

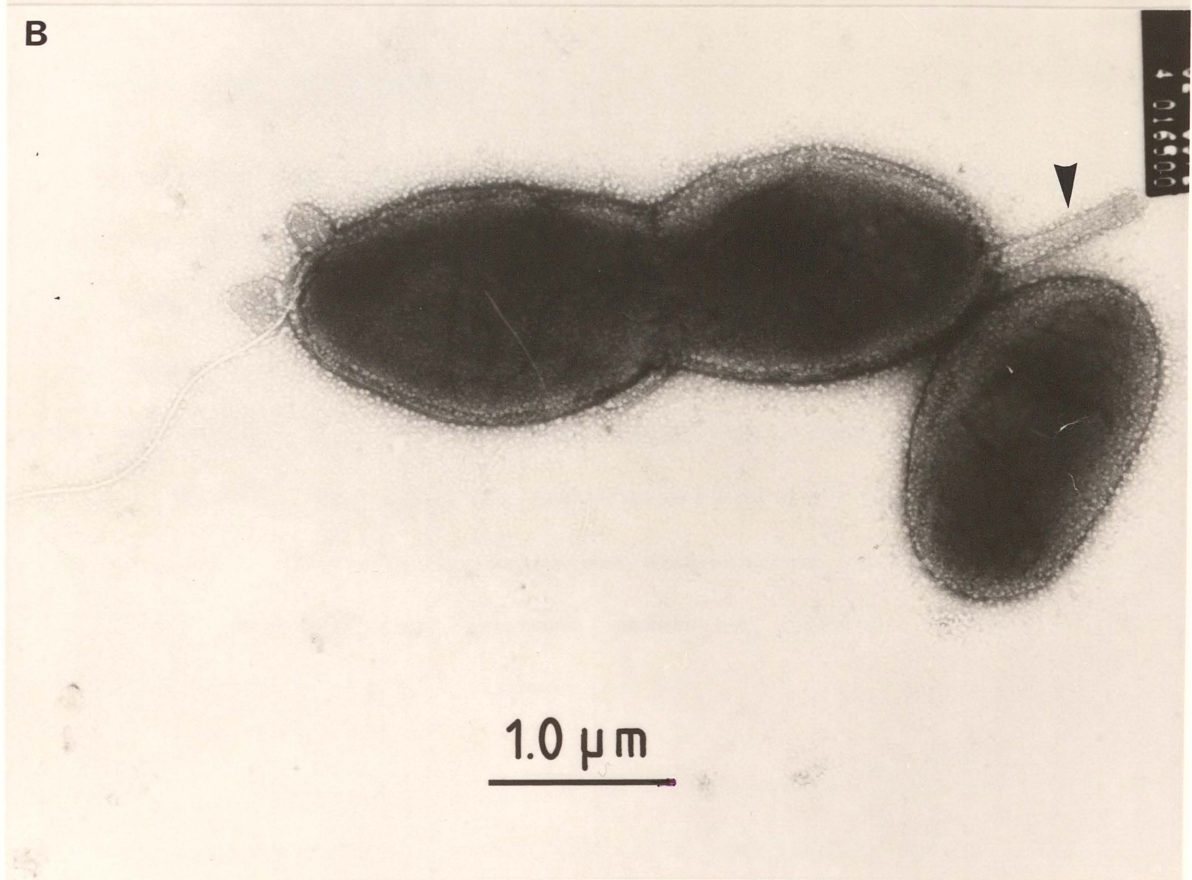
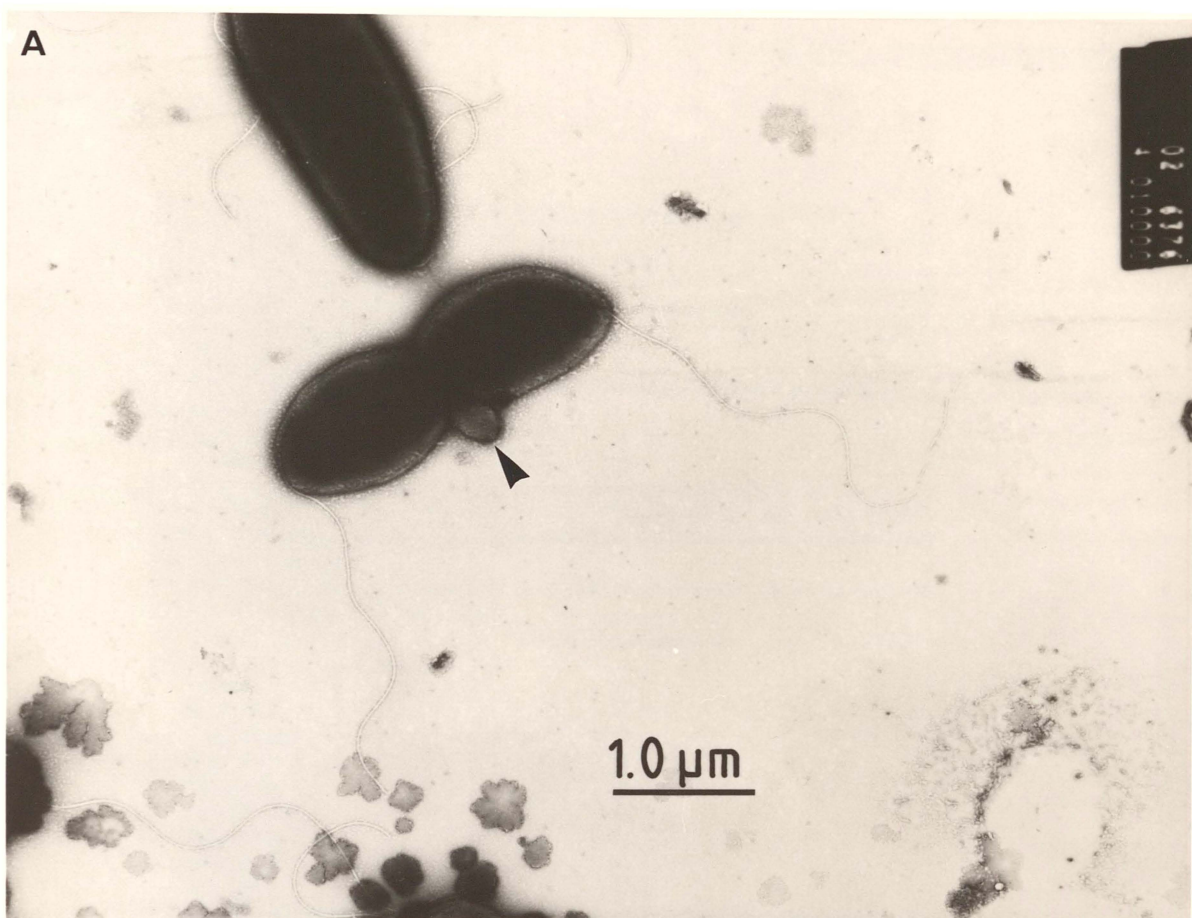
Colonies of Rt3-AB1 on Allen's medium were creamish white, translucent, convex and had smooth entire margins. Cells of Rt3-AB1 occurred singly, in pairs or in chains. The rods measured 1.2-2.0µm by 1-1.2µm. Motility was due to the presence of single, polar flagella (Fig 8.5). In addition protuberances of the cell were also observed under the electron microscope (Fig 8.5). Growth was better under aerobic conditions in TSB than under anaerobic conditions in TYEG medium. Slime was produced in both media. The isolate had a pH optimum for growth of 7.0 with a range from pH 5.0 to 9.5, grew optimally at 53°C with a maximum below 65°C. Rt3-AB1 was also capable of growth in 0.8% glucose and peptone water aerobically.

Rt3-AB1 possessed monopolar flagella, a feature common to Pseudomonas species. Egorova and Bogdanova (1974) have isolated thermophilic Pseudomonas species from Russian hot springs. However, all Pseudomonas species including the thermophilic species are strict aerobes and hence physiologically different to isolate Rt3-AB1. It would be more appropriate to group Rt3-AB1 in the Vibrionaceae family, which are monopolarly flagellated and some of which are anaerobes, until more is known about these organisms.

(c) Type 3 facultative anaerobes

A facultatively anaerobic, Gram variable sporulating organism tentatively identified as B. stearotherophilus was isolated from pool Rt8 and designated Rt8-AB2. The organism formed creamish white colonies and according to the properties (Table 8.12) it was placed in group 3 of

Figure 8.5 EM of isolate Rt3-AB1 showing the presence of a single polar flagellum on each of the divided rod (A) and the presence of cell protruberances, either adjacently from the cell (A) or terminally from the cell (B).



Walker and Wolfe's classification scheme for Bacillus stearothermophilus (Walker and Wolf, 1971).

Table 8.12 Properties of B. stearothermophilus Rt8-AB2

Morphology of spores	Oval
Swelling of sporangium	Definitive
Growth in 3% NaCl	-
Hydrolysis of:	
Starch	+
Casein	+
Litmus milk	Acid, clot and reduction
Gas from nitrate	-
Anaerobic growth in glucose	+
Acid but no gas from	
Xylose	+
Mannose	+
Arabinose	W
Sucrose	W
Glucose	+
Galactose	+
Rhamnose	-
Mannitol	-
Lactose	-

W = weak

8.3.5 SUMMARY AND CONCLUSIONS:

Probably the most widely studied groups of thermophilic bacteria are the aerobic genera growing in the 70°C to 80°C range. These organisms have not been extensively investigated in this thesis because it was not expected that such organisms would be unique or unusual. This has largely been borne out. The study has shown the widespread distribution of Thermus, and Bacillus species, and the presence of Thermothrix species in a few springs. The only organism not encountered was Thermomicrobium, but this may reflect the limited screening effort rather than its complete absence from the environment.

Generally the isolated species conformed to the major characteristics of type strains for the particular genus previously reported. The common occurrence of hydrogen oxidising bacteria in N.Z.

springs was surprising and might suggest that hydrogen is a common electron donor for thermophiles.

The demonstration of the presence of species in a pool conveys little information on the ecology of the habitat. It was beyond the scope of this study to determine density and distribution of each species within each spring. On selected springs this would undoubtedly be worthwhile, and it should be feasible to select springs with either a diverse or restricted flora for such a study.

APPENDIX 1

Microscopic Examination Of Geothermal samples:

Pool No.	Temp.(°C)	pH	Morphotypes Observed			
			Cocci	Rods	Filaments	Others
ROTORUA THERMAL AREA						
(A) Kuirau Park:-						
Rt1	85	ND	+	+	+	-
Rt2	82.5	7.66	-	+	-	-
Rt3	70	7.2	-	+	+	Spirochetes.
Rt4	87	7.2	-	+	+	-
Rt5	91	ND	-	+	+	-
Rt6	64	9.4	-	+	+	Spirochetes.
Rt37	63	4.95	-	+	-	-
Rt38	72.9	3.2	+	+	-	-
Rt39	61.8	3.96	+	+	+	-
Rt58	103	8.12	-	+	+	-
Rt59	103	7.6	-	-	+	-
Rt60	97.7	7.16	-	+	+	-
Rt66	78	6.12	+	+	+	-
Rt67	80	6.6	+	+	+	-
Rt68	95	7.52	-	-	+	-
(B) Whakarewarewa:-						
Rt11	76	7.1	-	+	-	-
Rt12	66	6.9	-	+	+	Rods with spheroids.
Rt13	98	7.4	-	-	+	-
Rt14	80	7.0	-	+	+	-
Rt15	96.4	8.7	-	+	+	-
Rt16	67	5.5	-	-	-	-
Rt17	96	5.2	-	+	-	-
Rt25	ND	2.1	+	+	-	-
Rt26	85	3.0	+	+	-	-
Rt29	97	5.0	+	-	+	-
Rt30	97	3.69	+	-	-	-
Rt34	75.7	6.53	+	+	+	-
Rt35	80	6.56	+	+	+	Cocci in chains.
Rt36	62	4.0	-	+	-	Thin rods & thick rods.
Rt42	97	7.3	+	+	-	-
Rt44	62	7.81	+	+	+	Rotund bodies. Rods with spheroids.

Microscopic Examination Of Geothermal samples:

Pool No.	Temp.(°C)	pH	Morphotypes Observed			
			Cocci	Rods	Filaments	Others
Rt46	90	2.76	-	+	+	-
Rt51	82	7.34	+	-	+	-
Rt52	68	6.16	-	+	+	-
Rt54	72.5	3.15	+	-	-	-
Rt56	90	7.87	+	+	-	-
Rt57	97	7.2	-	+	+	-
Rt61	94.5	8.1	+	+	+	-
Rt70	98.2	3.38	+	-	-	-
Rt71	98.5	3.32	+	-	-	-
Rt72	97	6.64	-	-	+	-
Rt78	ND	ND	-	-	+	-
Rt82	91	6.9	-	-	+	-
Rt84	ND	ND	-	+	+	-
Rt86	92	6.69	-	+	+	-
Rt92	ND	ND	-	+	-	-
Rt93	96	6.81	-	+	+	-
Rt94	37	2.6	-	-	-	Thiovulum & Thiospira.
Rt95	37	8.0	-	+	-	Three types of motile rods.
Rt97	78	7.14	-	+	+	-
Rt98	97	8.52	-	-	+	-
Rt99	94.8	4.75	-	-	+	Spiral.
Rt100	81	7.33	-	-	+	-
Rt101	66	2.71	+	+	-	-
(C) Arikapakapa:-						
Rt33	67.8	3.2	-	+	-	-
Rt47	100.5	3.2	-	+	-	-
Rt48	98	3.1	-	+	-	-
Rt49	98	3.0	+	+	-	-
Rt50	ND	3.1	0	0	0	0
(D) Sulphur flats:-						
Rt31	80.4	2.8	+	-	+	-
Rt32	80.4	2.8	+	-	-	-

Microscopic Examination Of Geothermal samples:

Pool No.	Temp.(°C)	pH	Morphotypes Observed			
			Cocci	Rods	Filaments	Others
(E) Government Gardens:-						
Rt8	70	9.1	-	+	+	Sporulating rods & rods with spherods
Rt9	84	8.4	-	+	-	-
Rt10	94	7.9	-	+	+	-
(F) Ohinemutu:-						
Rt7	91	7.3	+	+	+	-
WAIOTAPU THERMAL AREA						
(A) Waiotapu Tourist Reserve:-						
WP1	89.8	2.87	+	-	-	-
WP2	89.9	2.9	+	+	-	-
WP3	81.0	2.76	+	-	-	-
WP4	86.2	2.9	-	-	-	-
WP5	98.5	ND	-	-	-	-
WP6	100.5	ND	-	-	-	-
WP7	95.0	ND	-	-	-	-
WP8	83.6	6.8	-	-	-	-
WP9	56.0	2.86	+	+	-	Cocci in pairs or in tetrads
WP10	92.0	3.76	+	+	-	Small & large rods
WP11	76.1	3.36	-	-	-	-
WP12	79.4	3.48	+	+	-	-
WP13	76.4	4.54	+	+	-	-
WP14	76.2	5.74	+	-	+	-
WP15	57.0	4.29	-	+	-	-
WP16	75.5	4.01	+	+	-	-
WP17	81.7	3.96	+	+	-	-
WP18	87.2	3.58	+	+	-	-
WP19	78.8	2.0	-	-	-	-
WP20	74.8	5.55	+	-	+	-
WP21	67.7	6.58	+	-	+	-
(B) Waiotapu Forest Reserve:-						
WP22	93.0	1.41	-	-	-	-
WP23	96.0	6.98	+	-	+	-
WP24	78.0	3.8	+	+	-	-
WP25	84.0	4.4	-	+	-	-
WP26	82.0	2.88	+	-	-	-
WP27	98.0	ND	+	+	-	-

Microscopic Examination Of Geothermal samples:

Pool No.	Temp.(°C)	pH	Morphotypes Observed			
			Cocci	Rods	Filaments	Others
WP28	88.0	2.05	+	+	-	-
WP29	76.8	4.0	+	+	-	Rods with subterminal spores.
WP30	73.6	ND	-	-	-	
WP31	69.8	5.5	+	+	+	-
TIKITERI THERMAL AREA						
Tk1	45.0	2.12	-	+	-	Single or paired rods
Tk2	43.7	2.3	+	+	-	
Tk3	43.6	2.3	+	+	-	-
Tk4a	90.1	5.8	+	+	-	-
Tk4b	90.5	6.1	+	+	-	-
Tk5	61.0	5.6	+	+	-	-
Tk6	84.0	4.7	+	+	-	-
Tk7	54.8	5.4	-	+	-	-
Tk8	80.5	3.2	+	-	-	-
Tk9	86.2	3.12	+	-	-	-
Tk10	75.3	7.3	-	+	+	-
Tk11	45.3	4.49	+	+	+	-
Tk12	78.0	6.94	+	+	-	-
Tk13	47.6	4.46	+	+	-	-
ROKAWA THERMAL AREA						
Rk1	91.0	5.5	-	-	+	-
Rk2	89.0	3.5	+	+	+	-
Rk3	77.0	2.5	+	-	-	-
Rk4	93.0	2.5	-	+	-	-
KETATAHI THERMAL AREA						
Kt4	80.0	6.0	-	-	+	-
Kt5	80.0	3.0	+	+	-	-
Kt6	87.0	5.5	-	-	-	-
Kt7	ND	ND	+	+	+	-
Kt8	ND	ND	+	+	+	-
Kt9	ND	ND	+	-	-	-
Kt10	ND	ND	-	+	+	-
ORAKEI KORAKO THERMAL AREA						
OK1	80.2	4.8	+	+	+	-
OK2	57.0	7.6	-	+	+	Curved cells
OK3	81.0	8.6	+	+	-	

Microscopic Examination Of Geothermal samples:

Pool No.	Temp.(°C)	pH	Morphotypes Observed			
			Cocci	Rods	Filaments	Others
OK4	60.0	6.9	+	+	+	-
OK6	102.5	8.19	+	+	-	Short, fat rods
OK7	98.8	6.57	-	-	+	-
OK8	83.5	8.5	-	-	+	-
OK9	83.0	6.8	-	-	+	-
OK10	74.0	7.9	+	+	-	-
OK11	96.0	7.4	-	+	-	-
OK12	80.0	7.4	0	0	0	0
OK13	98.0	7.3	0	0	0	0
OK14	97.0	3.87	0	0	0	0
OK15	97.0	7.03	-	+	-	-
OK16	78.0	4.7	+	+	-	-
OK17	90.0	7.24	+	-	+	-
OK18	77.0	3.88	+	+	-	-
OK19	80.0	7.57	0	0	0	0
<u>WAIRAKEI AND HIPUAU THERMAL AREAS</u>						
(A) Wairakei:						
Wk1	97.0	3.15	+	-	-	-
Wk2	98.0	4.0	0	0	0	0
(B) Hipau:						
Hp1	>100.0	6.67	-	-	-	-
<u>WAIMANGU THERMAL AREA</u>						
Wa11	78.0	3.5	+	-	-	-
Wa12	89.0	3.5	+	-	-	-
Wa13	48.0	5.5	-	+	-	-
Wa14	56.0	ND	+	+	-	-
Wa18	85.0	3.03	+	-	-	-
Wa19	88.0	3.1	+	-	-	-
Wa110	91.0	7.4	-	+	+	-
Wa111	96.0	8.5	-	+	+	-
Wa113	92.0	6.5	+	+	+	-
Wa115	84.0	6.0	+	+	-	-
Wa117	80.0	8.5	-	+	-	-
Wa118	81.6	7.34	-	+	-	-
Wa119	79.0	7.7	+	+	+	-
Wa120	96.0	6.85	+	+	+	-

Microscopic Examination Of Geothermal samples:

Pool No.	Temp.(°C)	pH	Morphotypes Observed			
			Cocci	Rods	Filaments	Others
Wai21	67.0	6.5	-	+	+	-
Wai22	92.0	7.0	+	+	-	-
Wai23	97.0	7.7	+	+	+	+
<u>WHITE ISLAND THERMAL AREA</u>						
WIs1	52.8	2.5	+	+	-	-
WIs2	95.2	3.1	+	+	-	-
WIs3	83.7	2.9	+	+	-	-
WIs4	90-96	4.96	+	+	-	-
WIs5	64.5	3.5	-	+	-	-
WIs6	75.0	2.8	+	+	-	-
WIs7	75.0	3.24	+	+	-	-
WIs8	+105	ND	-	-	-	-
WIs9	89.7	2.6	+	+	-	-
WIs10	64.0	2.4	+	+	-	Vibriods.
WIs11	56.2	3.8	+	+	-	-
WIs12	91.4	2.87	+	+	-	-
WIs13	65.0	3.0	+	+	-	Vibriods.
<u>TAUPO THERMAL AREA</u>						
TP1	95.0	ND	-	+	-	-
TP2	95.0	ND	+	+	-	-
TP3	95.0	ND	-	+	-	-
TP5	89.0	6.47	-	+	-	-
TP6	78.0	7.28	-	-	+	-
TP8	48.0	ND	+	+	+	-
TP9	92.0	7.14	+	+	-	-
TP10	72.0	6.84	-	+	+	Sporulating rods.
TP11	96.0	6.6	-	+	+	-
<u>TOKANUU THERMAL AREA</u>						
Tok1	96.0	7.86	-	+	-	Small rods in twos.
Tok2	81.0	7.13	+	+	-	-
Tok3	76.0	6.93	-	+	+	-
Tok4	89.0	5.63	-	+	+	-
Tok5	52.0	5.93	-	+	+	-
Tok8	75-80	5.59	+	+	+	-
Tok9	86.0	7.0	-	+	-	-
Tok10	75.0	6.5	+	+	+	Sporulating rods.
Tok12	97.0	7.0	-	-	+	-

APPENDIX 2

EM SUPPORT FILM AND STAINING SOLUTIONS

1. Formvar Solution

Formvar - 0.5g (0.6, 0.7, 0.8g)
(Choloroform or
1,2, Dichloroethane) - 100 ml

2. Cellulose Acetate Solution

Cellulose acetate - 60 ml
Amyl acetate - 40 ml

3. Butvar Solution

Butvar B-98 - 0.25gm
Choloroform - 100 ml

4. 1% Phosphotungstic Acid Staining Solution

Phosphotungstic Acid - 1gm
Distilled water - 100ml
pH 7.0

5. 1% Uranyl Acetate Staining Solution

Uranyl Acetate - 1g
Distilled water - 100ml
adjust pH to 4.8.

APPENDIX 3

PREPARATION OF ANAEROBIC MEDIA

The anaerobic techniques first described by Hungate in 1950 have been modified in many ways in order to adapt them to the requirements of certain kinds of studies and to the particular needs of different groups of workers. However, a majority of the modifications have been directed towards modification of equipment (Behbehani et al, 1982; Miller and Wolin, 1974; Daniels and Zeikus, 1975; and Latham and Wolin, 1978) or to the use of reducing agents such as sodium thioglycollate, (Sijpesteijn, 1951) ascorbic acid, (Gill and King, 1958); sodium sulphide or cysteine hydrochloride either alone or in combination, (Bryant and Robinson, 1961); Titanium (III) citrate, (Zehnder and Wurhmann, 1976; Jones and Pickard, 1980); sodium dithionite, (Smith and Hungate, 1958); glutathione, (Bryant, 1958) dithiothreitol, (Hazelwood and Nugent, 1978) and Ferrous sulphide, (Brock and O'Dea, 1977), and as such, very little effort has been directed towards simplifying the method for dispensing anaerobic media without the use of expensive equipment. The original method of dispensing anaerobic media, as described by Bryant, (1972) has three major disadvantages: (1) Since the method uses mouth pipetting, reducing agents such as sulphide, or cysteine-HCl, regarded as toxic, should be used with extreme caution; (2) The technique for dispensing of media requires certain amount of practice to master especially for the newcomers in this field. (3) The method is labourious and time consuming.

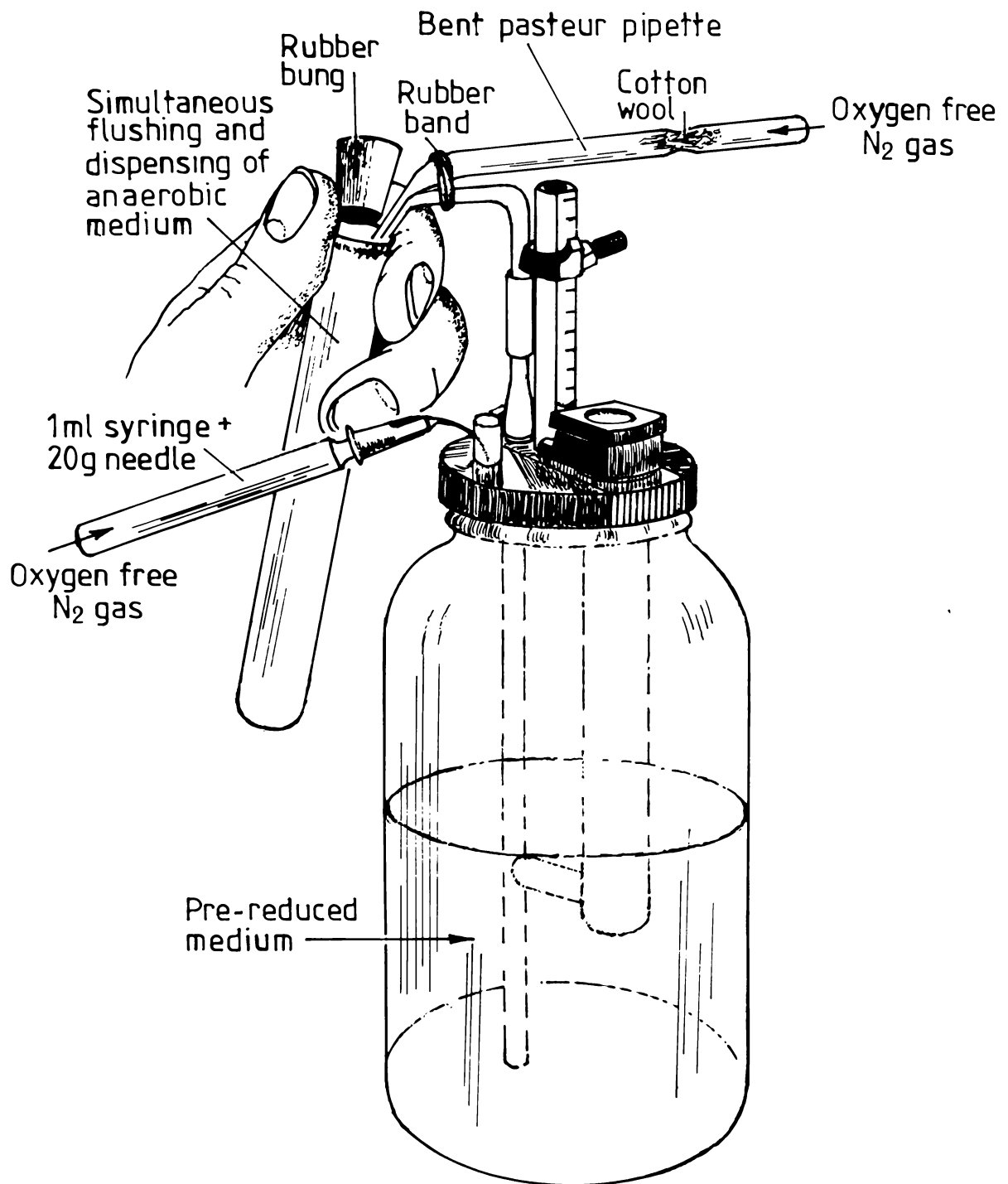
The modification of Oxford pipetteor, model S-A, as shown in the figure was used for simultaneous finishing and dispensing of media.

The method of Latham and Wolin, (1978) with a minor modification was used to deoxygenate the media. For this, the media was placed in the Oxford dispensing jar (model S-A) and autoclaved for 2-5 minutes, after which the medium was allowed to cool under a steady stream of oxygen free nitrogen. Depending on the nature of experiment, the media was either reduced with appropriate reducing agent(s) and dispensed, or was dispensed just as deoxygenated media, the process of dispensing done under a flow of oxygen free nitrogen.

This method does not require practice, and is extremely quick, (as many as 200 tubes can be dispensed in 30 minutes). Simultaneous flushing and dispensing of media can be achieved and since it does not involve mouth pipetting, the hazards of using toxic reducing agents are reduced. The volume of media required can also be changed quickly if so desired. Media required for growth of extremely oxygen sensitive organisms such as methanogens (Methanobacterium sp) or sulphur utilizing archaeobacteria (Desulfurococcus sp, Thermoproteus sp) have also been dispensed using this technique without any detrimental effect on the growth of these organisms.

Usually, for enrichment studies of glycolytic anaerobes, medium walled pyrex tubes either 152 cm x 12 cm or 126 cm x 12 cm containing 10 ml of pre-reduced media and fitted with '0' number stopper were used. For growth of archaeobacteria (Methanogens and sulphur reducers), Hungate tubes (of similar dimensions as above) filled with 5.0 ml or 10 ml of pre-reduced media, were used.

Figure 01 Method for simultaneously flushing and dispensing of anaerobic media.



APPENDIX 4MEDIA FOR GROWTH OF ARCHAEABACTERIA

CM Medium

1% Resazurin	- 1 ml
K ₂ HPO ₄	- 0.22 g
KH ₂ PO ₄	- 0.22 g
(2[N-Morphololino]ethane sulfonic acid	- 9.7 g
(NH ₄) ₂ SO ₄	- 0.22 g
1% FeSO ₄ , 7H ₂ O	- 120 ul
Yeast extract	- 0.5 g
Bacto tryptone	- 0.5 g
Sodium thioglycollate	- 0.3 g
NaCl	- 1 g
Trace element solution (Nitsch)	- 1 ml
KNO ₃	- 0.1 g
Cystene-HCl	- 0.3 g
Sodium sulphide, 9H ₂ O	- 0.1 g
L-glutamic acid	- 0.1 g
Distilled water	- 1000 mls

pH 6.5 to 6.8

Prepare and dispense medium using the anaerobic technique as described in Appendix 3

APPENDIX 5SCINTILLATION COCKTAIL

Toluene (Solvent Grade)	700 ml
Absolute Ethanol (Solvent Grade)	200 ml
2-Ethoxyethanol (Scintillation Grade)	100 ml
2,5-diphenyloxazole (Sigma)	6 gm

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LIST OF ERRATA AND ADDENDA

Chapter 3

Page 3-13, Figure 3.1 should read Figure 3.3 and should be on page 3-16.

Page 3-16, Figure 3.3 should read Figure 3.1 and should be on page 3-13.

Chapter 7

Page 7-15, Figure 7.2..Hatched areas indicate number of pools positive for extremely thermophilic glycolytic anaerobes and non-hatched areas indicate number of pools which were negative

PART 1. This part contains more information which has not been included in the thesis but has been added separately to form two publications (and are available in the back pocket cover of the thesis].

Chapter 8

Page 8-4, line 1 should readconfusion in the system of classification of.....

Page 8-4, line 22 should read under unfavourable conditions.....

Page 8-7, line 28 should readthermal areas was similar to that observed elsewhere and

Page 8-10, Figure 8.1, closed circles indicate Thermus- containing samples and open circles indicate Thermus- free samples. The hatched area indicates the higher frequency of obtaining Thermus from pools with pH of 5 to 10 and with temperatures of approximately 50 to 93 degrees centigrade.

References

Page 10- 4, include reference, "Brock, T. D. (1978). In, "Thermophilic microorganisms and life at high temperatures", Springer-Verlag, USA.

Page 10-15, include reference "Jasperse, P. (1984). Isolation and characterization of extremely thermophilic archaebacteria from New Zealand hot pools. M.Phil. thesis, University of Waikato, Hamilton, New Zealand.

Page 10-15, include reference Johnson, E. A., Madia, A. and Demain, A. L. (1981). Chemically defined medium for growth of the anaerobic cellulolytic thermophile *Clostridium thermocellum*. Applied and Environmental Microbiology, 41: 1060-1062.

Page 10-21, include reference, Martin, A. M. (1984). D.Phil. thesis, in preparation, University of Waikato, Hamilton, New Zealand.

Page 10-25, include the following two references:

Patel, B.K.C., Morgan, H.W. and Daniel, R.M. (1985a). Fervidobacterium odosum gen. nov. sp. nov., a new chemooragnotrophic caldoactive, lycolytic anaerobic bacterium. Archives of Microbiology (in press). Paper available in the pocket of the back cover.

Patel, B.K.C., Morgan, H.W. and Daniel, R.M. (1985b). Thermophilic anaerobic spirochetes in New Zealand hot springs. Federation of the European Microbiology Societies Letters. (in press). Paper available in the pocket of the back cover.

MAPS

Figure 1 A general sketch map of Whakarewarewa thermal area showing details of sampling sites (After Lloyd and Crafar, 1967a; Lloyd, 1975).

Figure 2 A sketch map of Kuirau Park thermal, Rotorua area showing details of sampling sites. (After Lloyd and Crafar, 1967b).

Figure 3 A general sketch map of Waimangu thermal area showing details of location of sampling sites. (Drawn from NZ tourist Publicity map).

Figure 4 A sketch map of Tikiteri showing details of sampling sites. (Drawn from the Tikiteri tourist map).

Figure 5 A general sketch map of Waitapu thermal area showing details of sampling sites

Figure 6 A sketch map of Orakei Korako thermal area showing general features and details of location of pools

Figure 7 A sketch map of White Island thermal area showing sampling sites.

Figure 8 A sketch map of thermal areas of Vanua Levu, Fiji. Wainggele (A), Nakama (B) and Savusavu (C). (After Cox, 1981a, b).

