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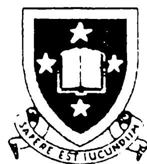
CHARACTERIZATION OF AN EXTREMELY THERMOPHILIC, ANAEROBIC SULPHUR-DEPENDENT ARCHAEBACTERIUM

A thesis submitted in partial fulfilment
of the requirements for the degree
of
Doctor of Philosophy in Biological Sciences
at the
University of Waikato
by

Karin Ursula Klages



University of Waikato
1991

Abstract

The sulphur-dependent archaebacterial isolate ANI was studied in aspects of its ecology, physiology, and enzymology.

Organisms closely resembling isolate ANI were enriched from New Zealand hot pools within a temperature range of 55-91°C and a pH range of 5.5-9.0 and a sodium content above 0.5g/ℓ. The concentration of sodium ions was found to be a third parameter determining the presence or absence of these organisms.

Isolate ANI is a neutrophilic, anaerobic extreme thermophile. In the laboratory, growth was observed within a pH range of pH 5.4 to 9.2 and a temperature range between 55 and 85°C. The organism is an obligate heterotroph with a requirement for peptone. In the presence of peptone, several amino acids, α-keto acids, glucose, lactose, casein, and starch stimulate growth. Sodium and sulphur are required for growth. Sodium can be replaced by lithium and sulphur by cystine and glutathione.

Sodium is required for utilization of several amino acids and for synthesis of ATP. Presence of a Na^+ gradient across the cytoplasmic membrane is essential for growth and ATP production. Ionophores affecting the Na^+ gradient show a strong inhibitory effect, while a protonophore or a K^+ ionophore exerted virtually no inhibition. In addition to a Na^+ gradient the existence of either a H^+ gradient or a membrane potential is required for growth and generation of ATP. The Na^+ gradient is possibly generated via a Na^+-translocating ATPase.

Utilization of branched-chain amino acids in the presence of sulphur results in production of NH_4, CO_2, H_2, sulphide, and branched-chain fatty acids. When valine is utilized the major fatty acid end product is isobutyrate and with leucine or isoleucine it is isovalerate or 2-methylbutyrate (which could not be distinguished by analytical techniques used here). The metabolic pathway of branched-chain amino acids was investigated. The proposed common metabolic pathway for valine,leucine, and isoleucine involves four steps: deamination and production of the α-keto acid, oxidation of the α-keto acid and production of the acyl-CoA, production of the acyl phosphate from the acyl-CoA, and production of the fatty acid from the acyl phosphate, the latter step being coupled to production of ATP from ADP and inorganic phosphate. The presence of amino acid aminotransferase, α-keto acid

oxidoreductase, and fatty acid kinase, which catalyze the first, second and fourth reaction respectively, was demonstrated in crude cell extracts, while the presence of phosphate acyltransferase, catalyzing the third step, was only suggested. Substrate-level phosphorylation is the major, if not the sole means of ATP generation in isolate ANI.
Reducing equivalents generated by amino acid oxidation are transferred to a postulated hydrogenase and sulphur reductase respectively and sulphide and H_2 are produced. No inhibitory effect of H_2 could be demonstrated and reduction of sulphur as means for H_2 detoxification does not appear to occur in isolate ANI. Instead, H_2 and sulphide are produced concomitantly during growth.
A type II restriction endonuclease of ANI, termed 'ANi I', was partially purified and its recognition sequence determined as CTAG. The cut site within the sequence is not known.

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List of Abbreviations

A_{280}	absorbance at 280nm
ADP	adenosine 5′-diphosphate
ATP	adenosine 5′-triphosphate
bp	base pairs
BSA	bovine serum albumin
CCCP	carbonyl cyanide *m*-chlorophenylhydrazine
CoA	coenzyme A
concn	concentration
DCCD	*N,N′*-dicyclohexylcarbodiimide
DSM	Deutsche Sammlung von Mikroorganismen
EDTA	ethylenediaminetetraacetic acid
EGTA	(ethylene-dioxy)diethylenedinitrilotetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononuleotide
h	hour(s)
HPLC	high performance liquid chromatography
Ket	Ketatahi
(α)KG	α-ketoglutarate
KIC	α-ketoisocaproate
KIV	α-ketoisovalerate
kPa	kilo pascals
ℓ	liter, used in "g/l" to avoid confusion with "1"
min	minute(s)
Mok	Mokai
MOPS	3-[N-morpholino}propane-sulfonic acid
MPN	most probable numbers
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
NADH	dihydronicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NBD-Cl	7-chloro-4-nitrobenzo-2-oxa-1,3,-diazole
nm	nanometers
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PID	2,3-di-*o*-phytanyl-*sn*-glycero-1-phosphoryl-1′-*myo*-inositol
PGD	phosphate glucosyl diether
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TBE	Tris borate EDTA
TEMED	*N,N,N′,N′*-tetramethylethylenediamine
TES	(N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid
Tok	Tokaanu
TPP	thiamine pyrophosphate
Tris	Tris(hydroxymethyl)aminomethane
(v/v)	volume for volume
Wai	Waimangu
Wkt	Waikite
Wtp	Waiotapu
(w/v)	weight for volume
YE	yeast extract

Chapter 1: Literature Review

1.1. Phylogeny

The archaebacterial kingdom encompasses organisms with a great diversity of morphology and physiology. Archaebacterial organisms are found in diverse habitats ranging from sewage sludge digestors to salterns and boiling mud pools. Organisms belonging to the archaebacterial kingdom were studied extensively before their fundamental difference to true bacteria was recognized. Originally, their idiosyncracies in morphology and physiology when compared to other bacteria, such as novel cell envelope structure, were interpreted to be adaptations to the 'extreme' environments these organisms were found in. Only in 1977 Woese and Fox proposed the new third kingdom of archaebacteria. Their proposal was based on partial homology studies of 16S ribosomal RNA (rRNA) sequences from eukaryotes, eubacteria, and archaebacteria (Woese and Fox 1977). Every organism carries within its genome its evolutionary history (Zuckerkandl and Pauling 1965). Comparative analysis of gene sequences between different organisms can be used to show their relatedness. The choice of genes to be compared is of great importance as different genes will be under varying degrees of evolutionary pressure. Use of 16S rRNA has shown to be very well suited. This part of the genome is found universally, highly constant in function and while some parts of its gene sequence change only very slowly with time permitting comparison between the most distantly related organisms, other parts change more rapidly, being useful when organisms of closer relation are compared (Stackebrandt and Woese 1981).

Since 1977 the idea of the archaebacterial kingdom has been strengthened and is now a generally accepted concept. Using sequences of 5S, 16S, and 23S rRNA species, the major archaebacterial lineages have been defined. Cataloguing of rRNA (Fox *et al*. 1980) have shown two major branches: one comprised of methanogens and halophiles, and the other of thermophilic sulphur-dependents (formerly 'thermoacidophiles'). Use of 5S rRNA has the advantage of being easier to sequence than the larger rRNAs, but its size is too small for detailed phylogenetic analysis, while secondary structure of 5S rRNA has been useful (Fox 1985; Wolters and Erdmann 1986). Sequences of 16S

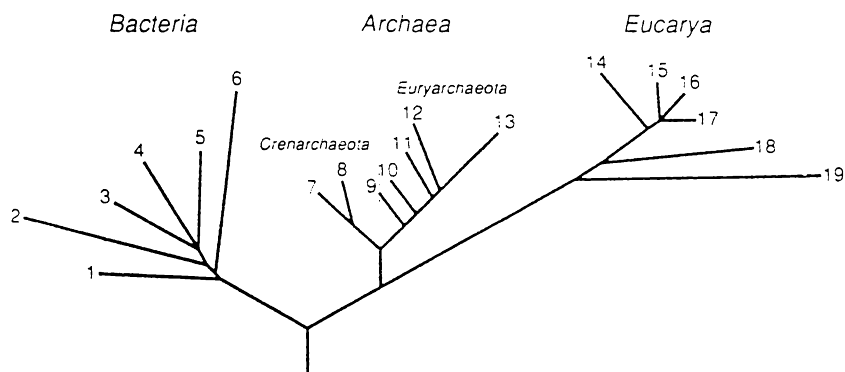
rRNA have been widely used for phylogenetic studies. While determination of entire sequences has become feasible only in the past few years, oligonucleotide catalogues have been used for defining the major branching orders among organisms. Sequence analysis of 16S rRNA (Yang *et al.* 1985; Olsen *et al.* 1985) allows determination of relationships between organisms more closely related and has been used for construction of phylogenetic trees (Woese and Olsen 1986; Woese 1987: Achenbach-Richter *et al.* 1988). Recently 23S rRNA has been used for phylogenetic analysis and was found more useful in studies on closely related organisms (Leffers *et al.* 1987).

The main difficulty in the construction of a phylogenetic tree is to account for variable evolutionary rates in different organisms as well as in different parts within a gene sequence. The unrooted archaebacterial phylogenetic tree derived by comparison of 16S rRNA sequences (Woese and Olsen 1986, Woese 1987) clearly separates *Thermococcus celer* from other sulphur-dependents (Fig.1.1.a). The *Methanomicrobiales* are the closest to the *Halobacteriales* and the *Halobacteriales* are thought to have arisen out of this group of methanogens (Jones *et al.* 1987), and both are apparently evolving rapidly. Within the methanogens, the *Methanococcales* are most distantly related and are closest to the imposed root which is consistent with buoyant density analysis of their ribosomal subunits (Cammarano *et al.* 1986; Teichner *et al.* 1986) which grouped them with sulphur-dependents. *Sulfolobus* appears to be the most rapidly evolving among the group of sulphur-dependents. *Thermococcus celer* represents the most slowly evolving line of archaebacteria. Its 16S rRNA places it in between the sulphur-dependents and methanogens. Eubacterial and eukaryotic lineages in this model intersect the archaebacterial tree between *Thermococcus* and other sulphur-dependents (Achenbach-Richter *et al.* 1988).

Recently Woese has proposed yet another new variety on the phylogenetic tree based on structure and sequence of rRNA (Woese *et al.*; Bown 1990). This tree is shown in Fig.1.1.a, showing a division of archaebacteria into two groups, the sulphur-dependents (except *Thermococcales)*, named crenarchaeota in one group, and the *Thermococcales*, methanogens, and halophiles in the other group, the euryarchaeota.

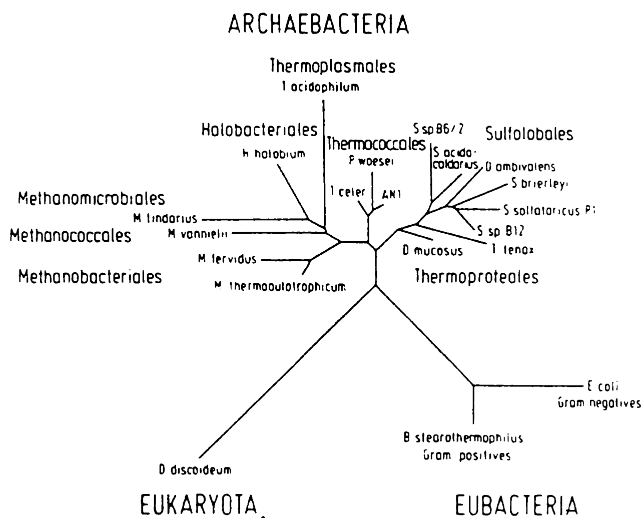
Fig.1.1 <u>Phylogeny of Archaebacteria: Phylogenetic trees based on DNA-rRNA cross-hybridization velocities and on comparison of rRNA species</u>

a)



The numbers on the branch tips correspond to the following groups of organisms. Bacteria: 1, Thermotogales; 2, flavobacteria and relatives; 3, cyanobacteria; 4, purple bacteria; 5, Gram-positive bacteria; 6, green nonsulfur bacteria. Archae: kingdom Crenarchaeota: 7, *Pyrodictium*; 8, *Thermoproteus*; and kingdom Euryarchaeota: 9, Thermococcales, 10, Methanococcales; 11, Methanobacteriales; 12, Methanomicrobiales; 13, extreme halophiles. Eucarya: 14, animals; 15, ciliates; 16, green plants; 17, fungi; 18, flagellates; 19, microsporidia.
(Woese *et al.* 1990)

b)



(Zillig *et al.* 1988)

DNA-rRNA hybridization has been used for phylogenetic studies (Tu *et al.* 1982; Zillig *et al.* 1987, 1988). The phylogenetic tree derived from DNA-rRNA hybridization (Fig.1.1.b) is very similar to the tree derived from 16S rRNA sequence analysis.
Structure of the DNA-dependent RNA polymerase has in general supported the deep dichotomy among archaebacteria (Zillig *et al.* 1982a, 1985a, 1985b; Gropp *et al.* 1986). The component pattern of the DNA-dependent RNA polymerases are distinct from both eukaryotes and eubacteria (Zillig *et al.* 1982a). Within the archaebacteria the component pattern has shown the division between methanogens on one side (the AB'B''CD pattern) and the sulphur-dependents on the other side (the BACD pattern). Construction of an unrooted phylogenetic tree based on the component pattern of the polymerase derives the eubacteria from the *Methanococcales* or *Methanobacteriales* or their immediate ancestor. The finding that the largest subunits of different eukaryotic DNA-dependent RNA polymerases show closer relationship to the corresponding component of archaebacterial polymerases than to one another indicates that archaebacteria are still closest to the ancestral precessor and evolved the slowest (Zillig *et al.* 1985b).
The structure, transcription, and organization of genetic material in archaebacteria and phylogenetic implications thereof have been reviewed (Zillig *et al.* 1988; Brown *et al.* 1989).

Lake *et al.* (1984, 1985; Lake 1986) proposed a reclassification of the archaebacteria into two kingdoms, separating the sulphur-dependents from the methanogens and halophiles and forming the new kingdom of 'eocytes'. As main criterion for this proposal they used the three-dimensional ribosomal structure. Lake's view has received much criticism (see Woese and Olsen 1986). Lake's model is however in agreement with the idea that the sulphur-dependent archaebacteria are more closely related to the universal ancestor of all extant life than any other organisms.
On the level of physiology, archaebacteria show unique features in their cell wall (Kandler 1982; Kandler and König 1985) and membrane lipid composition (Langworthy 1985; Langworthy and Pond 1986; De Rosa *et al.* 1986; De Rosa and Gambacorta 1988). Their cell wall lacks muramic acid and their membrane lipids are based on ether and not ester linkages and straight carbon chains are replaced by branched carbon chains.

Table 1.1. Distribution of shared (s) and unique (u) feature designs between archaebacteria (AB), eubacteria (EU), and eukaryotes (EK) (reprinted from Zillig *et al.* 1988)

Presence of feature in: +, all or most members of kingdom; (+), certain phyla of kingdom; ±, intermediate or divergent character of feature; (?), interpretation questionable

Feature	AB	EB	EK
Murein	–	(+)u	–
Outer membrane	–	(+)u	–
7S RNA	+?	–	(+)u
Isopranyl ether lipids	+u	–	–
Ester lipids	–	+s	+s
S^0 · reduction	(+)s	(+)s	–
SO_4^{2-} reduction	(+)s	(+)s	–
S^0 oxidation	(+)s	(+)s	–
Methanogenesis	(+)u	–	–
Nitrogen fixation	(+)s	(+)s	–
Photosynthesis (chlorophyll)	–	(+)u	– [a]
Operons	+s	+u	–
mRNA splicing	–	–	+u
tRNA splicing	(+)s	–	(+)s
CCA end of tRNA encoded	–	(+)u	–
fMet-tRNA	–	+	–
First base pair in initiator tRNA A · U	+s	–	+s
Aphidicolin-sensitive DNA polymerase	+s	–	+s
Reverse gyrase	+u	–	–
Promoter of pol II type	+s	–	+s
Promoter of eubacterial type	–	+u	–
80S ribosomes	±	–	+(s)
70S ribosomes	(+)s	+s	–
5.8S rRNA	–	–	+u
EFTu ADP-ribosylation possible	+s	–	+s
Ribosome binding sites	+s	+s	–
RNA polymerase of archaebacterial type	+s	–	+s
RNA polymerase of eubacterial type	–	+u	–
Specialized RNA polymerases	–	–	+u
Capping of mRNA			+u
Poly(A) tailing of mRNA			+u
Nucleus	–	–	+u
True chromatin (nucleosomes)	–	–	+u
Chromosomes			+u
Multi-cellular organisation	–	±	(+)u
Somatic differentiation	–	–(?)	(+)u
Cell division by septum formation	±s	+s	–
Cell division by other mechanisms e.g. constriction	±s	–	+s

[a] The chlorophyll found in eukaryotic cells is in chloroplasts of eubacterial origin.

The lack of murein in the cell wall, sensitivity to some eukaryotic antibiotics and resistance to some eubacterial antibiotics (Hilpert *et al.*1981; Böck and Kandler 1985) are the most easily determined features of archaebacteria.
In spite of deep branching within the archaebacteria, fundamental similarities on the molecular and physiological level are in support of one kingdom (Woese *et al.* 1978; Woese 1981; Woese and Olsen 1986; Gouy and Li 1989; Zillig *et al.* 1988; Kjems and Garrett 1990). Comparison of shared and unique features of archaebacteria, eubacteria, and eukaryotes is summarized in Table 1.1.

1.2. Archaebacterial diversity

The major three phenotypic groups of archaebacteria which largely also correspond to the phylogenetic groups are the halophiles, the methanogens, and the sulphur-dependent thermophiles.
There is only one order of halophiles the *Halobacteriales* which consists of two genera *Halobacterium* and *Halococcus*. These are mesophilic, aerobic chemoorganotrophs, growing at optimal NaCl concentrations of between 16 to 26% (reviewed by Kushner 1985). Halobacteria represent the only group of archaebacteria that is able to efficiently carry out oxidative phosphorylation, and transfer of gene sequences for an electron transport chain and enzymes for protection against O_2 toxicity may have occurred (Kerscher and Oesterhelt 1982).
Methanogens are all capable of methane formation from C_1 compounds and H_2. They occupy the most diverse habitats among all archaebacterial groups and are found in about every place where anaerobic biodegradation takes place (reviewed by Whitman 1985; Jones *et al.* 1987). There are three major groups of methanogens; the *Methanococcales, Methanobacteriales*, and the *Methanomicrobiales*. Several thermophilic methanogens have been described within the orders of *Methanococcales (Methanococcus thermolithotrophicus* [Huber *et al.* 1982], *Methanococcus jannaschii* [Jones *et al.* 1983], *Methanococcus igneus* [Burggraf *et al.* 1990a] and *Methanobacteriales* (*Methanobacterium thermoautotrophicum* [Zeikus and Wolfe 1972], *Methanothermus fervidus [Stetter et al.* 1981], *Methanothermus sociabilis* [Lauerer *et al.* 1986].

Table 1.2. Representative species of sulphur-dependent archaebacteria and some of their characteristics

Organism	growth temp. range (°C)	growth pH range	aerobic (ae)/ anaerobic (an)	S^0/H_2 autotrophy	habitat marine (m)/ solfatara (s)	%mol G+C	reference
Thermococcales							
Thermococcus celer	75-97	4.0-7.0	an	-	m	57	Zillig *et al.* 1983b
Pyrococcus furiosus	70-103	5.0-9.0	an	-	m	38	Fiala and Stetter 1986
Pyrodictiales							
Pyrodictium occultum	82-110	5.0-7.0	an	+	m	62	Stetter *et al.* 1983
Pyrodictium abyssum	80-110	4.7-7.5	an	-	m	60	Stetter *et al.* 1990
Thermodiscus maritimus	75-98	5.0-7.0	an	-	m	49	Fischer *et al.* 1983
Themoproteales							
Thermoproteus tenax	70-97	2.5-6.0	an	+[a]	s	56	Zillig *et al.* 1981a, 1981b
Thermophilum pendens	70-95	4.0-6.5	an	-	s	57	Zillig *et al.* 1983a
Pyrobaculum islandicum	74-103	5.0-7.0	an	+[a]	s	46	Huber *et al.* 1987
Pyrobaculum organotrophum	78-102	5.0-7.0	an	-	s	46	"
Desulfurococcus mobilis	70-95	4.5-7.0	an	-	s	51	Zillig *et al.* 1982b
Staphylothermus marinus	65-98	4.5-8.5	an	-	m	35	Fiala *et al.* 1986
Sulfolobales							
Sulfolobus acidocaldarius	60-85	1.0-5.0	ae	+[a]	s	37	Brock *et al.* 1972; Zillig *et al.* 1980
Metallosphaera sedula	50-80	1.0-4.5	ae	+[a]	s	45	Huber *et al.* 1989
Acidianus infernus	65-95	1.0-5.0	ae/an	+[b]	s	31	Segerer *et al.* 1986
Desulfurolobus ambivalens	70-87	1.0-3.5	ae/an	+[b]	s	32.7	Zillig *et al.* 1986
Other							
'*Caldococcus litoralis*'	55-100	5.9-7.0	an	-	m	41	Svetlichnyi *et al.* 1988
Hyperthermus butylicus	95-106	7 (opt.)	an	-	m	55.6	Zillig *et al.* 1990

[a]facultative autotroph
[b]facultative anaerobe and obligate autotroph

Recently extremely thermophilic methanogens from deep-sea hydrothermal vents have been isolated which grow at 110°C (Huber *et al.* 1989).
Thermoplasma is the only genus so far described of the *Thermoplasmales.* Two species are known; *Thermoplasma acidophilum* (Searcy 1982) and *Thermoplasma volcanium* (Segerer *et al.* 1988). These organisms lack a cell wall.
Discovery of sulphate-reducing archaebacteria has provided the possible 'missing link' between the sulphur-dependent and the methanogenic branches (Achenbach-Richter *et al.* 1987; Stetter *et al.* 1987). *Archaeoglobus* reduces sulphate, sulphite, or thiosulphate and produces a trace of methane. Two species have been isolated; *A. fulgidus* strain Z (Zellner *et al.*1989) and *A. profundus* (Burggraf *et al.* 1990b) from marine hydrothermal vents. The organisms grow at temperatures between 60° and 90°C between pH 4.7 and 7.5.
The sulphur-dependent archaebacteria are all thermophiles. There are four orders: *Thermococcales, Pyrodictiales, Thermoproteales,* and *Sulfolobales.* Representative species and some of their characteristics are summarized in Table 1.2.
Among the *Thermococcales* several other species have been described: *T. stetteri* (Miroshnichenko *et al.* 1989), *T. litoralis* (Belkin and Jannasch 1985; Neuner *et al.* 1990), and *P. furiosus* (Fiala and Stetter 1986) (see also Table 3.7). All validly named members of the *Thermococcales* are marine obligate heterotrophs.

The isolation of organisms with growth optima at 100°C and above has led to an ongoing quest for definition of the upper temperature limit for life (Stetter 1984). The 'hyperthermophiles', organisms which generally have an optimal growth temperature of 80°C and above and show no growth at 60°c or below, have recently been reviewed (Stetter *et al.* 1990).

1.3. Central metabolism

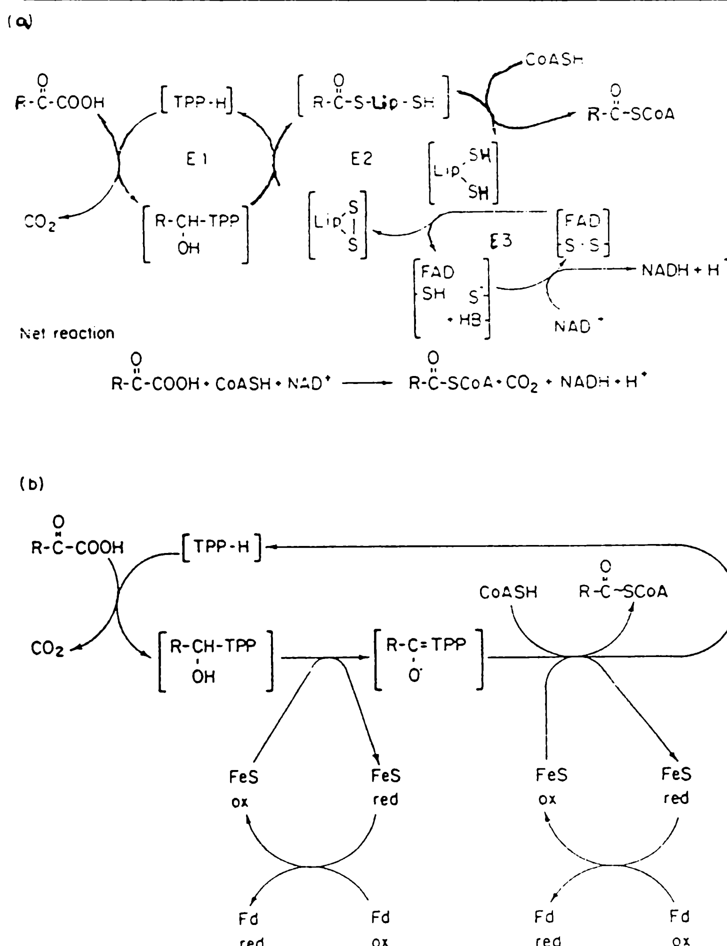
The metabolism of extreme thermophiles is of interest -initially to ascertain whether these organisms developed different metabolic strategies from those found in mesophiles for survival at such high temperatures. From an evolutionary point of view, studies on the

metabolism of archaebacteria will give an indication of the age and development of metabolic pathways of the eukaryotic, eubacterial, and archaebacterial kingdoms. To make comparisons between these, it has first to be determined which enzymes of central common metabolic routes are present in archaebacteria. Danson and co-workers have studied extensively the enzymes of archaebacteria involved in the metabolism of glucose and the citric acid cycle (Danson and Wood 1984; Danson *et al.* 1985; Budgen and Danson 1986; Smith *et al.* 1987a; reviewed by Danson 1988, 1989):

As methanogens are autotrophs growing on H_2 and CO_2 or some other C_1 compounds, the metabolism of glucose in these has been studied in the anabolic direction where evidence suggests the presence of an Emden-Meyerhof pathway acting in reverse. No evidence has been found in other archaebacteria for the presence of this pathway. In halophiles, the Entner-Doudoroff pathway is present although modified from the 'classical' (eubacterial) pathway and differing in the initial steps. Species of *Sulfolobus* and *Thermoplasma* appear to use a non-phosphorylated Entner-Doudoroff pathway. In *Thermoplasma* the pathway seems even further modified with conversion of glyceraldehyde into pyruvate via glycerol. Archaebacteria convert pyruvate into acetyl-CoA (Kerscher and Oesterhelt 1982). In *Thermoplasma* production of considerable amounts of acetate was observed and it has been suggested that the organism may derive energy from conversion of acetyl-CoA directly into acetate coupled to phosphorylation of ADP catalyzed by acetyl-CoA synthetase (ADP-dependent). Produced acetyl-CoA may be utilized via the citric acid cycle. Halophiles and *Thermoplasma* probably possess an oxidative citric acid cycle. The key enzymes have been found: citrate synthase and succinate thiokinase in halophiles, and isocitrate synthase, succinate thiokinase, and malate dehydrogenase in *Thermoplasma acidophilum*. *Sulfolobus brierleyi* and *Thermoproteus neutrophilus*, which are both facultative autotrophs seem to fix CO_2 via a reductive citric acid pathway. *Thermoproteus neutrophilus* appears to use a reductive citric acid cycle when it grows autotrophically, while it exhibits an incomplete cycle when it grows heterotrophically on acetate as the fumarate reductase activity is repressed. In methanogens anabolic variations of the citric acid cycle have been found. *Methanobacterium thermoautotrophicum* appears to possess an

incomplete reductive citric acid cycle while an incomplete oxidative citric acid cycle seems to be present in *Methanosarcina barkeri*
In many aerobic eubacteria, pyruvate and α-ketoglutarate are decarboxylated via the 2-oxo acid dehydrogenase complex with NAD as electron acceptor. The complex consists of three separate enzyme activities: 2-oxo acid decarboxylase, dihydrolipoamyl acyltransferase, and dihydrolipoamide dehydrogenase. The decarboxylase-catalyzed step is dependent on thiamine pyrophosphate (Fig.1.2.a). α-keto acid oxidoreductase is found in halophiles, methanogens, and sulphur-dependents. As electron acceptor either ferredoxin or cofactor F_{420} (in methanogens) is used. The α-keto acid is decarboxylated in a thiamine pyrophosphate-dependent reaction (Fig.1.2.b).

Fig.1.2. <u>Reaction mechanisms of the 2-oxo acid dehydrogenase multienzyme complexes and the 2-oxo acid:ferredoxin oxidoreductases (reprinted from Danson 1988)</u>



Reaction mechanisms of the 2-oxo acid dehydrogenase multienzyme complexes and the 2-oxo acid:ferredoxin oxidoreductases. (a) The 2-oxo acid dehydrogenase multienzyme complexes E1 (a 2-oxo acid decarboxylase), E2 (a dihydrolipoyl acyltransferase) and E3 (dihydrolipoamide dehydrogenase) represent the three enzymic activities that make up the complexes: TPP-H, thiamin pyrophosphate; Lip, lipoic acid; B is a base on the dihydrolipoamide dehydrogenase component. (b) The 2-oxo acid:ferredoxin oxidoreductases of the archaebacteria. TPP-H indicates thiamin pyrophosphate; Fd ferredoxin and FeS an enzyme-bound iron-sulphur cluster.

As all archaebacteria so far investigated possess either an α-keto acid oxidoreductase similar to that found in anaerobic eubacteria, or using F_{420} instead of ferredoxin as electron acceptor Kerscher and Oesterhelt (1982) have suggested that this enzyme existed before the divergence of archaebacteria, eubacteria, and the eukaryotic ancestor. Further, the presence in some archaebacteria of dihydrolipoamide dehydrogenase in the absence of the two enzymes normally found associated, belies any apparent function and may be of evolutionary significance (Danson *et al.* 1986; Smith *et al.* 1987b).

No other metabolic pathways in thermophilic anaerobic sulphur-dependent archaebacteria have been described. Much work is carried out on the isolation, purification, and often cloning of enzymes with industrial potential of the extreme thermophiles but these studies generally do not deal with the metabolism of these organisms as a whole.

1.4. Energy metabolism and ATPases in archaebacteria

Aspects of energy metabolism have been studied in organisms belonging to all major groups of archaebacteria.

In *Thermoplasma acidophilum*, a sulphate-stimulated ATPase has been described (Searcy and Whatley 1982). *T. acidophilum* was shown to be resistant to *N,N'*-dicyclohexylcarbodiimide (DCCD), a specific inhibitor of H^+-translocating ATPases, and to ouabain, which inhibits eukaryotic Na^+/K^+-ATPases. A b-type cytochrome and a quinone were detected and were the only components likely to constitute a respiratory chain for H^+ expulsion. Generation of ATP was proposed to most likely occur via substrate-level phosphorylation.

A membrane-associated ATPase has been reported and characterized in *Sulfolobus acidocaldarius* (Lübben and Schäfer 1987; Konishi *et al.* 1987). Results obtained by Lübben and Schäfer (1987) strongly suggest this ATPase to be involved in oxidative phosphorylation with H^+ as the coupling ion. Whole cells as well as purified ATPase show sensitivity to DCCD (Wakagi and Oshima 1986, 1987; Lübben and Schäfer 1987) and a F_0 related DCCD-binding proteolipid has been identified (Lübben and Schäfer 1989). This and other studied archaebacterial H^+-translocating ATPases (see below) are functionally related to F_0F_1-ATPases, as they catalyze synthesis of ATP. However, based on studies on sequence homology of ATPase subunits and immunological cross-reactivity with

other ATPases (Denda *et al.* 1988a, 1988b; Lübben *et al.* 1987; Konishi *et al.* 1990) archaebacterial ATPases appear related, yet distinct to both F_0F_1-type and vacuolar ATPases. Close relationship between the H^+-ATPase of *S. acidocaldarius* and vacuolar ATPases indicates that these share a common ancestor, and that the vacuolar ATPase may have been internalized from the plasma membrane by a process resembling phagocytosis (Nelson and Taiz 1989). Vacuolar and F_0F_1-type ATPases in turn are strongly related families. However, the studies on the three types of ATPases can only be to understand the underlying mechanism of these enzymes rather than to reconstruct a common hypothetical ancestor (Schäfer *et al.* 1990a)

Electron transport has been studied in *S. acidocaldarius* (Anemüller *et al.* 1985; Moll and Schäfer 1988; Schäfer *et al.* 1990a, 1990b). A model of a primitive respiratory chain with a branched electron transport system has been proposed. A NADH-dehydrogenase and a succinate-dehydrogenase containing an iron-sulphur/flavoprotein, the unique quinone 'caldariella quinone', and as terminal oxidase cytochrome aa_3 (Anemüller and Schäfer 1990) has been detected. Presence of a cytochrome *b/o* has been indicated (Schäfer *et al.* 1990a). No cytochrome *c* oxidase has been detected.

At present no reports on complete respiratory chains or ATPases are available in anaerobic sulphur-utilizing archaebacteria.

In halophiles, photophosphorylation has been well characterized (for reviews see Lanyi 1979; Stoeckenius and Bogomolni 1982; Stoeckenius 1985). Proton-translocating ATPases from halophiles have been described and characterized (Nanba and Mukohata 1987; Hochstein *et al.* 1987). As with the ATPase from *S. acidocaldarius*, ATPases from halophiles were found to differ from eubacterial F_0F_1-type ATPases (Mukohata *et al.* 1987; Stan-Lotter and Hochstein 1989).

Energy metabolism in methanogens has received much attention in the past decade. Studies have involved characterization of membrane-bound H^+ translocating ATPases of *Methanobacterium thermoautotrophicum* (Doddema *et al.* 1978) and *Methanosarcina barkeri* (Inatomi 1986). These ATPases were shown to be closely related to the ATPase of *S. acidocaldarius* (Inatomi *et al.* 1989). The ATPase of *Methanolobus tindarius* (Scheel and Schäfer 1990) also shares common characteristics with both vacuolar and F_0F_1 ATPases.