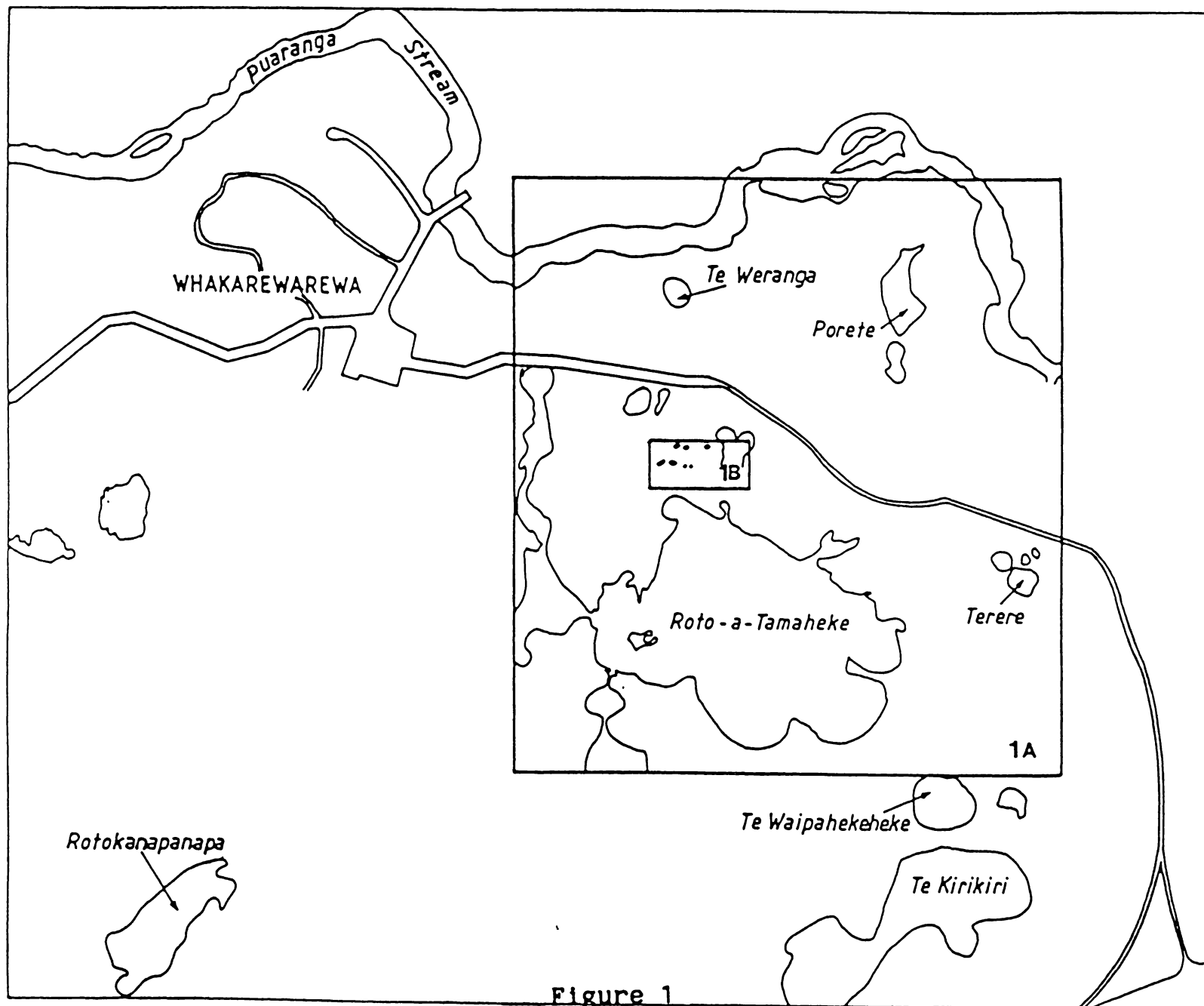
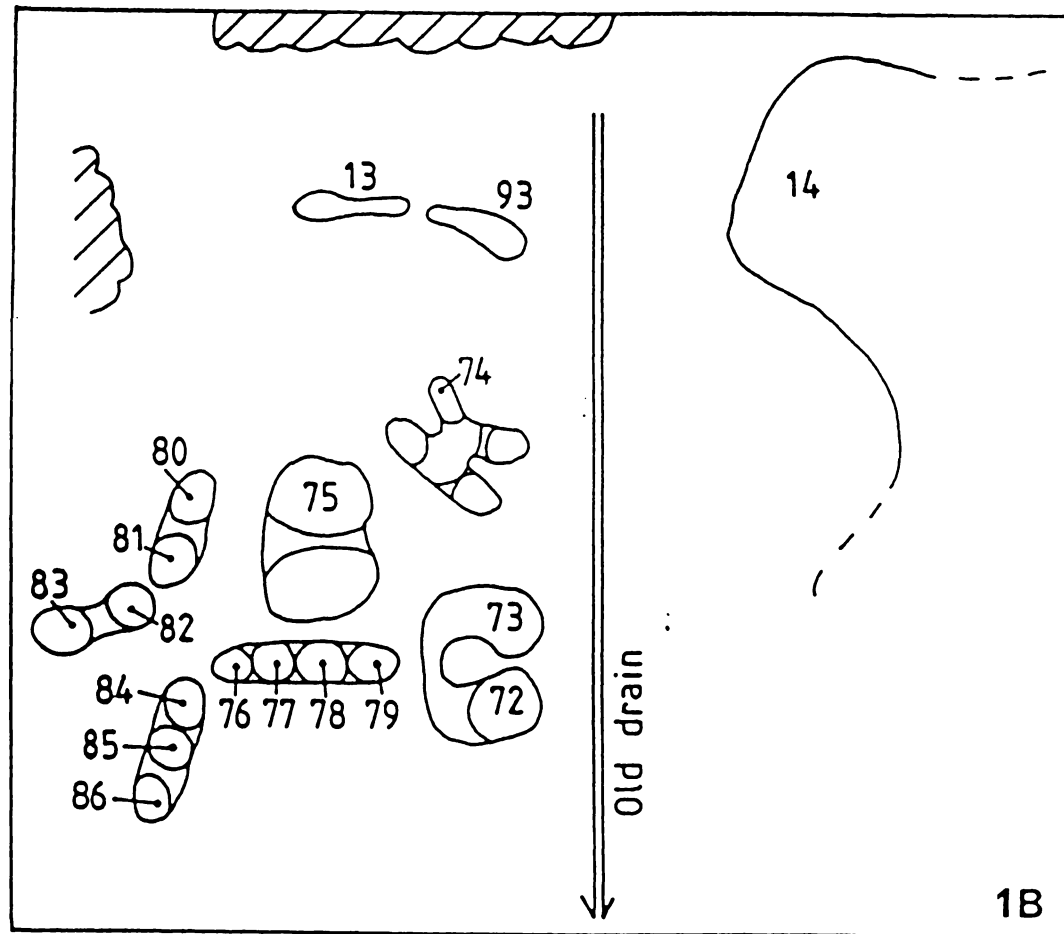
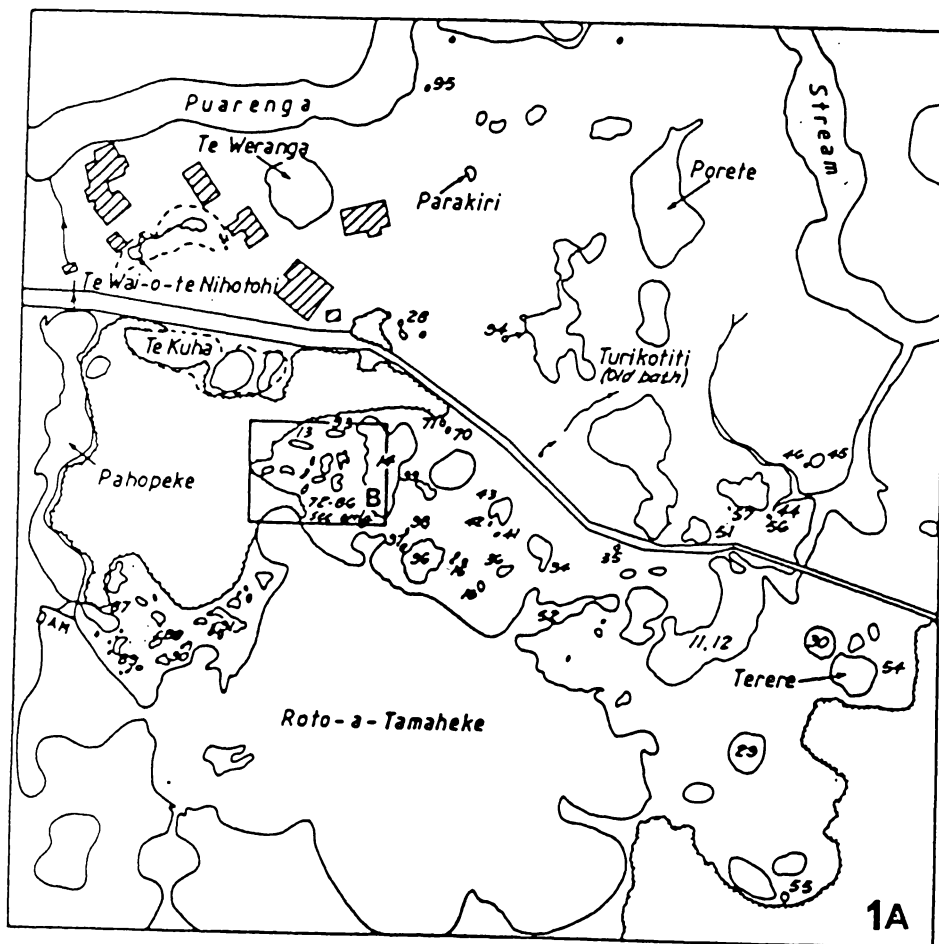


Figure 1



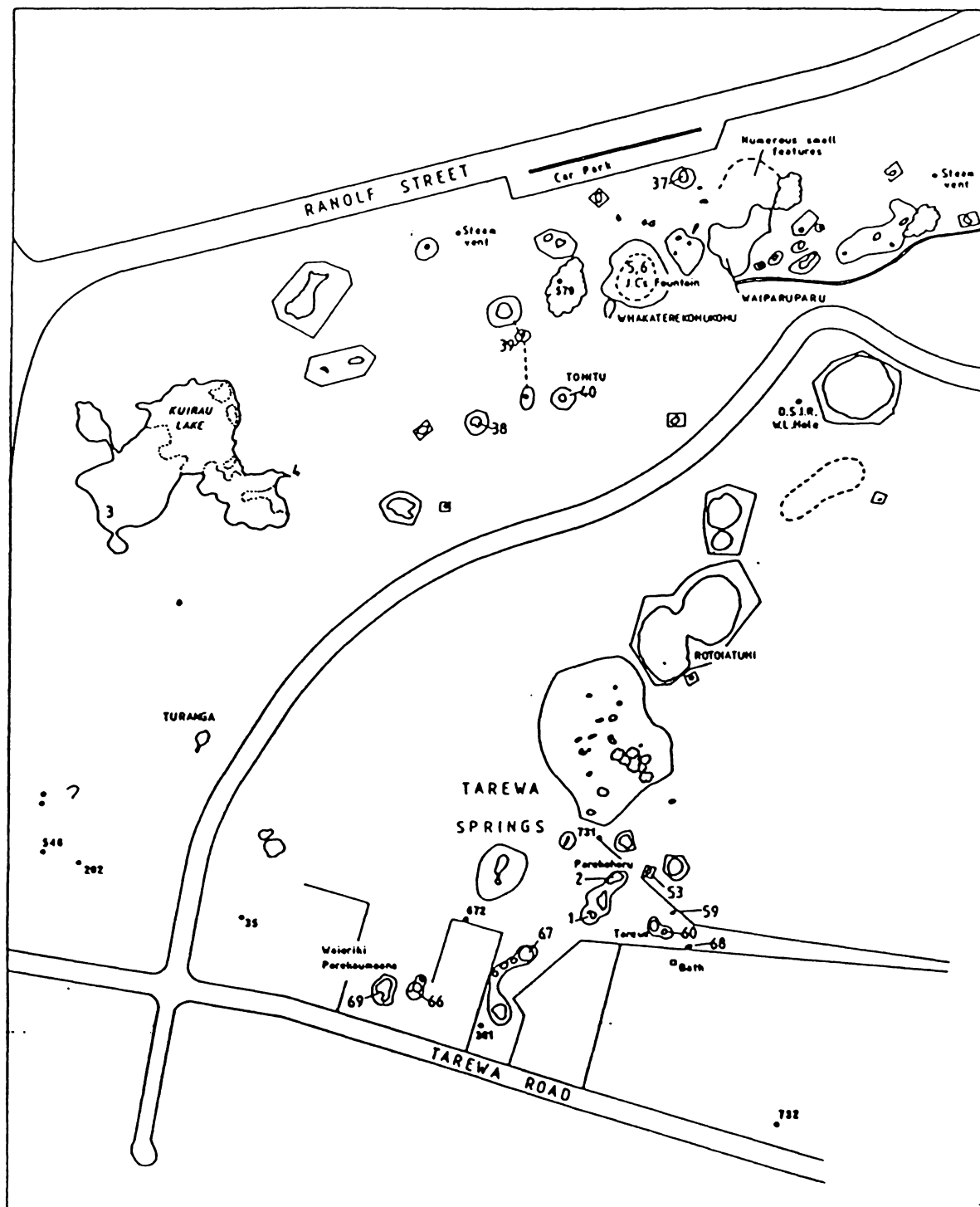


Figure 2

- ① Tearooms
- ② Southern Crater + Emerald Pool
- ③ Echo Crater
- ④ Waimangu Cauldron
- ⑤ Cathedral Rocks
- ⑥ Ngapuia - O - Te Papa
- ⑦ Waimangu Geyser site
- ⑧ Ruamokos Throat
- ⑨ Birds Nest Terrace
- ⑩ Iodine Pool
- ⑪ Warbrick Terrace
- ⑫ Lake Rotomahana

Waimangu Valley

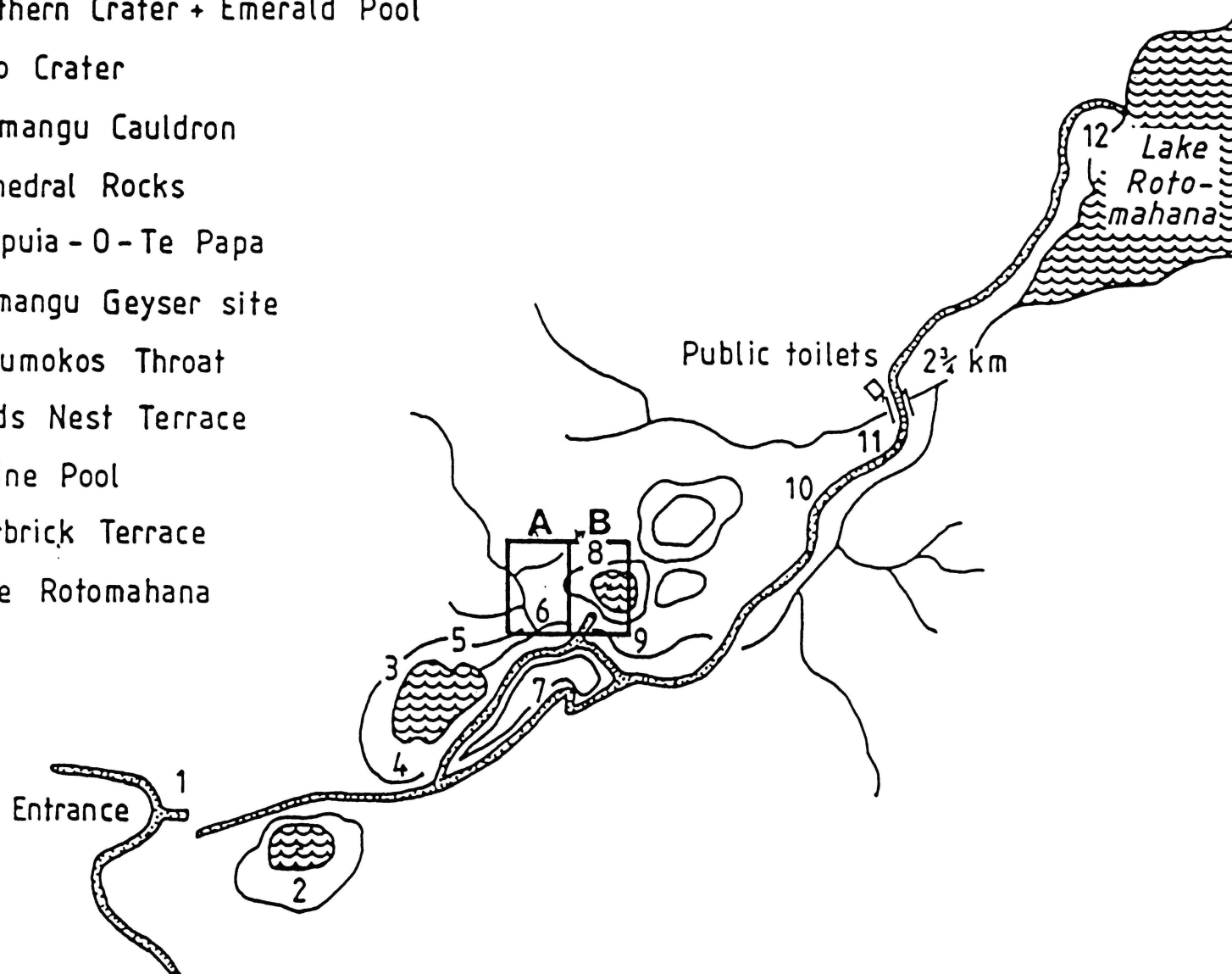
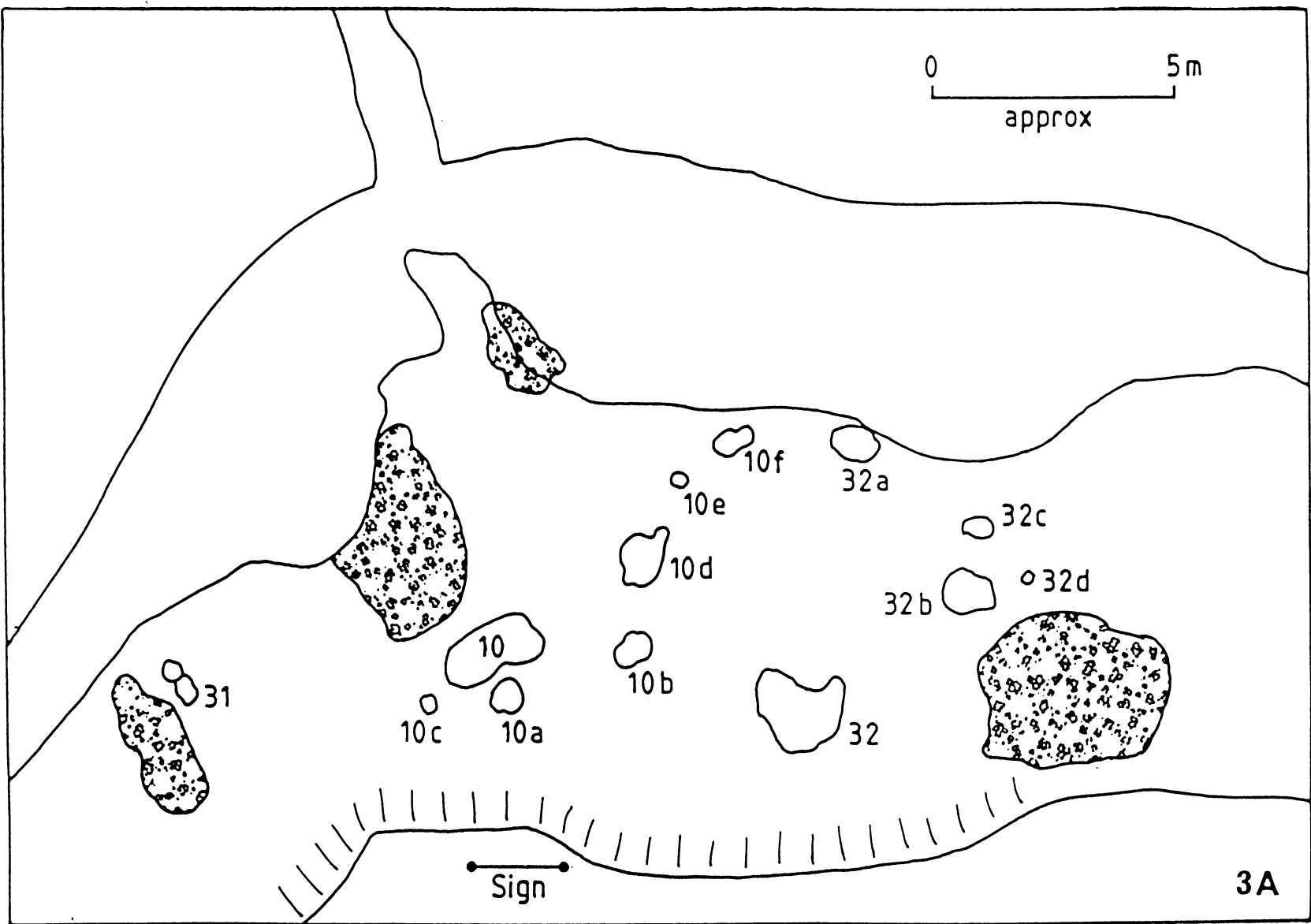
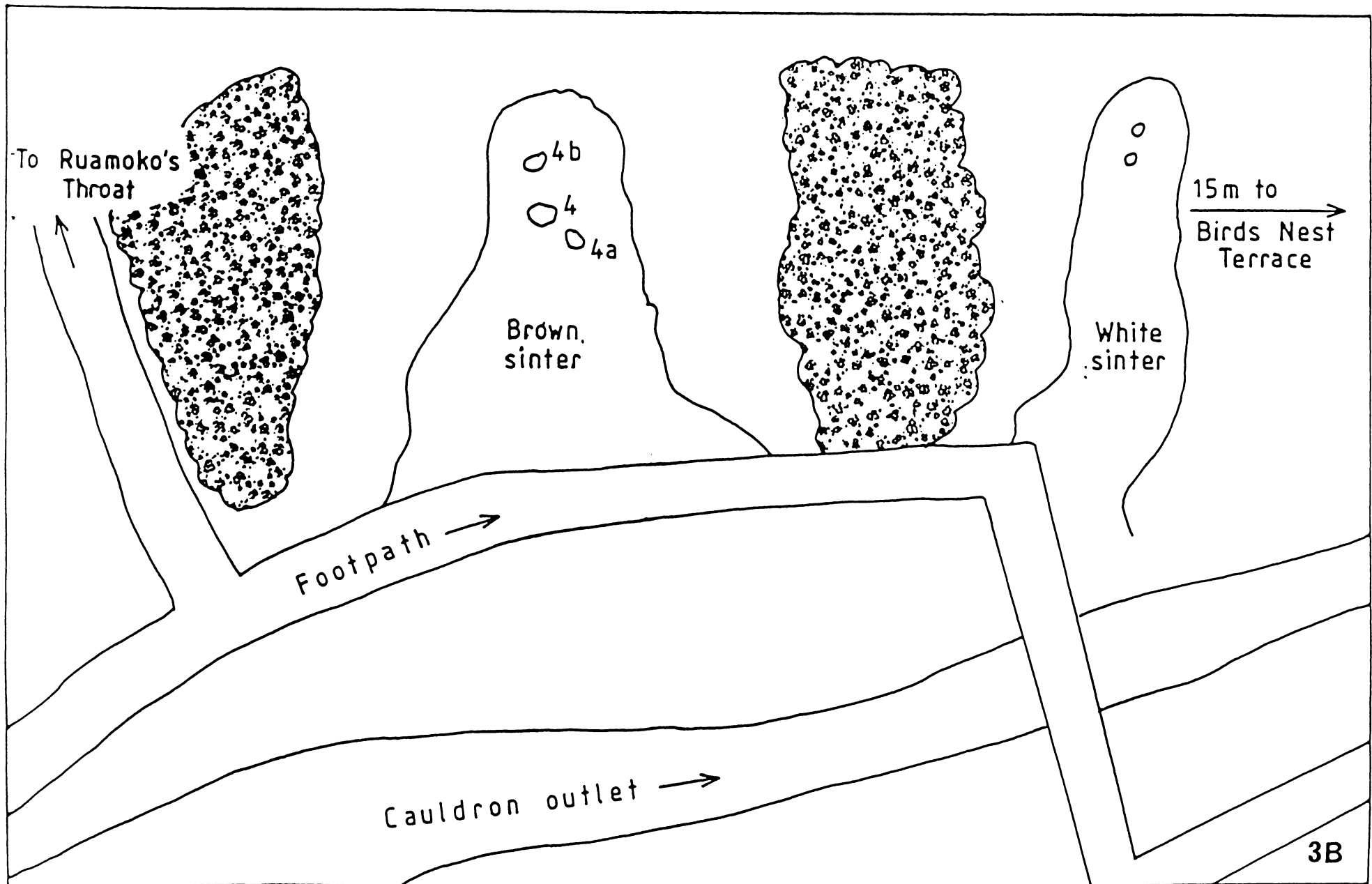


Figure 3





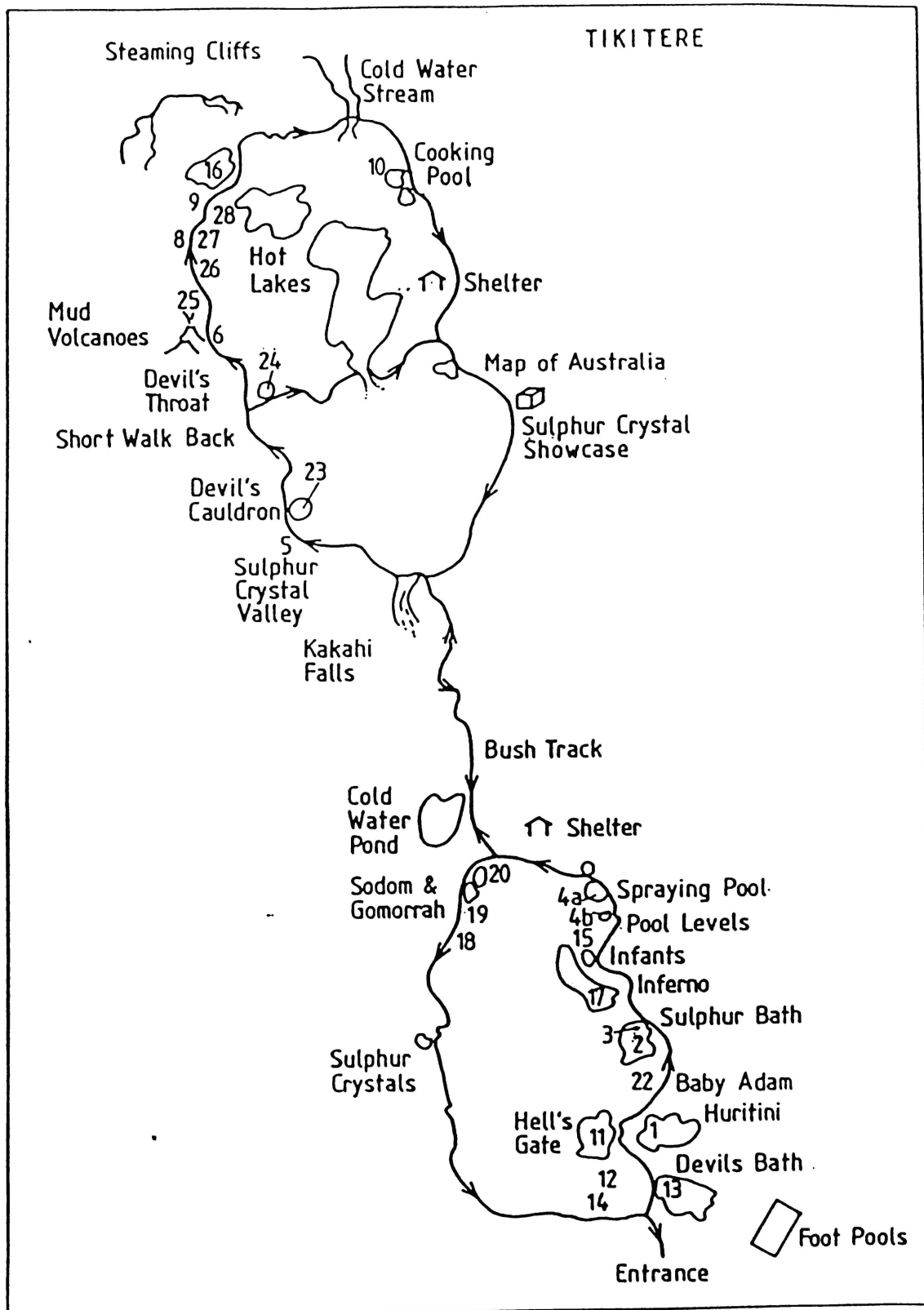


Figure 4

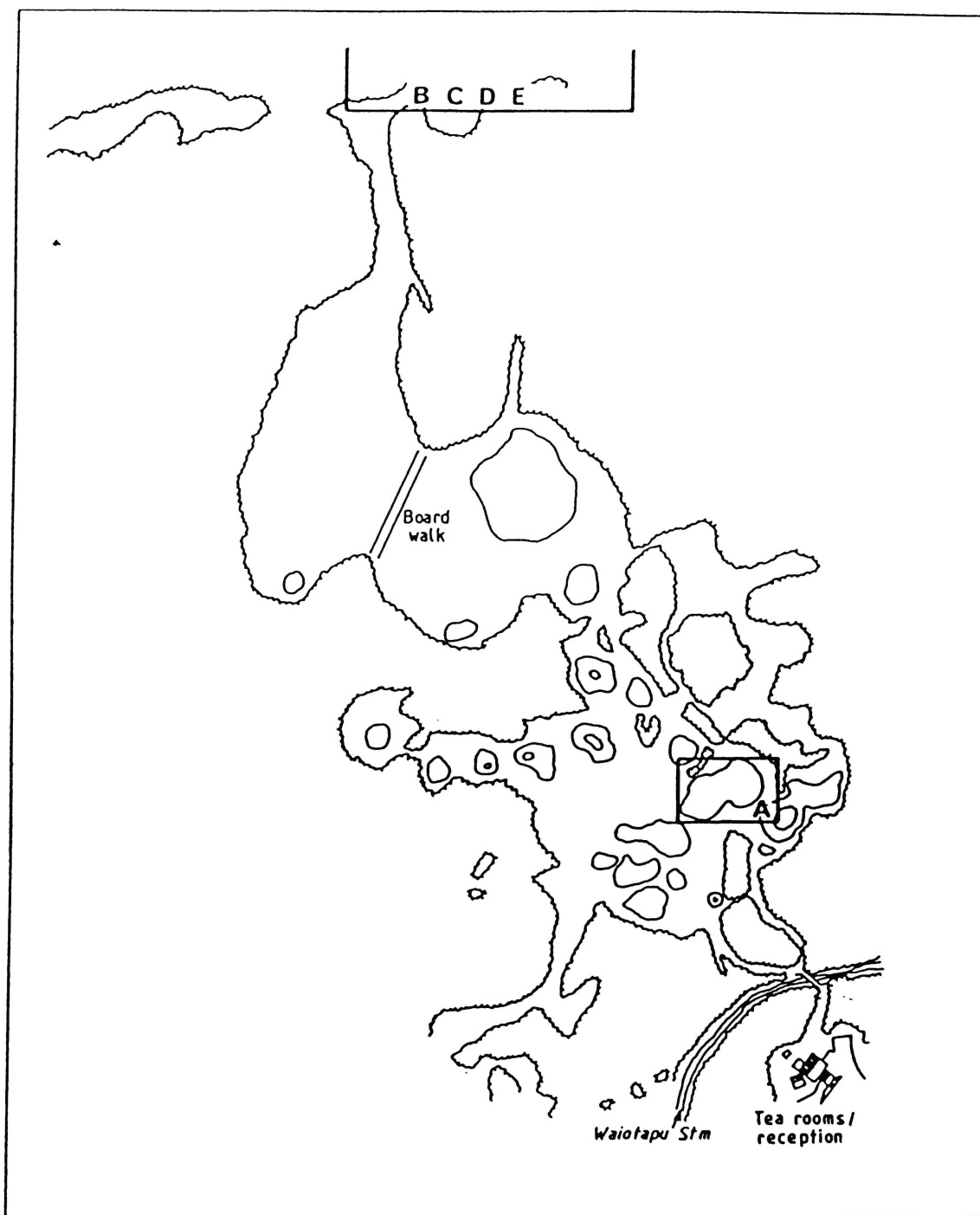
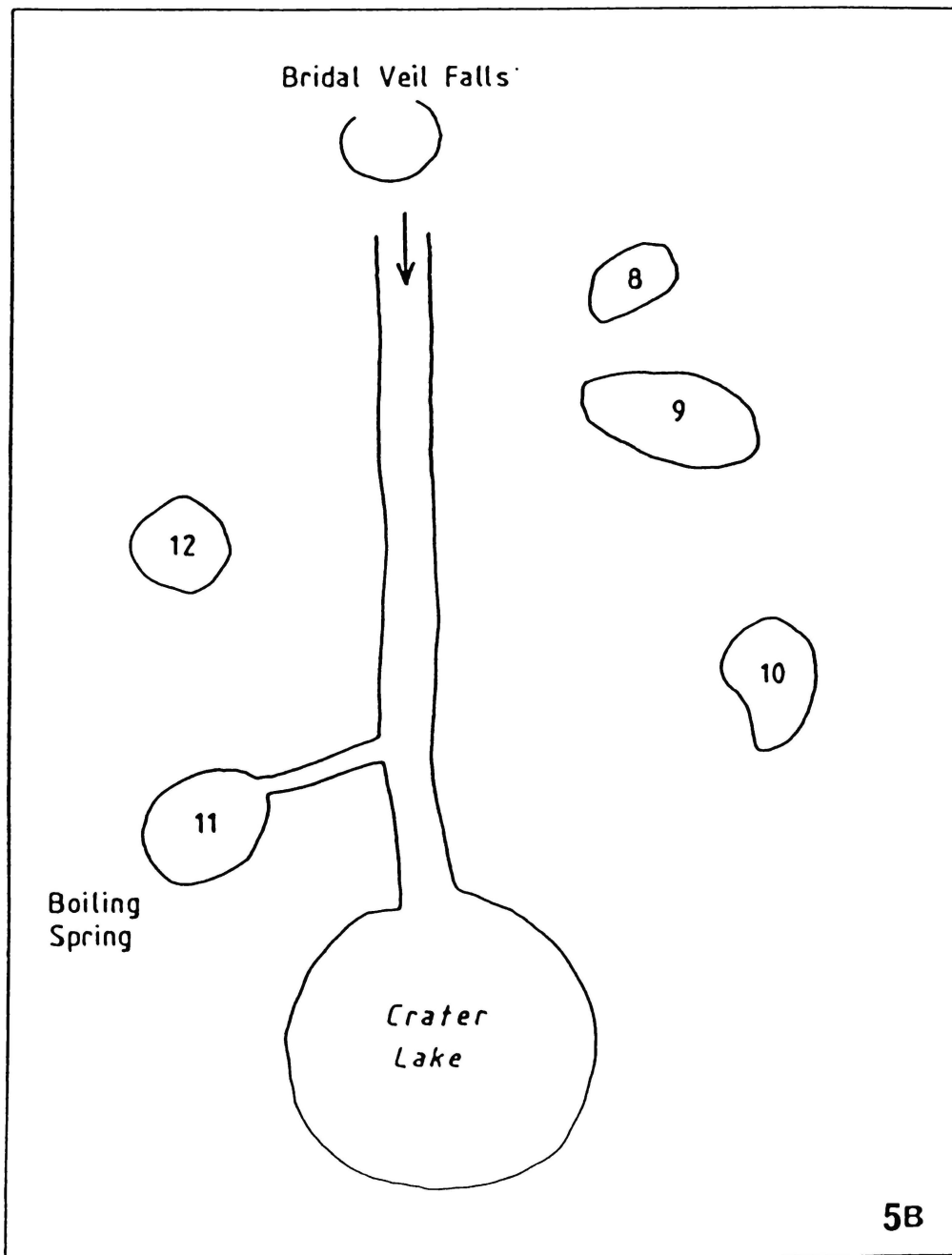
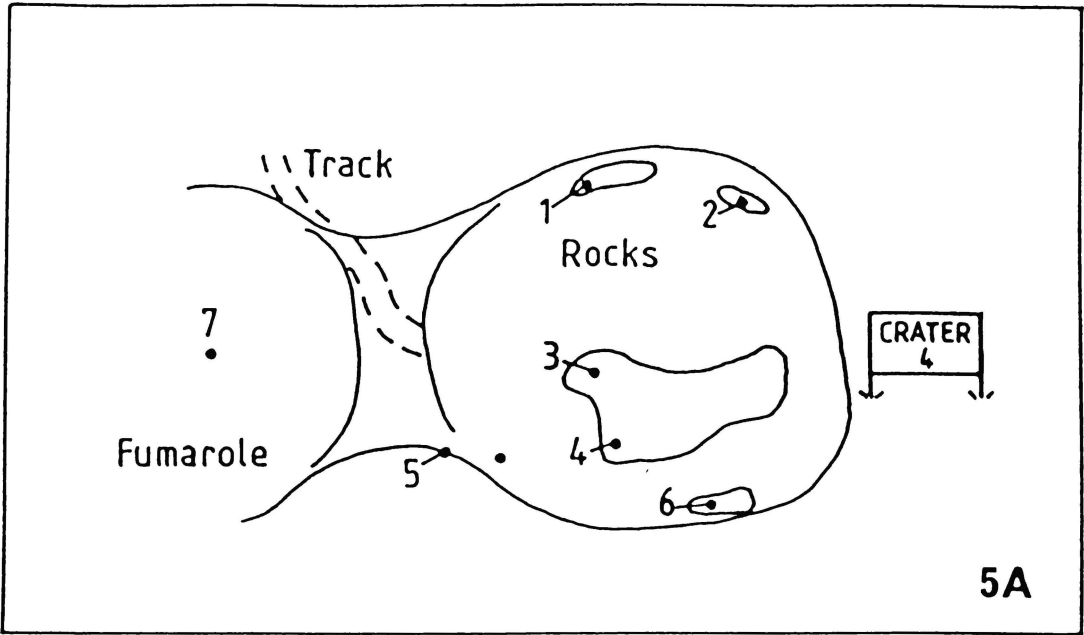
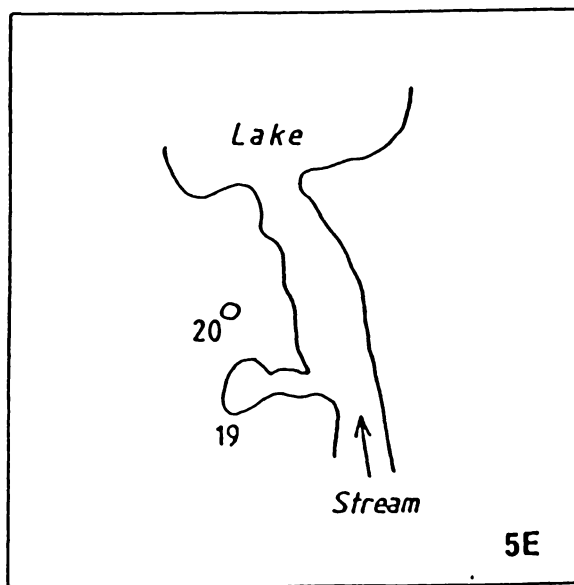
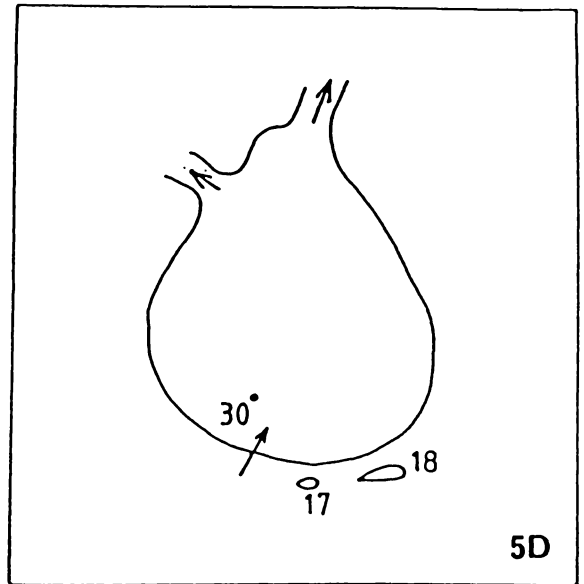
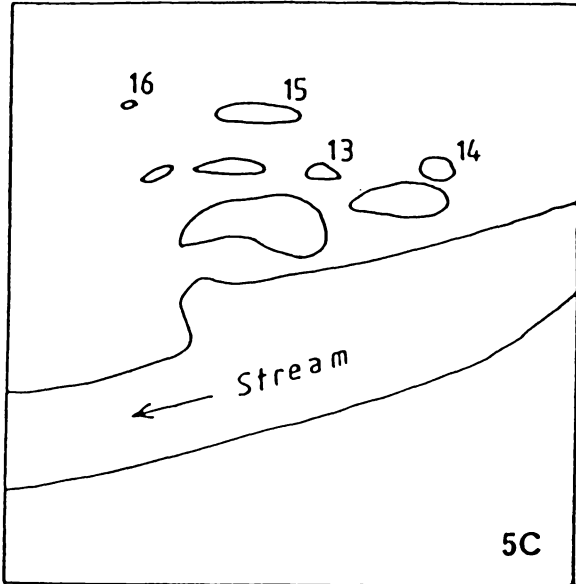