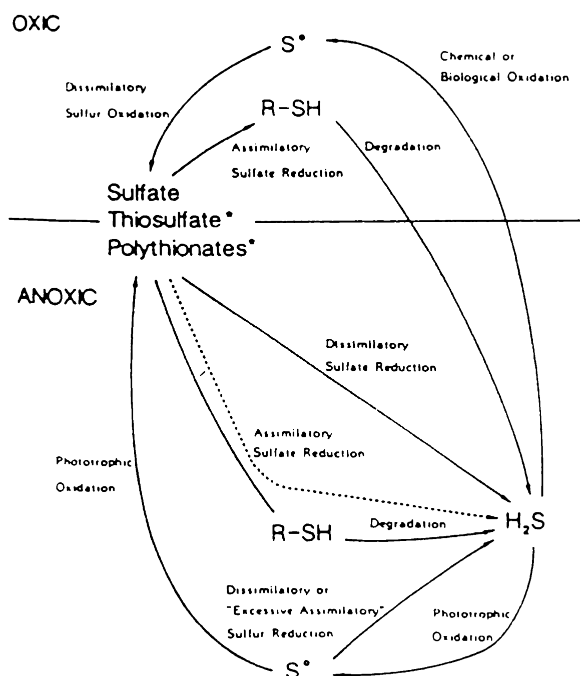
1.5. Sulphur metabolism

1.5.1 The sulphur cycle

The biological sulphur cycle (Fig.1.3) involves the conversions of elemental sulphur, sulphide, sulphite, sulphate, thiosulphate and polythionates. Bacteria of both aerobic and anaerobic habitats; eubacteria and archaebacteria are involved in this cycle.

Elemental sulphur is reduced by eubacteria and archaebacteria to hydrogen sulphide. This in turn is oxidized to S^0 anaerobically by green and purple sulphur bacteria or aerobically by bacteria like *Thiobacillus* or *Beggiatoa*, forming sulphate. Chemical oxidation of sulphide to S^0 or thiosulphate and sulphite in the presence of oxygen occurs in a rapid reaction. Elemental sulphur, sulphate, sulphite, and thiosulphate produced from oxidation are reduced in anaerobic environments to sulphide. These processes are summarized in Fig.1.3. For reviews see Widdel (1988) and Le Faou *et al.* (1990).

Fig. 1.3. <u>The biological sulphur cycle</u>
(reprinted from Le Faou *et al.*1990)



The biological sulfur cycle. * Thiosulfate and polythionates are not involved in all the represented metabolic pathways, however, as they are sulfur sources for both assimilative and dissimilative processes, they were placed concurrently with sulfate in the sulfur cycle.

1.5.2. Sulphur reduction

Sulphur reduction has been studied in eubacteria and descriptions of organisms and the enzymes and electron carriers involved have recently been reviewed (Widdel 1988; Le Faou *et al.* 1990). Most work reported has focussed on eubacteria growing on single organic substrates and sulphur, conserving energy by linking reduction of sulphur to oxidation of the organic substrate. Sulphur respiration has been studied in some detail in *Wolinella succinogenes* (Macy *et al.* 1986; Schröder *et al.* 1988; Wloczyk *et al.* 1989), in *Desulfuromonas acetooxidans* (Pfennig and Biebl 1976), and some other strains of eubacteria (Zöphel *et al* 1988). A novel genus of thermophilic eubacterium, *Desulfurella acetivorans* carrying out sulphur reduction linked to oxidation of acetate has been described recently (Bonch-Osmolovskaya *et al.* 1990a).

Several sulphate reducing eubacteria are also capable of using elemental sulphur as terminal electron acceptor (Pfennig and Biebl 1976; Biebl and Pfennig 1977; Widdel 1988), while no true sulphur reducers reduce sulphate.

While so far only two species of the single genus of sulphate reducing archaebacteria, *Archaeoglobus fulgidus* strain Z (Zellner *et al.* 1989) and *A. profundus* (Burggraf *et al.* 1990), have been reported, several archaebacteria from a variety of genera carry out reduction of elemental sulphur. Dissimilatory reduction of sulphur has been observed in several methanogens (Stetter and Gaag 1983; Stetter 1985; Le Faou *et al.* 1990) where addition of elemental sulphur led to production of H_2S and a great reduction in methane production in an almost complete switch to lithotrophic growth. Chemolithoautotrophic growth has been shown in various sulphur-utilizing archaebacteria (Table 1.2). *Pyrodicitum (Pd.) occultum* and *Pd. brockii* are strict sulphur-H_2 autotrophs (Stetter *et al.* 1983). Some species of *Thermoproteus* (Fischer *et al.* 1983) and *Pyrobaculum islandicum* (Huber *et al.* 1987) are facultative chemolithotrophs with H_2, CO_2, and elemental sulphur. Among the *Sulfolobales* several species of *Sulfolobus* are capable of chemolithotrophic growth with elemental sulphur while *Acidianus infernus* and *Desulfurolobus ambivalens* are obligate chemolithotrophs. No chemolithotrophic growth has been observed within the genera of *Thermofilum, Desulfurococcus,*

Staphylothermus, 'Caldococcus', Pyrococcus, or *Thermococcus* (Table 1.2). None of these archaebacteria have been found capable of utilizing sulphite, thiosulphate, or sulphate as electron acceptor (Le Faou *et al.* 1990). Most members of the *Thermoproteales* and *Themococcales* grow on organic substrates such as yeast extract and peptone and carry out "sulphur respiration" (Stetter 1985; Le Faou *et al.* 1990). The facultatively and obligately anaerobic heterotrophic sulphur reducing archaebacteria are thought to carry out "sulphur respiration" with H_2S and CO_2 as products. Alternatively, they may carry out fermentation with CO_2 and other products such as volatile fatty acids which may be linked to sulphur respiration. However, very little is known about these pathways.

The interaction of cells with sulphur has been studied in *Pyrococcus furiosus* (Blumentals *et al.* 1990). In this organism it was found that no direct contact with the sulphur particles was required for the production of H_2S. Polysulphides were required for solubilization of the sulphur, causing opening-up of the 8-atom ring structure of elemental sulphur and subsequent chain degradation and formation of H_2S. In their studies Blumentals *et al.* found that Na_2S, or H_2S, and cystine were potent solubilizing agents of elemental sulphur, which may explain earlier findings that traces of sulphide in the medium were required for growth with sulphide production (Zillig *et al.* 1983b). Sulphur metabolism of *P. furiosus* was suggested to be purely dissimilatory as no radiolabelled sulphur was integrated into cell protein. Further, sulphur did not seem to enter the cell in the reduction reaction which was assumed to be a membrane-bound process.

Not all sulphur reducing heterotrophs were found to have an obligate requirement for sulphur. *Pyrodictium abyssum, Desulfurococcus (D.) mobilis, D.mucosus,* and *D. amylolyticus, Pyrococcus (P.) furiosus* and *P. woesei, 'Caldococcus litoralis',* and *Thermococcus (T.) celer* and *T. litoralis* were found to grow in the absence of sulphur. (Le Faou *et al.* 1990). In all cases, absence of S^0 resulted in reduced growth although this affected growth rate in *T. celer* (Zillig *et al.* 1983b), *D. mobilis,* and *D. mucosus* (Zillig *et al.* 1982b), while in *T. litoralis* (Belkin and Jannasch 1985), *D. amylolyticus* (Bonch-Osmolovskaya *et al.* 1988), and *P. woesei* (Zillig *et al.* 1987) final growth yield was reduced. In *P. furiosus* (Fiala and Stetter 1986) growth rate and yield

were the same in the presence or absence of S^0 as long as as culture vessels were continuously flushed with N_2.

Among these heterotrophs which do not require sulphur for growth, activity of hydrogenase has been studied. *T. celer* grown in the absence of sulphur was found to contain high levels of hydrogenase activity (Adams 1990a). The hydrogenase of *P. furiosus* has been purified and characterized (Bryant and Adams 1989). The hydrogenase of *P. furiosus* is very thermostable. However, in contrast to all other hydrogenases it preferentially catalyzes H_2 production. This is likely to be its physiological role as *P. furiosus* is a fermentative organism and presumably has to evolve H_2 for re-oxidation of its reduced electron carriers. Functionally this hydrogenase is most closely related to hydrogenases of species of *Clostridium*, while structurally it resembles NiFe hydrogenases. It differs, however, from both types in its insensitivity to inhibitors (CO, nitrite, C_2H_2) (Bryant and Adams 1989; Adams 1990a). From an evolutionary point of view this hydrogenase is of interest as it shares characteristics with hydrogenases of aerobic and anaerobic eubacteria and with methanogenic hydrogenases catalyzing reduction of cofactor F_{420}. This is in support of evolutionary models placing the *Thermococcales* closest to the common ancestor (section 1.1). Ferredoxin is the natural electron donor to the hydrogenase. Ferredoxin has been purified and characterized from *Thermoplasma acidophilum, Sulfolobus acidocaldarius, Desulfurococcus mobilis* (Kerscher *et al.* 1982), and *P. furiosus* (Aono *et al.* 1989).

1.6. Sodium requirement in bacteria

A requirement for sodium has been reported in a diversity of bacteria for various functions. The great importance of sodium ions in some essential processes has become apparent only in the past decade, and sodium has emerged as the second most important cation next to protons, in transport and energetic processes involving bacterial cytoplasmic membranes.

A requirement for Na^+ has been demonstrated in respect to different areas:

1) Na^+/solute **co-transport**
2) Na^+ coupled energy conservation and transduction
3) pH homeostasis
4) activation of enzymes catalyzing Na^+ translocation

Na^+ appears essential for many bacteria of Na^+-rich environments; marine, alkalophilic, and rumen bacteria. Neutrophilic freshwater bacteria do not seem to require Na^+ to such an extent but an increasing number of examples are now evident of Na^+/solute transport systems in such organisms.

Sodium/solute transport systems appear wide-spread amongst bacteria. Several marine organisms require Na^+ for the transport of most if not all metabolites into the cell. *Alteromonas haloplanctis* requires Na^+ for uptake of every metabolite studied, *Vibrio fisheri* shows such requirement for all metabolites tested except for glucose (Droniuk *et al.* 1987). In *Vibrio alginolyticus* the transport of nineteen amino acids (cited in Wisse and MacLeod 1989) and of sucrose is Na^+-dependent (Kakinuma and Unemoto (1985). *Pseudomonas doudoroffi* 70 requires Na^+ for uptake of acetate, glutamate, and succinate (Wisse and MacLeod 1989). *Bacillus firmus* RAB (Krulwich *et al.* 1982) and *B. alkalophilus* (Guffanti *et al.* 1981) and other alkalophiles (cited in Krulwich 1983) require Na^+ for the uptake of metabolites via Na^+/solute symport systems. Sodium-dependent amino acid transport has been studied in several rumen bacteria (Russel *et al.*1988; Chen and Russel 1989; Chen and Russel 1990).

However, Na^+-dependent solute uptake is not restricted to bacteria of natural Na^+-rich environments. *Methanococcus voltae* possesses a Na^+-dependent transport system for isoleucine (Jarrell *et al.* 1984). In *Escherichia coli* the melibiose uptake system, the glutamate uptake system, and proline uptake are Na^+-dependent (Steward and Booth 1983 and references cited therein). In *Salmonella typhimurium*, uptake of proline via the major proline permease uptake system is absolutely-dependent on Na^+ and transport proceeds via a Na^+/proline symport (Cairney *et al.* 1984). Uptake of amino acids has been studied in one thermophilic bacterium, *Clostridium fervidus* (Speelmans *et al.* 1989). The uptake of twelve amino acids in membrane vesicles of *C. fervidus* was shown to be dependent on a Na^+ gradient across the membrane.

In Na^+/solute transport the solute is transported against its concentration gradient coupled to Na^+ influx. A transmembrane Na^+ gradient is required where $[Na^+_{in}] > [Na^+_{out}]$. For Na^+/solute symport a transmembrane charge gradient or membrane potential generally does not seem to be required, although it further stimulated solute transport in some of the uptake systems mentioned above.

A transmembrane Na^+ gradient can be formed in different ways: via secondary transport involving H^+/Na^+ antiporters or via primary transport involving Na^+ translocation coupled to a chemical reaction. Movement of Na^+ across the cytoplasmic membrane via H^+/Na^+ antiporters seems to be a widespread mechanism amongst bacteria (Lanyi 1979). Sodium extrusion via primary Na^+ pumps has been demonstrated in several organisms. The transport of Na^+ coupled to ATP hydrolysis and the presence of an inducible ATPase exchanging Na^+ for K^+ was reported in *Streptococcus faecalis* (Heefner *et al.* 1980; Heefner and Harold 1980, 1982; Kakinuma and Igarashi 1990a, 1990b), and a similar mechanism has been proposed in *Mycoplasma mycoides* var. Capri (Benyoucef *et al.* 1982a, 1982b). The Na^+-translocating ATPase differed from eukaryotic Na^+/K^+ ATPases and was not sensitive to the inhibitor ouabain. In both organisms a H^+/Na^+ antiporter has been proposed which appears to be constitutive in *S. faecalis* (Kakinuma and Igarashi 1990a). *S. bovis* has been reported to expel Na^+ from the cell via a Na^+-translocating ATPase which in contrast to that of *S. faecalis* was constitutive, K^+-independent, and not affected by changes in the pH between pH 6 and 8 (Strobel and Russel 1989).

A sodium-translocating ATPase has been reported in *Methanococcus voltae* (Carper and Lancaster 1986; Dharmavaram and Konisky 1987, 1989). This seems distinct in its physiological role and properties from ATPases described in other archaebacteria. The novel vanadate-sensitive ATPase was resistant to DCCD, ouabain, and oligomycin and has been classified as a P-type ATPase. It therefore differs fundamentally from other archaebacterial (H^+ translocating) ATPases mentioned above and is similar to cation-translocating ATPases as found in *Streptococcus faecalis* (see above). Its role is not generation of ATP but rather translocation of ions (Na^+) across the membrane at the expense of ATP. Translocation of Na^+ coupled to ATP hydrolysis would provide a Na^+ gradient which in this organism was found to be required for uptake of some amino acids (see below).

Activity of ATPase (ATP synthase) in *Methanobacterium*

thermoautotrophicum which was stimulated by Na^+ has been reported (Smigan *et al.* 1988). Cells grown in 50mM NaCl were more stimulated in their ATP production by addition of a further 50mM NaCl than cells grown in 5mM NaCl. DCCD (250μM) inhibited activity in either 5mM or 50mM NaCl by only up to 40%, while inhibition increased to about 60% when the same concentration of DCCD was added together with 50mM NaCl to cells grown either with 5mM or 50mM NaCl. Speculations of a possible similarity to the Na^+ translocating ATPase of *Propionigenium modestum* were made. Other primary Na^+ pumps expel Na^+ coupled to a decarboxylation reaction. Such mechanism was first reported in *Klebsiella aerogenes* (Dimroth 1982), *Veillonella alcalescens* (Hilpert and Dimroth 1982), and *Acidaminococcus fermentans* (Buckel and Semmler 1982).

In *V. alginolyticus* the Na^+ pump is coupled to the respiratory chain (Unemoto and Hayashi 1979).

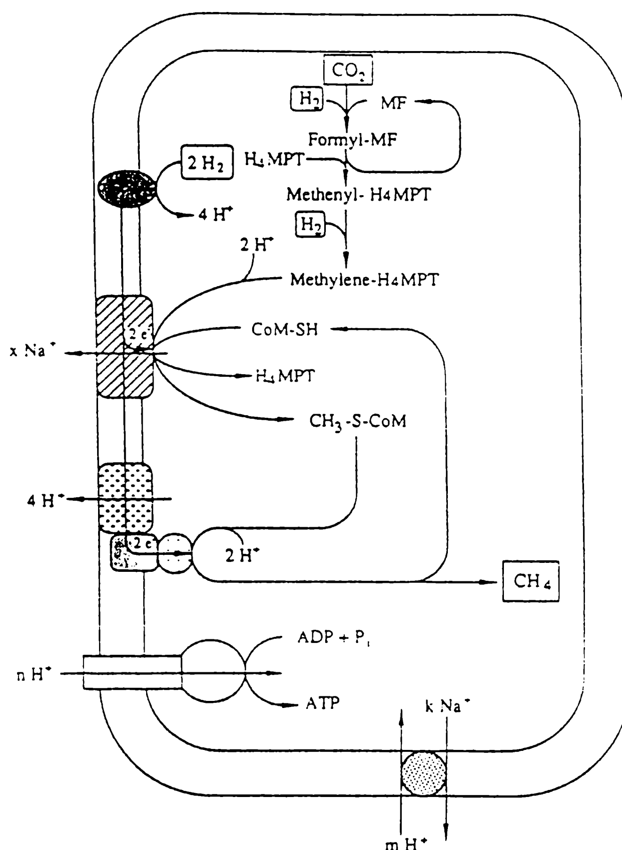
The pathways employed in methanogenesis are well studied in several species (for reviews see Jones *et al.* 1987; Thauer 1990; Gottschalk and Blaut 1990).

It has been known for a number of years that sodium is required for growth and methane formation of methanogens (Perski *et al.* 1981). The role of sodium has been elucidated (Schönheit and Perski 1983; Kaesler and Schönheit 1989a, 1989b; Gottschalk and Blaut 1990). A Na^+/H^+ antiporter has been described in *Methanobacterium thermoautotrophicum* (Schönheit and Beimborn 1985) and in *M. barkeri* (Müller *et al.* 1986). Electron transport driven sodium extrusion was found in *M. barkeri* (Müller *et al.* 1988a, 1988b).

Cycling of both H^+ and Na^+ across the membrane during methanogenesis has been proposed to couple endergonic and exergonic reactions (Kaesler and Schönheit 1989a, 1989b). The involvement of Na^+ in methanogenesis of *Methanosarcina barkeri* grown on methanol has been studied (Blaut *et al.* 1986; Müller *et al.* 1987). While originally, Na^+ was thought not to be involved in methanogenesis from methanol recent work (Müller et al. 1988a, 1988b) has shown the requirement for a secondary $\Delta\mu Na^+$.

In addition to proton translocation the sodium pump represents a second site of energy conservation. The current model of involvement of Na^+ gradients in methanogenesis is shown in Fig.1.4.

Fig.1.4. Ion translocation during the conversion of CO_2 and H_2 to methane (reprinted from Gottschalk and Blaut 1990) (H4MPT, tetrahydromethanopterin; CoM-SH, coenzyme M; MF, methanofuran)



Sodium and K^+ gradients across the membrane are suggested to be involved in the control of cytoplasmic pH homeostasis, especially in alkalophiles (Lanyi 1979; Padan *et al.* 1981; Krulwich 1983; Booth 1985; Krulwich and Guffanti 1989). These gradients are also thought to serve in energy storage (Skulachev 1978; Brown *et al.* 1983; Drachev *et al.* 1985).

In 1984 Hilpert *et al.* described ATP synthesis in *Propionigenium modestum*. The ATP synthase in this organism differed from all other F_0F_1-type ATPases described up to that time as it preferentially used Na^+ and not H^+ as coupling ion but otherwise shared common properties with other F_0F_1 ATPases (Laubinger and Dimroth 1987; Hoffmann *et al.* 1990). A proposed ATP synthase (utilizing Na^+ as the coupling ion) has already been mentioned for *Methanobacterium thermoautotrophicum*. A Na^+-translocating ATPase involved in ATP synthesis has been also reported in *V.*

alginolyticus. In this organism co-existence of Na^+- and H^+-motive ATPase activity has been detected and the possibility of one ATP synthase operating with both ions has been proposed (Dibrov *et al.* 1988; Skulachev 1989. In this organism a complete Na^+-cycle has been described (Dibrov *et al.* 1986a, 1986b). The roles of Na^+ in membrane-linked energy transduction and Na^+ cycling across the membrane have been reviewed (Skulachev 1984, 1985, 1989; Rosen 1986; Dimroth 1987).

1.7. Isolate ANI

At the onset of this study a preliminary characterization of this isolate had been given (Morgan and Daniel 1982). ANI was described as a thermophilic sulphur-dependent coccus of regular to irregular shape and 0.5-2.0 µm diameter. Sodium chloride and sulphur were required for growth. Cell wall analysis showed absence of muramic acid, galactosamine, and glucosamine. Resistance to antibiotics normally inhibiting cell wall and protein synthesis in eubacteria further supported the classification of ANI as an archaebacterium. It has been deposited with the Deutsche Sammlung von Mikroorganismen (DSM), (Braunschweig, Germany) as a *Desulfurococcus* species designated DSM 2770. In the past few years more information on this organism has become available.

Isolate ANI has been assigned to the *Thermococcales* by Zillig *et al.* (1987) who reported results on Ouchterlony gel-immunodiffusion tests on the DNA-dependent RNA polymerases from *Pyrococcus woesei, Thermococcus celer, and* isolate ANI. While *P. woesei* polymerase showed incomplete cross-reaction with the other two, both *T. celer* and ANI showed complete cross-reaction using both antisera.

Zillig *et al.* (1987) also presented a phylogenetic tree derived from DNA/rRNA cross-hybridization velocities (see Fig.1.1.c). Isolate ANI was grouped within the *Thermococcales*, more closely related to *T. celer* than to *P. woesei*.

The lipid composition of isolate ANI has been described previously (De Rosa and Gambacorta 1988; Lanzotti *et al.* 1989).

Lipids make up approximately 3.5% of the cell dry weight and 8-10% of lipid extract is a mixture of hydrocarbons, mainly isoprenoids (Lanzotti *et al.* 1989). As for *Thermococcus celer* and *Pyrococcus woesei*, isolate ANI virtually lacks phytanyl tetraether lipids. Forty

percent of the lipid is 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-1'-myo-inositol (PID), which constitutes 80% of lipids of *T. celer* (De Rosa *et al.* 1987), and 90% of *P. woesei* (Lanzotti *et al.* 1989). Forty-five percent of the lipid of ANI is accounted for by the phosphate glucosyl diether (PGD) which has not been reported in any other archaebacterium to date (Lanzotti *et al.* 1989).
Studies on reverse gyrase activity in archaebacteria has shown this enzyme to be present in ANI (Collin *et al.* 1988). Screening for extracellular enzymes from ANI has shown strong amylolytic and some proteolytic activity (Bragger *et al.* 1989).

1.8. Aim of this project

Although there is abundant information on archaebacterial organization on the molecular level, studies of the physiology of archaebacteria have in the main concentrated on methanogens and halophiles, possibly because these organisms have been isolated and cultured over many decades compared to the relatively recent isolation of sulphur-utilizing extreme thermophiles.
Nothing is known about the ecology of most of the sulphur-utilizing archaebacteria. Investigation of the distribution of isolate ANI in New Zealand hot pools was hoped to provide some results on which factors determine the presence or absence of these organisms.
While the metabolism of autotrophic anaerobic archaebacteria has been studied, there is really nothing known about the metabolism of the anaerobic heterotrophic sulphur-utilizing archaebacteria. In recent reviews by Stetter (1985) and Le Faou *et al.* (1990) energy yielding reactions of these organisms were shown to be either complete oxidation of an organic compound to CO_2 coupled to the reduction of sulphur, fermentation of 'yeast extract' to organic acids and CO_2, and "unknown anaerobic respiration" of 'yeast extract' with unknown products ("+CO_2?"). In recent publications on novel species, production of volatile fatty acids and CO_2 was reported (Fiala *et al.* 1986; Miroshnichenko *et al.* 1989; Zillig *et al.* 1990; Bonch-Osmolovskaya *et al.* 1990b) from growth on peptides. No metabolic pathways have been reported. Investigation into the pathways for formation of the end products detected seemed a worthwhile study. The aim of this project was to obtain an overall model of growth of isolate ANI; which substrates it utilizes, how the substrate is metabolized, and how the organism gains energy.

Chapter 2: Ecological Aspects

2.1.Introduction:

Hot pools represent one of the favoured habitats for the isolation of novel eubacteria and archaebacteria, and for ecological study. In New Zealand, the microflora of some hot pools has been described by Brock and Brock (1971). A variety of techniques have been used to study the diversity and abundance of microorganisms in hot pools. Methods fall into the two general categories of enrichment/subculture isolation and *in situ* enumeration/identification.

Enrichment/isolation methods are simpler and more traditional. They have been commonly used in investigations of novel habitats (e.g. Patel *et al.* 1986a; 1986b, Hudson *et al.* 1990). The result of this approach is the bulk of pure cultures of thermophilic bacteria described in the literature. The shortcomings of the procedure have been frequently commented upon. Generally, organisms which have a high growth rate on rich medium are favoured in isolation. Although it is possible to alter the selective pressure by adjusting conditions of one or more growth parameters, this is time consuming and haphazard since nutritional requirements of target organisms are generally unknown. Enrichment methods produce information on the presence or absence of a particular organism in a habitat and can be modified (by use of MPN) to give estimates of abundance of an organism in a habitat. The relationship of the abundance of a selected isolate to the total biomass of a habitat is often not achieved.

In situ methods may provide better estimates of bacterial abundance and the prevalence of particular types. Many approaches have been taken, e.g., *in situ* colonization (Patel 1984, Patel *et al.* 1986c), fluorescent antibody staining (Patel 1984), lipid fingerprints (Ward *et al.* 1985). The use of genetic markers for identification and enumeration of specific groups of bacteria without prior culture has been described by Pace *et al.* (1985) and Ward *et al.* (1990a, 1990b).

It seemed a reasonable starting point in the characterization of isolate ANI to determine how wide-spread the organism was and what determined its distribution. Isolate ANI was originally isolated from Kuirau Park, Rotorua, New Zealand (Morgan and Daniel 1982). Patel

(1984) and Martin (1985) reported on the distribution of 'ANI-like' organisms isolated from New Zealand hot pools. The study by Patel (1984) showed the distribution of archaebacterial cocci with a sodium requirement, growing at the same pH and temperature as ANI. Fluorescent antibodies prepared against ANI gave different fluorescent intensities when cross-reacted with these other isolates, indicating that the group was possibly composed of heterogenous and distinct serotypes. Different serotypes have been previously described for *Sulfolobus acidocaldarius* isolated from hot springs in Yellowstone Park (Bohlool and Brock 1974). It was found that some hot springs harboured more than one *Sulfolobus* serotype. Patel (1984) obtained samples from 50 pools and carried out enrichments for ANI-like organisms on them. Only 7 pools yielded positive enrichments (Patel 1984). However, most of the pools sampled were apparently of too acidic pH (as low as pH 2.1) for ANI-like organisms and from results presented one could only conclude that the organisms were restricted to pools with temperatures between 75°C and 96°C and of pH 5 to pH 8. Martin (1985) sampled 66 pools in her study. From 15 pools ANI-like isolates were obtained. ANI-types were found in pools of a wide temperature and pH range (68°C-"boiling" and pH 3.6-9.1). Many pools with temperature and pH values which were within the range suitable for ANI culture, did not yield positive enrichments. Thus, other factors apart from temperature and pH were involved in determining the distribution of ANI-like organisms. Martin (1985) suggested a regional factor to be involved in the distribution of ANI-like organisms in New Zealand as some thermal areas appeared to be devoid of such organisms. The 15 isolates obtained in Martin's study were analyzed using pyrolysis mass spectrometry. She found three distinct groups of organisms which were not in accord with geographical distribution. The strains all showed 90% homology (compared to 12.5% homology with *Thermococcus celer*) using that technique of analysis, differences between strains were most probably at sub-species level.

In laboratory culture isolate ANI has an obligate requirement for Na^+ for growth at concentrations limited to a non-marine environment (see section 3.3.6). The sodium content of pool water is a likely additional determinant of the occurrence of ANI-like organisms and was used in this study as a third parameter in defining the organism's distribution.

2.2.Methods:

2.2.1. Sampling

Samples were obtained from the North Island of New Zealand and the Azores (provided by HW Morgan). Samples from New Zealand originated from the the thermal areas of Rotorua (Rt), Waimangu (Wai), Waiotapu (Wtp), Mokai (Mok), Waikite (Wkt), Ketatahi (Ket), and Tokaanu (Tok), all located in the central North Island.

Samples from the Azores were taken from Praia do Fugo Caldera Grande and Caldera Grande at Furnas.

Pool temperatures ranged from 36° to 101°C, the majority being between 70° and 80°C. The pH ranged from pH 4.0 to pH 9.0, with most between pH 7.0 and pH 8.0.

2.2.2.Sampling Techniques

Samples for detection and enumeration of ANI-like organisms were injected into 25ml glass tubes sealed with rubber stoppers (Bellco). The tubes contained an oxygen-free nitrogen atmosphere and 0.2ml of a neutralized 10% Na_2S solution as reductant to ensure anaerobic conditions and were filled to the top with sample. Samples were transported and stored at ambient temperatures.

Culturing from samples usually occurred within two days apart from those from the Azores which were analysed two weeks after sampling.

Samples for Na^+ determination were taken using 100ml glass bottles which were submersed into the pool and filled with sample then stoppered.

The temperature, pH ,and conductivity of the pools were measured using a digisense pH/mV/°C meter.

2.2.3.Enrichment media

For media, solutions, and buffers listed in this and the following chapters all chemicals used were of highest grade available and from major suppliers. Gases were supplied by New Zealand Industrial Gases (Wellington, New Zealand). Unless otherwise stated, 'H_2O' always refers to distilled water.

The following media were used:

ANI medium: ($g\ell^{-1}$ H_2O) K_2HPO_4, 1.5g; $MgCl_2 \cdot 7H_2O$, 0.3g; NaCl, 2.5g; Wolin's Vitamin Solution (Wolin *et al.* 1963), 1ml; Zeikus Trace Elements (Zeikus *et al.* 1979), 5ml; trypticase peptone, 8g; sodium thioglycollate, 0.5g; resazurin (0.1%), 1ml. The vitamin solution of Wolin (1963) contained (mg.ℓ^{-1} H_2O) biotin, 2.0; nicotinic acid, 5.0;

pantothenic acid, 5.0; vitamin B_{12}, 0.1; ρ-aminobenzoic acid, 5.0; thioctic acid, 5.0. The trace elements of Zeikus contained (gl^{-1} H_2O) nitrilotriacetic acid, 12.8; $FeCl_3 \cdot 6H_2O$, 0.2; $MnCl_2 \cdot 4H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.017; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnCl_2$·, 0.1; $CuCl_2$, 0.02; H_3BO_4, 0.1; $NaMoO_4 \cdot 2H_2O$, 0.01; NaCl, 1.0; Na_2SeO_3, 0.02. The pH was adjusted to pH 7.4. The medium was sterilized by autoclaving (121°C for 20 minutes).

For preparation of Gelrite plates, per 1000ml of ANI medium $MgSO_4 \cdot 7H_2O$, 1g; and Gelrite (Merck, San Diego, CA, USA), 11g; were added. The medium was boiled prior to autoclaving ensuring that the Gelrite was completely dissolved.

To determine if growth of isolate ANI was inhibited by either sodium ion deficiency or by some other unknown factor, samples of pool water shown not to harbour isolate ANI were supplemented with all components of ANI medium except for NaCl and reduced. Aliquots of 10ml of this medium were distributed to Hungate tubes ((Kimble, Owens-Illinois, USA), autoclaved, and subsequently inoculated with 0.1ml of an actively growing culture of ANI. Further aliquots of the medium were supplemented with an additional 50mM NaCl prior to inoculation.

Db medium: $g\ell^{-1}$; $(NH_4)_2SO_4$, 1.3; $CaCl_2 \cdot 2H_2O$, 0.075; $MgSO_4 \cdot 7H_2O$, 0.28; KH_2PO_4, 0.28; yeast extract, 0.1; trypticase peptone, 2; Nitsch's Trace Elements (Brock *et al.* 1972), 1ml; $FeCl_3$ (0.02%), 1ml; L-cystine, 0.6g; resazurin (0.1%), 1ml. The pH was adjusted to pH 5.5. Db medium was not autoclaved, but instead incubated overnight at 88°C and any medium showing any sign of turbidity after that incubation was discarded. Before use, the medium was reduced by adding $1ml\ell^{-1}$ of a sterile, neutralized 10% Na_2S solution.

Yeast Extract (YE) medium: $g\ell^{-1}$ $(NH_4)_2SO_4$, 0.65; $CaCl_2 \cdot 2H_2O$, 0.074; $MgSO_4 \cdot 7H_2O$, 0.28; KH_2PO_4, 0.28; NaCl, 7; yeast extract, 5; MOPS, 4; Nitsch's Trace Elements (Brock *et al.* 1972), 1ml; Wolin's Vitamin Solution (Wolin *et al.* 1963), 1ml; resazurin (0.1%), 1ml. Nitsch's trace elements contained ($mg\ell^{-1}$ H_2O) $MnCl_2 \cdot 4H_2O$, 1.8; $Na_2B_4O_7 \cdot 10H_2O$, 4.5; $ZnSO_4 \cdot 7H_2O$, 0.22; $CuCl_2 \cdot 2H_2O$, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.03; $VoSO_4 \cdot 2H_2O$, 0.03; $CoSO_4 \cdot 7H_2O$, 0.01. The pH was adjusted to pH 7.0. The medium was autoclaved and prior to use reduced by addition of $1ml\ell^{-1}$ of a sterile, neutralized solution of 10% Na_2S.

Preparation of anaerobic media: All media were boiled to drive off oxygen and subsequently cooled under a stream of oxygen-free N_2. Media were dispensed in an anaerobic chamber (Coy, Inc., USA). For enrichments, media were dispensed in 9ml aliquots into 15ml Hungate

tubes and sealed with butyl rubber stoppers.
Medium containing Gelrite was boiled, autoclaved, and flushed with filter sterilized (0.22μm filter) N_2. Plates were poured in the anaerobic chamber. For incubation, metal anaerobic jars were used, slightly pressurised with N_2.