Figure 5





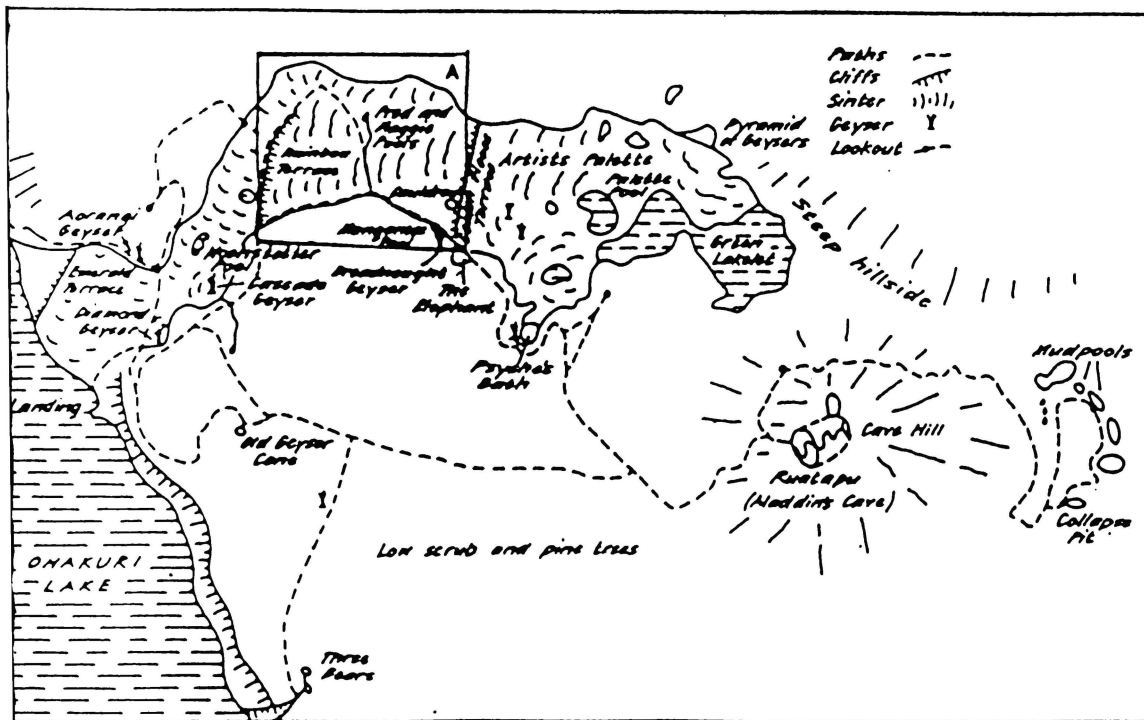
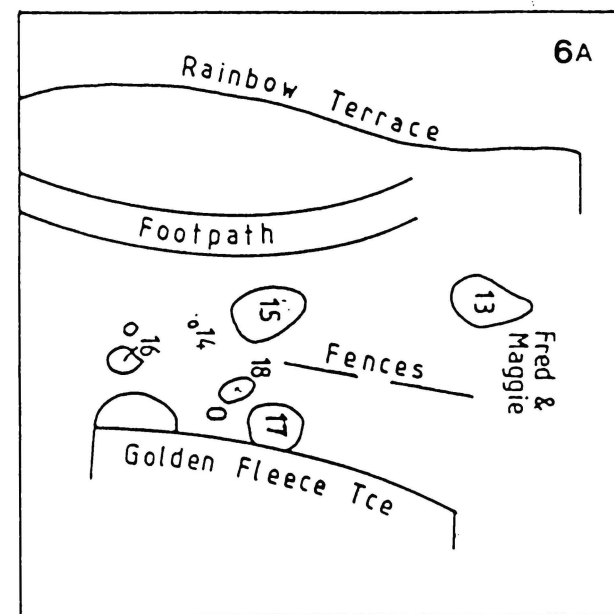


Figure 6



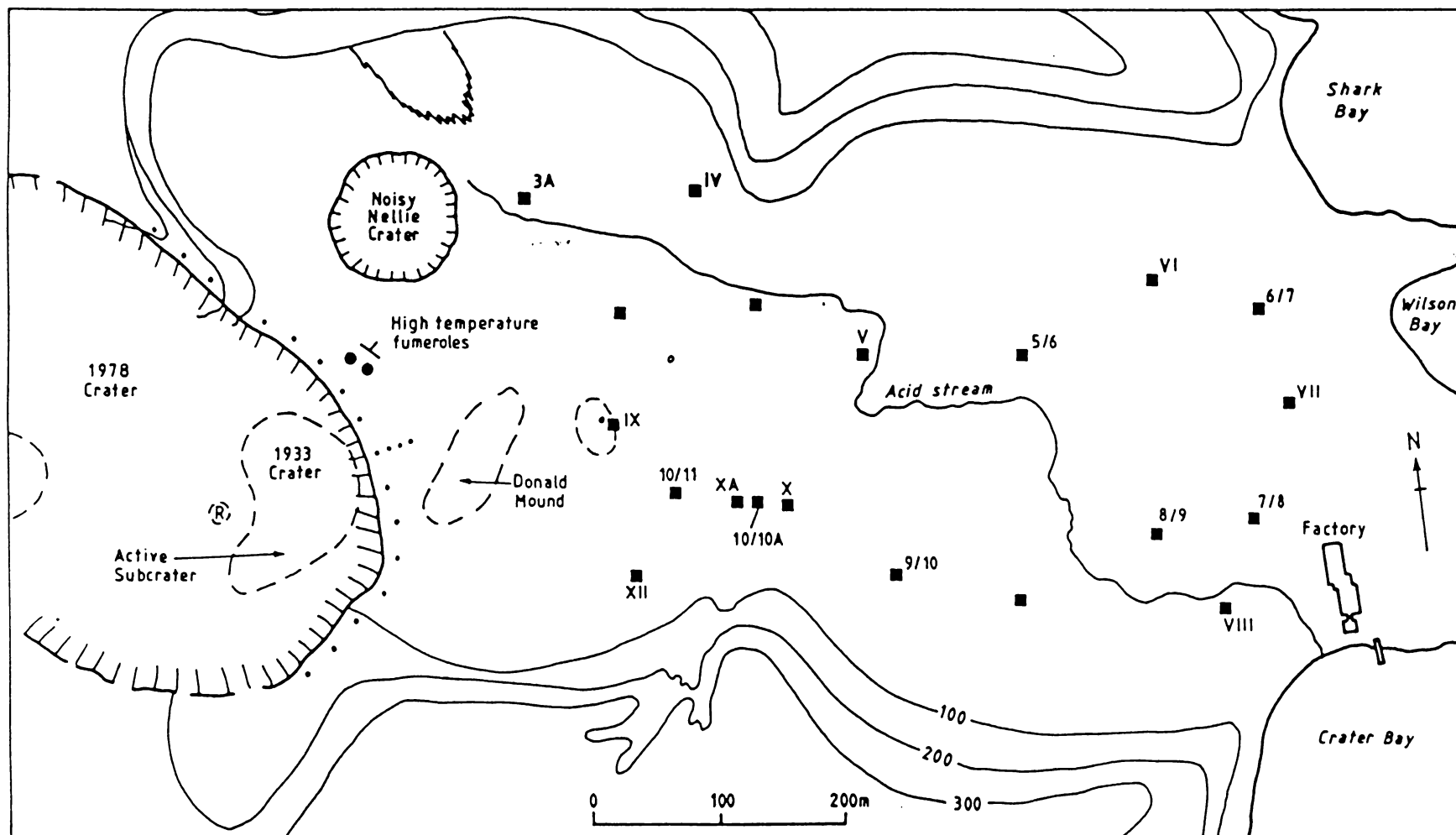


Figure 7

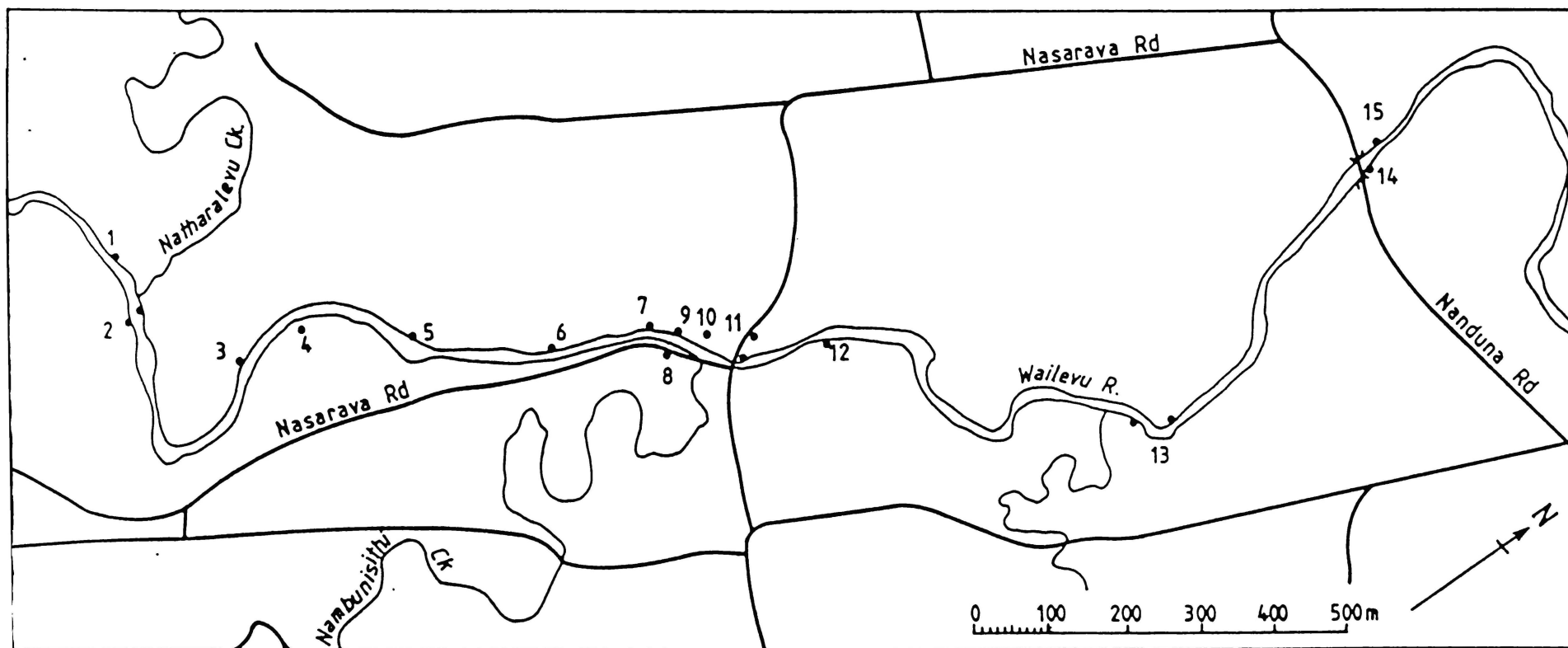
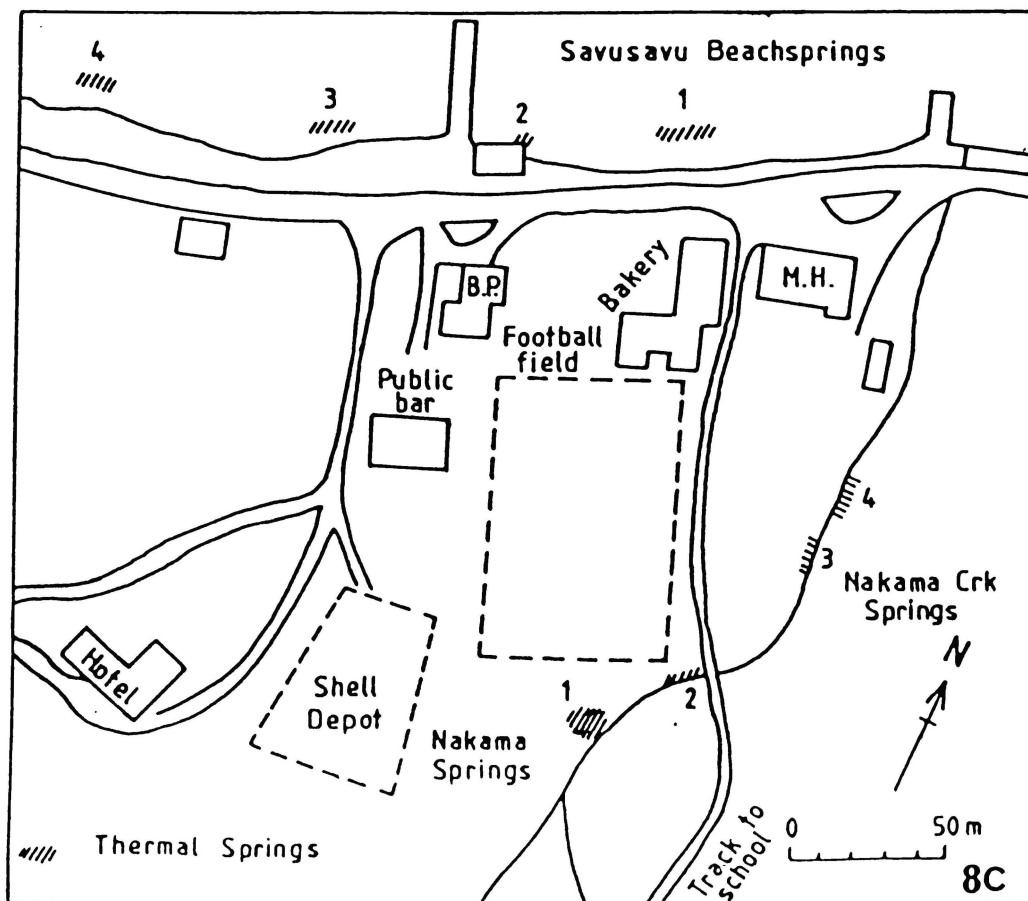
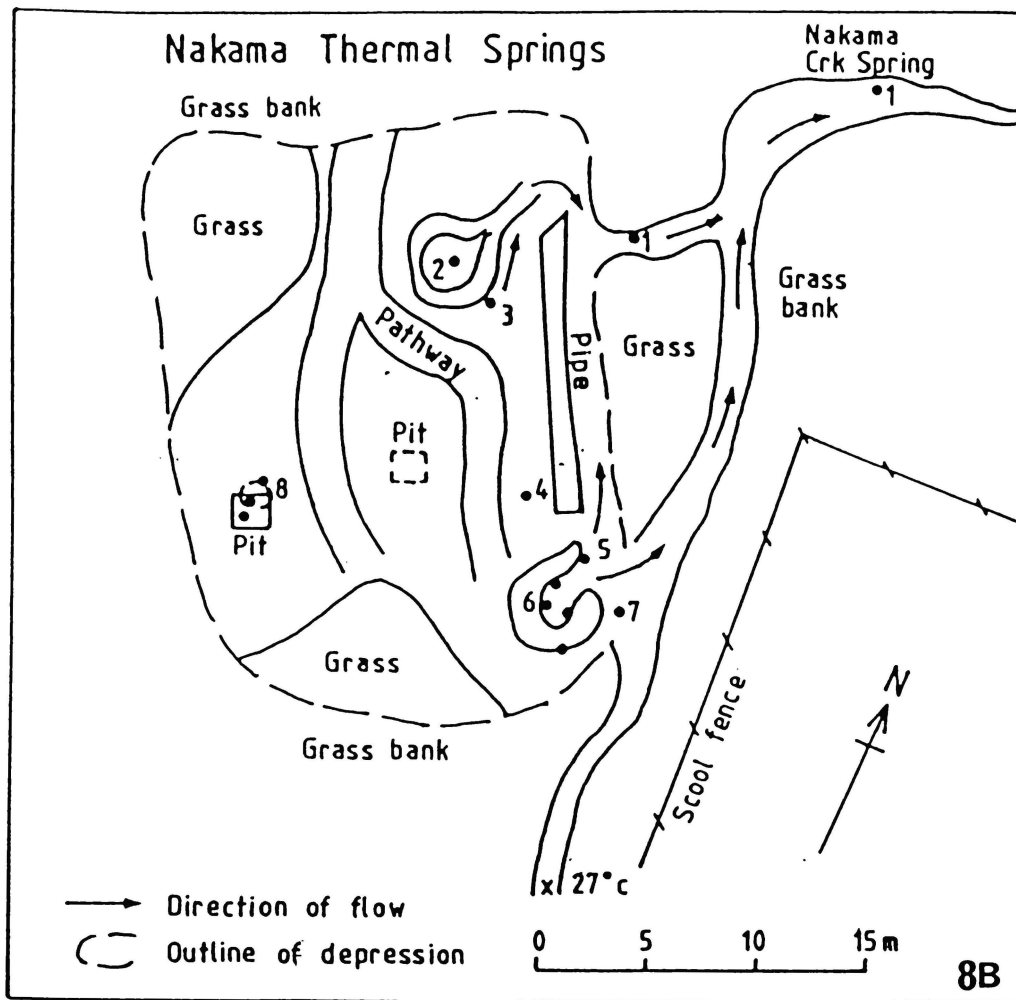


Figure 8A



PATEL ET AL., (1985a). ARCHIVES OF
MICROBIOLOGY (In the press). This paper
will be replaced by the original when it
comes to hand.

Fervidobacterium nodosum gen. nov. and spec. nov., a new
chemoorganotrophic, caldoactive, anaerobic bacterium.

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Department of Biological Sciences,
University of Waikato,
Hamilton, New Zealand

Abstract: A new species of extremely thermophilic, glycolytic anaerobic bacterium, *Fervidobacterium nodosum* isolated from a New Zealand hot spring, is described. *Fervidobacterium nodosum* strains were Gram negative, motile, non-sporulating obligately anaerobic rods that existed singly, in pairs or in chains. Electron micrographs of thin sections revealed a two layered cell wall structure. The outer layer of the cell wall produced "spheroids", which was a typical feature of this organism. The optimum temperature for growth was 65 to 70°C, the maximum 80°C and the minimum greater than 40°C. Growth occurred between a pH of 6.0 and 8.0 with the optimum being 7.0 to 7.5. The doubling time of *Fervidobacterium nodosum* at optimal temperature and pH, was 105 minutes. The DNA base composition was 33.7% guanine plus cytosine as determined by thermal denaturation. A wide range of carbohydrates including glucose, sucrose, starch and lactose could be utilized by the organism. Lactate, acetate, hydrogen, and carbon dioxide were the major end products of glucose fermentation with lesser amounts of ethanol being formed. Growth was inhibited by tetracycline, penicillin and chloramphenicol indicating that the organism was a eubacterium.

Keywords: *Fervidobacterium nodosum* - Genus, Species description - Obligate anaerobe - Lactic acid - Thermophile ecology and physiology.

During the past decade a number of thermophilic glycolytic anaerobic bacteria, capable of growth above 70°C, have been isolated. This includes *Clostridium thermohydrosulfuricum*, initially isolated from an Austrian sugar factory (Klaushofer and Parkkinen 1965) but subsequently isolated from mesophilic (Wiegel et al. 1979) and thermophilic environments including the thermal springs of Yellowstone National Park (Wiegel et al. 1979; Zeikus et al. 1980), *Thermoanaerobium brockii* (Zeikus et al. 1979), *Thermoanaerobacter ethanolicus* (Wiegel and Ljungdahl 1981), *Thermobacteroides acetoethylicus* (Ben-Bassat and Zeikus 1981) and *Clostridium thermosulfurogenes* (Schink and Zeikus 1983). All of these anaerobes have been isolated from algal-bacterial mat ecosystems, and to date little attention has been paid to the presence of glycolytic anaerobes in thermal springs with little or no biomass production or springs of extreme pH and temperature.

Keeping these factors in mind, we initiated studies to identify the ecological niches of caldoactive glycolytic anaerobes in New Zealand hot springs. A number of species, some of which were similar to strains described in the literature, and some novel, were isolated. We report here the isolation and characterization of a new species of caldoactive glycolytic anaerobic bacterium, *Fervidobacterium nodosum*.

Materials and Methods

Chemicals: All chemicals were reagent grade. Antibiotics were purchased from Sigma Chemical Company and oxygen free nitrogen (OFN) was obtained from New Zealand Industrial Gases Ltd.

Sampling Procedure: Samples from thermal regions of New Zealand (Rotorua, Waiotapu, Tikiteri, Waimangu, Orakei Korako, Wairakei, Taupo, Tokaanu and White Island) and Fiji were collected in sterile glass containers. The collection vessels were completely filled, usually with sediment and pool water, sealed with a gas tight septum and capped tightly, after which they were transported to the laboratory at ambient temperature. The samples were used immediately or stored at room temperature for future use.

Media and cultivation condition: TYEG medium as described by Zeikus et al. (1979) was used. The Hungate technique (Hungate 1950) as modified by Latham and Wolin (1978) with minor changes was used to prepare anaerobic media. Basal media contained per litre of distilled water NH_4Cl , 0.9g; MgCl_2 , 0.2g; KH_2PO_4 , 0.75g; K_2HPO_4 , 1.5g; trace mineral solution, 9ml; 10% FeSO_4 , 0.03ml; 0.2% resazurin, 1ml; 5ml of vitamin solution (Wolin et al. 1963); yeast extract, 3g; trypticase peptone, 10g and glucose 5g. Trace mineral solution contained per litre distilled water: 12.5g nitrilotriacetic acid neutralised to pH 6.5 with KOH, $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$, 0.2g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.017g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1g; ZnCl_2 , 0.1g; CuCl_2 , 0.02g; H_3BO_3 , 0.01g; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.01g; NaCl , 1.0g and Na_2SeO_3 , 0.02g. Media was autoclaved in an Oxford dispensing jar (model S-A) for 1 minute which was sufficient to reduce the medium as indicated by resazurin reduction. A steady stream of O_2 -free nitrogen gas was allowed to flow through the inlet of the dispenser via a bent syringe needle as the media cooled. 10ml of 10% $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ was added and mixed, and unless indicated 10ml of media was dispensed into tubes under a stream of O_2 -free nitrogen. For enrichments, medium walled pyrex tubes (size 12 x 152mm) fitted with '0' number stoppers and secured in a press before autoclaving (15 min, 121°C) were used. For all other studies, such as, nutritional, growth and antibiotic inhibition studies, 23 ml anaerobic culture tubes (Bellco) fitted with butyl rubber septums and caps, were used.

Primary enrichments were initiated by adding 0.1 to 0.2ml of sediment slurry to pre-reduced trypticase peptone yeast extract - glucose media (TYEG) and incubating at 70°C. After 1 to 3 days, tubes were examined both visually and microscopically for growth. Positive cultures were transferred and incubated at least three times in the same media after which they were purified using the end point dilution method in TYEG agar deeps (2% w/v of agar). Pure cultures were then stored at -20°C in a 50:50 glycerol-medium mixture.

For studies on nutrient utilization, yeast extract (YE) medium was used. YE medium was TYEG medium minus trypticase peptone and glucose. Additions of substrates were made to YE medium from 10% sterile stock solutions to a final concentration of 0.5%.

Source of Cultures: *Thermoanaerobium brockii* (ATCC 33075), *Thermobacteroides acetoethylicus* (ATCC 33265) and *Thermoanaerobacter ethanolicus* (ATCC 31550) were purchased from American Type Culture Collection, Rockville, M.D., U.S.A. *C. thermohydrosulfuricum* strain E100-69 was a gift from Professor F. Hollaus, Sugar Research Institute, Fuchsenbigl, Austria. All organisms were grown in TYEG medium at their optimal pH and temperature for each strain.

Growth and Metabolic Characterization: Growth was determined by measuring increase in turbidity at 600nm by inserting Bellco tubes directly into a Spectronic 20 (Bausch and Lomb, N.Y.). All growth experiments were performed in duplicate.

Fermentation end products were analysed by gas chromatography. Metabolic gases were analysed in a Pye Unicam PU4500 gas chromatograph equipped with a thermal conductivity detector. A stainless steel column (2.7m by 3mm outer diameter), packed with Carbosieve B, 120/140 mesh (Supelco Inc., Bellefonte, Pa.) was used for analysis of H₂ and CO₂ at an oven temperature of 180°C, detector temperature of 76°C and an injector temperature of 200°C. Oxygen-free nitrogen was used as a carrier gas at a flow rate of 8 ml/min. For analysis, 0.4ml from the headspace of the culture tube was withdrawn with a 0.5ml gas-tight syringe (Dynatec Precision Sampling Corporation, Louisiana) and injected into the gas

chromatograph. Concentrations of gases dissolved in the medium were obtained by calculation (Stephen and Stephen 1963). Volatile fatty acids, ethanol and lactate were determined in a Pye G.C.D. gas chromatograph equipped with a flame ionization detector. Chromosorb 101, 100-120 mesh (Supelco Inc., Bellefonte, Pa., U.S.A.) was packed in a glass column (1.8mm x 4.5mm) and the oven was run isothermally at 160°C with a detector temperature of 225°C and an injector temperature of 215°C for determination of ethanol or volatile fatty acids. Oxygen-free nitrogen was used as a carrier gas at a flow rate of 60 ml/min. Lactic acid was determined by the same method, but the column temperature was 200°C and the flow rate of nitrogen carrier gas was 52 ml/min. Propionic acid at a final concentration of 10mM was used as an internal standard for determination of volatile fatty acids and ethanol. 2 µl was injected for determination of fatty acids and alcohols.

Peak areas of all analysis were automatically integrated on a Hewlett Packard 3390A integrator.

Glucose was determined by Glucose GOD-Perid method. (Boehringer Mannheim GmbH, FRG.)

Cellular characterization: An Olympus BH phase contrast microscope was used for direct examination and cell size determinations. Thin sections of cells were prepared for electron microscopy by fixing an exponential culture grown in TYEG medium by the method of Sleytr and Glauert (1976). Fixed cells were dehydrated in a graded series of ethanol, embedded in Spurr's resin (Spurr 1969) and sectioned with glass knives on a Reichert ultramicrotome. Sections were placed on 400 mesh grids, stained with 1% uranyl acetate (pH. 4.0) and lead citrate. Negative staining was accomplished by floating 200 mesh butvar coated grids on a drop of bacterial culture for one minute. Excess culture was drawn off the grid with filter paper and the grid stained by applying a drop of 1% uranyl acetate (pH 4.0) for 30 seconds, removing excess stain and the grids (both negatively stained and thin sections) examined under a Phillips EM 400 electron microscope at an accelerating voltage of 80kV.

G+C content: Cells were lysed using the method of Owen and Hill (1979) and the DNA recovered to the first precipitation stage as described by Marmur (1961). Further purification of the DNA was achieved on a Sepharose 4B column (Gibson and Ogden 1979). The T_m of the DNA was measured in a Pye SP8-400 spectrophotometer with stepwise heated polyethylene glycol 4000 circulating through the heating block. The temperature of the DNA solution was measured directly in the cuvette with a YSI 427 thermistor probe (Yellow Springs Instrument Co., Ohio, U.S.A.) inserted through a stopper and read directly on a Digisense thermometer (Cole Palmer Inst. Co., Illinois, U.S.A.). *E. coli* DNA (Sigma Chemicals) was used as a standard. The %G+C of *E. coli* standard was 51.2.

Results

Enrichment Isolation and Cultivation

Positive enrichments for glycolytic anaerobes were obtained from 51 out of the 266 pool samples following incubation at 70°C for 48 hours in TYEG medium. Microscopic examination revealed, in addition to the conventional bacterial morphologies such as rods and filaments, the presence of unique rods with terminal swellings in nine of the 51 enrichments (Table 1). We have called these terminal swellings spheroids. All the 9 springs were located in the Rotorua and Waimangu thermal areas. Other thermal areas including Taupo, Tokaanu, White Island, Waiotapu, Tikiteri, Orakei Korako, Wairakei and Fiji did not yield spheroid bearing organisms.

Additionally, investigations of a number of pools by electron microscopy of butvar coated grids colonised *in situ* and microscopic examination of colonised glass and pool water also revealed the presence of spheroid bearing organisms (B.K.C. Patel, Ph.D. thesis).

Spheroid bearing bacteria were not restricted to pools with an algal-bacterial mat, and the nine pools from which they were enriched covered a temperature range of 56°C to 95°C and pHs from 4.9 to 9.0. In three of the enrichments (the two unnamed mud pools and outflow of Waimangu

Cauldron) spheroid bearing bacteria were the only morphotype observed and pure cultures were obtained by the end point dilution method in TYEG agar shake tubes. Colonies in agar shake tubes were approx. 1 mm in diameter, uniformly round and creamy white after 48 hours incubation at 65°C. Colonies failed to develop on TYEG agar plates even when plating procedures were carried out in an anaerobic glove box indicating that they were strict anaerobes. Isolation of pure cultures was not attempted from enrichments containing mixed cultures obtained from outflow of pool 426, though we subsequently observed that supplementing the TYEG medium with 0.8% NaCl produced enrichments with spheroid bearing bacteria as the only morphotype. Strains Rtl6-B1, Rtl7-B1 and Wai4-B1 belong to the species *Fervidobacterium nodosum* with Rtl7-B1 regarded as the type strain. Stock cultures of the strains for further studies were prepared by transferring single well isolated colonies from agar shakes into fresh anaerobic TYEG medium followed by incubation at 70°C for 16-24 hours. Cultures remained viable for over two years when stored anaerobically in TYEG medium at room temperature.

Morphology: Cells of *Fervidobacterium nodosum* were Gram negative, motile, straight to slightly curved rods occurring singly, in pairs or in chains (Fig. 1) and rarely in filaments. The average size of Rtl7-B1 cells was 1.0 to 2.5 μm long by 0.50-0.55 μm wide. At all temperatures tested (45-75°C) and throughout the growth curve, 80-85% of cells in a culture produced spheroids. Only one spheroid was produced per cell. Spheroids occurred terminally, varied from 1.0 - 2.0 μm in diameter, and were observed during all stages of growth. Spheroids lysed immediately on addition of SDS (10%); however the cells themselves remained intact for one to two hours during which time granulation of cell content occurred. Whether the cells were viable after such a treatment was not investigated.

In addition to spheroids, rotund bodies were also frequently observed. The rotund bodies were membrane bound structures containing as many as 5-7 cells within. The size of the rotund bodies was between 5 - 8 μm in diameter.