2.2.4. Evaluation of presence/abundance of ANI-like organisms and some other bacteria

Numbers of ANI-like organisms in pools were determined using Most Probable Numbers (MPN) dilution series (Collins and Lyne, 1976) in ANI medium. Inoculations were in triplicate over the dilution range from 10^{-1} to 10^{-5} and incubated at 80°C for 48 h. Growth was measured by visual assessment of turbidity and all tubes showing turbidity were checked microscopically using a phase contrast microscope (Olympus BH-2; Olympus Optical, Japan). Water samples (1ml of a 10^{-1} dilution in ANI medium) were also plated out onto ANI medium- Gelrite plates, incubated for four days, and checked for growth daily.
In addition, 10^{-1} dilutions of samples were made into Db and YE medium, and incubated at 88°C, and ANI medium without NaCl, and incubated at 80°C, all for 48 h.
ANI-like organisms were identified by morphological characteristics such as regular coccal shape at all stages of growth, an inclusion body observed at later stages of growth which is thought to be sulphur (see section 3.3.1), cell size, which was about 0.5μm in early stages of growth and up to 2.0μm in diameter at later stages of growth (after 48 h incubation). Sulphide was produced by all ANI-like organisms which was detected by smell at the end of growth. Several randomly selected enrichments positive for ANI-like organisms were also analysed for volatile fatty acid production and checked for inhibition of growth in the presence of the antibiotics chloramphenicol (100μg/ml), penicillin G (10μg/ml), and mevinolin (50 μg/ml).

2.2.5.Determination of the Na^+ content of pool water

The Na^+ content was measured using a Flame Photometer (Pertacourt PFP-1). Standard NaCl concentrations were used from 0.1 to 7.5 $g\ell^{-1}$ and yielded a linear response from 0 to $2.5 g\ell^{-1}$.

2.3. Results and Discussion:

A) Distribution of ANI-like organisms in thermal areas sampled

Table 2.1. Temperature, pH, and sodium content of pools sampled and Most Probable Number counts for ANI-like organisms

Area	Pool	Temperature	pH	$Na^+ (g\ell^{-1})$	(MPN)
Waiotapu	Wtp 1	77°C	5.5	0.85	15
	Wtp 2	98°C	5.5	1.10	NG
	Wtp 3	98°C	4.0	1.25	NG
	Wtp 4	76°C	5.4	2.65	28
Waimangu	Wai 1	88°C	7.2	0.85	75
	Wai 2	98°C	8.2	0.75	NG
	Wai 3	72°C	8.2	1.20	4
	Wai 4	72°C	7.9	1.05	NG
	Wai 5	60°C	7.8	1.00	43
	Wai 6	75°C	8.8	1.00	93
	Wai 7	75°C	8.8	1.25	NG
Rotorua	Rt 1	75°C	7.5	0.75	150*
	Rt 1AB	70°C	7.4	0.80	150*
	Rt 2	64°C	7.1	0.80	93*
	Rt 4	92°C	7.6	0.70	4
	Rt 8	70°C	8.2	0.45	NG
	Rt 358	79°C	7.3	1.25	750*
	RtOH 1	91°C	8.7	0.80	15*
	RtOH 2	80°C	9.0	0.85	7*
	RtOH 3	74°C	3.2	0.20	NG
	RtOH 4	76°C	2.8	0.15	NG
	Rachel Spring	76°C	7.3	1.5	2100*
Waikite	Wkt 1	95°C	6.9	0.30	NG
	Wkt 2	88°C	8.0	0.35	NG
	Wkt 3	78°C	7.2	0.30	NG
	Wkt 4	92°C	6.8	0.25	NG
	Wkt 5	84°C	7.8	0.35	NG
	Wkt 6	75°C	8.2	0.30	NG
Mokai	Mok 1	46°C	6.4	0.35	NG
	Mok 2	58°C	6.4	0.20	NG
	Mok 4	68°C	5.2	<0.10	NG
	Steam Vent	Var.	7.4	3.10	NG
	Mok 7	36°C	3.1	<0.10	NG
	Mok 20	63°C	6.3	<0.10	NG
	Mok 21	60°C	6.3	<0.10	NG
	Mok 28A	71°C	6.5	0.90	NG
	Mok 28D	46°C	9.0	0.85	NG
	Mok 28E	60°C	6.5	0.75	NG
	Mok 28F	59°C	5.8	0.70	NG
	Mok 38	65°C	6.6	-	NG
	Mok 40	61°C	7.0	0.75	NG
	Mok 41	66°C	6.0	0.80	NG
	Mok 42	71°C	6.5	0.90	NG

Area	Pool	Temperature	pH	$Na^+ (g\ell^{-1})$	(MPN)
Tokaanu	Tok 1	80°C	7.0	4.90	4
	Tok 2	76°C	7.7	4.55	NG
	Tok 3	68°C	6.6	4.70	9
	Tok 4A	77°C	5.8	3.80	150*
	Tok 4B	91°C	5.7	3.05	NG
	Tok 5	60°C	5.8	2.95	15
	Tok 7	67°C	5.9	4.80	NG
	Tok 8	53°C	5.8	3.80	NG
	Tok A	66°C	6.3	4.05	NG
	Tok B	60°C	6.4	1.55	460*
	Tok C	55°C	6.0	1.65	93
Ketatahi	Ket 2	85°C	5.5	<0.10	NG
	Ket 4	93°C	6.2	<0.10	NG
	Ket 8	101°C	4.9	<0.10	NG
	Ket 10	51°C	2.5	<0.10	NG
Azores	Lago de Furnas	75°C	5.8	NA	NG
	Caldeira Grande (Furnas)	75°C	7.1	NA	NG
	Runoff 40m from Caldeira Grande	72°C	8.2	NA A	NG
	Runoff below Caldeira Pero Botellio	65°C	6.5	NA	NG
	Small sulphur spring below C. Pero Botellio	66°C	6.3	NA	NG
	Black lower pool below Nasceute da Aqua	81°C	6.8	NA	NG
	Geothermal Station exit of cooling flume	82°C	8.8	NA	NG
	Praia do Fogo	72°C	6.2	NA	NG

NG- no growth of ANI-like organisms after 24, 48, or 72 hours incubation at 80°C
*-growth of ANI-like organisms on Gelrite plates (at 10^{-1} dilution only)

B) Verification of ANI-like Organisms

Isolate ANI is morphologically distinct from other described archaebacterial cocci. Organisms assigned to be 'ANI-like' all had the same morphology as isolate ANI. All produced sulphide. Ten randomly selected enrichments tested for antibiotic resistance were resistant to chloramphenicol and penicillin G, and sensitive to mevinolin. Those

tested for volatile fatty acid end products, and sodium requirements gave the same or very similar results to isolate ANI which were distinct from other described archaebacterial cocci (Table 2.2.).

Table 2.2. Comparison of ANI to Other ANI-like organisms, New Zealand *Desulfurococcus* isolate Tok12 S.1, and *Desulfurococcus mobilis*

Isolate	Growth on ANI Medium	Growth on DB Medium	NaCl Req.	Growth at 88°C	Fatty Acids[b] (mM) Ac.[c]	IB.[d]	IV.[e]	Sulphide[b] (µmoles/ml)
ANI	+	-	+	-	5.1	2.9	6.0	13.4
Rt358	+	-	+	-	3.7	2.7	7.5	14.2
Rt2	+	-	+	-	4.1	2.4	4.9	11.4
Tok3	+	-	+	-	5.1	3.2	6.7	14.4
Tok4A	+	-	+	-	3.2	0.9	2.7	10.1
Tok5	+	-	+	-	4.5	2.9	5.6	13.6
Wai5	+	-	+	-	3.1	1.0	2.9	8.2
Wtp4	+	-	+	-	4.2	1.9	5.1	11.2
Tok12.S.1[a]	-	+	-	+	1.2	0.2	0.5	1.2
D. mobilis	-	+	-	+		ND[f]		ND

[a]*Desulfurococcus* is the only genus of extreme thermophilic sulphur-utilizing archaebacterial cocci described with species from non-marine environments
[b]after 48 h. at 75°(80°C for Tok12.S.1)
[c]acetate
[d]isobutyrate
[e]isovalerate
[f]not determined

In order to claim that all these isolates belong to the same species, more detailed studies would be required. However, differences are probably on a sub-species level as was suggested by Martin's study (1985) on the isolates she obtained.

C) Distribution of ANI-like organisms

Pools from which ANI-like organisms were enriched fell within the range of pH 5.5 to 9.0, temperatures between 55° and 91°C, and Na^+ content above 0.5 g/ℓ. Assuming these parameters to be the limits for conditions permitting survival of ANI-like organisms, enrichments from

samples of many pools were negative because conditions of either pH, temperature, or Na^+ content were outside these limits.

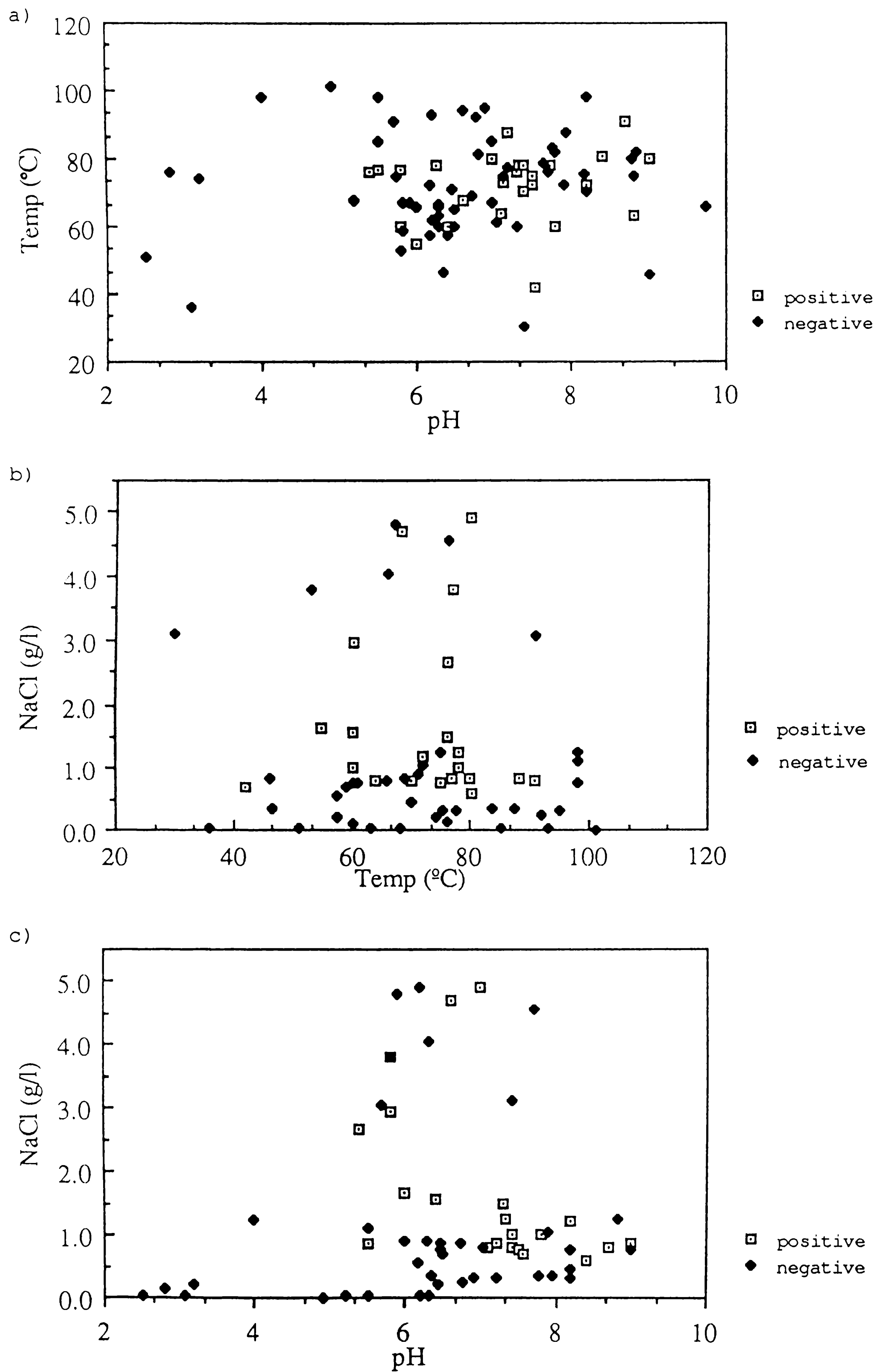
Occurrence of ANI-like organisms in New Zealand pools is shown in Figs.2.1.a-c. The figures show the limits of pH, temperature, and Na^+ content for these organisms. However, when only two of the three measured parameters are considered, some pools yielding positive enrichments seemed well outside 'suitable conditions' for ANI-like organisms while for other pools whose parameters were within the range for ANI growth, enrichments were negative.

From samples within the temperature and pH range of ANI-like organisms 49% (20 out of 41) yielded positive enrichments. Of the negative pools 33% had an Na^+ content below 0.5 g/ℓ. From samples of pools within the temperature and Na^+ content range 63% (20 out of 32) were positive; all negatives within this range were within the pH range permitting survival of these organisms. Fifty-six percent (20 out of 36) of pools within the Na^+ content and pH range yielded positive isolates while 75% of negative pools within this range (12 out of 16) were outside the temperature limits for ANI-like organisms. Generally, positive enrichments were obtained from these pools when the pH was above 7.0. However, a regional factor appeared to be involved, as none of the Mokai pools possessed ANI-like organisms while their presence could not be ruled out on the basis of temperature, pH, or Na^+ content.

The upper limit of Na^+ concentration for ANI-like organisms is not known from this study. No samples of pools were of a Na^+ content above 4.9g/ℓ, and that sample was positive. One can only assume that no such organisms would be found at temperatures and pH values outside the range of this study, by looking at previous studies (Patel 1984; Martin 1985). Of course, it is possible that all pools sampled in their studies were of too high or low Na^+ content, although it seems very unlikely. Data obtained from laboratory growth experiments on ANI supports the optima and ranges for temperature and pH suggested in this study (see section 3.3.6).

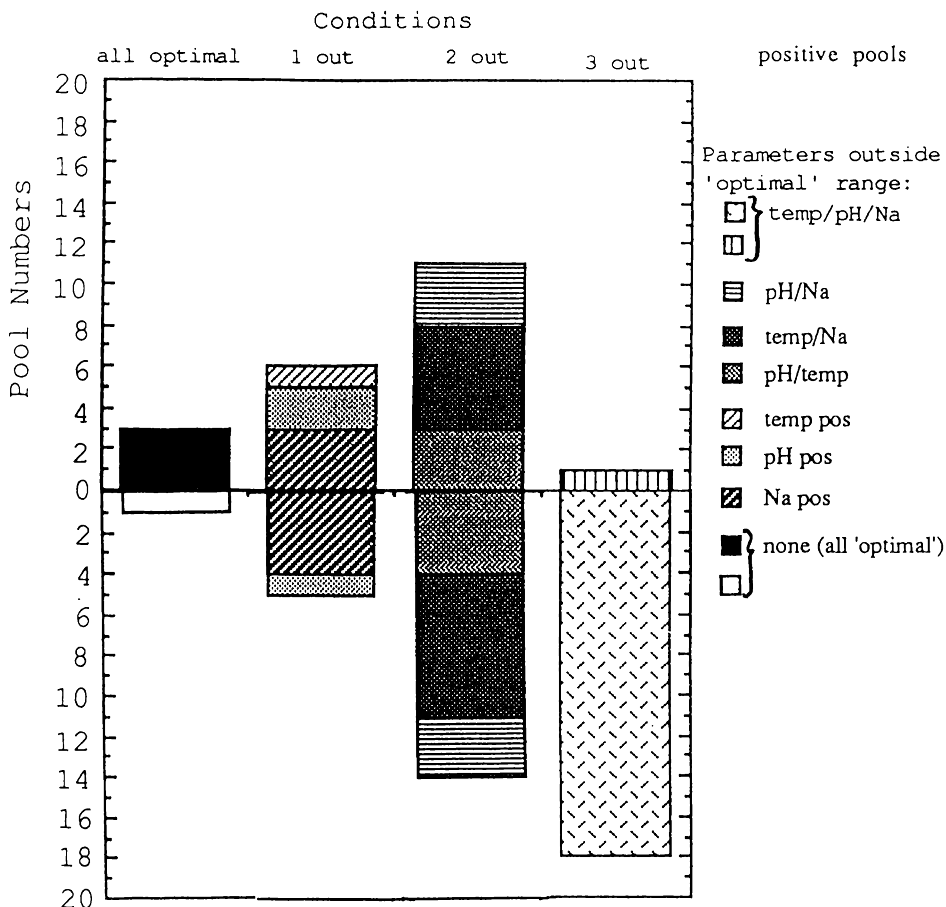
A reasonable number of samples within the range of pH, temperature, and Na^+ content required for growth of ANI-like organisms did not yield positive enrichments. However, conditions of many of these pools were distant from optimal conditions for isolate ANI. Results were, therefore, re-classified: conditions of hot pools were rated as to whether they were in the range of supporting >50% final growth yield after 48 hours of isolate ANI in the laboratory (see section 3.3.6).

Fig.2.1. Occurrence of ANI like organisms in New Zealand hot pools of various temperature, pH, and Na+ content



Thus, the pH range narrowed to pH 6.6 to 8.5, temperature to 70° to 82°C, and Na^+ content to 1.25 to 5.4 g/ℓ. It appeared that these organisms could tolerate extreme conditions of one or two of the three parameters tested if conditions of at least one parameter were within the 'optimal' (i.e. supporting >50% growth) range. Pools were grouped according to the number of parameters lying within and outside this range. Results are shown in Fig. 2.2.

Fig. 2.2. Distribution of positive and negative pool samples of one, two, or all three parameters at 'optimal' conditions



Only a small number of pools were of optimal conditions for all three parameters. Still, 3 out of 4 gave positive enrichments. Pools where one of the three parameters was outside this range and no ANI-like organisms were detected were all within the optimal temperature range; most were too low in their Na^+ content (bearing in mind that the Na^+ content was the unknown parameter at the time of selecting pools to sample). Fifty percent of pools sampled in this group yielded positive enrichments. Of pools where two of the three parameters were not within the optimal range, 41% yielded positive enrichments. From only 5% (1 out of 19) of pools where all three parameters were outside the optimal ranges positive enrichments were obtained. It appears that these organisms can tolerate a wider range of conditions as long as at least one of the three parameters tested is within the optimal range for ANI. There was little in the percentage of positives from pools where conditions of either one or two of the parameters lay outside the optimal range, and MPN counts were similar as well (Table 2.2.). In contrast, samples of two of the pools with optimal conditions for all three parameters gave far higher MPN counts. Both pools were in Rotorua. The other two pools in this group giving a low MPN count and negative enrichment respectively were both in the Tokaanu thermal area.

Table 2.2. Most Probable Number Counts from pools of various optimal condition (area is given in ( )

	All Optimal	1 out	2 out	3 out
MPN counts	2100(Rt)	150(Rt)	93(Rt)	15(RtOH)
	750(Rt)	150(Rt)	93(Wai)	
	4(Tok)	150(Tok)	75(Wai)	
		28(Tok)	43(Wai)	
		9(Wai)	15(Wtp)	
			7(RtOH)	
			4(Rt)	

Other factors not considered here appear to be involved in determining the distribution of these organisms since some pools within the temperature, pH, and [Na^+] range permitting survival of ANI-like organisms did not yield positive enrichments.
Water of these pools may have been deficient in some factor required for growth or may have contained substances which inhibited growth of

such organisms, e.g. oxygen or metabolites.
Several pools not harbouring ANI-like organisms were assessed for their ability to support growth of ANI after addition of supplements to pool water. ANI was able to grow in most pool samples when these were supplemented with peptone and inoculated (Table 2.3). Absence of such organisms in these pools may, therefore, have been due to the lack of one or more of the components of trypticase peptone rather than to the presence of some inhibitor. NaCl further improved growth in many pools and water from Mok 28D became positive, indicating the low NaCl concentration to be a major cause of the absence of ANI-like organisms. Samples of two pools did not allow growth of ANI and it seems likely that either an inhibitory compound was present in the water or another growth parameter not considered here was below the limits of growth of these organisms. This was not investigated further.

Table 2.3. Growth of ANI in anaerobic pool water in the presence of 0.8% trypticase peptone, and S^0
(after 36 h incubation at 75°C)

	Growth of ANI	
Pool	+NaCl	-NaCl
Rachel Spring	+++	++
Wai 4	+	+
Wai 7	+++	+++
Wkt 3	+++	+
Wkt 5	-	-
Wkt 6	+++	++
Mok 28A	+	+
Mok 28D	++	-
Mok 28E	++	++
Mok 28F	++	+
Mok 40	++	+
Mok 41	++	+
Mok 42	++++	+
Tok 2*	+	++
Tok 7*	+	+
Tok A	-	-

*Cells observed in Tok 2 and Tok 7 medium were much smaller than normally (only about 0.5µm in diameter after 36 h incubation while cells of other cultures of that age were 1.5-2µM in diameter).
growth was rated from ++++ good growth to + for poor growth, and - for no growth

Since reasonable growth was observed in all samples of the Mokai area (and very good growth in Mok 42 with added NaCl) low Na^+ content and/or absence of a required component of peptone may be the main reason for

apparent absence of ANI-like organisms from this entire area.
One point of importance regarding all pools sampled is the fact that all but two pools were sampled on one occasion only. No data is available on fluctuation in temperature, pH, or Na^+ content of these pools. It is possible that pools with conditions suitable for ANI-like organisms at the time of sampling were at other times at different conditions as was indeed observed for pool Rt 8 (A. Hudson pers.comm.)
Especially pools with conditions on the apparent extreme of those suitable for ANI-like organisms would not have to vary much in their conditions to prove an unsuitable environment for such organisms. Therefore, repeated sampling of pools as well as sampling of a greater variety of pools in respect to the three parameters investigated would provide more definite results on the importance of these factors to the distribution of ANI-like organisms.
This study has given some insight into the distribution of ANI. The presence of this organism in hot pools is dependent on temperature, pH, and Na^+ content of the water.
In most areas sampled there is a good correlation between the three parameters measured and the presence of ANI-like organisms.
Due to the relatively small number of samples within a limited range of temperature, pH, and Na^+ content, no more detailed statistical analyses were possible. No definite clusters of positive pools within certain conditions were identified.
One factor not taken into account in this study is the fact that isolates from pools of different pH and/or temperature will probably be under selection pressure for the different conditions. Samples were, however, all incubated in the same medium at the same temperature, making no allowances for the various origins. This will have some influence at least on the values obtained for MPN counts. Mosser *et al.* (1974) reported on different temperature strains of *Sulfolobus acidocaldarius* in Yellowstone Park. The strains had temperature optima between 68°C and 80°C. Such a variance would definitely influence results of studies as presented here.
In order to gain a better understanding on the distribution of ANI-like organisms a water analysis of pools would be required . More information on the needs of this organism would be gained by comparing pools from which it was isolated with pools from which it was absent in spite of close to optimal conditions for temperature, pH and Na^+ content. Metal ions, sulphur compounds and types and nature of available nutrients would be of interest. Gaining a few more clues

about stimulatory and inhibitory factors in the natural environment of ANI would no doubt be useful for more successful culturing of the organism.

From the Azores not enough sample was available to determine the Na^+ content of pools. Therefore the absence of ANI-like organisms could be due to an inhibitory concentration of Na^+ or other unknown factors. The possibility of a regional factor being involved in the distribution pattern of ANI-like organisms is possible as pools in different areas will vary in their composition of available nutrients, trace elements, and other factors. However, a variety of New Zealand pools from which no ANI-like isolates were isolated previously (Martin, 1985) yielded positive enrichments in this study. It would be very interesting to obtain more samples from these and other pools outside New Zealand in order to study the global distribution of this organism. Other sulphur-metabolizing archaebacteria have been reported from various sites from different continents: *Sulfolobus* has been reported from the USA (Brock 1972), from Japan (Inatomi *et al.* 1983), and from New Zealand (Martin 1985), *Thermoproteus* from Iceland (Zillig *et al.* 1981a,b) from New Zealand (Patel 1984, Patel *et al.* 1986b), and from Kamchatka (Bonch-Osmolovska *et al.* 1990b) and *Desulfurococcus* from Iceland (Zillig *et al.* 1982b), from Kamchatka and Kunashir Island (Bonch-Osmolovska *et al.* 1988), from deep-sea hydrothermal vents (Jannasch *et al.* 1988) and from New Zealand (Patel 1984, Patel *et al.* 1986). No organisms resembling isolate ANI have been isolated from Iceland, New Mexico, or Fiji (Morgan, pers. comm.).

D) Other organisms

As mentioned above, enrichments of all samples were also set up into DB, YE, and ANI(-NaCl)-medium. None of the organisms in any of these enrichments were isolated. Rather, these enrichments were carried out as an indication of diversity (within the limited range of these enrichment media) in pools.

A list of organisms observed using phase contrast microscopy is given in appendix I.

Chapter 3: Characterisation and Growth of Isolate ANI

3.1. Introduction:

From studies on the ecology of isolate ANI (chapter 2) it is evident that the organism is viable at a temperature between 55°C and 92°C, within a pH range of 5.5 to 9.0, and NaCl at concentrations of at least 0.7 g/ℓ. The organism was enriched in medium containing 0.8% trypticase peptone.

The following chapter provides a description of morphological and some cytological characteristics of ANI, the organism's requirements for growth and its metabolic end products.

3.2. Materials and Methods

3.2.1 Culture conditions

The medium used for growth of isolate ANI was as described in section 2.2.3. When L-cystine was used as substitute for S^0, the medium was prepared at double-strength. L-cystine was prepared separately as a double-strength solution (2 g/ℓ unless otherwise stated) by raising the pH to 11 and subsequently adjusting it to pH 7.5 with 1N HCl. When S^0 or L-cystine was omitted from the medium, the medium was reduced using sterile titanium-nitrilotriacetic acid (Ti-NTA) (Moench and Zeikus 1983) and sodium thioglycollate was omitted.

For determination of substrate utilization media were prepared in 50% (or 10%) increased strength medium to allow for dilution upon addition of substrate stock solutions. To 5ml (or 9ml) of medium 5ml (or 1ml) of substrate stock solution was added.

Cultures sampled over a time course were grown in 170ml or 150ml (total volume) serum bottles containing 50ml medium sealed with butyl rubber stoppers.

When larger quantities of cells were required the organism was grown in 1ℓ or 2ℓ glass bottles (Schott, Germany) containing 800ml or 1600ml medium respectively. Schott bottles were sealed with screw caps with teflon and rubber seals under a stainless steel blank with a small aperture for inoculation and sampling. The medium was autoclaved and subsequently cooled under a stream of sterile N_2.

All cultures were grown under a N_2 headspace of 1 atmosphere pressure unless otherwise stated.

In order to determine the pH optimum for growth, the pH of the medium was adjusted by the addition of 1M H_2SO_4 or 1M NaOH.
For experiments involving the addition of various concentrations of sulphur (to determine the effect of different amounts of S^0 or cystine in the medium), medium was heated to 100°C for 15 min instead of autoclaving to prevent the sulphur from melting and coalescing into large globules.
Cultures were routinely transferred every three days into 10ml medium in Hungate tubes and incubated at 75°C for 24 hours. Stock cultures were stored at room temperature.

3.2.2.Microscopy

For routine phase contrast microscopy and for determination of cell morphology under different growth conditions an Olympus BH-2 (Olympus Optical, Tokyo, Japan) microscope was used.
Phase contrast micrographs were obtained using a Reichert-Jung Polyvar microscope (C. Reichert, Vienna, Austria) with FP4-135 film (Ilford, Cheshire, UK). Cultures for photomicrography were immobilised on agar-coated slides as described by Pfennig and Wagener (1986): Quality agar (2g) was washed four times in 500ml H_2O. For each wash the agar suspension was magnetically stirred for several minutes, then allowed to settle, and the supernatant was decanted and replaced with fresh H_2O. After the last wash, the agar was suspended in 100ml H_2O to give a final concentration of approximately 2% (w/v). The agar suspension was autoclaved (121°C/15min). Microscope slides were cleaned and placed on an even horizontal surface. While the autoclaved agar was kept molten in a 98°C waterbath, 2ml of agar were removed with a 5ml graduated pipette. Starting from one corner of the slide, the agar was distributed from one end to the other of the slide, moving the pipette in zig-zag fashion. Care was taken not to let the agar run over the edge. Slides were dried for 2 weeks in a dust-free cupboard. For photomicrographs, droplets of cell cultures of approximately 0.025ml were placed onto the agar-coated slides and immediately covered with a clean coverslip. Slides were left for 5-10min to allow the liquid to be soaked up by the agar and cells to settle.
Electron microscopy was carried out as described by Patel *et al.* (1985): cell cultures were harvested by centrifugation in a benchtop centrifuge (10min/5000rpm). The supernatant was decanted and pellets were resuspended in a solution of 1% (w/v) OsO_4 in veronal acetate buffer pH 6.1 (Hayat 1972) with addition of 0.1M $CaCl_2$. Cells were fixed in OsO_4 for 60min at room temperature. Fixed cells were

centrifuged for 5min /5000rpm. The supernatant was aspired off, and the pellet resuspended in veronal acetate buffer, centrifuged again, and the supernatant was discarded. The pelleted cells were resuspended in approximately 250µl of a 1% (w/v) solution of agar (Difco noble agar) at 50°C (agar was previously melted in a microwave oven and allowed to cool to just above setting point). The suspension of fixed cells in the agar was pipetted onto a clean glass microscope slide and allowed to set. Small blocks of agar containing clumps of cells were excised with a scalpel (size of block approximately $3mm^2$ x 0.5-1mm thick). Blocks were dehydrated through a series of ethanol solutions from 50% to absolute ethanol (30min in each solution at room temperature with intermittent mixing). Then two additional exchanges with absolute ethanol were followed by 30min at 20°C in ethanol:Spurr's embedding resin (1:1)(supplied by Agar Scientific Ltd., England) and then followed by 60min in Spurr's resin and finally for 16h (overnight) in Spurr's resin at 4°C. The agar blocks were trimmed to approximately 0.5mm x 0.5mm x 1mm with a scalpel blade and embedded in Spurr's resin in Beem capsules (Agar Scientific Ltd., England). The resin was left to polymerize for 24h at 70°C. The blocks were trimmed and cut on a Reichert ultramicrotome using glass knives made on a LKB 7800 knifemaker (LKB, Sweden). Sections were cut to a thickness (90nm) showing interference colour of silver to gold. Sections were collected on copper grids(200 mesh; Polaron Equipment Ltd., England) and stained with uranyl acetate (1% aq.) for 15min at 20°C, followed by a milli-Q water rinse, then stained in lead citrate (prepared by dissolving 1.33g $Pb(NO_3)2$ and 1.76g Na-citrate in 30ml milli-Q water, shaking the solution for 30min, then adding 8ml of 1N NaOH and adjusting the volume to 50ml with milli-Q water) for 15min/20°C, another rinse in milli-Q water, and air dried. Grids were viewed using a Philips EM 400 transmission electron microscope.

3.2.3.Cytological characteristics

Cytochrome scan: Cell-free extracts were prepared and a cytochrome scan performed according to Janssen and Harfoot (1990). Approximately 800ml of cells were harvested at late log to early stationary phase and washed twice in 100mM sodium phosphate buffer, pH 7.0 (20°C). The cell pellet was resuspended in 1-2ml of buffer and disrupted by sonication (3-5 minutes in 20 second intervals) with a sonic dismembrator (Dynatech), during which time the sample was kept in a small plastic tube in an ice-water bath. The sonicate was centrifuged at maximum

speed in a Runne microcentrifuge (H. Rehm, Germany) for 5 min. The supernatant was examined for cytochromes by an oxidized-versus-reduced spectra scan from 400 to 700nm. Samples were oxidized by vigorous shaking and reduced by addition of a few small granules of Na-dithionite. Scans were performed on a Shimadzu UV-250 UV-visible recording spectrophotometer with a Shimadzu PR-1 graphic printer. As a control, a scan was performed on cell-free extracts of *Desulfovibrio baculatus* strain H.L.21 (DSM 2555) which had been grown in freshwater medium plus lactate and sulphate as described by Janssen and Harfoot (1990).

Isolation of DNA: DNA was isolated using the phenol:chloroform:isoamyl alcohol extraction method as described in the manual by Sambrook *et al.* (1989): A cell culture (800ml) was harvested at late log-phase by centrifugation (25,000 x g for 20 min.) and washed twice in 20mM Tris(hydroxymethyl)aminoethane (Tris) and 0.9% NaCl, pH 7.4. The cell pellet was resuspended in 5ml of the same buffer plus 10ml of 100mM Tris, 1% NaCl, and 100mM ethylenediamine tetraacetic acid (EDTA). To the cell suspension 20% sodium dodecyl sulphate (SDS) to a final concentration of 1% and proteinase K (250µg/ml) were added and the mixture was incubated at 50°C for 2 hours. The mixture was transferred to an ice-water bath for 15 min and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The sample was mixed gently, then centrifuged at 1500 x g for 15 min. The phenol layer was taken off the sample which was extracted again. The sample was dialysed against 50mM Tris: 10mM NaCl; 10mM EDTA, pH 8.0 in 5 x 500ml. The dialysate was treated with RNase (100µg/ml) for 1 hour at 37°C. The sample was extracted with phenol:chloroform:isoamyl alcohol twice more and dialysed against 10mM Tris; 1mM EDTA, pH 8.0. The DNA was concentrated by addition of $^1/_3$ volume 10M NH_4-acetate and subsequent gentle addition of 2 volumes 100% ethanol. The sample was stirred gently with a glass rod and DNA was 'spooled' off the ethanol/NH_4-acetate interphase. The DNA was rinsed 4 x in 80% ethanol, transferred to a microfuge tube and vacuum dried. DNA was resuspended in Tris-EDTA (TE) buffer (10mM Tris-HCl; 1mM EDTA, pH 8.0) or 1 x or 0.1 x saline citrate buffer (SSC: 15mM NaCl, 1.5mM sodium citrate, pH 7.0). DNA purity was estimated spectrophotometrically by using the ratio of absorbance at 260nm and 280nm.