Spores were never observed in cultures Rtl7-B1 and Wai4-B1 during studies on their physiology. The conditions used included variations in pH, temperatures and substrates (Table 3 provides the substrates tested for sporulation). Additional attempts to use special media such as GC medium (Pheil and Ordal, 1967), xylose-soil extract medium (Hollaus and Sleytr, 1972) and the special method as used by Wiegel et al., 1979 designed to induce sporulation, also failed and spores were never observed. However cultures of *C. thermohydrosulfuricum* El00-69 which were used as controls sporulated under these special growth conditions.

Cell Wall Ultrastructure: Electron microscopy of thin sections of *F. nodosum* Rtl6-B1, Rtl7-B1 and Wai4-B1 revealed that the cell envelope was made of two layers, which were connected with regular junctions (Fig. 2B) and thereby distinguishing it from the cell wall structure of *T. ethanolicus* (Fig. 2D). Both layers of Rtl6-B1, Rtl7-B1 and Wai4-B1 had an irregular convoluted structure. The outer layer protruded to form spheroids and this was the layer which was found to be susceptible to lysis by SDS (Fig. 2A). The cell was capable of growth and replication within the spheroid envelope thus forming rotund bodies (Fig. 2C).

Antibiotic sensitivity: Data on antibiotic sensitivity of Rtl7-B1 together with *Ta. Brockii* and *T. ethanolicus* is presented in Table 2. All the strains were totally inhibited by 100 µg/ml of penicillin, chloramphenicol and tetracycline. This suggests that *F. nodosum* is a eubacterium and not an archaebacterium.

Rtl7-B1 was unique in its complete resistance to D-cycloserine, an antibiotic inhibiting D-alanine incorporation into cell walls, pronounced resistance to streptomycin and its sensitivity to 500 µg/ml of sodium azide.

Growth optima: *F. nodosum* did not grow aerobically in stationary or shaken tubes containing TYEG (minus the reductant) medium, and required and Eh of less than -110 mV, as indicated by resazurin reduction, for growth. This indicated that it was a strict anaerobe.

The pH range over which growth occurred was 6.0 to 8.0 for all strains, the pH optima for Rtl7-B1 and Wai4-B1 was 7.0 whereas for Rtl6-B1 it was 7.5. All three strains grew at 47°C but not at 40°C or 85°C. The optimum temperature for growth for Rtl7-B1 was 70°C whereas for Rtl6-B1 and Wai4-B1 it was 65°C.

In TYEG medium and at optimum temperature and pH, Rtl7-B1 had a generation time of 105 minutes (Fig.3).

Nutritional Characterization: Table 3 summarizes the nutritional versatility of Rtl7-B1, Wai4-B1 and Rtl6-B1. Rtl7-B1 could be distinguished from the morphologically similar isolates Rtl6-B1 and Wai4-B1 by its inability to ferment pentoses. The isolates also differed in their ability to utilise sucrose, pyruvate and rhamnose.

None of the isolates were able to degrade cellulose and none grew on acetate, lactate, dextrin, xanthan or citrate. Rtl7-B1 did not grow on serine, glycine, glutamate, arginine, succinate, formate, fumarate, xylitol, carragenan, inulin, karaya gum and oxalate. No growth occurred in a sulphate and lactate or sulphate and acetate medium.

End Product Formation: The main products of glucose fermentation by all three strains of *F. nodosum* were acetate, lactate, CO₂, H₂, and ethanol. Traces of n-butyric and n-valeric acid were also formed. Typical end product formation kinetics during glucose fermentation by *F. nodosum* Rtl7-B1 are given in Fig.3. All end products increased in response to growth and glucose consumption. A fermentation balance typical for glucose metabolism by *F. nodosum* Rtl7-B1 and determined after 13 hours of growth at 70°C is shown in Table 4.

DNA Base Composition: The melting temperature (T_m) for the DNA from *F. nodosum* Rtl7-B1 was at 83.2°C which corresponded to a mol % guanine plus cytosine (G+C) of 33.7.

Discussion

The discovery of *Fervidobacterium nodosum* further extends the known diversity of extremely thermophilic bacteria in nature. Unlike *Ta. Brockii* (Zeikus et al., 1979), *Tb. acetoethylicus* (Ben-Bassat and Zeikus, 1981) and *T. ethanolicus* (Wiegel and Ljungdahl, 1981), *F. nodosum* can be isolated from springs devoid of algal-bacterial mats. *F. nodosum* is geographically restricted in nature since it could be isolated from Rotorua and Waimangu thermal areas but not from pools within the same pH and temperature range from other thermal areas of New Zealand or Fiji. Presumably properties other than pH or temperature of a spring influence the distribution of this species.

The temperature optimum for growth of *F. nodosum* is 70°C, which is very similar to that of *C. thermohydrosulfuricum*, *Ta. Brockii* and, *T. ethanolicus*. Similarly the moles % G+C content of DNA of all the organisms are very similar (30-34%).

The end product spectrum from glucose fermentation by *F. nodosum* is also very similar to that of *Ta. Brockii*, *C. thermohydrosulfuricum* and *T. ethanolicus*. However, the ratios of the end products formed are very different. For example, ethanol was a major end product from *Ta. Brockii*, *T. ethanolicus* and *C. thermohydrosulfuricum* but is always a minor end product (<2mM) in *F. nodosum*. Furthermore the production of spheroids, rotund bodies and the resistance to D-cycloserine differentiates *Ta. Brockii*, *T. ethanolicus* and *C. thermohydrosulfuricum* from *F. nodosum*. Additionally, *F. nodosum* is Gram negative and has a double layered cell wall which differentiates it from *Ta. Brockii*, a Gram positive extreme thermophile possessing a monolayered cell wall. The inability of *F. nodosum* to sporulate differentiates it from *C. thermohydrosulfuricum*.

Protuberances are produced by *T. ethanolicus* during stationary phase leading to cell lysis. However the protuberances (blebs) of *T. ethanolicus* and the protuberances (spheroids) of *F. nodosum* are clearly different. Spheroids are produced terminally and only one is produced per cell. Spheroids are produced due to protrusion of the outer cell wall, contain no cellular material (figure 2A) and hence appear

very phase light (Figures 1A and 1B). Blebs of *T. ethanolicus* on the other hand occur on any part of the cell, are protrusions of the cell membrane and cell wall, contain cellular material within and hence are phase dark (Figure 1D). Furthermore blebs are produced in stationary phase as a result of initiation of a lytic phase. Since the spheroids of *F. nodosum* occur throughout all stages of growth and since they have also been observed on cells *in situ* they are a natural part of the cell and are not produced due to lytic process of the cell. Additionally the cell wall structure of *T. ethanolicus* (Figure 2D) and *F. nodosum* (Figure 2B) are different.

Members of the family *Thermoproteaceae* isolated from hot springs of Iceland (Zillig et al., 1981, 1983) and New Zealand (B.K.C. Patel, Ph.D. thesis) also produce terminal protruberances (golf clubs). However, their inability to undergo binary division, failure to produce volatile fatty acids and alcohols, inhibition by high concentrations of carbon substrates, the presence of a distinct cell wall subunit structure and a high G+C content of DNA (55.5 moles %) clearly differentiate these organisms from *F. nodosum*. Furthermore, the members of *Thermoproteaceae* are archaeobacteria but the antibiotic inhibition spectrum suggests that *F. nodosum* is a eubacterium.

F. nodosum is similar to *Tb. acetoethylicus* in a number of features. Both have a restrictive pH range for growth, are not inhibited by D-cycloserine and have a double layered cell wall structure. However, *Tb. acetoethylicus* has never been observed to produce spheroids or rotund bodies and produces no lactate on glucose fermentation.

Rotund bodies have not been reported from any species other than *Thermus* (Brock and Freeze 1969). The arrangement of cells within rotund bodies of *Thermus* is more regular than those observed within *F. nodosum* rotund bodies and the morphology, G+C content and physiology of *F. nodosum* clearly differentiates it from *Thermus* species.

Since these unique anaerobic spheroid bearing organisms cannot be identified as belonging to any known bacterial genus and species classification, we feel justified in placing these strains in the proposed new genus and species, *Fervidobacterium nodosum*, gen. nov. spec. nov.

Description of the new genus and species is given below. The description of the species is the same as for the genus since, *Fervidobacterium nodosum* is so far the only species of the genus.

Fervidobacterium nodosum gen. nov. spec. Fer. vi. do. bac. ter. i. um.
L. adj. *Fervidus* hot; L. masc. n. *bakterion* a small rod; L. neut. n. *Fervidobacterium*, rod which grows at higher temperatures. *nodosum*. L. adj. *nodosum* knotty or swollen.

Morphology: Rods occur singly, in pairs and in chains, are 0.5 - 0.55 μm , by 1.0 - 2.5 μm , stain Gram negative, do not sporulate, are motile. A majority of the cells produce terminal swellings, called spheroids. Spheroids are 1.0 - 2.0 μm in diameter and only one spheroid is produced per cell. Rotund bodies formed due to multiple divisions of the cell within the spheroid are also frequently observed.

Colony morphology: TYEG agar deep (2%; 65°C) colonies are 1mm in diameter, uniformly round and creamish white after 48 hours incubation.

Physiology: Chemoorganotrophic, strictly anaerobic eubacterium. Growth occurs between 41°C and 79°C and from pH 6 to 8.0 with temperature optimum of 70°C and pH optimum of 7.0. Utilises various hexoses; inhibited by tetracycline, penicillin and chloramphenicol but resistant to D-cyloserine. Fermentation end products from glucose are lactate, CO_2 , H_2 , acetate with minor production of ethanol.

G+C Guanine plus cytosine content of the DNA: 33.7 Mol % as determined by the thermal denaturation method.

Habitat: Slightly acidic, neutral and alkaline hot springs of Rotorua and Waimangu thermal areas of New Zealand.

Type strain: *Fervidobacterium nodosum* Rt17-B. (ATCC35602)

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Table 1: Thermal features yielding *Fervidobacterium* type isolates

DSIR Pool No and/ or description of pool	Temperature (°C)	pH	Strain Designation
<u>ROTORUA:</u>			
602; Kuirau Park Lake ^a	70°C	7.2	-
Outflow from a vent in Government Gardens ^a	64°C	9.0	-
<u>Rotorua Whakarewarewa Tourist Reserve</u>			
428; Outflow of Roto-a-Tamaheke	66°C	6.9	-
358; Ororea	80°C	7.0	-
Unnamed mud pool	69°C	5.5	Rtl6-B1
Unnamed boiling mud pool	95°C	5.2	Rtl7-B1
Outflow from 426 ^a	62°C	7.8	-
353	78°C	7.1	-
<u>WAIMANGU:</u>			
Outflow of Waimangu Cauldron ^a	56°C	4.9	Wai4-B1

^a = Algal and/or bacterial mats were present. The outflows of Government Garden vent and pool 426 and Ororea were sampled on several occasions and similar organisms were obtained.

DSIR = Department of Scientific and Industrial Research, New Zealand.

Table 2: Comparison of antibiotic sensitivity of *Fervidobacterium nodosum* Rt17-B1 with *Ta. Brockii* and *T. ethanolicus*

Antibiotic	Concentration	Strains (% inhibition) ^a		
		<i>F. nodosum</i> Rt17-B1	<i>Ta. Brockii</i>	<i>T. ethanolicus</i>
D-CLYCLOSERINE	10 µg/ML	0	20	0
	100 µg/ml	0	100	56
NEOMYCIN SULPHATE	10 µg/ML	0	NA	49
	100 µg/ML	50	NA	97
STREPTOMYCIN SULPHATE	10 µg/ML	0	20	94
	100 µg/ML	83	100	97
TETRACYCLINE-HCl	10 µg/ML	100	100	100
PENICILLIN	10 µg/ML	100	100	100
CHLORAMPHENICOL	10 µg/ML	95	100	100
SODIUM AZIDE	250 µg/ML	0	31	70
	500 µg/ML	100	36	94

NA = not analysed

^a All strains were grown in TYEG at 65°C overnight. 0% inhibition of growth represents a final A₆₀₀ increase of 0.65 for *F. nodosum*, 1.0 for *Ta. Brockii* and 0.4 for *T. ethanolicus*.

Table 3: Nutritional spectrum of *Fervidobacterium nodosum*

Substrate	Rtl7-B1	Rtl6-B1	Wai4-B1
Glucose	+	+	+
Raffinose	+	ND	+ (Slow)
Galactose	+	+	+
Mannose	+	+	+
Fructose	+	+	+
Arabinose	+ (Slow)	+	+
Sorbitol	+	ND	+
Xylose	-	+	+
Ribose	-	+	+
Rhamnose	<u>+</u>	-	+
Lactose	+	+	+
Maltose	+	+	+
Sucrose	+	-	+
Pectin	+	+	+
Glycerol	+ (Slow)	ND	+
Pyruvate	<u>+</u>	-	+
Nitrate broth	+	-	-

All strains were grown for 30 hours at 65°C. 0.5% substrate was added to yeast extract (YE) medium.

ND = not determined.

Table 4: Glucose fermentation products of *F. nodosum* Rt17-B1:

Product	Amount Produced
Ethanol	23
Acetate	115
Lactate	162
CO ₂	120
H ₂	160
Glucose degraded	133
O/R index	0.86
C ₁ /C ₂	0.87
C - recovery	1.1

Results are given as total micromoles of product formed or glucose consumed per tube after 13 hours of incubation at 70°C in TYEG medium, pH7.0.

Fig. 1. Phase contrast microscopy of *F. nodosum* demonstrating the presence of and variations in size of spheroids (A,B) and the formation of a chain of cells (C) Magnification: X4200. Bleb formation in *T. ethanolicus* (D) Magnification: X2000.

Fig. 2. Ultrastructure features of *F. nodosum* demonstrating the presence of (A) spheroid as a protruberance of the cell wall (B) the cell envelope layers; *CM* = cytoplasmic membrane, *O* = outer layer, *I* = inner layer, *J* = junctions joining inner and outer layers (C) a rotund body containing cells dividing within and (D) the cell wall structure of *T. ethanolicus* showing a middle dense layer (arrow) surrounded by an inner and outer layer.

Fig 3. Relationship of end product formation to growth of *F. nodosum*. Anaerobic culture tubes containing 10 mls of TYEG, pH 7.0 were incubated at 70°C. Results are given as total micromoles formed per tube at indicated times. Symbols: ■ Lactate; ● H₂; Δ CO₂; ○ acetate; ▼ ethanol; □ growth; x glucose utilised.