Determination of G+C content: The G+C content of DNA was determined using melting point analysis (Marmur and Doty 1962). A Pye Unicam spectrophotometer equipped with a heating cuvette holder was used. A temperature probe , previously calibrated against a standard reference thermometer, was placed into the sample cuvette. DNA was redissolved in 1 x or 0.1 x SSC buffer. Samples were appropriately diluted to an absorbance of 0.3-0.4 at 260 nm. Their absorbance was followed over a rise in temperature from 60°C to 98°C (1°C per minute). Absorbance was recorded every 0.5°C. The DNA melting temperature was determined from the temperature at which half the hyperchromic shift in absorbance occurred. Using the following equation, the %mol G+C was calculated:

%mol G+C = $2.44T_m - 169$ (for 1 x SSC);

= $2.08T_m - 106.4$ (for 0.1 x SSC) (Owen and Pitcher 1985)

As controls, melting curves of DNA from *Escherichia coli* and *Clostridium perfringens* (Sigma Chemical Co., USA) in 0.1 x SSC and 1 x SSC respectively were produced.

Determination of *dam*-methylation of DNA: DNA (1µg/ml) was incubated with the restriction endonucleases Mbo I, Dpn I, and Sau 3A (Bethesda Research Laboratories Life Technologies, Inc.). The reaction mixtures contained: 1µl DNA, 1µl 10x appropriate reaction buffer, 1µl restriction enzyme (4 U/µl), 7µl milli-Q water. The reaction buffers were (10x concn.): Mbo I: 500mM Tris-HCl pH 8.0, 100mM Mg Cl_2, 500mM NaCl (REact 2 ™); Dpn I and Sau 3A: 200mM Tris-HCl pH 7.4, 50mM $MgCl_2$, 500mM KCl (REact 4™). Incubations were carried out at 37°C for 60min. Following incubation, a quarter volume of 15% Ficoll and 0.125% bromophenol blue in Tris-borate-EDTA (TBE) buffer (89mM Tris, 89mM boric acid, 2.5mM EDTA-di-sodium salt), filtered through glass fibre filter) was added to the DNA and the sample was loaded and run on a 1% agarose gel (70ml; 10 x 10cm) in 450ml TBE buffer at constant voltage (70 V) for 3.5 hours. Agarose gels were prepared by melting agarose (ultrapure agarose, electrophoresis grade,; Bethesda Research Laboratories, USA) in TBE buffer (for 1% gel; 0.7g agarose in 70ml TBE) using a heating mantle. The agarose solution was cooled to ~50°C and poured onto a gel platform. The platform had plastic dams sealed by 1.5% agar at both ends. A teflon comb was inserted at one end and the gel set. Electrophoresis buffer (TBE) was poured over the set gel to 0.5cm above the top of the gel and the comb and dams were removed

At the end of the electrophoresis run, the agarose gel was stained in 0.02% ethidium bromide for $^1/_2$ hour and subsequently destained in distilled water for at least 15 min. The DNA on the gel was visualized

under a Fotodyne Ultraviolet (UV) transilluminator (UV light at 366nm). A photograph was taken with a Polaroid Land Camera using Polaroid 667 film (ASA 75) at an F stop of 4.7 for 15 seconds. The negative was fixed in 18% Na_2SO_4 for 30 min and rinsed in H_2O.

3.2.4. Substrate utilisation

All anaerobic stock solutions were prepared in distilled water, boiled and subsequently cooled under N_2 prior to sterilization.

Trypticase peptone, yeast extract, casein, casamino acids, and skim milk were added to the culture medium to a final concentration of 0.8%. All carbohydrates were added to a final concentration of 0.2 and 0.5%. Organic acids were added to a final concentration of 10mM and all amino acids to 10mM and 50mM final concentration. All carbon sources tested were added to sterile medium from neutralised anaerobic stock solutions which had been sterilised either by autoclaving or filter sterilisation into sterile Bellco tubes or serum vials. All amino acids were prepared in 100mM stock solutions, pH adjusted to pH 7.4, and filter-sterilized using 0.22μm filters enclosed in a Sartorius Minisart NML disposable syringe filter holder (Sartorius, Germany). Valine, leucine, isoleucine, alanine, threonine, norleucine, and norvaline were alternatively autoclaved at 34.47kPa/15min. Most organic acids were added from filter-sterilized 100mM stock solutions. Tiglic acid was added directly to single-strength medium and was autoclaved at 34.47kPa/15min. Carbohydrates were added from filter-sterilized 10% stock solutions.

The amino acid mixes used had the following composition:

Amino Acid Mix I: Glu, Arg, Asp, Ile, Leu, Pro, Trp (2mM);
Val, Thr, Ala (5mM);
Met, Cys, Lys, His, Phe, Tyr (1mM)

Amino Acid Mix II: Gly, Val, Ile, Arg (3mM);
Phe, Trp, Leu, His, Met, Pro (1mM)

Modified Amino Acid Mix I: Val, Ile, Thr, Ala (5mM;)
Glu, Asp, Arg, Pro, Trp, Cys, His, Phe (1mM)

Composition of these mixes was based on findings of growth stimulation by single amino acids (section 3.3.5).

Unless otherwise stated, L-isomers of organic acids and amino acids and D-isomers of carbohydrates were used.

Salts tested as substitutes for the organism's requirement for NaCl were added from autoclaved (121°C/15min) anaerobic stock solutions (5M).

Potential electron acceptors were added to sterile medium, containing 0.1% trypticase peptone and 10mM L-leucine, to a final concentration of 10mM or 30mM from neutralised sterile anaerobic stock solutions.
Medium supplements and carbon sources were added to the appropriate medium by disposable syringes and hypodermic needles.

3.2.5. Metabolic end products

Acetate, propionate, isobutyrate, isovalerate/2-methylvalerate, H_2, and CO_2 were detected using gas chromatography according to the method described by Patel *et al.* (1985). Volatile fatty acids were detected using a Pye Unicam GCD chromatograph equipped with a flame ionization detector. Chromosorb 101 (Supelco, USA) was packed in a glass column (1.8m by 4.5mm). The oven was run at a constant temperature of 170°C with detector temperature of 225°C and injector temperature of 190°C. O_2-free nitrogen was the carrier gas at a constant flow rate of 48ml/min. Peaks were integrated by a Spectra Physics SP 4100 computing integrator. Samples of cell-free supernatant were acidified by addition of 1N H_3PO_4 (1:1 (vol/vol)). Samples of 2µl were injected using a SGE 10 microliters syringe (Scientific Glass Engineering PTY. Ltd., Australia). Standard solutions of acetate, propionate, isobutyrate, isovalerate, and 2-methylbutyrate of 1, 2, 5, and 10mM each were run and standard curves were prepared and these were linear. After every 8-10 sample runs, a mixture of either 5mM or 2mM standards was run.
Carbon dioxide and H_2 were detected via gas chromatography using a Pye Unicam PU 4500 gas chromatograph equipped with a thermal conductivity detector. A stainless steel column packed with Carbosieve B, 120-140 mesh (Supelco) was used. The oven was at 188°C, with detector temperature of 75°C, and injector temperature of 200°C. O_2-free nitrogen was the carrier gas at a constant flow rate of 30ml/min. Peak heights were recorded by a Sekonic SS-250F chart recorder. As standard pure hydrogen at 1 atmosphere pressure was used. Prior to H_2 determination all cultures were cooled to room temperature. Samples were removed from the headspace of cell cultures using a Pressure-Lok^R 1ml syringe (Precision Sampling Corp., USA) and 0.2ml of sample was injected.
Sulphide was determined colorimetrically according to Trüper and Schlegel (1964). A 1ml or 0.1ml sample of cell culture was added to 20ml of 2% zinc acetate solution in a 100ml volumetric flask. About 60ml of H_2O and then 10ml of a dimethyl-*p*-phenylenediamine solution

(0.2% in 20% H_2SO_4)were added. The solution was immediately mixed by gentle swirling. Last, 0.5ml of a $NH_4Fe(SO_4)_2 \times 12H_2O$ solution (10% in 2% H_2SO_4) was added and the mixture was shaken and allowed to stand for 10min. The flask was filled up to 100ml with H_2O, and the absorbance at 670nm was read against a reagent blank. Standards were prepared using Na_2S. Crystals of Na_2S were washed under H_2O and loose or brownish parts removed. The crystals were blotted dry in paper and used. Na_2S was assumed to be $Na_2S \times 9H_2O$. The standard curve was linear to 2µmoles/ml. When required, samples were diluted appropriately with H_2O.

The method described by Weatherburn (1967) (modified to fit microfuge tubes) was used to measure NH_4. Two reagents were used (reagent A: phenol, 5g; Na-nitroprusside; 25mg; H_2O, 500ml; reagent B: NaOH, 2.5g; 15% Na-hypochlorite, 4.2ml; H_2O, 500ml), stored at 4°C in amber bottles for maximally one month. Of each reagent 500µl was added to 100µl sample, mixed, and incubated for 20 min. at 37°C. Absorbance was read at 625nm. A standard curve was prepared using NH_4Cl standards. The curve was linear to 1µmole/ml of NH_4. Samples were diluted with H_2O when required.

For tests on end product inhibition of growth of ANI, 0.8% peptone + S^0 medium was supplemented with 10mM acetate, 5mM isobutyrate, and 10mM isovalerate before inoculation and incubated at 75°C for 48 h. Inhibition of end products was also tested by growing cells in 0.8% peptone + S^0 for 24 and 48h. at 75°C in 10ml medium, anaerobically centrifuging the medium in a benchtop centrifuge (Hettich, Germany) to remove cells, transferring the cell-free supernatant to sterile anaerobic Hungate tubes, supplementing the cell-free supernatant with 0.8% peptone from a sterile anaerobic stock solution, inoculating the medium, and incubating it for a further 48h.

3.2.6. Antibiotic sensitivities

Monensin and rifampicin were added from ethanolic stock solutions (5mM and 10mM respectively). To controls the same volume of 100% ethanol was added. Other antibiotics were added from filter-sterilized aqueous stock solutions.

3.3. Results

3.3.1. Morphology

Under phase-contrast microscopy the cells appeared as motile, regular cocci. They were of 0.5µm-1.0µm in diameter in early to mid-log stages of growth. In late-log and stationary phase their size increased up to 2.0µm. While in early and mid-log phase cells were frequently in pairs and phase-dense, they were predominantly single and became phase-transparent in later stages in growth (Fig.3.1.a-c). Cells divided by constriction (Fig.3.1). Cytoplasmic connections as described for *Thermococcus celer* (Zillig *et al.* 1983b) were observed. At later stages in growth a refractile inclusion body was observed frequently when cells were grown in the presence of S^o (indicated in Fig.3.1.c). Such an inclusion body has been reported in *Desulfurococcus amylolyticus* and described as sulphur (Bonch-Osmolovskaya *et al.*, 1988). When S^o was replaced by L-cystine, no such inclusion was seen. At the end of stationary phase (after 40-70h depending on growth medium and incubation temperature) cell lysis occurred.

3.3.2. Cytological characters

Thin sections of ANI revealed the presence of a cell envelope which was of 14-28nm in thickness and consisted of a definite inner layer and a more amorphous outer layer of variable width (Fig.3.2.a,b). Attached on the exterior and, in some cases, the interior surface was electron-dense material. This outer layer increased in width when grown with L-cystine instead of S^o (Fig.3.2.c.).

No cytochromes were detected in crude cell extracts of ANI grown in medium containing 0.8% peptone and S^0. An absorbance scan from 400nm to 700nm gave a steady line with no distinct peaks (see also Fig.6.1).

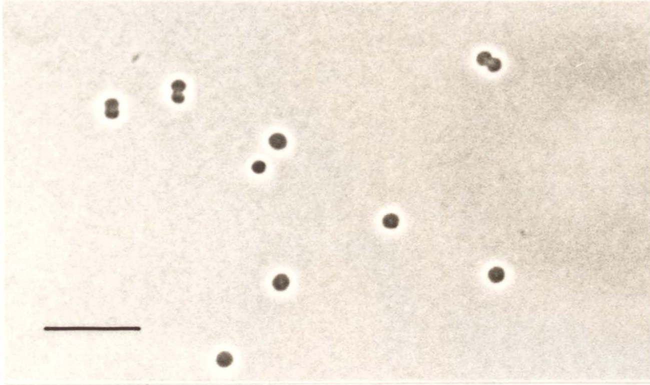
From DNA melting curves the %mol G+C was determined. In 1 x SSC buffer the control *Clostridium perfringens* DNA showed a T_m=82.3°C. The %mol G+C was given as 27.5% by the supplier (Sigma, USA). Here, the %mol G+C = 2.44 x 82.3 - 169 = 31.8%; i.e. an error of +4.3%.

The *Escherichia coli* DNA in 0.1 x SSC had a T_m=77.6°C. The %mol G+C = 2.08 x 77.6 - 106.4 = 55.0%. The %mol G+C was given to be 50.0% by the supplier (Sigma). The error here was +5.0%.

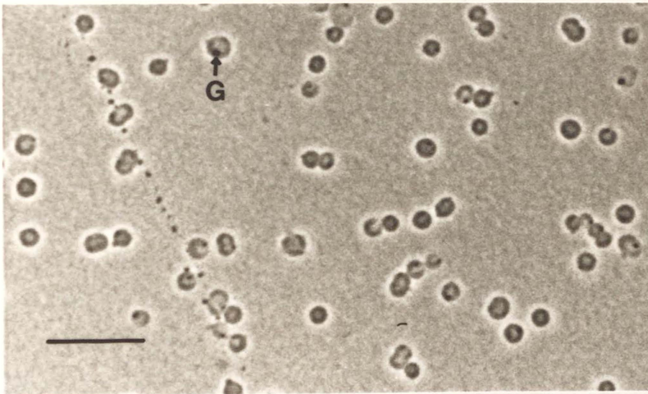
In 1 x SSC ANI DNA had a T_m=90.1°C; %mol G+C = 2.44 x 90.1 - 169 = 50.8%; accounted for error in *Cl. perfringens* control: = 46.5%.

Fig.3.1. Cell morphology of ANI observed under the phase-contrast microscope at various stages of growth in 0.8% peptone medium with S^0 (Bar=10μm)

a) after 12 hrs. incubation at 75°C



b) after 36 hrs. incubation at 75°C



G - granule observed later in growth assumed to be sulphur

c) after 62 hrs. at 75°C

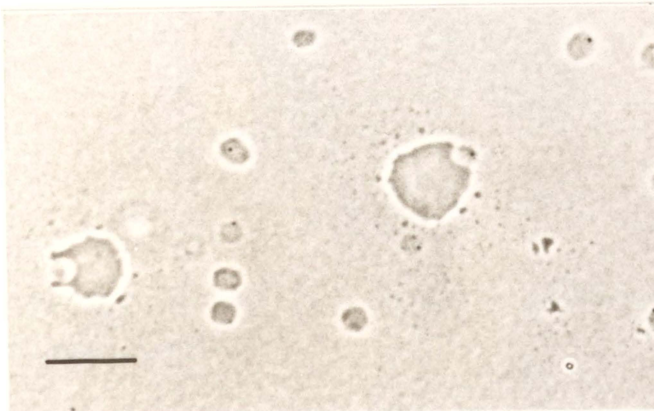
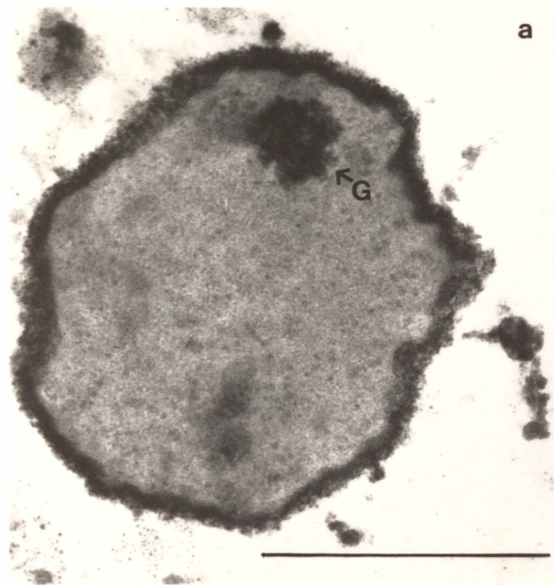
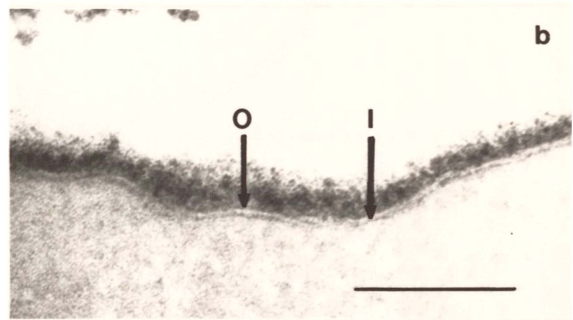


Fig.3.2. <u>Electron micrographs of thin sections of cells of ANI</u>

a,b) cells grown in the presence of S^0 (30hrs/75°C)

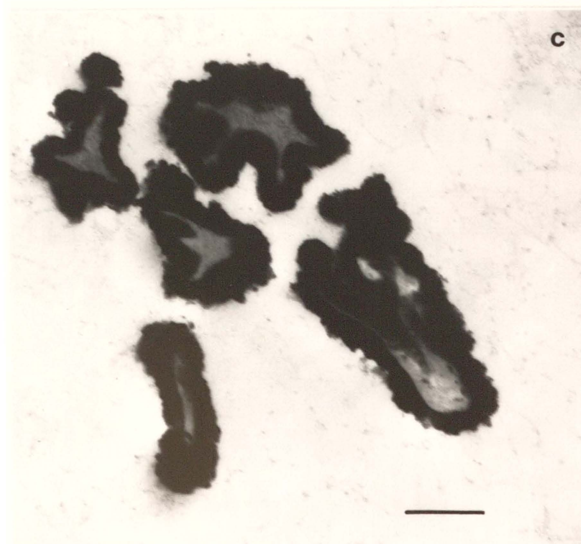


a) note the 'sulphur' granule (G) (Bar=0.5μm)



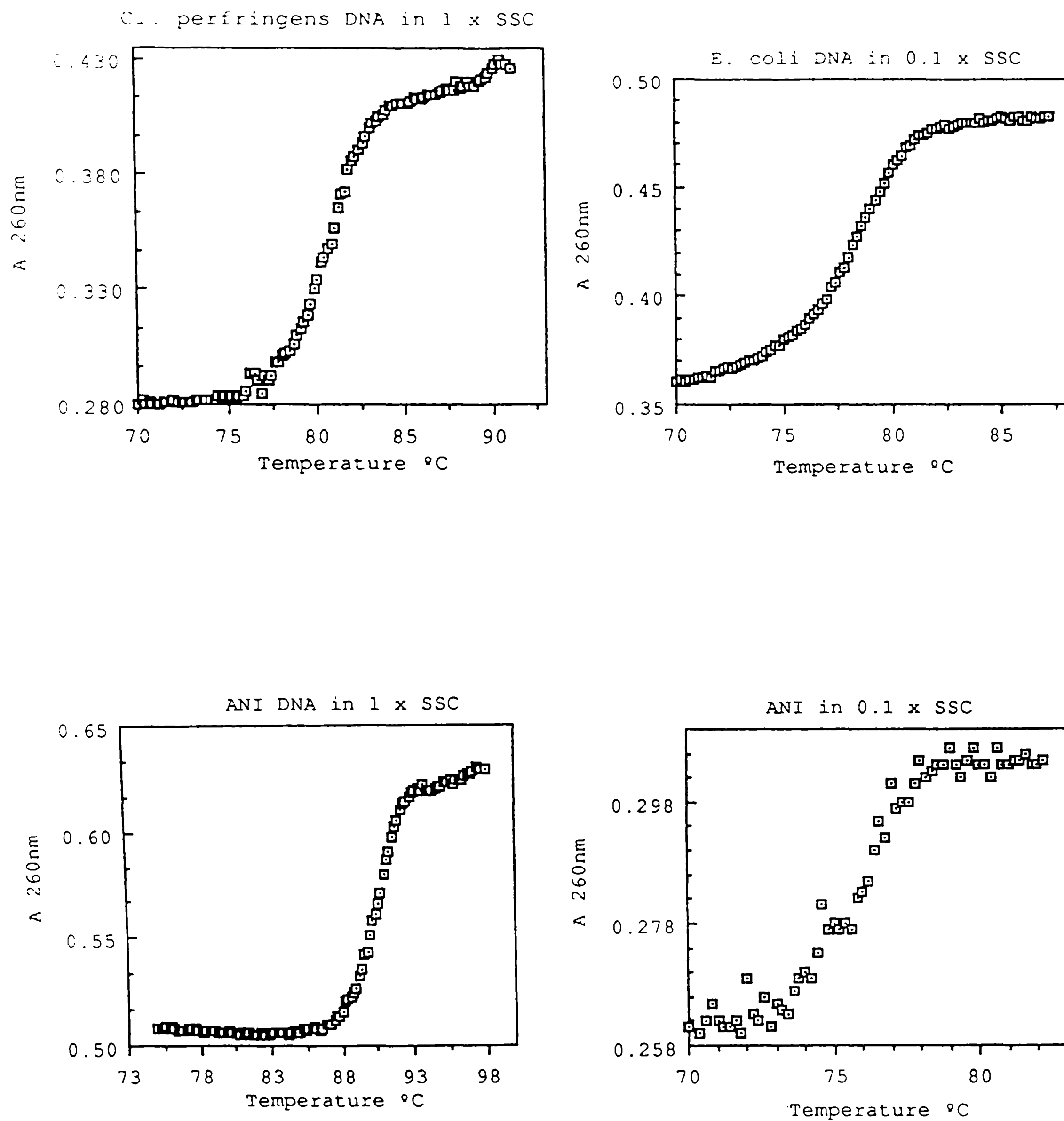
b) note the two-layered cell envelope structure: a defined inner layer (I) and amorphous outer layer(O) (Bar=0.1μm)

c) cells grown in the presence of L-cystine (30hrs/75°C)



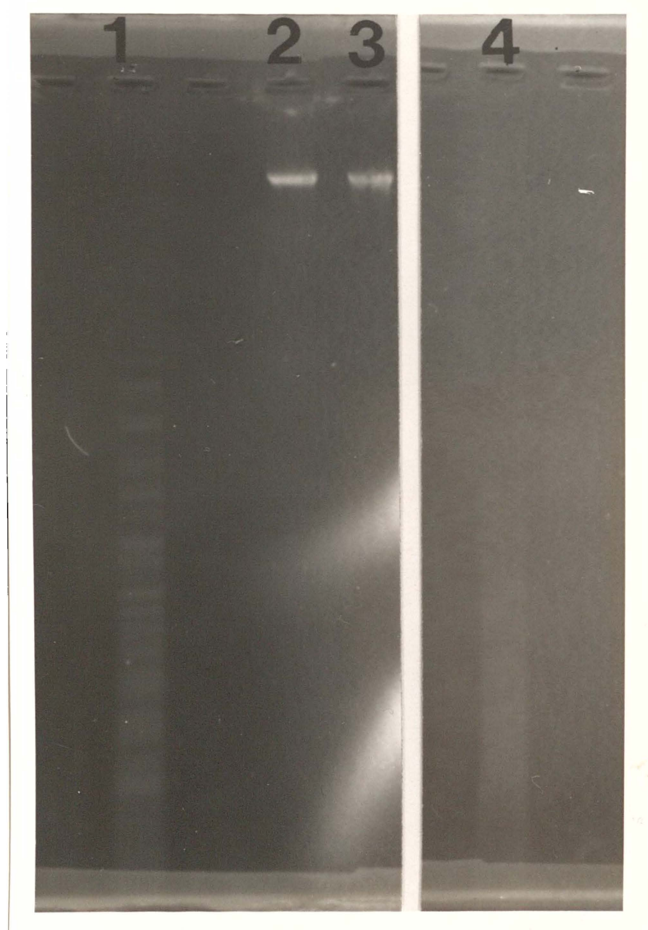
note absence of 'sulphur' granule and electron-dense material on the cell exterior (Bar=5μm)

Fig.3.3. Melting curves of DNA of ANI, *Cl.perfringens*, and *E.coli*



In 0.1 x SSC ANI DNA had a T_m=75.6°C;
%mol G+C = 2.08 x 75.6 - 106.4 = 50.8%; accounted for error in *E. coli* control: = 45.8%.
The %mol G+C ratio was 46.15 ± 0.35% by thermal denaturation. Melting curves of the controls and of ANI DNA in 1 x SSC and 0.1 x SSC buffer are shown in Fig.3.3.
DNA of isolate ANI was digested with the restriction endonucleases Mbo I, Dpn I, and Sau 3A in order to test for *dam* methylation (methylation of the N^6 position of the adenine residue within the DNA sequence GATC). The three endonucleases used for digestion of DNA all cut the same sequence, GATC. Mbo I does not cut the sequence when the A residue is N^6 methyladenine, while Dpn I only cuts when the A residue is methylated. Sau 3A cuts the sequence whether the A residue is adenine or N^6-methyladenine and acts as a control. DNA digested with each of the the three restriction endonucleases was run on an agarose gel. DNA was cut by Sau 3A and Dpn I but not by Mbo I (Fig.3.4). The DNA is, therefore, assumed to contain a N^6-methyladenine in the sequence GATC, and isolate ANI is Dam^+.

Fig.3.4. Restriction endonuclease digests of ANI DNA



Lane 1: ANI DNA / Dpn I

Lane 2: ANI DNA / Mbo I

Lane 3: ANI DNA uncut control

Lane 4: ANI DNA / Sau 3A

3.3.3. Cultural characters

Isolate ANI was obligately anaerobic. Growth was completely inhibited in the presence of O_2 (when resazurin in the medium turned pink). Cultures stored in the dark at room temperature remained viable and could serve as inoculum after at least two years.

3.3.4. Nutritional requirements

The organism grew on trypticase peptone, yeast extract, or casein but not on skim milk. Peptone was the preferred substrate. After 36 hours incubation at 75°C in medium containing 0.8% trypticase peptone an OD_{650} of 0.42 was reached compared to one of 0.37 on 0.8% yeast extract, and 0.20 on 0.8% casein. A variety of organic acids, amino acids, and carbohydrates was tested for utilization. However, no growth was detected on any in the absence of peptone. In the presence of 0.1% trypticase peptone several substrates stimulated growth (Table 3.1.). No stimulation of growth was observed in medium containing 0.1% peptone, 2mM acetate, and S^0 under H_2/CO_2 atmosphere compared to a N_2/CO_2 atmosphere.

Of the substrates stimulating growth, D- and L-valine, D- and L-leucine, L-isoleucine, L-norleucine, DL-norvaline, L-threonine, L-alanine, and L-arginine had the biggest effect. These amino acids in combinations and in mixtures with other amino acids which on their own did not stimulate growth were used in further growth studies. In Fig.3.5 the stimulation of growth by these amino acid combinations added to various peptone concentrations is shown.

Fig.3.5. Stimulation of growth at various concentrations of peptone (48h./75°C)

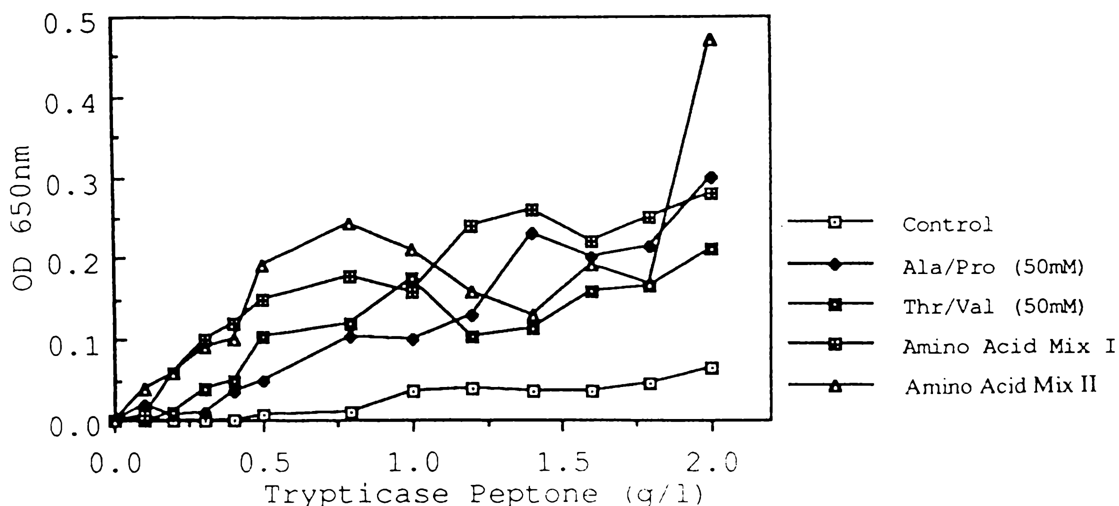


Table 3.1. Substrates stimulating/not stimulating growth of isolate ANI in the presence of 0.1% peptone (in order of decreasing stimulation)

a. Substrates stimulating growth

Substrate	Concentration	% Growth (OD_{650}) 42h/75°C (growth on 0.8% peptone is 100%)
α-Ketoisocaproate	50mM	63
α-Ketoisovalerate	"	52
L-Isoleucine	"	45
L-Leucine	"	45
L-Valine+L-Threonine	25mM each	45
Casamino acids+ L-Isoleucine	0.1%+ 10mM	39
L-Valine	50mM	38
Pyruvate	50mM	31
L-Arginine	"	28
L-Alanine	"	28
L-Threonine	"	25
DL-Norvaline	10mM	25
L-Alanine+Glycine	25mM each	23
2-amino-n-butyrate	50mM	23
L-Leucine	10mM	23
L-Valine+L-Leucine	5mM each	23
L-Valine+L-Leucine+ L-Isoleucine	3mM each	23
L-Tryptophan	50mM	20
L-Glutamate	"	18
L-Norleucine	10mM	18
L-Ornithine	50mM	18
L-Cystine	"	17
D-Leucine	10mM	17
L-Aspartate	50mM	15
Glucose	0.5%	15
Casamino acids	0.1%	14
L-Valine	10mM	13
L-Isoleucine	"	10
L-Lysine	50mM	10
D-Threonine	10mM	10
L-Threonine	"	10
D-Valine	"	10
L-Methionine	50mM	8
L-Asparagine	"	5
L-Serine	"	5
Starch	0.5%	5
L-Proline	50mM	4
Lactose	0.5%	3

b. Substrates not stimulating growth

Substrate	Concentration
Acetate	50mM
β-Alanine	"
Arabinose	0.5%
Cellulose	"
L-Cysteine	50mM
Galactose	0.5%
L-Glutamine	50mM
Glycine	"
Homoserine	"
α-Hydroxy-n-valerate	"
Isobutyrate	"
Isocitrate	"
Isovalerate	"
Malate	"
Maltose	0.5%
Mannose	"
Oxalate	50mM
Phenylalanine	"
Succinate	"
Tartrate	"
Tiglic acid	"
Xylose	"

Optimal growth was reached at far lower peptone concentrations in the presence of supplementary amino acids, but peptone was still required for growth in low quantities. Growth increased in the presence of low levels of peptone when a mixture of amino acids (amino acid mixes I and II) was added.

3.3.5. Metabolic end products

Detected metabolic end products were H_2, CO_2, NH_4, sulphide, acetate, propionate, isobutyrate, and isovalerate/2-methylbutyrate (Table 3.2). Volatile fatty acids, H_2, and sulphide were produced throughout growth at a consistent ratio to each other (see Fig. 3.11).

No endproduct inhibition in growth was detected in medium with fatty acids added or in re-inoculated medium. The $OD_{(650)}$ reached after 48h was 0.44 and 0.43 respectively (control was 0.43).

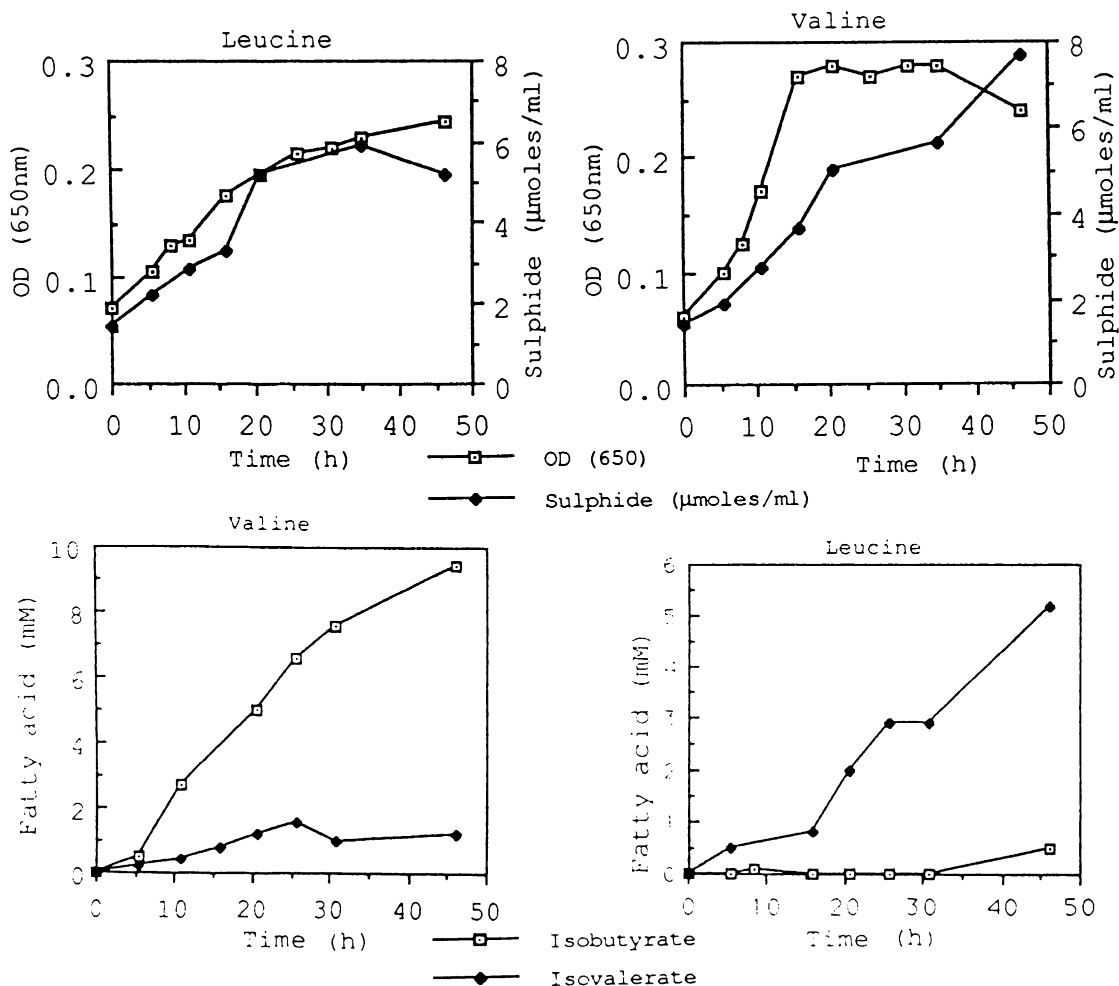
The ratios of different fatty acids produced varied when the organism was grown on reduced amounts of peptone and added single amino acids (Table 3.3; Fig.3.6).

Table 3.2. End products of isolate ANI on 0.8% peptone, S^0 under N_2 after 48 h at 75°C

End product	Amount (µmoles/ml culture)
H_2	22.1 µmoles/ml
CO_2	not determined
NH_4	26.8 µmoles/ml
sulphide	13.4 µmoles/ml
acetate	5.1 µmoles/ml
propionate	0.7 µmoles/ml
isobutyrate	2.9 µmoles/ml
isovalerate/ 2-methylbutyrate	6.0 µmoles/ml

Growth on 10mM leucine or valine in the presence of 0.1% peptone and S^0 was monitored over a period of 46h and end products were measured (Fig.3.6).

Fig.3.6. Growth on 0.1% peptone and S^0 with 10mM leucine or 10mM valine and production of sulphide, isobutyrate and isovalerate



Growth in the presence of valine showed a slightly higher yield over 35h incubation than in the presence of leucine. However while the OD of cultures with valine declined towards 40h incubation it was still rising in cultures with leucine. Leucine as substrate resulted in a slower growth rate (see also Fig.5.1). Production of sulphide followed growth, although relative to the OD of the cultures more sulphide was produced in the presence of leucine until late stages in growth. Ammonium was measured only at the beginning and the end of the growth curve. At the beginning of the incubation the NH_4 concentration in medium with 10mM leucine was 1.1mM and 1.2mM in medium with 10mM valine. After 46h incubation in medium with 10mM leucine the concentration of NH_4 was 14.0mM and in medium with 10mM valine the concentration was 16.0mM.

Production of isobutyrate and isovalerate in cultures with leucine or valine showed that with leucine as major substrate, isovalerate was the major end product and production of isobutyrate is much reduced while isobutyrate was the major fatty acid end product when valine served as major substrate.

Table 3.3. Production of NH_4 and volatile fatty acids from amino acids

Amino Acid Added[a]	NH_4	End product (mM) Acetate	Propionate	Isobut.[b]	Isoval.[c]/ 2-Methylbut.[d]	OD ($_{650nm}$)
10mM L-leucine + 10mM L-valine	5.2	0.7	0.4	1.1	2.3	0.095
10mM L-valine	3.5	0.7	0.3	3.0	<0.1	0.05
10mM D-valine	2.4	1.4	0.3	0.3	0.4	0.04
10mM L-leucine	6.0	1.1	0.2	<0.1	4.8	0.095
10mM D-leucine	4.4	1.2	0.2	0.2	1.8	0.07
10mM L-isoleucine	3.1	1.0	0.2	0.0	2.3	0.04
10mM DL-norvaline	6.9	1.4	0.4	0.1/5.0[e]	<0.1/0.3[f]	0.10
10mM L-norleucine	6.2	0.7	<0.1	<0.1/0.3[e]	0.2/3.5[f]	0.075
10mM L-threonine	6.2	7.8	0.5	<0.1	0.6	0.04
10mM L-alanine	5.3	4.0	0.2	0.2	0.5	0.10

[a]media contained 0.05% trypticase peptone
[b]Isobutyrate
[c]Isovalerate
[d]2-Methylbutyrate
[e]*n*-Butyrate
[f]*n*-Valerate

To confirm a hypothesis that volatile fatty acids formed were products of the breakdown of these amino acids (isobutyrate from valine, isovalerate/2-methylbutyrate from leucine/isoleucine, and acetate primarily from alanine and threonine), isolate ANI was grown on media with reduced amounts of trypticase peptone (0.05%) and high concentrations of branched-chain amino acids (D- and L-isomers), norleucine, norvaline, alanine, and threonine (Table 3.3).

3.3.6. Growth parameters

a) Temperature and pH

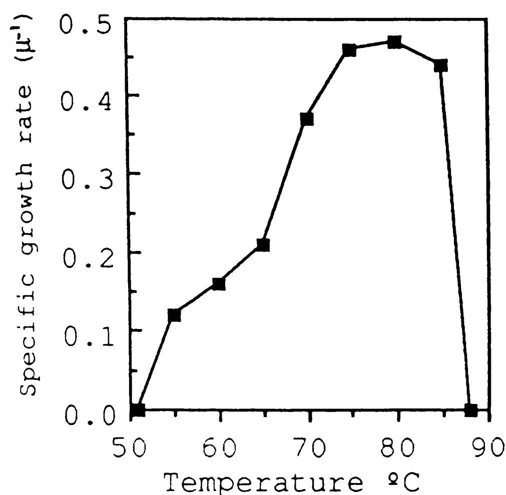
Isolate ANI grew at temperatures ranging from 55°C to 85°C with optimum growth between 75°C and 80°C. No growth could be detected at either 50°C or 90°C (Fig.3.7.a).

The organisms grew at pH values ranging from pH 5.4 to 9.2 with optimum growth at pH 7.4. No growth could be detected at pH 5.2 or pH 9.4 (Fig.3.7.b).

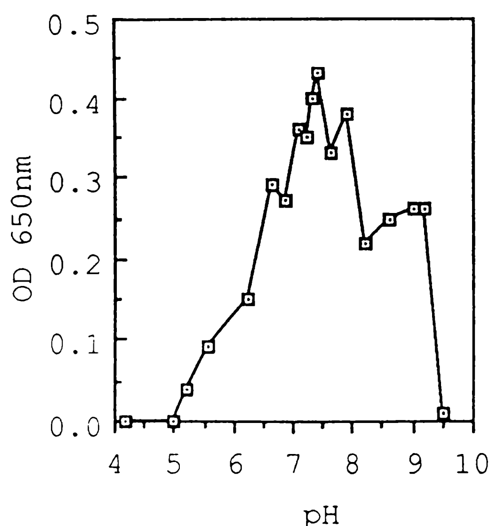
At 75°C, pH 7.4, and 50mM NaCl the specific growth rate on 0.8% peptone medium in the presence of S^0 (2g/l) was 0.46 h^{-1} corresponding to a doubling time of 1.5h.

Fig.3.7. Temperature and pH range and optima for ANI (in 0.8% peptone with S^0)

a) Specific growth rate of ANI at different temperatures



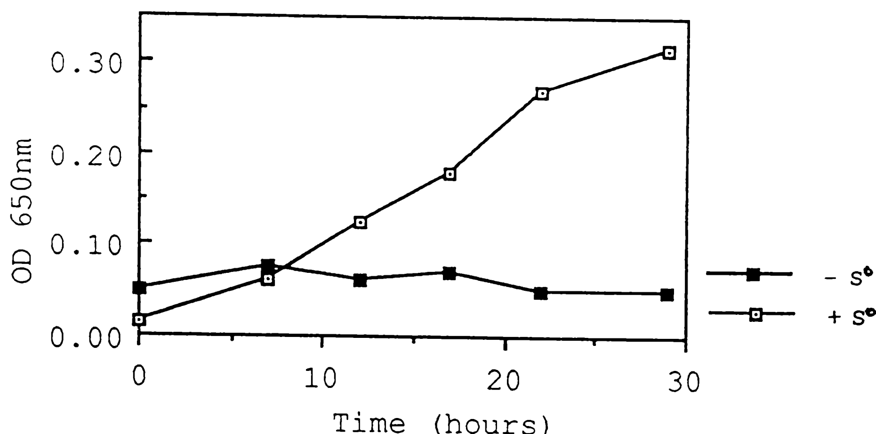
b) Growth yield of ANI at different pH values (36h./75°C)



b) Sulphur requirement

Isolate ANI obligately required sulphur for growth. Sulphur was reduced to sulphide. Medium without sulphur, reduced with Ti-NTA instead of thioglycollate, did not support growth (Fig.3.8).

Fig.3.8. Growth of ANI on 0.8% peptone in the presence and absence of S^0



Elemental sulphur could be replaced by L-cystine or glutathione but not by a variety of other substances tested (Table 3.4.).

Table 3.4. Substitution for S^0 as electron acceptor in ANI in 0.8% peptone medium

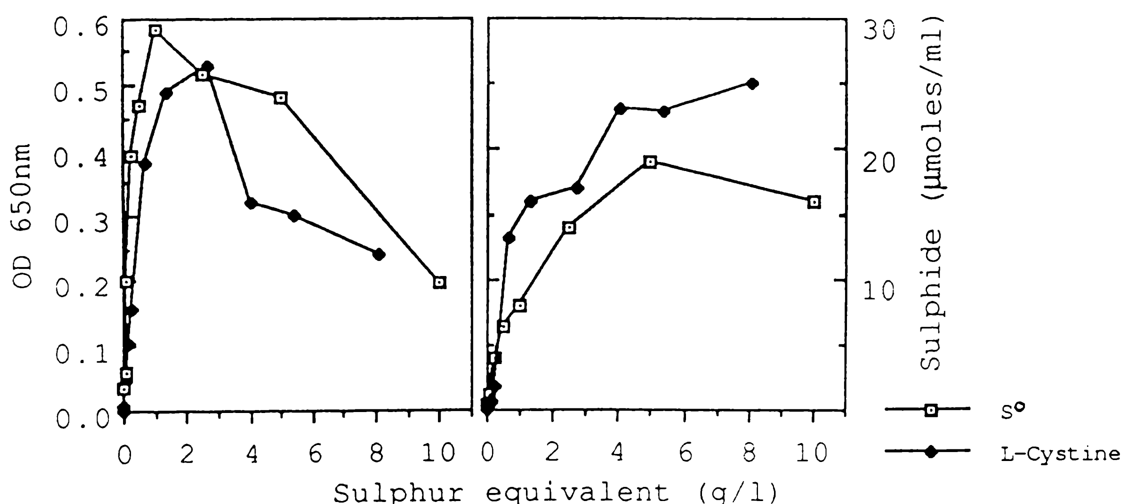
Substrate		Growth (OD_{650}) (75°C/26h incubation)
S^0	(0.5 g/l)	0.46
SO_4^{2-}	(20mM)	0.00
SO_3^{2-}	"	0.07
$S_2O_3^{2-}$	"	0.05
NO_3^-	"	0.00
malate	(10mM, 50mM)	0.00
Fe^{3+}	(1.5mM)	ND[a]
homocystine	(0.3 g/l sulphur equivalent)	0.00[b]
L-cystine	"	0.12[b]
glutathione	"	0.09[b]
S (tert butylthio) L-cystine	"	0.00[b]
propyl disulphide	"	0.00[b]
ethyl disulphide	"	0.00[b]
isopropyl disulphide	"	0.00[b]
2-hydroxyethyl disulphide	"	0.00[b]
thioctic acid	"	0.00[b]

[a]not determined as medium was very cloudy. The medium was examined under the phase-contrast microscope and no cells were observed.
[b]incubated for 48h

Isolate ANI did not grow in medium containing 0.05% peptone with 20mM acetate and amino acid Stickland pairs (Nisman 1954; Gottschalk 1986) alanine/glycine, alanine/proline, or valine/proline (25mM each). After 24 and 48 hours incubation at 75°C the OD_{650} in all inoculated cultures had not increased compared to uninoculated controls.

The effect of different concentrations of elemental sulphur and L-cystine on the growth of isolate ANI were investigated and the results are illustrated in Fig.3.9. With S^0, growth yield increased up to a concentration of 1g/ℓ, at higher concentrations growth inhibition became pronounced. Cystine gave a similar result but the optimum equivalent sulphur concentration was 2.7g/ℓ (1g/ℓ sulphur equivalent is present in 3.75g/ℓ cystine). Maximum growth yields of ANI on optimum concentration of either S^0 or cystine were essentially the same. Surprisingly, for both sulphur compounds, the production of sulphide increased along with increased concentration of sulphur equivalent, i.e., whereas sulphide production was proportional to growth up to the optimum sulphur equivalent concentration, at higher concentrations of sulphur where growth was inhibited, increased amounts of sulphide were formed.

Fig.3.9. Growth yield and sulphide production on 0.8% peptone medium containing a range of amounts of elemental sulphur and L-cystine respectively (36 h. incubation at 75°C)



The reason for inhibition at the higher concentration is not known. One possibility was the production of sulphide abiotically by interaction between sulphur and medium constituents. This does occur but under the conditions used in this experiment a maximum of 1.8μmoles/ml sulphide were produced abiotically on incubating the medium at 75°C for 48h in the presence of 10g/ℓ S^0. In fact, an additional 10μmoles/ml of sulphide was produced as the elemental sulphur concentration was increased from 1 to 5g/ℓ at which stage growth inhibition was marked. Thus the increased production of sulphide must be a result of ANI metabolism even though the organism was showing growth inhibition at these concentrations. In effect the amount of sulphide produced per unit of biomass had increased between 3 and 5 fold.

The decrease in growth of ANI at higher sulphur concentrations is similarly not due to sulphide reaching inhibitory concentrations. Firstly, at the onset of growth the initial sulphide concentration was always low; even at 20g/ℓ sulphur sulphide concentrations at inoculation did not exceed 3.2μmoles/ml (compared to 0.06μmoles/ml in medium with 1g/ℓ sulphur). When medium was supplemented with sodium sulphide such that the concentration at inoculation was 18μmoles/ml very little inhibition of growth was observed. After 28 hours incubation at 75°C, inoculated medium with Na_2S added had an OD_{650} of 0.36 compared to 0.40 in medium without Na_2S addition. However, complete inhibition of growth was observed when Na_2S was added to a final concentration of 30μmoles/ml. The pH of media containing 1g/ℓ and 20g/ℓ of S^0 was measured after heating to 100°C and was found not to vary much (pH 7.4 in the presence of 1g/ℓ and 7.3 in the presence of 20g/ℓ S^0).

It had to be clarified whether the stimulation of growth in the presence of increased amounts of L-cystine was due to increased availability of disulphide bonds or due to the utilization of L-cystine as an energy source. L-cystine stimulated the growth yield of ANI to 17% of growth yield obtained with 0.8% peptone when added to medium with reduced amounts of peptone (section 3.3.4). In the case of catabolic breakdown of L-cystine, increased amounts of acetate compared to other fatty acids would be expected. Acetate increased in relation to other end products (Table 3.5).

Table 3.5. Volatile fatty acid end products from growth on 0.8% peptone in the presence of 10g/ℓ L-cystine or 2g/ℓ S^0 (autoclaved) (48 h at 75°C)

Fatty acid	Production (mM)	
	10g/ℓ cystine	2g/ℓS^0
Acetate	25.3	5.1
Propionate	0.8	0.7
Isobutyrate	1.6	2.9
Isovalerate/ 2-Methylvalerate	5.1	6.0

Stimulation of growth rate and yield by increased availability of sulphur was examined using S^0 in media which had been autoclaved (where sulphur was in one solid lump) or only heated to 100°C (where sulphur remained as small suspended particles). As is apparent from the results (Fig.3.10), growth was greatly stimulated when sulphur was more freely available. Not considered are possible chemical reaction modifying the sulphur upon autoclaving. After 25h there was no sulphur visible in cultures with a sulphur suspension, while the lump of sulphur in cultures with autoclaved sulphur was still clearly visible.

Fig.3.10. Growth in 0.8% peptone with 0.5g/ℓ S^0 autoclaved (121°C) vs. heated (100°C)

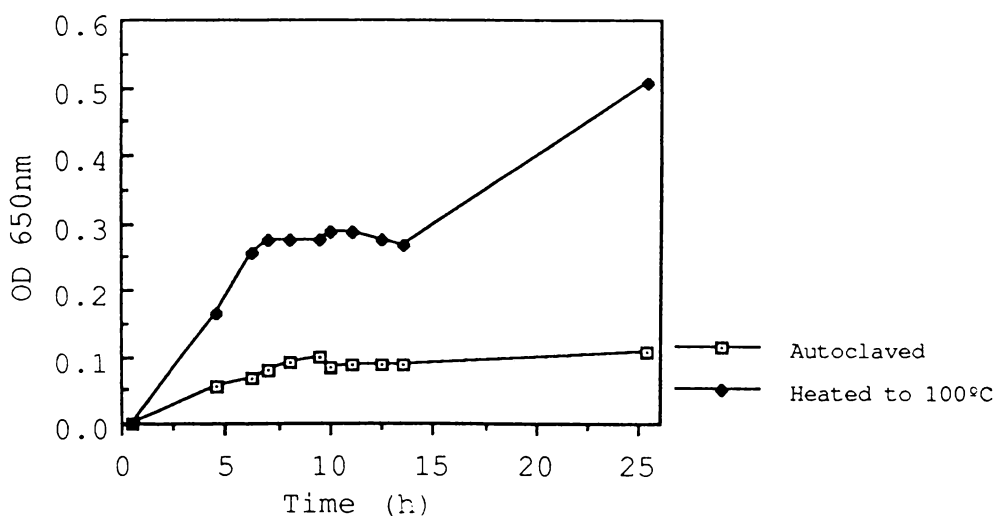
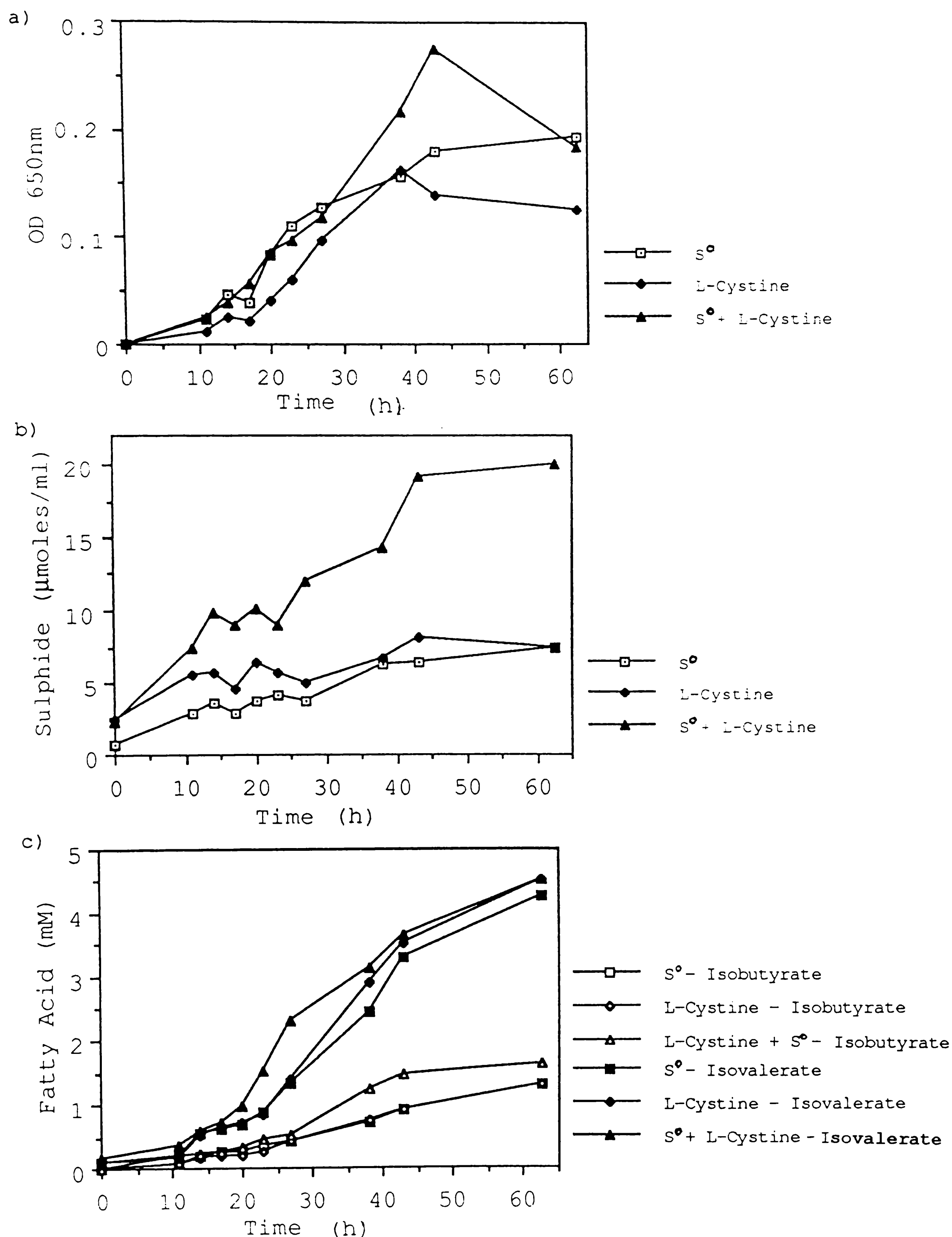


Fig.3.11. Growth and production of sulphide, isovalerate, and isobutyrate on 0.8% peptone with S^0 (2g/ℓ), L-cystine (1g/ℓ), or S^0 (2g/ℓ) plus L-cystine (1g/ℓ)

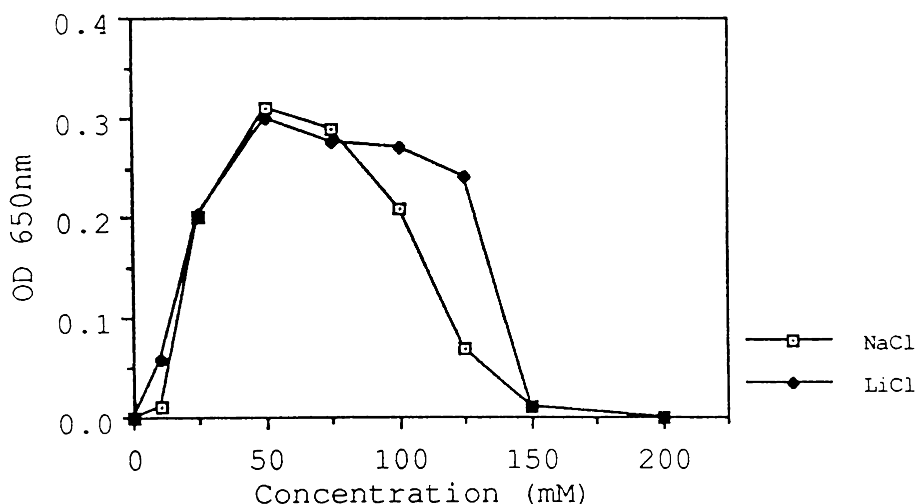


Isolate ANI was grown in 0.8% peptone medium in the presence of S^0 (2g/ℓ), cystine 1g/ℓ), or both. This was done in order to test for different effects on growth of either constituents over time. Both were added together to see if growth would be stimulated. Growth and sulphide production showed an increase when both S^0 and cystine were added (Fig.3.11). No increase in production of isobutyrate or isovalerate in cultures with S^0 and cystine was found compared to cultures with either only S^0 or only cystine. Acetate was not measured in this experiment.

c) Sodium requirement

The organism obligately required sodium for growth. No growth was observed below 10mM or above 150mM of NaCl or similar concentrations of LiCl (Fig.3.12).

Fig.3.12. Range and optimum concentration of NaCl and LiCl for growth in 0.8% peptone (36 h. at 75°C)



The requirement was shown to be for Na^+ rather than Cl^-, as Na_3-citrate and Na-sulphate but not KCl could serve as a substitute for NaCl. Na^+ could be substituted for by Li^+, but not by K^+, Rb^+, Cs^+, Mg^{2+}, or Ca^{2+}. NaCl could not be substituted for by $NaNO_3$ (Table 3.6).

The requirement was not of a purely osmotic nature as sucrose at concentrations between 10 and 200mM, which corresponds to osmolality of NaCl between 3.7 and 115mM NaCl did not support growth at all. (Osmolality was calculated from standard tables [Wolf *et al.* 1982].)

Table 3.6. Substitution of NaCl as a growth requirement (added at 50mM final concentration)

Substrate	% Growth yield[a] (48 h./75°C)
NaCl	100
KCl	0
LiCl	92
RbCl	0
CsCl	0
$MgCl_2$	5
$CaCl_2$	0
Na-citrate	83
$NaSO_4$	35
$NaNO_3$	15

[a]where 100% growth was OD_{650} of 0.43

The organism's requirement for sodium was further investigated and is discussed in more detail in chapter 4.

3.3.7. Antibiotic sensitivities

Isolate ANI was sensitive to the antibiotics mevinolin (5μg/ml) and monensin (1μg/ml). No inhibition was observed in the presence of chloramphenicol (100μg/ml), penicillin G (10μg/ml), or rifampicin (100μg/ml). Penicillin G has however been shown since to lose its potency rapidly at 72°C (Peteranderl *et al.* 1990).

3.4. Discussion

Isolate ANI is a novel member of the *Thermococcales*. In contrast to other described species of this order, ANI was isolated from a non-marine environment.

In its physiology, ANI resembles species of *Thermococcus*. The mol% G+C of DNA of isolate ANI lies well within the range reported for *Thermococcus* of between 38-40 mol% for *T. litoralis* and 56 mol% for *T. celer*. Lodwick *et al.* (1986) investigated *dam* methylation in some archaebacteria (mainly methanogens and halophiles). From their data it appears that the Dam^+ phenotype is present in all species of one genus but not in all genera of the same family. Of the thermophilic sulphur-utilising archaebacteria, only *Sulfolobus solfataricus* was tested. Knowledge of the Dam phenotype of other members of the *Thermococcales*

would be of interest as further indication of their relatedness.
Compared to other *Thermococcales*, ANI has a more alkaline pH optimum. Its optimum growth temperature was below that of other *Thermococcales* by 10°C or more (except for *T. stetteri* which has a similar optimum to ANI [Miroshnichenko *et al.* 1989]).

Isolate ANI has an obligate growth requirement for peptone, yeast extract, or casein. Peptides have been reported to be stimulatory in the uptake of nonpolar amino acids in *Fusobacterium varium* (Gharbia *et al.*1989). Stimulation of growth of several other anaerobic bacteria by peptides has been attributed to poor capacity to utilize amino acid nitrogen compared to peptide nitrogen. An obligate requirement for oligopeptides has been shown in *Thermoplasma acidophilum* (Smith *et al.* 1975). For this organism peptides were suggested to be involved in ion scavenging and protection of the organism from its acidic environment. Yeast extract has been suggested to serve as a surface-active agent on elemental sulphur, by helping to increase the surface area available for interaction of the cell with sulphur (Parameswaran *et al.* 1987, 1988).

No growth was observed on carbohydrates, organic acids, or amino acids which is similar to findings in other *Thermococcales* (see Table 3.7 for references). Some amino acids stimulated growth in the presence of low levels of peptone. Maximum growth yield was reached at lower levels of peptone when amino acids were added. The requirement for peptone seems to be primarily that of a growth factor and amino acids seemed to be suitable alternative substrates. Growth on peptone and production of fatty acids suggests growth by fermentation. Amongst sulphur-utilizing archaebacteria production of volatile fatty acids has so far been reported in *Staphylothermus marinus* (Fiala *et al.* 1986), *Hyperthermus butylicus* (Zillig *et al.* 1990), and *Themoproteus (Tp.) tenax* and *Tp. uzoniensis* (Bonch-Osmolovskaya *et al.* 1990b), and in *T. stetteri* (Miroshnichenko *et al.* 1989); *S. marinus* producing acetate and isovalerate and the latter three organisms producing acetate, isobutyrate, and isovalerate.

In isolate ANI, formation of acetate from alanine (Table3.3) was probably via pyruvate as intermediate (see also section 5.4.4).

Threonine is degraded to propionate, H_2, and CO_2 in organisms like *Clostridium propionicum* and *Peptostreptococcus prevotii* (Gottschalk 1986). However, acetate formation from growth on threonine medium has been shown in strains of *Clostridium histolyticum* (Elsden and Hilton 1978). A suggested pathway for acetate formation from threonine in these organisms involved action of threonine aldolase followed by an oxidoreduction between the products acetaldehyde and glycine to form acetate and ammonia. Metabolism of neither amino acid was investigated further.

In respect to branched-chain amino acids, the results shown above (Fig.3.6, Table 3.3) demonstrate that production of isobutyrate and isovalerate/2-methylvalerate is due to breakdown of valine and leucine/isoleucine respectively. When leucine and valine were added together, less of each respective fatty acid was produced (Table 3.3). Final growth yield was the same as on leucine alone and the amount of isobutyrate and isovalerate produced combined was similar to the amount of isovalerate produced when only leucine was added. The two amino acids appeared to exert some degree of inhibition on utilization of one another. A similar effect was observed with leucine and α-ketoisocaproate (section 5.3.1). Growth yield on the D-isomers was reduced and they were utilized only to a limited degree as reflected in low production of the respective fatty acids. As found with the L-isomers, D-leucine stimulated growth yield to a greater degree as did D-valine. Norleucine and norvaline both served as suitable substrates. Formation of butyrate and valerate indicates that these two amino acids were degraded in a fashion analogous to the branched-chain forms. In contrast to their branched-chain counterpart, norvaline was a superior substrate over norleucine. Production of NH_4 corresponded quite well with volatile fatty acid production, indicating that for the amino acids examined per mole of amino acid degraded (assuming NH_4 determination to be close to 100% recovery) one mole of fatty acid is formed. As peptone was present in all media, not all fatty acid detected would have been the product of the single amino acid added. When D-leucine or norleucine were added the amount of NH_4 detected exceeded the sum total of fatty acids detected (in mM) by at least 1mM and such large increase in NH_4 over fatty acids was not detected with other amino acids used here. When threonine was added more acetate was produced than NH_4. Discrepancies between NH_4 and fatty acid production with other amino acids were probably due in part to utilization of NH_4

and catabolites in production of biomass. Also, other minor end products may not have been detected (e.g. hydroxy acids).

In contrast to described *Thermococcales* (except for *T. stetteri*), isolate ANI could not be grown in the absence of sulphur (S^0, L-cystine, or glutathione). The organism had an obligatory requirement for S^0 but at concentrations above 1g/ℓ this became progressively inhibitory. The reason for this inhibition was not further investigated. In the presence of 10g/ℓ L-cystine almost nine times more sulphide was produced than in the presence of 1g/ℓ L-cystine. Growth yield increased almost four times. When ANI was grown in the presence of greater amounts of S^0 or L-cystine added or in the presence of both S^0 and L-cystine, the relative increase in sulphide production was greater than the increase in growth yield. The observed increase in growth yield in the presence of increased amounts of L-cystine and S^0 up to a degree was probably due in part to increased access to sulphur bonds (increased surface area) which resulted in production of more sulphide. When S^0 was less accessible (when medium was autoclaved) growth yield was reduced considerably (at 0.5 g/ℓ S^0 added). In contrast to S^0, cystine served both as substrate and as 'electron' acceptor. A greater increase in sulphide production in the presence of a sulphur equivalent of 1g/ℓ or more of cystine compared to S^0 was observed. From the amount of acetate produced in the presence of 10g/ℓ cystine (Table 3.5) it appears that about 9mM cystine were catabolized (if all acetate produced above the amount produced in the 2g/ℓ S^0 control culture without cystine was from degradation of cystine and each molecule of cystine yielded two of acetate). The increase in sulphide production in the presence of cystine compared to S^0 may therefore be due to increased metabolic activity. Or, more cystine may have been utilized as substrate than as acceptor of reducing equivalents and sulphide was a product of metabolic degradation. However, the lack of increase in growth yield in the presence of cystine compared to S^0 indicates that if cystine was used as growth substrate, little or no net energy gain (ATP production) was coupled to its catabolism. Cystine and alanine could serve as substrate and cystine could serve as a substitute for S^0. Cysteine however could not be utilized either as growth substrate nor as substitute for S^0. Production of acetate may have occurred via pyruvate (maybe catalyzed by cysteine desulfhydrase) (see also section 5.4 and Fig.5.6). Production of sulphide and H_2 was greater than production of NH_4 or

fatty acids on 0.8% peptone medium indicating that sulphide (and H_2) produced is not solely related to the breakdown of amino acids. This may indicate that not all sulphide produced is directly related to growth yield. Production of sulphide even at stationary phase and to levels not related to increased growth has been observed in *Pyrodictium (Pd.) occultum* and *Pd. brockii* (Parameswaran *et al.* 1987, 1988) where yeast extract and H_2 partial pressure respectively were strong stimulants of sulphide production. In this study abiological production did not appear to be a major cause for the great increase in sulphide observed. Studies by Belkin *et al.* (1985) have shown that abiological sulphide production is strongly temperature dependent but occurs only at low levels at temperatures below 85-90°C in the medium used in their studies. The requirement of sulphur and production of sulphide are discussed further in chapter 6.

An obligate requirement for sodium was found in ANI which confirms results shown on the ecological distribution of ANI-like organisms (chapter 2).

All *Thermococcus* species so far described exhibit sensitivity to rifampicin (Miroshnichenko *et al.* 1989; Neuner *et al.* 1990). While originally *T. celer* was described as rifampicin resistant (Zillig *et al.* 1983b) in a later publication it is referred to a rifampicin sensitive (Neuner *et al.* 1990). A variety of archaebacteria have been screened for their resistance to antibiotics with different target sites in eubacteria (Hilpert *et al.* 1981). Rifampicin has been shown to be non-inhibitory on the DNA-dependent RNA polymerase of archaebacteria tested. Inhibition by rifampicin of the growth of halophiles has been attributed to a possible detergent effect. Inhibition of growth of *Thermococcus* species may be due to similar reasons. Resistance against rifampicin demonstrated in isolate ANI may be due to its unique major lipid component (Lanzotti *et al.* 1989).