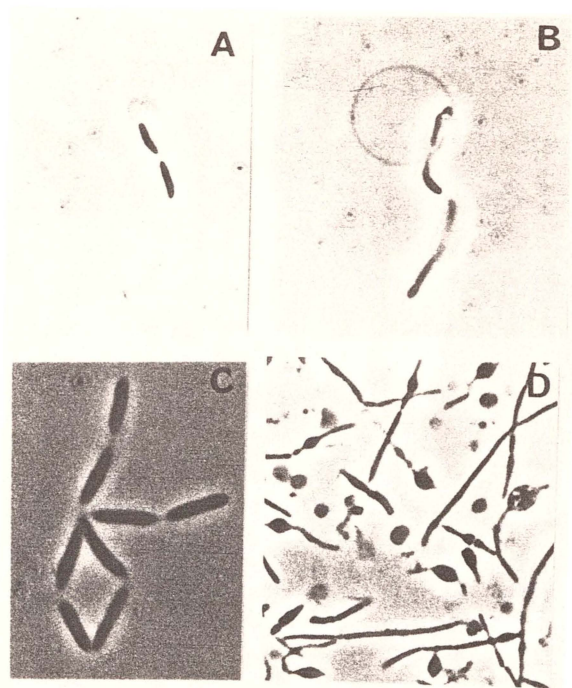
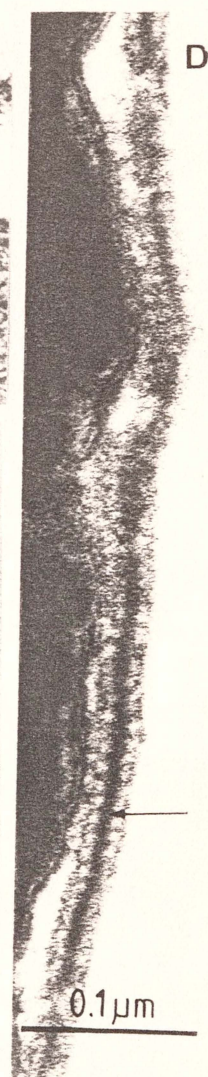
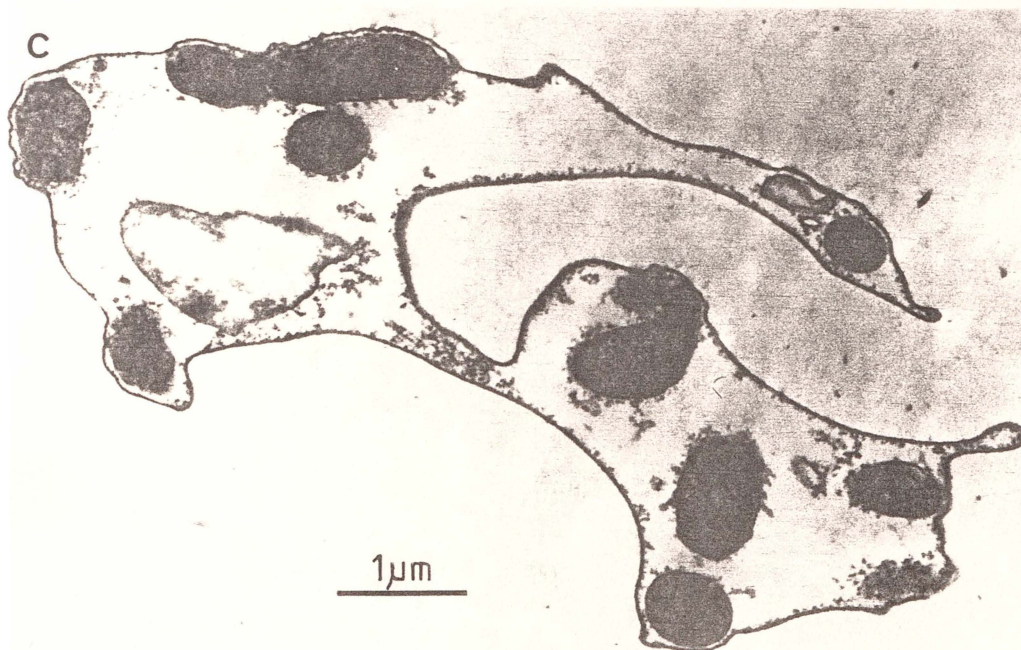
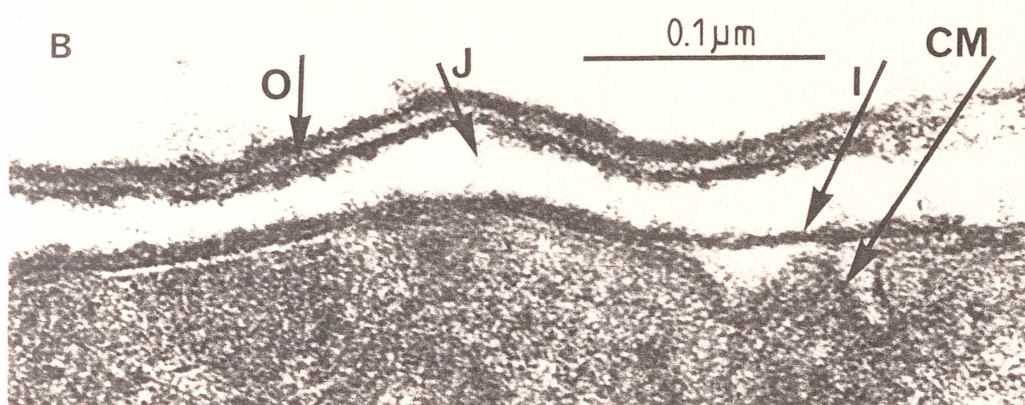
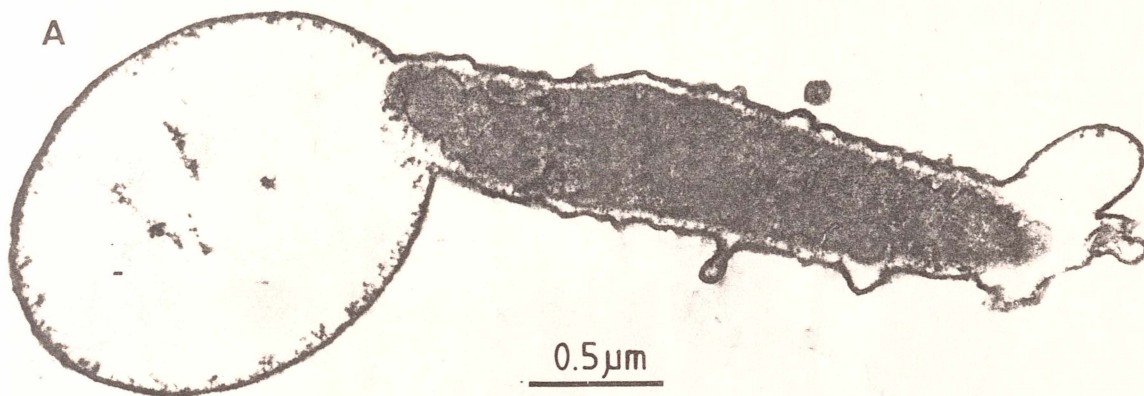


Figure 1



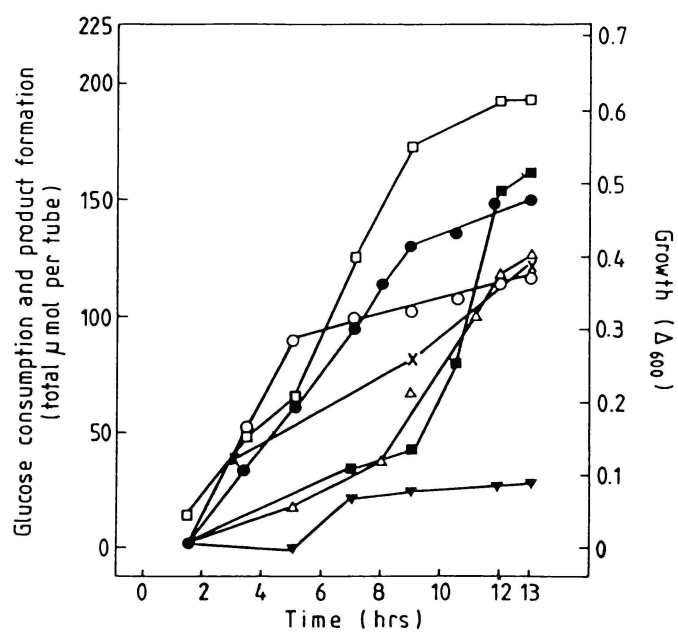


Figure 3

Thermophilic Anaerobic Spirochetes in New Zealand Hot Springs

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Key Words: Thermophilic spirochetes- obligate anaerobes- ecology- physiology- phage.

PATEL ET AL., (1985b). FEMS MICROBIOLOGY LETTERS
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THE ORIGINAL WHEN IT COMES TO HAND.

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1. SUMMARY

Electron and light microscopy revealed the presence of spirochetes in New Zealand thermal springs. The spirochete population in one spring studied (Kuirau Lake) was affected by fluctuations in temperature and/or pool level. A pure culture of the strictly anaerobic bacterium revealed that it grew optimally at a temperature of 45-50°C, with no growth occurring above 60°C, and a pH of 7.0-7.5 with no growth occurring at pH 5.5 or 8.5. Growth was inhibited by chloramphenicol, penicillin, streptomycin, tetracycline and neomycin but not by D-cycloserine, novobiocin or phosphomycin at 10µg/ml. A wide range of carbohydrates were utilized but not organic acids. Acetate was the major end product of glucose fermentation with substantial amounts of ethanol and traces of lactate being produced.

2. INTRODUCTION

Both obligately and facultatively anaerobic members of the genus Spirochaete have been isolated from mesophilic aquatic habitats such as sediments of ponds, lakes, rivers, oceans and marshes and from the rumen. At present, six species of spirochetes have been described, all of which are saccharolytic (1). All spirochetes except Spirochaete halophila which grows very poorly at 45°C, have a maximum temperature for growth below 40°C (2). Recently, spirochetes were isolated from the deep sea thermal vents of the Galapagos Rift (3). The optimal temperature for the growth of the vent spirochetes was between 25-30°C. Further studies on the species were thwarted because of its slow growth rate, low yields and lack of consistent growth in any liquid medium tested.

A novel "in situ" electron microscopy technique had earlier revealed the presence of spirochetes in New Zealand hot springs (B.K.C. Patel, Ph.D thesis). The purpose of the study reported here was to isolate and characterize these spirochetes.

3. MATERIALS AND METHODS

Enrichment cultures were initiated in spirochete enrichment (SE) medium. SE medium consisted of Allen's salts (4) plus (in grams per litre of deionized distilled water) yeast extract 0.5; trypticase peptone, 0.5; glucose, 2; cellobiose, 2; HEPES (N-2-hydroxyethyl piperazine- N-2-ethanesulfonic acid), 2.4 and resazurin, 0.002. The pH was adjusted to 7.2. Media was autoclaved in an Oxford dispensing jar (model S-A) for 1 minute which was sufficient to reduce the medium as indicated by resazurin reduction. A steady stream of oxygen free nitrogen was allowed to flow through the inlet of the dispenser via a bent syringe needle as the media cooled. 7ml of 10% Na₂S; 9H₂O was added, mixed and 10 ml of media dispensed into anaerobic culture tubes (23 ml; Bellco). Tubes were sealed with butyl rubber septums, capped and autoclaved. Filter-sterilized rifampicin at varying concentrations was added to the autoclaved medium as a selective agent for spirochete enrichment (5, 6).

Pure cultures from enrichments were obtained by the end point dilution technique in SE medium containing 10µg/ml rifampicin (Sigma Chemicals Co., USA) and fortified with 1% agar. Attempts were also made at purifying cultures using the filtration technique (7).

For studies on substrate utilization and antibiotic inhibition, yeast extract medium (YEM) was used. YEM consisted of LPBM salts plus 0.3% yeast extract and fermentable substrates (8). Addition of substrates was made to YEM from 10% sterile stock solutions to a final concentration of 0.5%. Growth was determined by observing turbidity visually and by phase contrast microscopy.

Fermentation end products were analysed by gas chromatography. H₂ and CO₂ were analysed in a PYE PU4500 gas chromatograph equipped with a thermal conductivity detector. A stainless steel column (2.7m by 3mm outer diameter)

packed with Carbosieve B 120/140 mesh (Supelco Inc., Bellefonte Pa.) was run at an oven temperature of 76°C and an injection temperature of 200°C. Oxygen-free nitrogen was used as a carrier gas at a flow rate of 8 ml/min. Volatile fatty acids, ethanol and lactate were detected in a Pye GCD gas chromatograph equipped with a flame ionization detector. Chromosorb 101, 100-120 mesh (Supelco Inc., Bellefonte, Pa.) was packed in a glass column (1.8m by 4.5mm) and the oven was run isothermally at 160°C with a detector temperature of 225°C and an injector temperature of 215°C for determination of ethanol or volatile fatty acids. Oxygen-free nitrogen was used as a carrier gas at a flow rate of 60ml/min. Lactate was determined by the same method but with a column temperature of 200°C and nitrogen carrier gas flow rate of 52ml/minute.

An Olympus BH phase contrast microscope was used for direct examination and cell size determination. For electron microscopy, negative staining was accomplished by floating 200 mesh butvar coated grids on a drop of bacterial culture for a minute. Excess culture was drawn off the grid with filter paper and the grid stained by applying a drop of 1% uranyl acetate (pH 4.0) for 30 seconds before removing excess stain. The grids were examined under a Phillips EM400 electron microscope at an accelerating voltage of 80kV.

4. RESULTS

4.1 Ecological Observations

36 New Zealand thermal springs were examined for microbial presence by a novel "in situ" electron microscopy method (B.K.C. Patel, unpublished Ph.D. thesis, 1984). Though a diverse microbial flora existed in the springs, the most commonly observed morphotype was rods and in only four pools were spirochetes present. Their identification (Fig. 1) was based on the presence of periplasmic filaments (PF), a protoplasmic cylinder (PC) and an outer

sheath (OS) (1). Table 1 presents data on pH and temperature of the springs in which spirochetes were observed.

During the course of these studies, the temperature of Kuirau Lake fell from 70°C to 50°C which resulted in substantial changes in microbial flora. At 70°C, the predominant microbial flora was rods with very few spirochetes present. However, at 58°C, the rod-shaped bacterial population was replaced largely by the spirochete population. Subsequently, a further drop in temperature to 50°C together with a drop in pool level occurred. Microscopic examination of the pool water, colonized slides and enrichment studies which were performed during the course of these changes, repeatedly failed to reveal the presence of spirochetes at the lower temperature.

4.2 Enrichment and Isolation

At an incubation temperature of 52°C and 56°C but not at 60°C and with 10µm/ml of rifampicin added to SE medium, Kuirau lake samples yielded spirochetes in addition to motile rod-shaped bacteria. However at 0µm/ml and 30µm/ml of rifampicin only the motile rods but not spirochetes were enriched. Since rifampicin at 10µg/ml was the concentration at which most spirochetes were obtained, this concentration was used to enrich spirochetes from Warbrick Terrace outflow. Similarly in all other studies, of enrichment and isolation 10µg/ml of rifampicin was used.

Phage particles (Fig. 2) were observed attached to spirochetes in enrichment cultures containing 10µg/ml rifampicin. Frequently upto 5 phage per bacterial cell were seen in cultures, probably indicating that they were infective to spirochetes. Phages were not observed in enrichments without rifampicin or containing 30µg/ml rifampicin and were also not observed attached to rods.

Initial pure cultures of spirochetes from Kuirau Lake were obtained by filtering the enrichment culture through a 0.45µm cellulose ester filter membrane (Millipore). To avoid the possibility of phage contamination of pure cultures, subsequent pure cultures were prepared from colonies growing in rifampicin containing SE agar.

Colonies of spirochetes in SE agar shakes obtained from Kuirau Park and Warbrick Terrace outflow enrichments were similar in appearance. The colonies were cream, 1mm in diameter and were fluffy.

4.3 Morphology and Growth Characteristics

The pure cultures of spirochetes obtained from Kuirau Lake and Warbrick Terrace outflow, designated Rt3-BS1 and Wai21-BS1 were similar in morphology. Both consisted of tightly coiled cells (Fig. 3A) with a wavelength of 0.4-0.5µm, an amplitude of 0.15-0.2µm and of dimension 0.15-0.3µm by 15-25µm. The axial filament of both strains exhibited a 1-2-1 arrangement (Fig. 3B).

Spherical bodies generally 1-2µm in diameter were observed under stationary growth phase or under unfavourable growth conditions e.g. in the presence of traces of oxygen.

Rt3-BS1 and Wai21-BS1 were strict anaerobes and did not grow aerobically as evidenced by their inability to form colonies in the top oxidised 1cm of SE agar shakes. Growth did not occur if oxygen free nitrogen was replaced by air in SE liquid medium.

The optimum temperature for growth of Rt3-BS1 was between 45-50°C and the maximum temperature for growth below 60°C. Growth occurred at room temperature. The optimum pH for growth was between 7.0-7.5 with no growth

occurring at 5.5 or 8.5.

Growth of Rt3-BS1 was totally inhibited by chloramphenicol, penicillin, streptomycin, tetracycline and neomycin at 10µg/ml and by novobiocin and D-cycloserine at 100µg/ml, when incubated in SE medium at 50°C, however, it was not inhibited by phosphomycin at 100µg/ml or by sodium azide at 250µg/ml. Sodium azide at 500µg/ml was slightly inhibitory.

4.4 Utilization of Carbohydrates

Rt3-BS1 grew on a wide range of carbohydrates including maltose, sucrose, cellobiose, glucose, mannose, mannitol, adonitol and maize starch. However much better growth occurred with disaccharides than with monosaccharides. The maximum optical density reached by Rt3-BS1 when grown on maltose was never higher than 0.15-0.2 under optimum growth conditions. Rt3-BS1 did not grow on casein hydrolysate or organic acids such as lactate, acetate, fumarate and benzoate.

4.5 Fermentation end products

Acetate was the major end product formed during glucose fermentation. Ethanol, CO₂ and H₂ were also produced in substantial amounts together with traces of lactate.

5. DISCUSSION

The presence of spirochetes was demonstrated in 4 hot springs of Rotorua and Waimangu. The temperatures of the springs were between 58-70°C. The observation that more spirochetes were present in Kuirau Lake at 56°C than at 70°C, together with the fact that under laboratory conditions the growth

temperature maximum of spirochetes was below 60°C, indicates that though spirochetes were present at 70°C in their natural environment, they actually grew at much lower temperatures, possibly in much cooler parts of the spring. Additionally, the spirochete population was susceptible to drastic changes in their environment (fluctuations in temperature and/or pool level). Such changes have not affected growth of archaeobacterial communities present in other springs (B.K.C. Patel, Ph.D thesis, 1984). This was perhaps because spirochetes were more fragile and much more sensitive to environmental changes than were other microorganisms.

The phage present in spirochete enrichment cultures were morphologically distinct from the tailed phages of the Thermus species from Japan (9) or from New Zealand (B.K.C. Patel, Ph.D. thesis 1984). This is the first evidence of phage in spirochete cultures.

The thermophilic spirochete, Rt3-BS1, was very similar to S. stenostrepta (10). Both are morphologically similar and produced the same end products from glucose fermentation, but Rt3-BS1 produced far more acetate in relation to ethanol than S. stenostrepta. Similarly, Rt3-BS1 has a higher temperature optimum and maximum for growth than S. stenostrepta.

An obligately anaerobic spirochete designated GS-2, has been recently isolated from the hydrothermal deep sea vents (3). However, in contrast to the New Zealand isolates, GS-2 had an optimum temperature for growth between 25-30°C and it also had an obligate requirement for sodium chloride. To our knowledge, this is the first report on the isolation and characterisation of a thermophilic spirochete from thermal springs and their presence further extends the known diversity of thermophilic anaerobes in geothermal habitats.

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Table: 1 Features of thermal springs in which spirochetes were observed and/or enriched and isolated:

Pool	Temperature (°C)	pH	Strain designation
Kuirau Lake	58-70°C	7.2	Rt3-BS1
Outflow of Jaycee's fountain	64°C	9.4	-
Puara pool	70°C	7.2	-
Outflow of Warbrick Terrace	67°C	6.5	Wai21-BS1

All springs except Kuirau Lake contained extensive algal and/or bacterial mats.

Wai21-BS1 and Rt3-BS1 were morphologically similar isolates and most studies were restricted to isolate Rt3-BS1 only.

Fig. 1. Fine structure of a spirochete from Kuirau Lake showing periplasmic filaments (PF) wound around the protoplasmic cylinder (PC) and the outer sheath (OS).

Fig. 2. Electron micrograph of a phage attached to a spirochete (A) and ultrastructure of a free phage (B) present in a spirochete enrichment culture containing 10µg/ml rifampicin.

Fig. 3. Morphology of the spirochete Rt3-BS1 showing the tightly coiled nature (A) and a 1-2-1 arrangement of the the periplasmic filament of the bacterium (B).