In Table 3.7 isolate ANI is compared to other archaebacterial sulphur-utilizing cocci in some of its characteristics. Isolate ANI appears to be more closely related to *Thermococcus* than *Pyrococcus* from %mol G+C and characteristics of growth and physiology. Sequencing of its 16s RNA, however, would be required for a judgement of whether it is a new species of *Thermococcus* or whether it represents a new genus within the

Thermococcales. As little is known about general growth characteristics of these organisms, a thorough comparison on the level of their physiology cannot be made. Isolate ANI differs from species described for the genus *Thermococcus* in characteristics such as optima for temperature, pH, and NaCl, resistance to rifampicin, and presence of PGD as major cell lipid component.

Table 3.7. Comparison of ANI to other thermophilic archaebacterial sulphur-metabolizing cocci

a) Thermococcales

	ANI	Thermococcus celer	Thermococcus stetteri	Thermococcus litoralis	Pyrococcus woesei	Pyrococcus furiosus
Temperature°C						
Range	55-85	55-93	55-94 (98)	55-98	70-104	70-103
Optimum	75-80	88	75 (88)	88	100-103	100
pH Range	5.5-9.0	NA	5.7-7.2	6.0-8.5	NA	5.0-9.0
Optimum	7.4	5.8	6.5	7.2	6.0-6.5	7.0
G+C mol%	46.0 +/-0.4	56.6	50.2	39.1	37.5	38
S^0 Required	+	strongly stimulatory	+	stimulatory	_	stimulatory (H_2 toxic)
NaCl Required (optimum)	+ (2.5g/l)	+ (38g/l)	+ (25g/l)	+ (25g/l)	+ (30g/l)	+ (20g/l)
Morphology	regular coccus	irregular coccus	irregular coccus	irregular coccus	irregular coccus	regular-irregular coccus
Flagella	+	+	- (+)	-	+?	+
Cell Lysis at Critical Concentration	+	+ in closed vessels	-	NA	+	NA

Nutrition (sole carbon source)	peptone, yeast extract, casein	peptone, tryptone, casein	peptone, starch, pectin	peptone, yeast extract, tryptone 'marine broth'	yeast extract, tryptone, starch	yeast extract, tryptone, starch, maltose meat/bacterial extract (feeble growth on casamino acids)
Metabolic Products	H_2, CO_2, H_2S, NH_4, acetate, propionate, isobutyrate. isovalerate	NA	H_2, CO_2, H_2S, isobutyrate, isovalerate	NA	H_2, CO_2, H_2S	H_2, CO_2, H_2S
Lipids (major component in %)	diethers (PGD*, 45%) (PID**, 40%)	diethers (PID, 80%)	NA	NA	diethers (PID, 90%)	diethers (NA)
Isolation Source	Rotorua, New Zealand	Vulcano, Italy	Northern Kurils	submarine thermal spring	Vulcano, Italy	Vulcano, Italy
Reference	Morgan and Daniel, 1982; this study	Zillig et al., 1983b	Miroshnichenko et al., 1989	Belkin and Jannasch, 1985	Zillig et al., 1987	Fiala and Stetter, 1986

*phosphate glycosyl diether
**2,3,-di-*o*-phytanyl-*sn*-glycero-1-phosphoryl-1'-myo-inositol

Table 3.7.b. Other sulphur-metabolizing archaebacterial extremely thermophilic cocci

	Desulfurococcus mucosus	D. mobilis	D. amylolyticus	D. strain S	Caldococcus litoralis	Staphylothermus marinus	Strain ES-1
Temperature°C							
Range	NA	NA	NA	50-95	55-100	70-92	50-91
Optimum	85	85	90-92	90	88	85-92	82
pH Range	4.5-7.0	4.5-7.0	5.7-7.5	5.5-8.0	5.9-7.0	4.5-8.5	6.0-8.5
Optimum	6.0	6.0	6.4	7.5	6.4	6.5	7.2
G+C mol%	51.3	50.8	42.1	52.01	42 +/- 2	35.3	
S^0 Required	strongly stimulatory	strongly stimulatory	stimulatory stimulatory	strongly stimulatory	strongly	+	-
NaCl Required (Optimum)	NA	NA	- (2g/l inhibitory)	+ (23-26g/l)	+ (25g/l)	+ (15g/l)	+ (20g/l)
Morphogy	regular coccus	regular coccus	irregular coccus	irregular coccus	oval, irregular coccus in clusters	regular/slightly irregular coccus	irregular coccus
Flagella	-	+	non-cellular appendages	+	monopolar filamentous bundles resembling flagella	-	-
Cell Lysis at Critical Concentration	NA	NA	NA	NA	when H_2S is not removed (7mM or above)	NA	NA

Nutrition (sole carbon source)	YE bactotryptone, casein,tryptic casein digest	YE bactotryp- tone, casein di- gest, casein	peptides glycogen pectin starch (feeble growth on amino acids)	peptone tryptone yeast extract	yeast extract, peptides, casein,casam- ino acids-and hydrolysate (feeble growth on amino acids)	yeast extract, peptone, meat extract, bacterial extract	yeast extract, peptone, casein hydro- lysate
Metabolic Products	NA	NA	NA		NA	CO_2, H_2S, acetate, isovalerate	NA
Lipids	diethers, tetraethers	diethers, tetraethers	NA tetraethers	di-and tetraethers novel C30 lipid	NA tetraethers	diethers,	diethers,
Isolation Source	hot springs, Iceland	hot springs Iceland	thermal springs Kunashir Island, Kamchatka	deep-sea hydrother- mal vents	hydrothermal vents,hot beach, Kunashir Island	beach,Vulcano, Italy, submarine hydrothermal vent,East Pacific Rise	polychaete worm from submarine hydrothermal vent, Juan De Fuca Ridge
Reference	Zillig et al. 1982b	Zillig et al. 1982b	Bonch- Osmolovska al. 1988	Jannasch et al. 1988	Svetlichnyi et al. 1988	Fiala et al. 1986	Pledger and Baross 1989

***isolate strain SY described in the same publication differed from isolate strain S in its temperature optimum (85°C)and range(50-90), pH range (5.5-7.5) and optimum (pH 7.0), and absence of flagella

Chapter 4: Investigation into the nature of the sodium requirement

4.1. Introduction

In chapter 3 a requirement for sodium for growth of ANI was shown. As stated in the review of the literature (section 1.6), there are different functions sodium ions can perform in an organism. This chapter describes experiments which were carried out to elucidate the nature of the sodium requirement in ANI. Experiments focussed on a possible role of Na^+ in solute uptake and in energy conservation and transduction. A possible involvement of Na^+ in pH homeostasis, a general function in membrane integrity or enzyme stability of the organism were not investigated. Sodium may be involved in any one or combinations of such functions in ANI.

From the literature it is apparent that many studies regarding the sodium requirement of organisms for solute uptake or energy generation involve the use of inhibitors of antiport systems or of membrane-bound ATPases, and ionophores which disrupt gradients of cations across the membrane. The coupling of sodium ion gradients for energy generation and solute uptake has already been reviewed (section 1.6); the effect of inhibitors and ionophores on this coupling will now be described, with particular reference to the involvement of sodium ion gradients in amino acid transport and primary transport systems such as cation-driven ATPases. The ionophores and inhibitors and their actions which are used in this study are shown in Fig.4.1.

In the introduction and in the results section dealing with ionophores possible effects on a potential secondary transport (here Na^+/amino acid symport) are discussed first and then possible primary transport systems like H^+- or other cation-translocating ATPases.

Ionophores have been defined as antibiotics which form organosoluble complexes with alkali cations and mediate their transport across lipid barriers (Rees 1979).

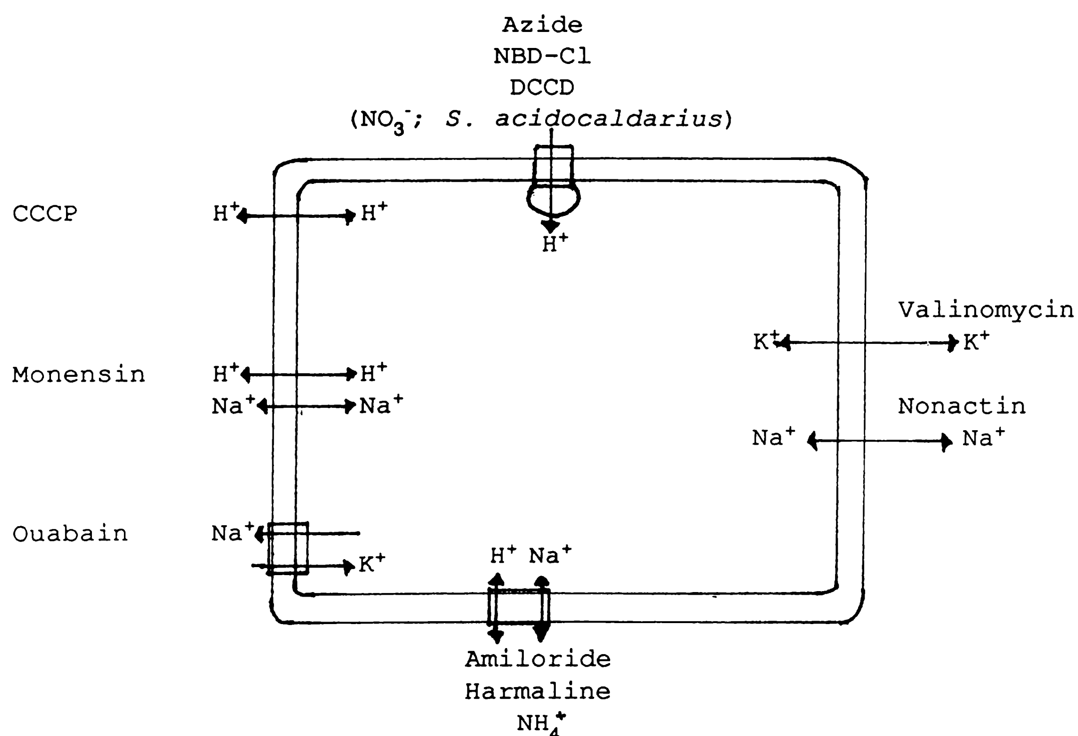
Monensin is a carboxylic ionophore. It catalyzes the exchange of Na^+ or K^+ for H^+ with a selectivity of Na^+:K^+ of 10:1 (Schönheit and Beimborn 1986). Therefore, monensin disrupts concentration gradients of both Na^+ (K^+) and H^+ but not the charge gradient since one positive

charge is exchanged for another. Monensin would, therefore, affect Na^+/solute symport systems and the results of Na^+/H^+ antiporter activity or primary Na^+ pumps.

Valinomycin is a neutral ionophore. In contrast to carboxylic ionophores, neutral ionophores do not catalyze the exchange of alkali cations for H^+ but carry only the alkali cation across the membrane, therefore, altering the membrane potential. The preference of valinomycin for K^+ over Na^+ is at least 400 times (Harold 1970). In respect to involvement of Na^+ in primary or secondary transport systems, valinomycin is used to dissipate the membrane potential (in the presence of external K^+) without directly interfering with either Na^+ or H^+ gradients.

Nonactin is another neutral ionophore. Its action is similar to that of valinomycin, but its selectivity for K^+ over Na^+ is not as great. In addition to dissipation of the membrane potential nonactin disrupts the Na^+ gradient (in the presence of Na^+). However, in the presence of nonactin ΔpH is still intact

Fig.4.1. Action/sites of action of ionophores and inhibitors on the cytoplasmic membrane



Carbonyl cyanide m-chlorophenylhydrazine (CCCP) is a protonophore, catalyzing the transfer of protons across the membrane. It will, therefore, interfere with both H^+/solute symport systems and generation of ATP via membrane-bound H^+-translocating ATPases. CCCP does not directly disrupt the Na^+ gradient. Its effect on the membrane potential is pH dependent.

Monensin, nonactin, valinomycin, and CCCP have all been used in studies on amino acid uptake. The uptake of radiolabelled amino acids in cell suspensions of *Corynebacterium glutamicum* was found to be mediated by a Na^+ symport system (Ebbighausen *et al.* 1989). Monensin (2µM) inhibited amino acid uptake. This was assumed to be due to abolition of the Na^+ gradient by the ionophore. Valinomycin (10µM) and CCCP (50µM) inhibited uptake as well. The inhibitory effect of valinomycin was ascribed to a requirement for the membrane potential in amino acid uptake. In the presence of valinomycin and K^+ (10mM) the membrane potential was dissipated. In cell suspensions of *Methanococcus voltae* isoleucine uptake was inhibited by monensin (20µM) and CCCP (20µM) (Jarrell *et al.* 1984).

Sodium/amino acid symport was studied in a ruminal bacterium (Chen and Russell 1990). Growth of the ruminal bacterium was inhibited completely by monensin (5µM) or absence of NaCl (8.2mM). Valinomycin (5µM) or the protonophore TCS (10µM) caused a delay in growth but similar final growth yield to control cultures. DCCD (5µM) had no effect on growth. Uptake of glutamate was measured in cell suspensions under conditions of dissipated Na^+ gradient and/or membrane potential. Absence of a Na^+ gradient and of the membrane potential resulted in complete inhibition, while presence of the membrane potential only, allowed a small initial rise in uptake. Presence of the Na^+ gradient in absence of the membrane potential allowed uptake although not quite as efficiently as in the presence of both the Na^+ gradient and the membrane potential. The experiments were carried out over 2min only, and dissipation of gradients was performed using cells loaded with Na^+ or K^+ in K^+ buffer or Na^+ plus K^+ buffer. It has been proposed that the ruminal bacterium possesses a primary Na^+ pump coupled to decarboxylation of glutaconyl-CoA, and a Na^+-translocating ATPase.

Nonactin inhibits the uptake of some amino acids in *Bacillus stearothermophilus* (De Vrij *et al.* 1990). As nonactin catalyzes an electrogenic transport across the membrane, a ΔpH still exists.

Therefore, transport of amino acids inhibited by nonactin is Na^+ dependent as the Na^+ gradient is destroyed but the H^+ gradient is still intact. Monensin on the other hand, increased the steady-state level of uptake of amino acids which are taken up via Na^+ symport as it converts the ΔpH into a $\Delta\mu Na^+$ in the presence of sodium.

Monensin has been an important tool in the demonstration of primary Na^+ pumps coupled to decarboxylation reactions in several eubacteria. Monensin inhibited Na^+ transport at 0.51μM in *Acidaminococcus fermentans* (Buckel and Semmler 1982). In *Propionigenium modestum* demonstration of the Na^+-translocating ATP synthase in reverse (measuring Na^+ uptake in membrane vesicles in the presence of ATP) was inhibited by the presence of monensin (50μM) (Hilpert *et al.* 1984). Decarboxylases acting as primary Na^+ pumps have been discussed previously (section 1.6). The effects of ionophores on methanogenesis and energy-related processes were studied in *Methanobacterium bryantii* (Jarrell and Sprott 1983). Monensin (20μM), CCCP (20μM), and valinomycin (20μM) were used in these studies. Monensin strongly inhibited methanogenesis but did not influence the membrane potential. CCCP inhibited methanogenesis (in absence of cysteine) but did not significantly reduce the membrane potential (at pH 6.82). Valinomycin in absence of K^+ lowered the membrane potential only slightly and did not interfere with synthesis of methane. However, valinomycin plus K^+ (0.5M) dissipated the membrane potential and inhibited methanogenesis by 40%. Cysteine (1-3mM) was found to block the action of CCCP. An apparent insensitivity to CCCP has been observed in *Methanobacterium thermoautotrophicum* (Kaesler and Schönheit 1988). The insensitivity has been ascribed in part to ether lipids of the membrane and in part to the cell wall hindering proton translocation by the protonophore, and to incomplete dissipation of the electrochemical proton potential $\Delta\mu_{H+}$. A Na^+/H^+ antiporter was demonstrated in *M. thermoautotrophicum* (Schönheit and Beimborn 1985, 1986). Two inhibitors of Na^+/H^+ antiport systems, amiloride and harmaline were used in these studies. Although these compounds had previously been used mainly in eukaryotic systems (Aaronson and Bounds 1980; Kinsella and Aaronson 1981), their inhibitory effect on methane formation in *M. thermoautotrophicum* was the first report of these inhibitors acting on prokaryotic systems

(Schönheit and Beimborn 1985). The inhibition by harmaline was relieved by the addition of NaCl which acted as competitive inhibitor. Monensin has also been tested for its effect on methane formation. While it had no effect on *M. thermoautotrophicum* in the presence of NaCl and high KCl, it stimulated methane formation at low NaCl (>0.2mM) and high K^+ (>25mM) concentration.

N'N-Dicyclohexylcarbodiimide (DCCD) covalently interacts with a proteolipid portion of the F_0F_1-ATPase. Interaction of one molecule of DCCD with one DCCD-binding subunit per ATPase complex inhibits the enzyme. DCCD has also been shown to interact and inhibit also vacuolar ATPases, NAD transhydrogenase, cytochrome c oxidase, the cytochrome bc_1 complex, and gastric H^+/K^+ ATPase (Solioz 1984). DCCD has been used as a possible inhibitor in most studies on prokaryotic or vacuolar ATPases. Growth of *Sulfolobus acidocaldarius* was inhibited by 1mM DCCD (Wakagi and Oshima 1986). The membrane-bound ATPase of *Methanosarcina barkeri* lost 90% of its activity in the presence of DCCD (10μM), while ouabain (100μM) caused a 19% loss, and 7-chloro-4-nitrobenzo-2-oxa-1,3,-diazole (NBD-Cl) (1mM) caused a 29% loss in activity (Inatomi 1986). Demonstration of an electrogenic sodium-translocating ATPase in *Methanococcus voltae* involved use of valinomycin (10μM), a protonophore (SF6847) and monensin (10μM) (Carper and Lancaster 1986). Cellular ATP levels in cell suspensions were monitored over 20 to 30 second intervals over periods of 5min or less upon additions of sodium and ionophores. Addition of NaCl (400mM) to cell suspensions resulted in a slow rise in cellular ATP which was increased 4-fold by addition of valinomycin plus NaCl while valinomycin plus monensin plus NaCl or valinomycin plus KCl (100mM) plus NaCl inhibited an increase in ATP levels. This effect was explained to be due to the membrane potential being induced by valinomycin-catalyzed K^+ efflux (which is inhibited in the presence of external K^+). The increase in ATP levels was attributed to possible stimulation of an ion-translocating ATPase (involving some ion other than Na^+) or to the electrogenic movement of Na^+ through such an ion pump. Since addition of monensin prevented the increase in ATP (as it allows Na^+ to move into the cell instead of moving through through the ATPase into the cell) the increase in ATP was not due to presence of Na^+ in the medium but to transport of Na^+. Addition of a protonophore plus valinomycin did not reduce the stimulatory effect of valinomycin alone significantly, showing that H^+

served no obligatory role in this process. Sodium alone could not drive ATP synthesis to a significant degree; however when a permeant charge counter ion (here sodium tetraphenylborate) was added, the increase in cellular ATP was about 50% of that induced by a membrane potential (addition of valinomycin). The action of the counter ion prevents formation of a reverse membrane potential (set up by electrogenic movement of Na^+, driven by the chemical Na^+ gradient) which would inhibit further movement of Na^+.

Table 4.1.: Ionophores and Inhibitors of Membrane-linked Processes

Compound	Action	Reference
Monensin	electroneutral Na+/H^+ antiporter	Pressmann (1976)
Valinomycin	electrogenic exchange of K^+	"
Nonactin	electrogenic exchange of Na^+	"
CCCP*	protonophore	Heytler (1979)
DCCD**	inhibitor of F_0F_1-ATPases, archaebacterial H^+-translocating ATPases	Linnet and Beechey (1979)
Ouabain	inhibitor of eukaryotic Na^+/K^+ ATPases	Benyoucef *et al.* (1982)
NBD-Cl***	inhibitor of V-type, F_0F_1-type, and archaebacterial H^+-translocating ATPases	Linnet and Beechey (1979)
NO_3^-	inhibitor of V-type and archaebacterial ATPases	Nelson and Taiz (1989)
Azide	inhibitor of F_0F_1-type ATPases and electron transport	Linnet and Beechey (1979)
Amiloride	inhibitor of Na^+/H^+ antiporters	Schönheit and Beimborn (1985)
Harmaline	inhibitor of Na^+/H^+ antiporters	"
NH_4^+	inhibitor of Na^+/H^+ antiporters	"

* carbonyl cyanide m-chlorophenylhydrazine
** *N,N'*-dicyclohexyl-carbodiimide
*** 7-chloro-4-nitrobenzo-2-oxa-1,3,-diazole

The abovementioned ionophores and inhibitors were tested in their effect on growth and cellular ATP levels of ANI. By finding out which substances had an inhibitory effect on ANI, it was hoped that a certain pattern would emerge giving some indication of the nature of the sodium requirement for growth of ANI. The possible inhibitors used are listed in Table 4.1.

4.2.Materials and methods

4.2.1. Media

Media for culturing isolate ANI were as described in sections 2.2.3 and 3.2.1. For inhibition studies cultures were grown in 50ml 0.8% peptone medium in 120ml or 150ml serum bottles in the presence of S^0 (2g/ℓ) under a N_2 atmosphere at 75°C unless stated otherwise.

4.2.2. Cell suspensions

For preparation of cell suspensions, cultures were grown in 400ml or 800ml 0.8% peptone medium plus S^0 under N_2 in glass bottles. Late log phase cultures were harvested after 22-24 hours incubation at 75°C by centrifugation at 27000 x g, and washed twice in sterile anaerobic 100mM 3-[N-morpholino]propane-sulfonic acid (MOPS) buffer. The pH of 100mM MOPS buffer was adjusted to 7.4 (at 20°C) with 10M KOH. As E_h indicator 1ml/ℓ of 0.1% (w/v) resazurin was added. As a reducing agent, either 1g/ℓ L-cysteine was added prior to autoclaving or 1ml/ℓ of sterile, neutralized 10%Na_2S solution was added after autoclaving. Autoclaved MOPS buffer was cooled under sterile N_2. Between each centrifugation step, cells were resuspended in buffer in an anaerobic chamber (Coy Inc., USA). Cell suspensions were finally resuspended at approximately six-fold of their original density in lots of 10ml in 50ml serum bottles. To each bottle, nonsterile S^0 (1g/ℓ) was added anaerobically. Care was taken to exclude air from vessels at all times.

4.2.3. Inhibitors

Inhibitors used and the solvents and concentrations for stock solutions are given in Table 4.2. The stock solutions, except for nonactin and valinomycin, were prepared fresh on the day. Stock solutions of valinomycin and nonactin were kept at 4°C for up to 10 days. When using ionophores and inhibitors from ethanolic stock solutions, as

controls the same volume of 100% ethanol was added to control cultures or cell suspensions.

4.2.4. Supplements

NaCl, KCl, CsCl, trypticase peptone, and amino acids were added from sterile, anaerobic stock solutions. Salt solutions were sterilized by autoclaving at 103.42kPa/15min; amino acids and trypticase peptone at 34.47kPa/15min. Stock solutions of salts were 50mM-5M concentration, amino acids were 100mM , and peptone was a 8% solution.

Table 4.2. Ionophores and inhibitors used: their solvents and concentrations of stock solutions

Substance[a]	Solvent	Stock solution
Monensin	100% ethanol	1-5mM
Nonactin	"	10mM
Valinomycin	"	10mM
CCCP	"	10-20mM
DCCD	"	25-100mM
NBD-Cl	"	20mM
NO_3^-	H_2O	5M
Azide	"	1M
NH_4^+	"	5M
Amiloride	"	10mM
Harmaline	"	15mM

[a]for abbreviations see Table 4.1

4.2.5. Measurement of cellular ATP

The method used for the measurement of ATP was based on that described by Blaut and Gottschalk (1984). A 0.5ml sample of cell suspension was added to 0.2ml ice-cold perchloric acid and kept on ice for 90 min. It was then neutralized by the addition of 0.2ml 3M KOH and 0.1ml 0.4M N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), pH7.4 (at 20°C). The TES buffer was filtered through 0.22µm filters prior to use. The $KClO_3$ formed was removed by centrifugation (5min top speed in benchtop microcentrifuge) and the supernatant analysed for its ATP content using firefly extract (FLE-50, Sigma). Fifty mg of firefly extract were hydrated in 25ml reconstitution solution (Pridmore *et al.*

1982). The reconstitution solution contained ($g\ell^{-1}$) bovine serum albumin, 1g; EDTA, 0.37g; $MgSO_4 \cdot 7H_2O$, 2.48g; pH 7.8. The cell supernatant was diluted 10 fold in assay buffer (20mM glycylglycine; 5mM Na-arsenate; 4mM $MgSO_4 \cdot 7H_2O$; pH 8.0.; filtered through a 0.22µm filter). To 100µl sample in assay buffer in a glass tube (0.5 x 6.0cm; Kimble, USA), 200µl of firefly extract was added. Luminescence was measured immediately using an integrating photometer (SAI Technology, Co., model 3000) following Pridmore *et al.* (1982); settings used for assays were integration mode with a 10 second delay followed by a 10 second assay period. The 'zero' was set using an assay buffer blank with firefly extract. Readings were taken in triplicates.
Although cells were harvested at the same stage in growth for each set of experiments, variance in initial ATP levels was observed. However, in experiments which were repeated the relative ATP levels of extracts from different treatments were close, so the relative effects of salts, amino acids, ionophores, and inhibitors used were comparable between different experiments while absolute values of ATP levels can only be compared within each experiment.

4.3. Results and discussion

4.3.1. Cell cultures

Growing cultures of isolate ANI were investigated for their sensitivity to various ionophores and inhibitors.
The effect of monensin and CCCP was investigated to see whether disruption of the Na^+ and H^+ gradient respectively would have any effect on the cell. In the first experiment monensin (0.5µM) and CCCP (100µM) were added to cultures which had been incubated for three hours at 75°C. Growth (OD_{650}) and production of sulphide were measured periodically over twenty hours (Fig.4.2.a,b). In a second experiment cultures were incubated for twelve hours before additions of CCCP (100µM) and/or monensin (0.1µM) were made. Cellular ATP levels and sulphide production were measured at intervals and results are illustrated in Figs.4.2.c,d. Growth of ANI was not affected by addition of CCCP, while monensin had a strong inhibitory effect (Fig.4.2.a). Cells treated with monensin were examined microscopically. They had increased in their phase-translucency and in their volume to about five to six times their normal size (Fig.4.3).

Fig.4.2. Growth, production of sulphide, and cellular levels of ATP in the presence of CCCP, monensin, or both

(additions indicated by arrows)

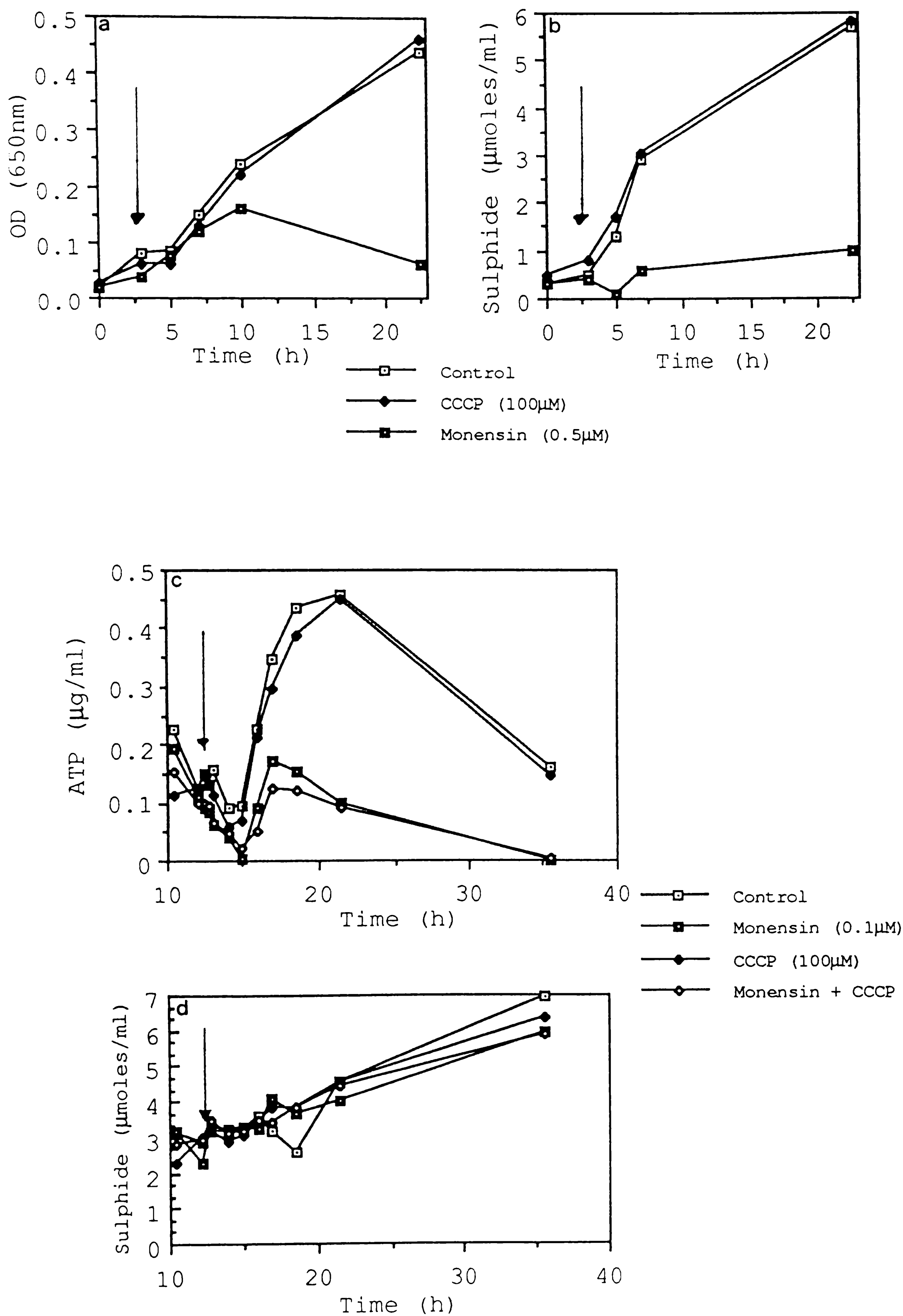
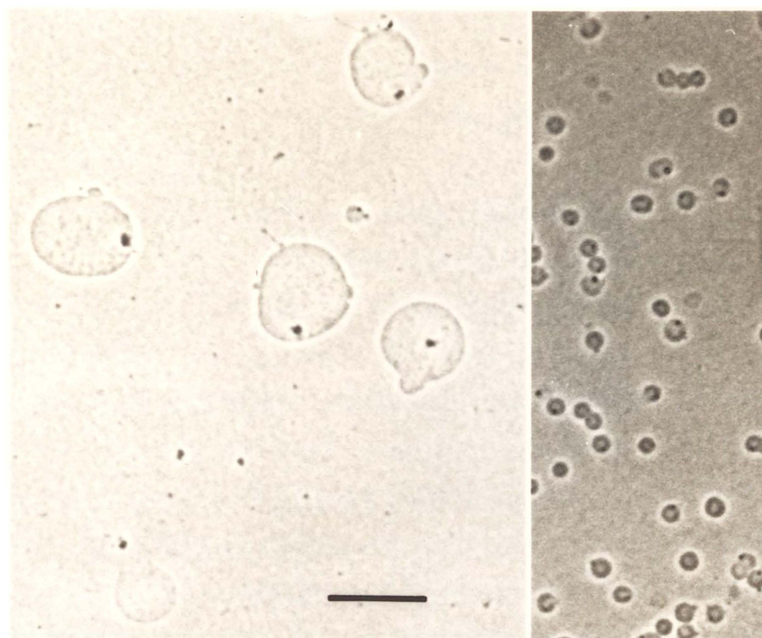


Fig.4.3. <u>Effect of monensin on the cell morphology of ANI</u>



after 28hrs in the presence of 0.5μM monensin
note the enlarged cells and their increased phase-translucency compared to normal-size cells of a control culture (shown on right)
(Bar=10μm)

Fewer cells per field of view and cell fragments were observed. In contrast, CCCP-treated cells appeared normal when examined microscopically. The cellular ATP levels in cell cultures treated with monensin, CCCP, or both, reflected the effect observed on the OD of the cultures in the previous experiment, i.e. CCCP had no inhibitory effect while monensin inhibited ATP synthesis (Fig.4.2.c) . In the second experiment monensin and CCCP were also added together to see whether the inhibitory effect of monensin observed in the first experiment was further enhanced by CCCP.

As monensin catalyzes electroneutral exchange of Na^+ and H^+ across the membrane while CCCP transports H^+ across the membrane in an electrogenic mode, addition of both together may affect more gradients across the membrane (i.e. Na^+, H^+, and possibly the charge gradient) than each added separately. However, the inhibitory effect on cellular ATP levels in the presence of monensin and CCCP was similar to that of monensin alone. Sulphide production was also monitored (Figs.4.2.b,d). In the first experiment, cultures with monensin added showed only about 20% of the amount of sulphide produced in control cultures and cultures with CCCP added. In the second experiment however sulphide production continued almost unaffected after ionophore addition (after 12h) regardless of which ionophore was added (cultures with monensin or monensin and CCCP added showed only a 20% decrease in sulphide levels after 40 h. Therefore, the time at which monensin was added to cultures seems to affect production of sulphide. In the first experiment, monensin and CCCP were added 3h after inoculation while in the second experiment cultures were incubated for 12h unperturbed prior to additions. Observations in the second experiment are similar to previous results (section 3.3.6) showing sulphide production to continue even when growth, or the level cellular ATP decreases. This suggests that sulphide production is uncoupled from energy metabolism at least under some conditions. Further experiments were carried out to determine the effect of monensin on sulphide over a short term in cell suspensions (see section 4.3.2).

Inhibition of growth and consequent morphological aberrations observed in cells upon treatment with monensin and the lack of these effects when CCCP was added, imply that a Na^+ gradient but not a H^+ gradient across the membrane is essential for ANI. At neutral pH the gradient between inside and outside of the cell may not be large enough for CCCP to dissipate the membrane potential, as the internal pH in neutrophiles is between pH 7 and 8 (Padan *et al.* 1981; Booth 1985). Normal growth

medium of ANI is at pH 7.4. To test for inhibition by CCCP at lower pH, ANI was grown at pH 6.0. If CCCP has some effect on the organism at pH 6.0 then it can be assumed that lack of inhibition at pH 7.4 was not due to failure of CCCP to interact with the membrane of ANI. Cultures were grown at pH 6.0 and 7.4. To a control culture ethanol was added. After 3h incubation CCCP was added (100µM and 300µM). Some inhibition of growth was found when ANI was grown in the presence of CCCP at pH 6.0; at pH 7.4 no inhibition was observed.

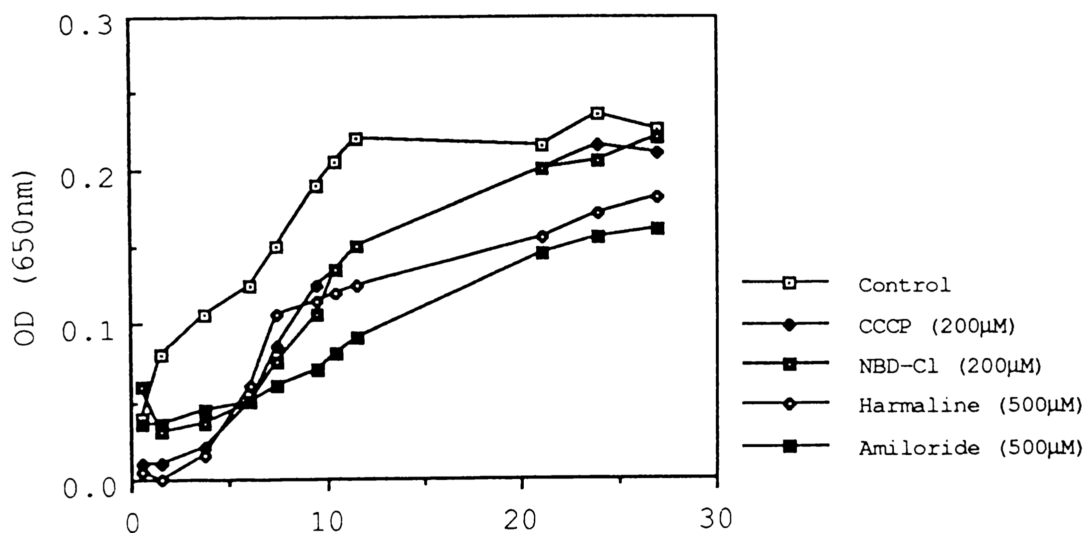
After 24h incubation the OD_{650} was read (shown in Table 4.3). While 100µM CCCP did not inhibit growth at either pH, growth was completely inhibited at 300µM at pH 6.0. It is assumed that CCCP can act on ANI and that a H^+ gradient plays a role in metabolism at least at lower pH..

Table 4.3. Effect of CCCP on growth of ANI at pH 6.0 and 7.4

	Growth yield (OD_{650})	
CCCP	pH 6.0	pH 7.4
100µM	0.32	0.41
300µM	0.00	0.28
none (ethanol)	0.34	0.45

Other compounds were tested for their possible inhibitory effect on cultures of ANI. Results are summarized in Fig.4.4. The compounds tested were added at the start of incubation.

Fig.4.4. Inhibition of ANI by various compounds



No growth was observed in the presence of Nonactin (10µM) or DCCD (1mM). Results shown in Fig.4.4 show that some inhibitors used had no apparent effect on growth of ANI while others severely inhibited it. Cultures to which an increased concentration of CCCP (200µM) had been added showed a lag period of a couple of hours which was not observed in control cultures. However, once growth entered log phase, it paralleled the controls,and cultures reached a final growth yield close to that of control cultures.

Final growth yields were assessed in the presence of other potential inhibitors (Table 4.4).

Table 4.4. Growth yield with potential inhibitory compounds in 0.8% peptone medium

Compound	% Growth yield (OD_{650nm}) (control=100%)
CCCP (100µM)	102
CCCP (200µM)	84
CCCP (300µM)	62
monensin (0.5µM)	13
monensin (10µM)	0
CCCP (100µM) + monensin (0.5µM)	0
valinomycin (100µM)	90
valinomycin (100µM) + CCCP (100µM)	0
nonactin (10µM)	0
harmaline (500µM)	80
amiloride (500µM)	71
NBD-Cl (200µM)	98
DCCD (250µM)	65
DCCD (1000µM)	0
ouabain (500µM)	80
NH_4Cl (50mM)	100
KNO_3 (50mM)	100
azide (10mM)	95

Nonactin, which catalyzes electrogenic transfer of Na^+ across the membrane, completely inhibited growth. In contrast to monensin, which is an artificial 'diffusion Na^+/H^+ antiporter', nonactin does not

interfere with the H^+ gradient, but dissipates the membrane potential (ANI-medium contains 50mM NaCl). Inhibition by nonactin supports the concept of a major importance of Na^+ gradients for cell growth. Additionally, dissipation of the membrane potential may be inhibitory to growth.

Harmaline and amiloride, which inhibit the Na^+/H^+ antiporter in *Methanobacterium thermoautotrophicum* (Schönheit and Beimborn 1985, 1986) showed some inhibition of growth of ANI. Inhibition may have been only limited because of high Na^+ concentration in the medium, as these inhibitors compete with Na^+ for the binding site (and their inhibitory effect could be relieved by addition of NaCl to *M. thermoautotrophicum* [Schönheit and Beimborn 1985]).

In respect to the presence of a Na^+/ amino acid symport system, the complete inhibition of growth caused by nonactin and monensin indicates a necessity for a Na^+ gradient. Partial inhibition by amiloride and harmaline indicates involvement of a Na^+/H^+ antiporter. In section 3.3.6.c it was shown that the requirement for Na^+ could be relieved by Li^+ but not by any other alkali cations. Substitution of Na^+ by Li^+ is commonly observed in Na^+/H^+ antiport systems (Krulwich 1983).

In the presence of valinomycin, which acts as an electrogenic carrier of K^+ across the membrane growth yield was close to that of control cultures after 24h incubation (Table 4.4). In the presence of K^+, valinomycin dissipates the membrane potential. In ANI medium approximately 17mM of K^+ are added as K_2HPO_4. Either the membrane potential was not completely dissipated or the organism can grow even when this gradient is perturbed. In contrast to lack of inhibition of growth by either valinomycin or CCCP alone , addition of both together caused complete inhibition of growth (Table 4.4). In the presence of both the H^+ gradient and the membrane potential would have been disrupted.

Inhibitors of various types of ATPase were also tested. DCCD completely inhibited growth at 1000µM concentration, while at 250µM growth yield was inhibited by 35%. DCCD is a reactive substance and complete inhibition observed in the presence of 1mM DCCD could be due to a number of reasons (Solioz 1984). Cultures to which NBD-Cl had been added showed similar growth to cultures with CCCP (200µM): a lag period, followed by growth similar to control cultures with an almost identical final growth yield. Lack of inhibition by azide indicates absence of F_0F_1-type AtPase and terminal section of the electron transport chain (the latter also being supported by the absence of

cytochromes [section 3.3.2]). Ouabain, an inhibitor of eukaryotic Na^+/K^+ ATPases had no effect on growth. Nor did NO_3^-, which has been shown to inhibit archaebacterial H^+-translocating ATPases and vacuolar ATPases (Nelson and Taiz 1989). Results obtained with potential inhibitors of ATPases indicate the absence of an F_0F_1-related ATPase as none of these compounds showed inhibition at concentrations generally used in studies of these ATPases (see section 4.1).

4.3.2. Cell suspensions

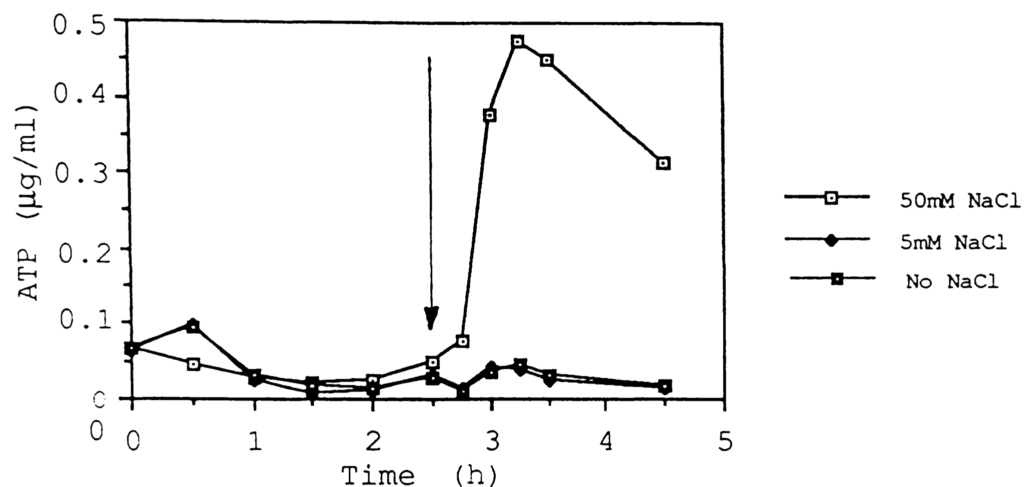
Cell suspensions of ANI were used in further experiments. Use of suspensions rather than growing cultures of cells had some advantages: cells could be at a higher concentration so that changes in the ATP level could be more easily detected, and interference from undesired medium components was excluded.

4.3.2.1. ATP measurements involving dependency of amino acid uptake on the presence of Na^+

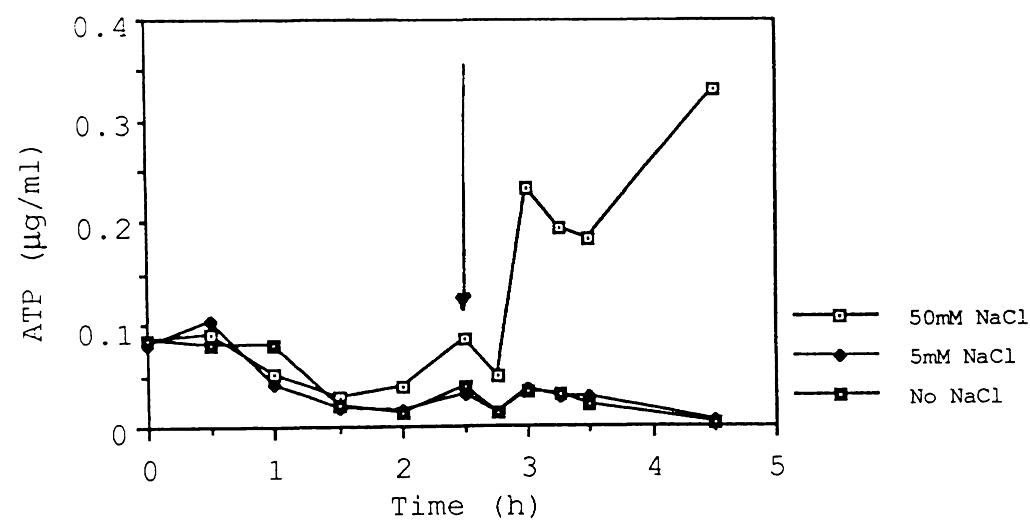
Experiments were carried out to determine the period of incubation required to deplete the cells' endogenous level of ATP and thereby maximize any stimulatory effect of added substrate. A starvation period of 2.5h was chosen. At that time cellular ATP was sufficiently depleted without loss of cell viability (in the presence of 50mM NaCl) (see Fig.4.5)
Cell suspensions were incubated for 2.5h in the presence of 50mM, 5mM, or no NaCl. In all cases a decline in ATP levels was observed although samples containing 50mM NaCl showed less reduction than other samples. Upon addition of 10mM leucine, valine or 0.8% peptone (Figs.4.8.a-c), ATP levels in samples containing 50mM NaCl increased rapidly while virtually no response was observed in samples containing 5mM NaCl or no NaCl. Addition of leucine resulted in a greater initial increase in ATP than of valine. A preference for leucine over valine has been observed in several other experiments with ANI (see section 3.3.4). Increases in ATP levels by the addition of peptone were slower than when either amino acid was added. It appears that Na^+ (at a concentration above 5mM) is required for (substrate utilization and concomitant) ATP production.

Fig.4.5. Stimulation of ATP formation by addition of amino acids or peptone (additions indicated by arrows)

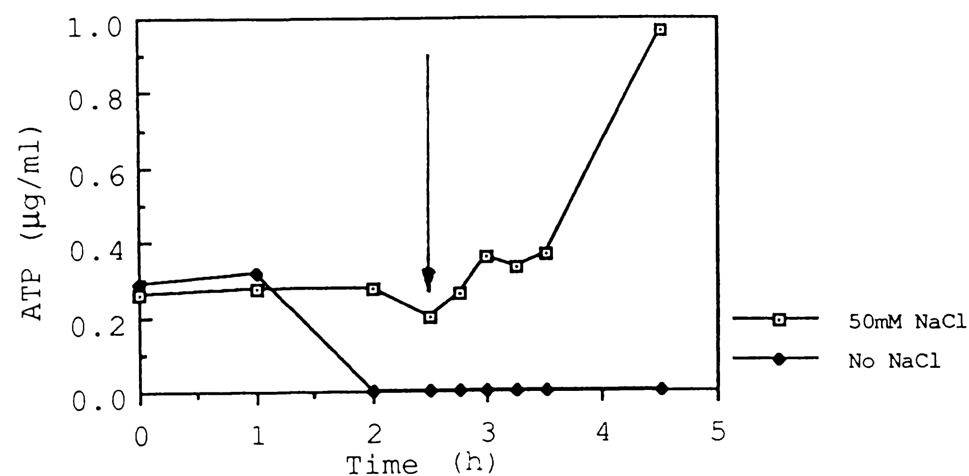
a) 10mM Leucine



b) 10mM Valine



c) 0.8% Peptone



If the requirement for Na^+ was purely for substrate uptake, i.e. for Na^+/substrate symport, a concentration of 5mM would be expected to be sufficient to allow uptake, albeit at a possible lower rate than in the presence of 50mM (see for example: Jarrell *et al.* 1984; Ebbighausen *et al.* 1989). As ATP levels did not increase at all in the 5mM samples, functioning of possible symport systems is unlikely to be the only site of Na^+ requirement. Sodium is, therefore, required for "ATP synthesis" in a broad sense; substrate can be utilized for ATP production only if Na^+ at concentrations above 5mM is present.

Cell suspensions with 10mM valine and different amounts of NaCl or no NaCl were also sampled to test for cell viability. Samples of all cultures were taken at T_0 (at start of incubation time of cell suspensions), after 2.5h (when valine was added), and after 22h incubation at 75°C. On each sample at each sampling time dilutions of 10^{-1}, 10^{-3}, and 10^{-5} were carried out in ANI medium (0.8% peptone plus 2g/ℓ S^0) and these dilutions were incubated at 75°C for 24h after which time growth was assessed by turbidity. Results are shown in Table 4.5.

Table 4.5. Viability of cells in suspensions in the presence of 50mM, 5mM, or no NaCl after different periods of incubation time

Sample time	Cell growth after 24h/75°C		
(h)	50mM NaCl	5mM NaCl	No NaCl
0	positive at 10^{-5}	positive at 10^{-5}	positive at 10^{-5}
2.5	"	positive at 10^{-3}	positive at 10^{-3}
22.0	"	positive at 10^{-1}	negative at 10^{-1}

NaCl is required not only for cell growth but also for cell viability. Even in the presence of 5mM NaCl cell viability was affected within 2.5h of incubation.

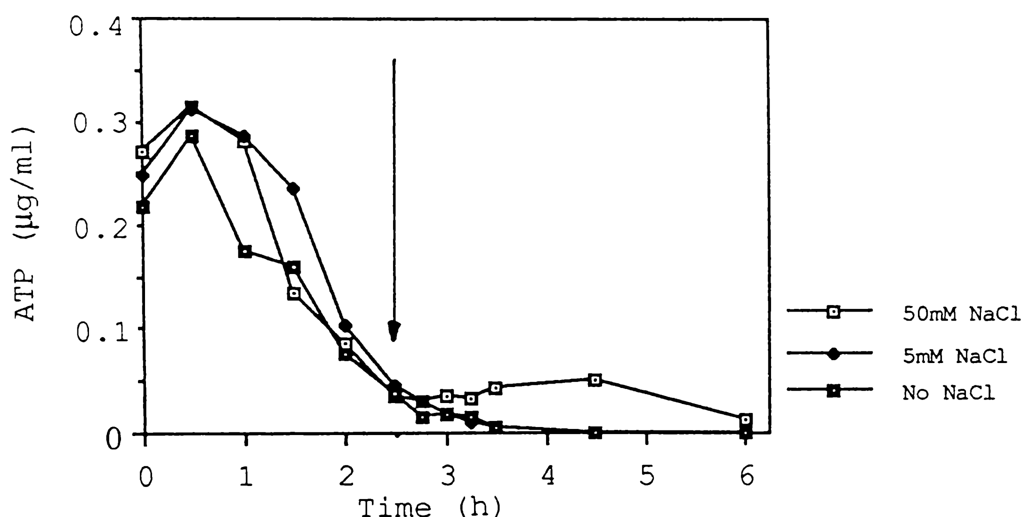
When substrate was added to cell suspensions at the beginning of incubation in the absence of NaCl it was not utilized for ATP synthesis (Fig.4.6). Addition of NaCl after 2.5h did not result in an increase in cellular ATP levels which by that time had dropped close to nil. Thus, cells appear to become irreversibly damaged in the absence of Na^+, incapable of recovering. Sodium appears to be crucial for the preservation of cell viability even in the absence of substrate when the metabolism would be slowed down.

Direct stimulation of ATP synthesis by Na^+ in the absence of substrate was investigated. Cell suspensions of ANI were incubated for 30min in the absence of sodium chloride or carbon source. After 30min NaCl was added to a concentration of 50mM and the effect on ATP concentration monitored and compared to suspensions which had no NaCl addition. Results are shown in Fig.4.7.

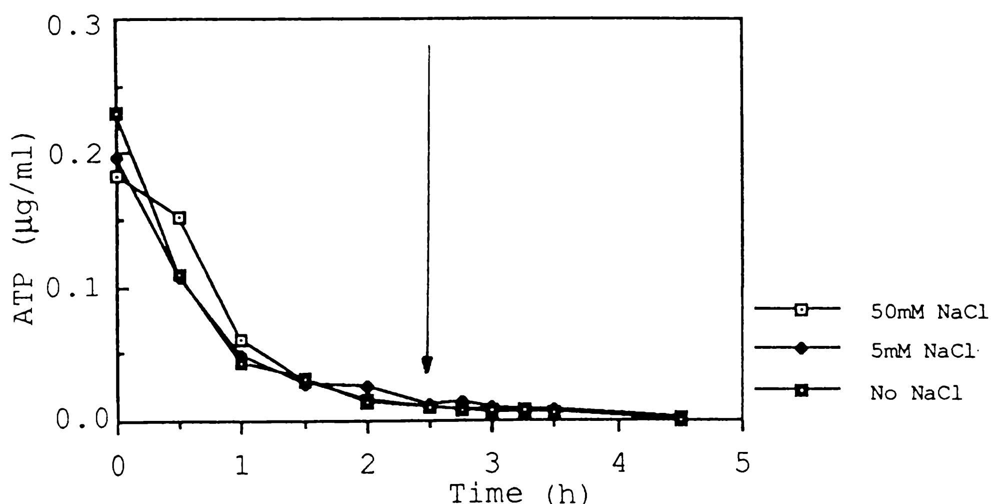
Fig.4.6. <u>Incubation of cell suspensions in the presence of 10mM leucine or 0.8% peptone in the absence of NaCl (NaCl added after 2.5h)</u>

(additions are indicated by arrows)

a) 10mM leucine / 50mM NaCl after 2.5h



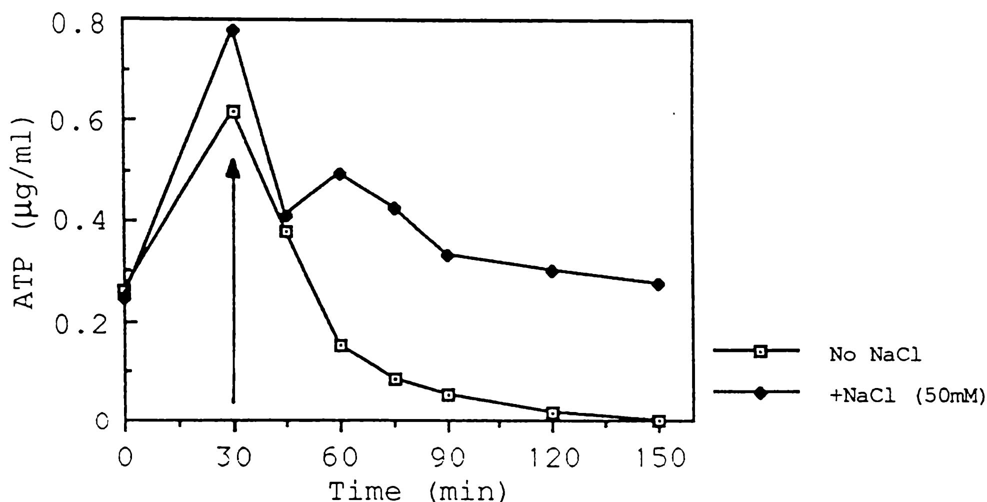
b) 0.8% peptone / 50mM, 5mM, or no NaCl added after 2.5h



The stabilizing effect of Na^+ on ATP levels implies a central role for Na^+ in the 'metabolism' of this organism. If Na^+ was required solely

for uptake, no such effect would be anticipated in the absence of any substrate. Thus, Na^+ appears to be required for the general well-being of the cell in addition to uptake of (some) amino acids.

Fig.4.7. Addition of NaCl to cell suspensions in the absence of substrate (additions are indicated by arrow)
(all additions included 10mM leucine final concentration)

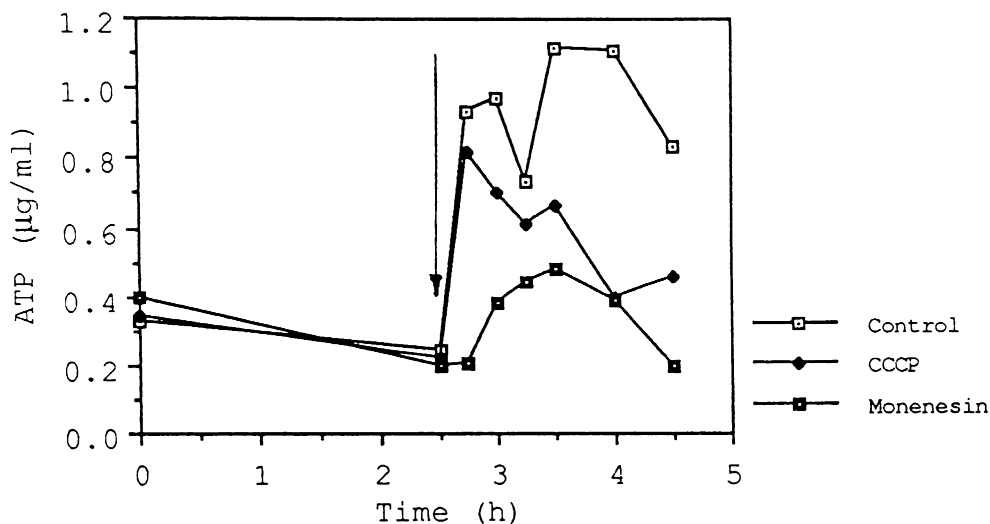


4.3.2.2 Effects of ionophores and potential inhibitors on cellular ATP levels

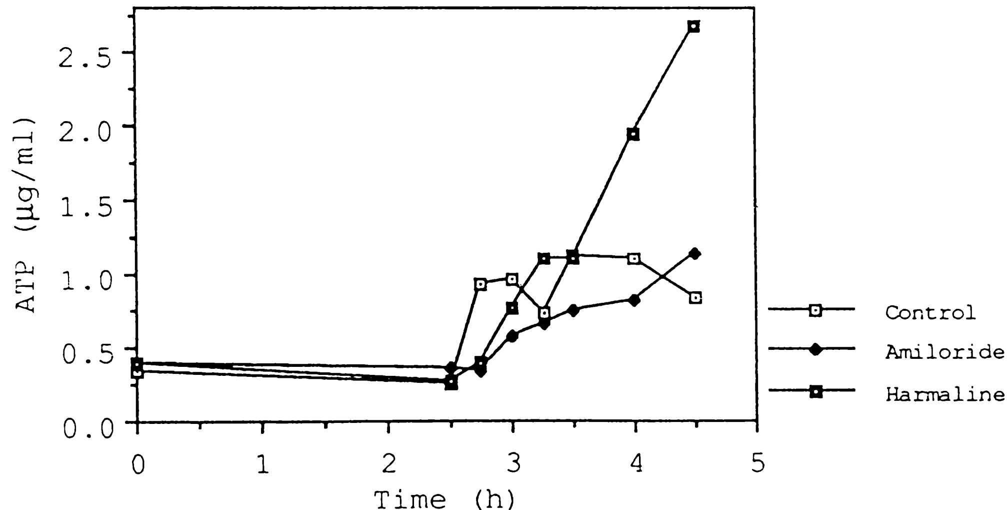
Although all ionophores and inhibitors used had already been tested on growing cell cultures, their effect was also determined on cell suspensions of ANI. The advantages of using cell suspensions have already been stressed. Further, short-term effects are easier to interpret in relation to cell energetics, as distinct from growth.
To cell suspensions starved for 2.5h in the presence of 50mM NaCl, additions of 10mM (final concn.) leucine and either CCCP (100μM), monensin (50μM), harmaline (200μM), or amiloride (100μM) were made. The concentration of monensin was increased from that used in cell cultures to enhance any short-term effect. The concentration of 50μM is still within the range commonly used by other workers (section 4.1).
CCCP and monensin additions to cell suspensions using leucine as substrate both had an inhibitory effect on ATP synthesis (Fig.4.8a). Suspensions with monensin showed a small increase in ATP from 0.2μg/ml to 0.48μg/ml in the first 75min, followed by a decline in ATP. Inhibition was not complete immediately upon addition of monensin and leucine.

Fig.4.8. Effect of CCCP, monensin, valinomycin, harmaline ,and amiloride on cellular ATP levels in cell suspensions in the presence of NaCl (additions are indicated by arrows)
(all additions included 10mM leucine final concentration)

a) CCCP and Monensin



b) Harmaline and amiloride



Results were similar to those obtained in growing cell cultures where addition of monensin did not result in immediate reduction in cellular ATP (Fig. 4.1.c). CCCP had a temporary inhibitory effect on cellular ATP production in cell suspensions. Following an initial rise in cellular ATP, levels declined, but stabilized in the last two samples taken. The inhibitory effect observed may correspond to the lag phase

observed in cell cultures when 200µM of CCCP had been added (Fig. 4.2). In cell suspensions as in growing cell cultures, monensin inhibited synthesis of ATP in the presence of leucine and NaCl, apart from a small temporary increase. Initial rise in cellular ATP upon addition of CCCP shows that leucine was taken up and metabolized. Uptake of leucine via a H^+/solute symport is not possible as it would be inhibited by CCCP. The observed initial rise further indicates that if a H^+-translocating ATPase was present it would not be the primary (or essential) source of ATP.

Harmaline and amiloride had no inhibitory short-term effects on cellular ATP levels in cell suspensions (Fig.4.8.b). While both substances had some inhibitory effect on final growth yield of ANI, they had a stimulatory effect on cell suspensions. Suspensions with amiloride showed a slower rise in cellular ATP levels than the control suspensions, but exceeded the levels of ATP reached after 4.5h. Harmaline too had an inhibitory effect in the first 15min after addition, but over the following 105min harmaline-treated suspensions reached more than triple the level of cellular ATP of controls. In growing cell cultures (Fig.4.4) cells in the presence of harmaline also showed a higher growth rate than controls over the initial 7.5h of incubation, which was followed by a levelling-off in growth. No such initial stimulation was found with amiloride-treated cell cultures. The different effects of these two substances may be due to varying interaction with their target binding site(s). Not much is known about the action of either. Harmaline was added at a higher concentration to cell suspensions which may have been one determinant in the varying effects (but see below).

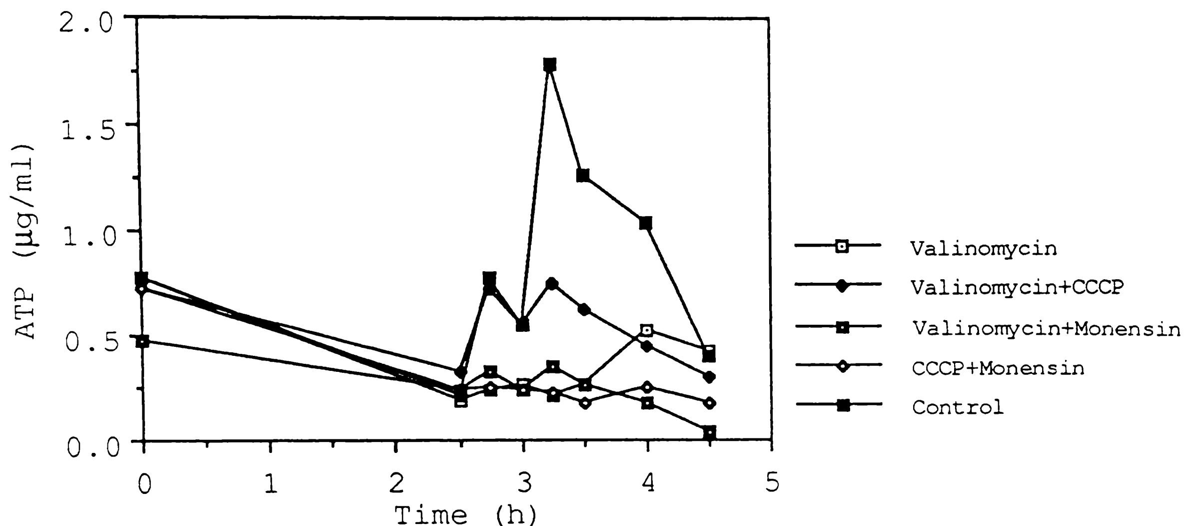
In respect to Na^+/solute symport the effect of these substances on cellular ATP cannot be explained directly. However, the results could be interpreted as a reduction in ATP 'expenditure' leading to intracellular build-up of ATP. Although, as the mode of harmaline inhibition is not known in detail, it may inhibit an energy-requiring Na^+ pump (e.g., a Na^+-translocating ATPase), used for export of Na^+ and generation of a Na^+ gradient, by competing with Na^+ for the binding site. This would lead in the short-term to substrate uptake, metabolism, and synthesis of ATP. In the longer term it would inhibit growth as the Na^+ gradient would not be regenerated. Similarly, if harmaline inhibited a Na^+/H^+ antiporter, gradients of either cation are required for the antiporter to function and generation of the gradient may involve ATP hydrolysis. If such was the action of harmaline on ANI, then growth in cultures would be expected to be inhibited

completely after an initial 'burst" in growth, as no substrate could be taken up. The rate of growth yield dropped after 7.5h incubation but no complete growth inhibition was observed. However, limited growth may have been still possible if other less favoured amino acid components of the peptone medium were utilized (for alanine a possible Na^+ independent utilization is mentioned in section 5.4.2).

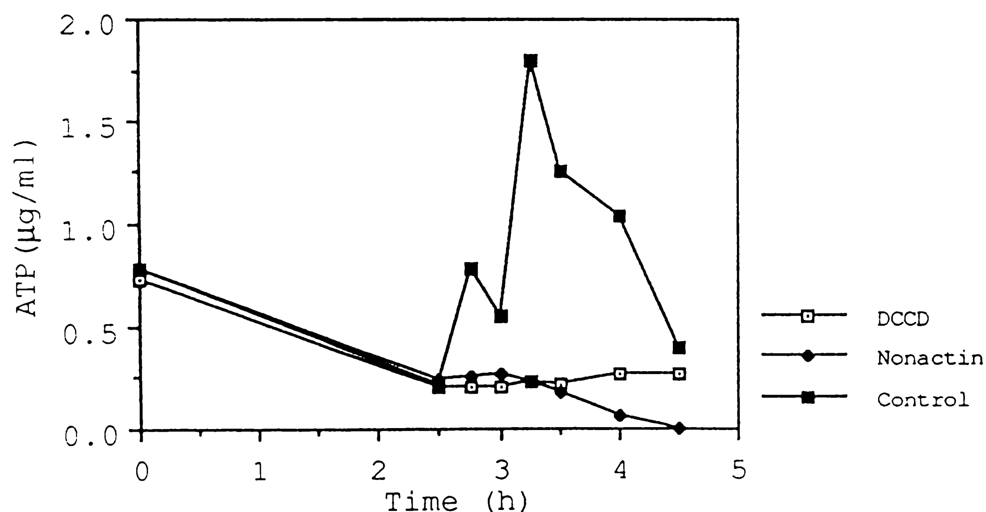
Valinomycin (100μM), CCCP (100μM), and monensin (50μM) were added in combinations to cell suspensions. As in the previous experiment, cultures were starved for 2.5h in the presence of 50mM NaCl. After 2.5h the ionophores and leucine (10mM final concn.)were added (Fig.4.9.a). In contrast to cell cultures (Fig. 4.1.c), in cell suspensions there was increased inhibition when CCCP plus monensin were added compared to monensin alone: while ATP levels in the presence of monensin rose initially (Fig.4.9.a) no such increase was observed when CCCP was added as well. The effect of valinomycin plus monensin was similar to that of CCCP and monensin. The increased inhibition when either was added with monensin compared to monensin alone indicates that the membrane potential, the H^+ gradient, possibly the K^+ gradient, or a combination of these 'enhances' ATP production (substrate uptake or ATP synthesis) but is not sufficient for ATP generation and growth in the absence of a Na^+ gradient. Valinomycin alone did not inhibit final growth yield of cultures (Table 4.4). Cell suspensions with valinomycin showed some increase in cellular ATP 90min after additions were made. The different effect of valinomycin and CCCP alone on initial ATP levels in suspensions shows that they interfere with different components of transmembrane gradients. The importance of K^+ gradients cannot be estimated. The major effect of valinomycin is dissipation of the membrane potential. The external concentration of K^+ was above 25mM as the MOPS buffer had always been neutralized with KOH. CCCP dissipates the membrane potential if there is a sufficient pH gradient. Inhibition of ATP production and growth by both added together compared to temporary inhibition in cellular ATP levels and no reduction in growth yield when added singly, may be due to either not completely dissipating the membrane potential while this occurs when both are added, or due to valinomycin dissipating the membrane potential, while CCCP primarily interferes with the H^+ gradient. Neither dissipation of the membrane potential nor of the H^+ gradient may be inhibitory on its own as long as the other is still intact.

Fig.4.9. Effect of valinomycin, alone and combinations of valinomycin, CCCP, and monensin, or of nonactin or DCCD on cellular ATP levels in cell suspensions in the presence of NaCl
(additions are indicated by arrows)

a) Valinomycin CCCP, and monensin



b) Nonactin , DCCD



Although valinomycin and CCCP added together caused inhibition of ATP production and growth, ATP levels rose in the initial 15min after addition. This supports the idea that neither the membrane potential nor the H^+ gradient is of primary importance for ATP generation, i.e. substrate uptake and metabolism, but at least one is required for growth (e.g. maintenance of membrane gradients, viability).

Maintenance of a Na^+ gradient required to drive Na^+/solute symport is influenced by both the H^+ gradient, probably via a Na^+/H^+ antiporter, and the membrane potential. The importance of presence of either the H^+ gradient or the membrane potential is highlighted in the increased initial inhibitory effect when monensin was added with either valinomycin or CCCP compared to monensin alone.

If the Na^+ gradient across the membrane was generated by a Na^+-translocating ATPase and the Na^+ cycle was simple and not dependent on a charge gradient, i.e., Na^+ enters the cell via Na^+/solute symport and is expelled via a Na^+-translocating ATPase then dissipation of membrane potential and H^+ gradient together would not have such a strong inhibitory effect (if they were not involved in other essential cell functions).

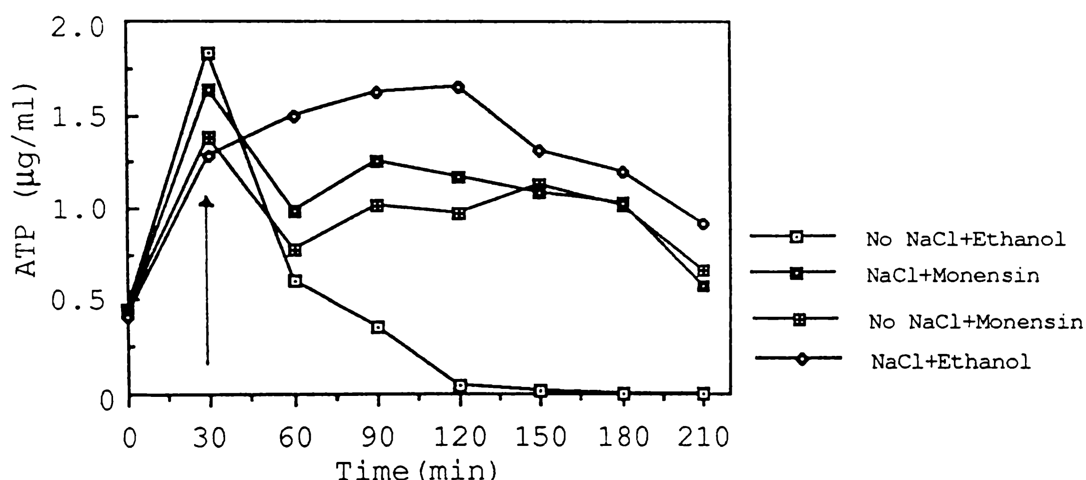
Nonactin (100μM) or DCCD (500μM) added to cell suspensions inhibited ATP synthesis over the time period sampled. The effect of nonactin on growing cell cultures was complete inhibition of growth, and in cell suspensions nonactin had a total inhibition of ATP synthesis. In the presence of nonactin there was no initial rise in cellular ATP levels as observed with monensin. Nonactin dissipates the membrane potential in the presence of Na^+ (as it catalyzes electrogenic transport of Na^+ across the membrane). The absence of an initial rise in ATP levels which was observed with monensin could be due dissipation of the membrane potential. Upon addition of valinomycin alone there was no initial ATP synthesis either. While cells 'recovered' from the effect of valinomycin, this did not occur in the presence of nonactin as the Na^+ gradient was disrupted. The action of nonactin is, therefore, similar to that of monensin and valinomycin added together (apart from any possible effect of monensin on the H^+ gradient) and had the same effect on ATP levels in cell suspensions (compare 'valinomycin + monensin' in Fig.4.9.a and 'nonactin' in Fig.4.9.b)

The short-term effect of DCCD on cellular ATP was inhibition of ATP synthesis. This is in contrast to final growth yield (Table 4.4). The amount of DCCD was high (500μM) and may not have been specific for a F_0F_1 -type ATPase. Since CCCP, NBD-Cl, and azide had no inhibitory effect on ATP generation (apart from temporary inhibition soon after addition) or final growth yield, the presence of a H^+-translocating ATPase as a major component of the organism's ATP generation system is not likely.

The effect of monensin on cellular ATP was investigated in the presence and absence of NaCl. As shown earlier (Fig.4.6), absence of NaCl in cell suspensions caused continuous decrease in cellular ATP and led to loss of viability even in the presence of substrate. In the absence of NaCl, monensin would cause an outward flux of Na^+. If Na^+ was expelled via a Na^+-translocating ATPase, presence of monensin in absence of NaCl may lead to some increase in ATP, as less or no ATP would be hydrolyzed to expel Na^+.

Fig.4.10. Cellular ATP levels in cell suspensions treated with monensin in the presence and absence of NaCl

(arrows indicate additions)



This increase in cellular ATP would only be transient, similar to that observed in the presence of harmaline, as the Na^+ gradient would not be regenerated. A possible Na^+-translocating ATPase may not be regulated in its activity by the external Na^+ concentration, but only by the internal Na^+ concentration, which would initially be higher in the absence of monensin. In its natural environment, the organism was not found in hot pools with a sodium content below 0.5g/ℓ (chapter 2) and the organisms most closely related to ANI are from marine habitats (section 1.2).

Cell cultures were incubated for 30min in the absence of NaCl or substrate. After 30min, leucine (10mM final concn.) was added to all suspensions. Monensin (50µM) with and without NaCl (50mM) was added.

One control contained NaCl plus ethanol while the other had only ethanol added. Cellular ATP levels were monitored over the following 180min. (Fig.4.10).

All suspensions showed an initial steep increase in cellular ATP. Such a rise was also observed in some other suspensions (Fig.4.11) when no NaCl was present, while it did not occur in others (Fig.4.6). It is not known why such initial increases occurred, but it did not seem to interfere with results obtained from cellular ATP levels upon additions made.

In the absence of NaCl, monensin exhibited a stabilizing effect on cellular ATP. While cellular ATP in control suspensions without NaCl steadily declined, cellular ATP levels in the presence of monensin were similar whether NaCl was present or not. The lower inhibitory effect by monensin (in the presence of NaCl) observed here compared to earlier experiments (Fig. 4.8.a) was probably due to the shorter initial starvation period (30min compared to 2.5.h). There is no known explanation for the stabilizing effect by monensin in the absence of NaCl. Radiolabelled Na^+ would be required to follow the movements of Na^+ across the membrane in the presence and absence of external NaCl and monensin.

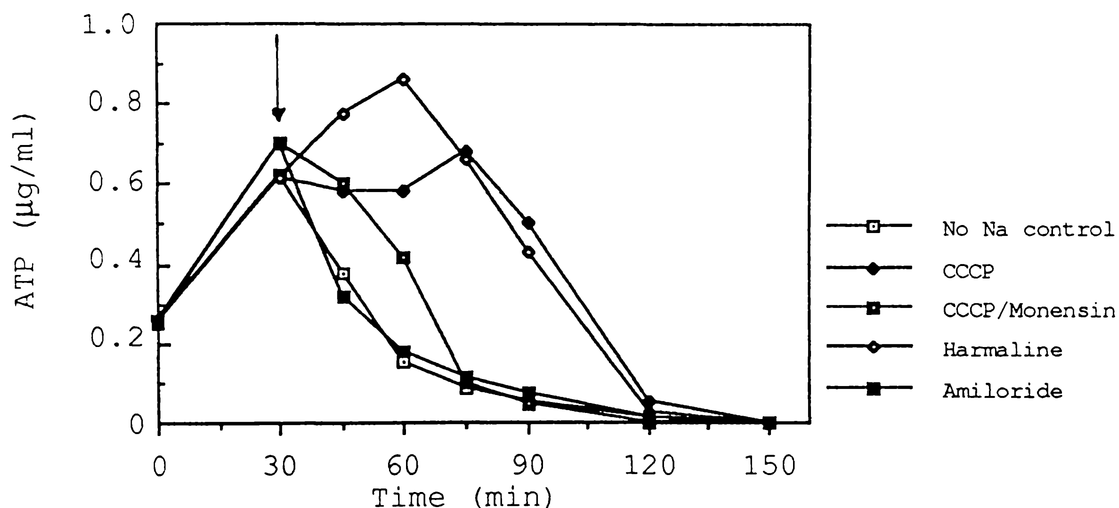
A stabilizing effect of monensin and other Na^+ ionophores has been observed in *Methanobacterium thermoautotrophicum* (Schönheit and Beimborn 1986). In their study it was found that under extracellular K^+ concentrations ≥25mM and Na^+ concentrations <0.2mM methane formation and ATP production were stimulated by the addition of Na^+ ionophores. The authors could not explain their results.

Since monensin showed an 'interesting' (although inexplicable) effect on cellular ATP levels in the absence of NaCl, CCCP (200μM) and the Na^+/H^+ antiport inhibitors amiloride (100μM) and harmaline (100μM) were tested for their effect in the absence of NaCl. The experiment was performed as previously; cell suspensions were starved for 30min, then the potential inhibitor and leucine (10mM final concn.) were added.

Addition of CCCP stabilized intracellular ATP levels over the next 45min following additions. After 45min ATP levels declined. This initial stabilizing effect cannot be explained either. When CCCP and monensin (50μM) were added together, the stabilizing effect occurred only over the first 15min. Whatever the effect of either is in the absence of NaCl allowing temporary stability in ATP levels, monensin and CCCP appeared to cancel out the stabilizing effect of one another.

Amiloride had no detectable effect on cellular ATP levels. Cellular ATP levels declined steadily, as in control suspensions. Suspensions with harmaline showed an initial increase in cellular ATP levels upon addition of the inhibitor and leucine. This increase was over 30min after which time the ATP levels steadily decreased. Harmaline, therefore, did not have as marked a stabilizing effect on ATP levels as monensin (or NaCl itself). The initial stimulatory effect, which had also been observed in the presence of NaCl (Fig.4.8.b) was therefore independent of NaCl in the suspension, as would be the case if it was due to inhibition of an energy-requiring mechanism for Na^+ expulsion. Results obtained in the absence of Na^+ show that harmaline and amiloride do not exert the same effect on ANI.

Fig.4.11. Effect of ionophores and inhibitors on cellular ATP levels in the absence of NaCl

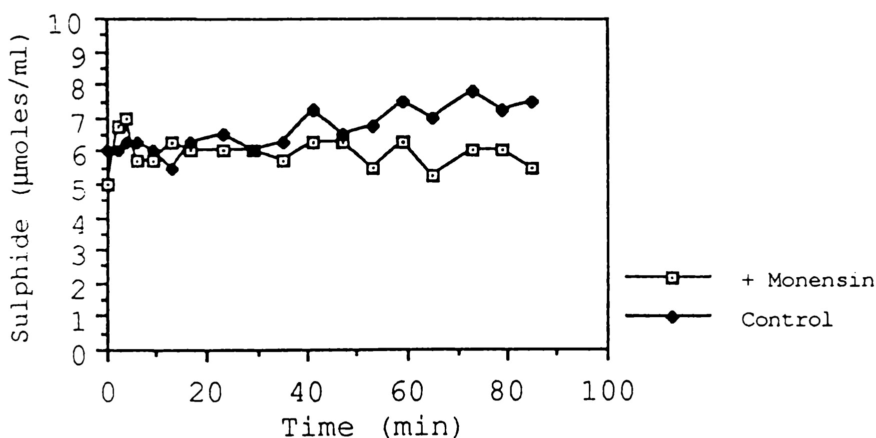


4.3.2.3. Attempted measurement of the direct effect of monensin on sulphide production

An experiment using cell suspensions was carried out to show the effect on sulphide production by monensin. If Na^+ was directly involved in the synthesis of ATP (via a Na^+-translocating ATP synthase), then disruption of the Na^+ gradient across the membrane would result in

uncoupling of metabolic turnover from ATP synthesis. Thus, it was investigated whether treatment with monensin would result in an increased short-term production of sulphide (and substrate turnover) prior to cell death. Also, this experiment was a measure of metabolic activity in cell suspensions in the presence of ionophores, as has been done with methane formation in methanogens (section 4.1). Such measurements may be of advantage over determinations of cellular ATP levels in cases where an inhibitor may block a major part of the metabolic activity and cells are unable to either take up substrate or to metabolize it. In the experiment, cell suspensions with additions of 50mM NaCl, 0.8% peptone, and 50μM monensin (or ethanol as control) were incubated in a 75°C waterbath. Samples for determination of sulphide were taken at intervals. Readings obtained are plotted in Fig.4.12. However, from results obtained, it was impossible to determine an increase in sulphide production as samples taken at different times showed no consistent trend. Short-term experiments of the kind undertaken in much of the literature cited were not possible under conditions employed here due to the high background and high variability between measurement of samples.

Fig.4.12. <u>Sulphide and production in cell suspensions in the presence of monensin</u>



4.4. Conclusion

Sodium plays a central role in the metabolism and growth of ANI. In cell suspensions Na^+ is required at concentrations above 5mM for ATP

production from added amino acids. Although labelled amino acids (and Na^+) would be required for direct demonstration of uptake,amino acid uptake via a Na^+/solute symport is probable as dissipation of the H^+ gradient or the membrane potential, did not inhibit amino acid uptake (assuming that an increase in cellular ATP is the direct result of uptake and metabolism of amino acids), while dissipation of the Na^+ gradient inhibited ATP generation. A requirement for Na^+ for general cell functioning was shown (Fig.4.7) since even in the absence of substrate NaCl had a stabilizing effect on ATP levels, while absence of NaCl led to loss of viability.

It is not clear how the Na^+ gradient is maintained. Na^+ appears to be transported out of the cell in an energy-requiring mechanism (indicated by the effect of harmaline and of monensin in the absence of NaCl), which is also dependent on the presence of either the membrane potential or the H^+ gradient (Fig.4.11a). If a Na+/H^+ antiporter is present in ANI, energy may be required for its functioning.

The effects of most of the ionophores and inhibitors tested on ANI were similar to those found in a Gram-positive, monensin-sensitive ruminal bacterium (Chen and Russell 1990) (see section 4.1.). As in the ruminal bacterium (taking ATP production in ANI analogous to glutamate uptake in the ruminal bacterium) Na^+ was required for amino acid uptake (ATP synthesis). Absence of the Na^+ gradient and the membrane potential resulted in immediate complete inhibition of 'uptake', while in the presence of the Na^+ gradient in the absence of a membrane potential, 'uptake' took place, although less efficiently. Effects of valinomycin and the respective protonophore were similar in the ruminal bacterium and ANI. There is no evidence in ANI for a primary Na^+ pump coupled to a decarboxylation reaction as suggested for the ruminal bacterium.

Studies on membrane fractions of ANI would be required to demonstrate possible primary Na^+ pumps coupled to other chemical reactions. If a Na^+-translocating ATPase was present it may be similar to the ATPase described in *Methanococcus voltae* (Dharmavaram and Konisky 1987, 1989) as its resistance to NO_3^-, NBD-Cl, azide, and limited inhibition only in presence of high concentrations of DCCD indicate absence of a vacuolar-type, F_0F_1-type,or 'archaebacterial' ('*Sulfolobus*'-type ATP synthase) ATPase.

Results obtained are included in a general model of ANI (chapter 8).

Chapter 5: Branched-Chain Amino Acid Catabolism

5.1. Introduction

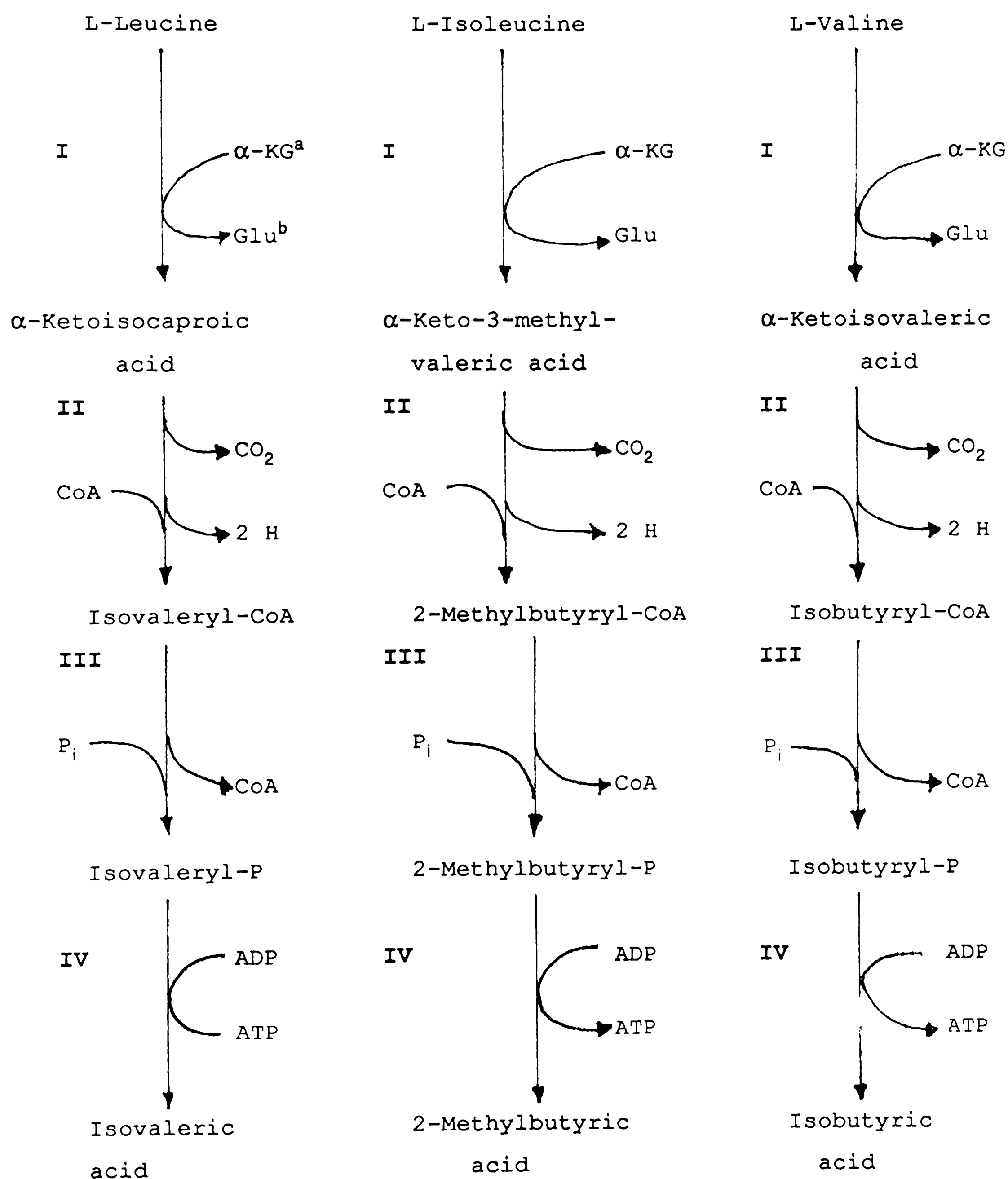
In the previous chapter Na^+/amino acid symport was proposed as a likely pathway for the uptake of (branched-chain) amino acids. In chapter 3 the detection of volatile fatty acids and NH_4 as metabolic end products from growth with branched-chain amino acids was described. This chapter deals with the steps involved in between, the catabolic pathways of leucine, isoleucine, and valine.
Growth experiments have shown that amongst the major metabolic end products of isolate ANI grown on trypticase peptone medium are acetate, isobutyrate, and isovalerate/2-methylbutyrate. Leucine, isoleucine and valine stimulated growth when added singly to medium containing reduced amounts of peptone (chapter 3). Since isobutyrate and isovalerate/2-methylbutyrate are likely end products from metabolism of valine and leucine/isoleucine respectively, possible catabolic pathways of these amino acids were investigated. Experiments performed in respect to the sodium requirement of isolate ANI showed that ATP synthesis occurred upon addition of L-leucine in the presence of NaCl. While the concept of ATP generation via a membrane-bound ATPase cannot be completely discarded, synthesis via substrate-level phosphorylation is a possible alternative or additional possibility. A fermentative pathway for branched-chain amino acids coupled to ATP synthesis has been described for the marine spirochete MA-2 (Harwood and Canale-Parola 1981). To determine whether such a pathway operates in ANI, cell-free extracts were assayed for the presence of enzymes involved, as shown in Fig.5.1.

5.2. Materials and methods

5.2.1. Media

Growth media and amino acid supplements were prepared and added as described under 3.2.1. and 3.2.5.
For growth curve experiments, cells were grown in 170ml serum bottles, and for preparations of extracts cells were grown in 1ℓ glass bottles in 800ml medium. Unless otherwise stated, growth medium contained 0.8% peptone, 0.2% S^0 under a N_2 atmosphere. Cultures were incubated at 75°C.

Fig.5.1. Enzymatic pathways of branched-chain amino acid fermentation by spirochete MA-2



I-Branched-chain amino acid aminotransferase

II-α-Keto acid oxidoreductase

III-Phosphate acyltransferase

IV-Fatty acid kinase

(from Harwood and Canale-Parola 1981)

[a]α-ketoglutarate
[b]glutamate

5.2.2. Analytical methods

Cellular ATP was measured as described in section 4.2.5.

Metabolic end products were determined as in section 3.2.6.

Protein content of crude cell extract was assayed by the Biuret method (Scopes 1982). Samples were diluted appropriately with H_2O. A standard curve was prepared using bovine serum albumin (globulin-free; Sigma). The standard curve was linear from 0 to 5mg of protein/ml.

High performance liquid chromatography (HPLC) was used for detection of α-ketoisocaproate according to Patel *et al.* (1987) using an Aminex HPX-87H organic analysis column (300mm x 7.8mm) (Bio-Rad, USA) fitted with a micro-guard ion exclusion guard column (Bio-Rad, USA). The mobile liquid phase was 0.01N H_2SO_4 at a flow rate of 0.5ml/min. The HPLC column was operated at 50°C using a temperature control module (Waters associates, USA). Elution was monitored by a Varian R1-4 refractive index detector (Varian, USA) and data was collected via a series 760 interface (Nelson Analytical Inc., USA) linked to an Exzel XT computer (Computer Imports Ltd., New Zealand) using 3000 series chromatography data system software (Nelson Analytical Inc., USA). Standards of 2, 5, and 10mM of α-ketoisocaproate in H_2O were used. A 10mM standard was run after every 20 samples. The volume injected was 100μl.

5.2.3. Enzyme assays

All assays were carried out on crude cell extract of ANI. Cultures (800ml) were grown in 0.8% peptone medium with S^0 at 75°C for 24 h. Cells were harvested by centrifugation (20 min. at 26000 x g at 4°C) and washed twice in 25mM MOPS buffer, pH 7.4. Cell pellets were resuspended in 2-3ml buffer and sonicated as described earlier (section 3.2.3). The sonicate was centrifuged for 30 min. at 13529 x g at 4°C, and the supernatant kept on ice until used in assays. For assays of branched-chain amino acid aminotransferase and fatty acid kinase cell pellets had been kept at -20°C prior to sonication while freshly grown cells were used for assays of α-keto acid oxidoreductase and phosphate acyltransferase assays.

Assays for detection of activity of branched-chain amino acid aminotransferase, α-keto acid oxidoreductase, and fatty acid kinase were performed as described by Harwood and Canale-Parola (1981). α-Keto acid oxidoreductase and phosphate acyltransferase assay conditions were not strictly anaerobic. All assay components were prepared in

water which had been boiled to drive off the O_2 and cooled under N_2, but assays were incubated in the presence of air.

α-Ketoglutarate-dependent branched-chain amino acid transaminase was assayed as described by Wong and Lessie (1979). Assays were performed in 1ml reaction mixtures containing: 100mM Tris, pH 7.5; 20μg pyridoxal phosphate; 15mM α-ketoglutaric acid; 40mM branched-chain amino acid; 100μl diluted crude cell extract/(1-2mg protein/ml). Reaction mixtures were preincubated at 75°C for 5 min prior to addition of crude cell extract. Assays were carried out at 75°C for 5, 10, and 15 min. The reaction was terminated by the addition of 0.1ml of 15% trichloro acetic acid (TCA). Two ml each of toluene (99.5% pure) and of 0.3% dinitrophenylhydrazine (in 2N HCl) were added to the acidified reaction mixtures while the mixture was vigorously swirled. The entire sample was shaken for 5min to extract the dinitrophenylhydrazones of the branched-chain keto acids as they formed. Samples were centrifuged for 1 min top speed in a bench top centrifuge. Branched-chain keto acid dinitrophenylhydrazones were in the toluene phase while the dinitrophenylhydrazone of α-ketoglutaric acid stayed in the aqueous phase (Duggan and Wechsler 1973). Branched-chain keto acid dinitrophenylhydrazones from 1ml of the toluene phase were extracted into 5ml of 10% Na_2CO_3, mixed rapidly for 1min and centrifuged for 1 min. To develop colour of the dinitrophenylhydrazone 0.5ml of the aqueous phase was added to 0.5ml of 2N NaOH. Samples were incubated at room temperature for 10 min to allow colour development then measured at 450nm. Dinitrophenylhydrazone standards were prepared from α-ketoisocaproic acid. The rate of reaction was linear for periods of at least 5min for all samples except for cell extracts grown on either leucine or valine with leucine as substrate (Table 5.2) where the rate was no longer linear between 5 and 10min. Therefore the activity of aminotransferase of these samples may be higher than was determined.

Assays for α-keto acid oxidoreductase were performed according to Harwood and Canale-Parola (1981). Reaction mixtures (1ml) contained: 100mM Tris-HCl, pH 7.5; 8mM α-keto acid; 150μM thiamine pyrophosphate; 9mM dithiothreitol; 150μM electron acceptor. Electron acceptors added were: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP). Reaction was started by addition of 200μl of crude cell extract (0.4-0.6mg protein). Continuous assays were performed by measuring the decrease in absorbance at 450nm for

flavin nucleotides or the increase in absorbance at 340nm for nicotinamide nucleotides. Assays were carried out at 55°C using a Shimadzu UV-250 UV-visible recording spectrophotometer with a Shimadzu PR-1 graphic printer and a Shimadzu TCC temperature controller. Addition of twice the amount of substrate or electron acceptor did not increase the rate, showing that the reaction was not limited by either either of these factors. Addition of less extract resulted in reduced activity (where 100μl extract added gave about half the activity).

Phosphate acyltransferase assays were carried out as described by Harwood and Canale-Parola (1981). Reaction mixtures for the back reaction contained in 0.8ml H_2O: 100mM Tris, pH 8.0; 3mM dithiothreitol; 200μM CoA-SH; 10mM acetyl phosphate; 100μl crude cell extract (0.3-0.5mg protein). Reaction mixtures were incubated for 5 min at 55°C. One ml of potassium arsenate was added (50μmoles) and the mixtures were incubated for a further 5, 10 or 15 min. The reaction was terminated by the addition of 0.2ml of neutralized hydroxylamine solution (4M hydroxylamine hydrochloride and 4M KOH mixed in a 1:1 ratio). Samples were incubated for 10 min. at 25°C to allow formation of hydroxamates, then 1ml of 10% TCA added to precipitate protein. Samples were centrifuged for 3min in a benchtop centrifuge (Hettich, Germany) to remove protein. The supernatant was added to 4ml of 1.25% (w/v) $FeCl_3$ in 1N HCl. Acyl hydroxamates were determined by measuring the absorbance at 540nm. Standards were prepared from acetyl phosphate.

Reaction mixtures for the forward reaction contained: 10mM isovaleryl-CoA (0.1ml); 500mM Tris-HCl pH 8 (0.2ml); 30mM dithiothreitol (0.1ml); 100mM K_2HPO_4 (0.1ml); 2M neutralized hydroxylamine (0.2ml); H_2O (0.1ml); cell extract (0.1ml). Reaction mixtures were incubated at 55° or 75°C for 5, 10, 15, and 30 min. Hydroxamates were detected as above.

Phosphate acyltransferase was also assayed as follows (Lamed and Zeikus 1980). The reaction mixture contained: 100mM Tris-HCl, pH 7.2; 3mM $MgCl_2$; 2mM glucose; 500μM NADP; 1 U hexokinase (Sigma); 1 U glucose-6-phosphate dehydrogenase (Sigma); 1mM ATP; 4mM acetyl phosphate; 200μl crude cell extract (0.62mg protein) in total volume of 1ml. The absorbance change at 340nm was continuously monitored. Assays were performed at 55°C using a Shimadzu spectrophotometer as above.

Phosphate acyltransferase activity was also assayed according to Bergmeyer *et al.* (1974). The reaction was carried out in reverse with the acyl-CoA as substrate. The 1ml reaction mix contained: 100mM Tris-HCl, pH 7.4; 2mM reduced glutathione; 400μM CoA-SH; 2mM acyl-CoA;

20mM ammonium sulphate; 100µl crude cell extract (0.5mg protein). Assays were carried out at 50°C and 55°C measuring the change in absorbance at 260nm using a Pye Unicam spectrophotometer as described earlier (3.2.3).

Fatty acid kinase activity was measured in the reverse direction by measuring the formation of acyl phosphates from fatty acids in an assay as described by Harwood and Canale-Parola (1981). The 1ml reaction mixtures contained: 100mM Tris-HCl, pH 7.5; 4mM $MnSO_4$; 10mM ATP; 2ml neutralized hydroxylamine (350mM); 60mM fatty acid (neutralized with 1N HCl); 100µl crude cell extract (0.3mg protein). Reaction mixtures were incubated at 75°C for 10, 15, 20, and 30min. Reactions were terminated by the addition of 1ml 10% TCA. Samples were centrifuged for 3 min. in a bench-top centrifuge to separate precipitated protein. Formation of hydroxamates was measured by the method described above . The reaction rate was linear over at least 30min.

Coenzyme A was removed from cell extracts by the addition of extracts to a thick slurry of Dowex-1 (Sigma) in H_2O in a ratio of 1:1 (v/v). The Dowex-1 particles had previously been washed in 7 x 500ml H_2O (until the A_{233} of the wash was equivalent to water blanks). The suspension was shaken for 15 min under N_2 and centrifuged for 5 min in a benchtop centrifuge (H. Rehm, Germany) at top speed to separate resin from cell extract. Successful binding of CoA-SH was monitored in controls to which free CoA-SH had been added by monitoring the decrease in absorbance at 260nm.

5.3. Results

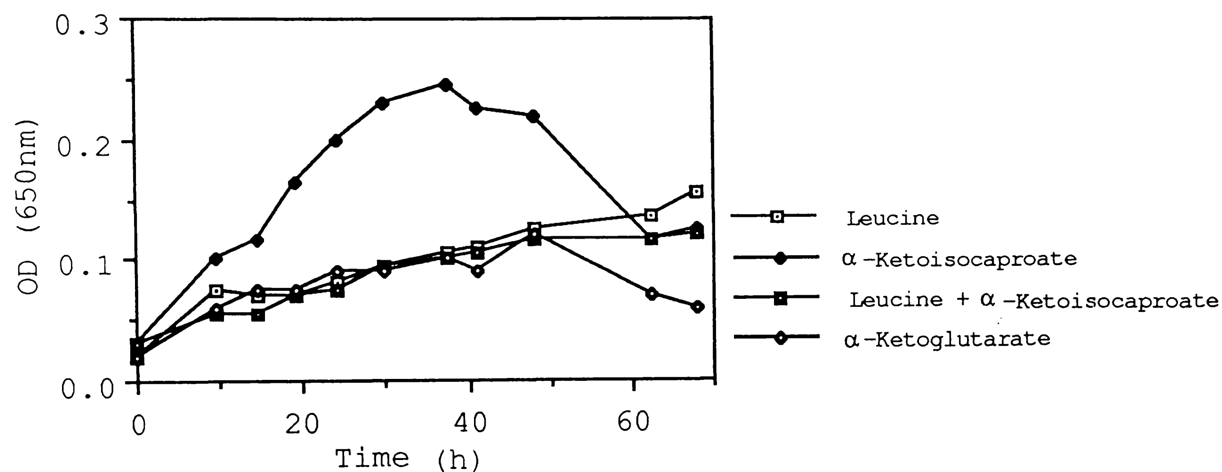
5.3.1. Growth on leucine and α-ketoisocaproate

Growth and end product formation was monitored on cells growing on 10mM L-leucine and/or 10mM α-ketoisocaproic acid in 0.05% peptone medium with S^0. To a control culture 10mM α-ketoglutarate was added. Results (Fig.5.2) show that α-ketoisocaproate supported better growth than leucine (Fig.5.2.a.). Growth on leucine was very slow (see also section 3.3.5, Fig.3.6). After 40 hours incubation cells on α-ketoisocaproate had reached stationary phase, possibly due to exhaustion of the α-keto acid substrate earlier. When both leucine and α-ketoisocaproate were added growth was not stimulated to the same degree as with α-ketoisocaproate alone.

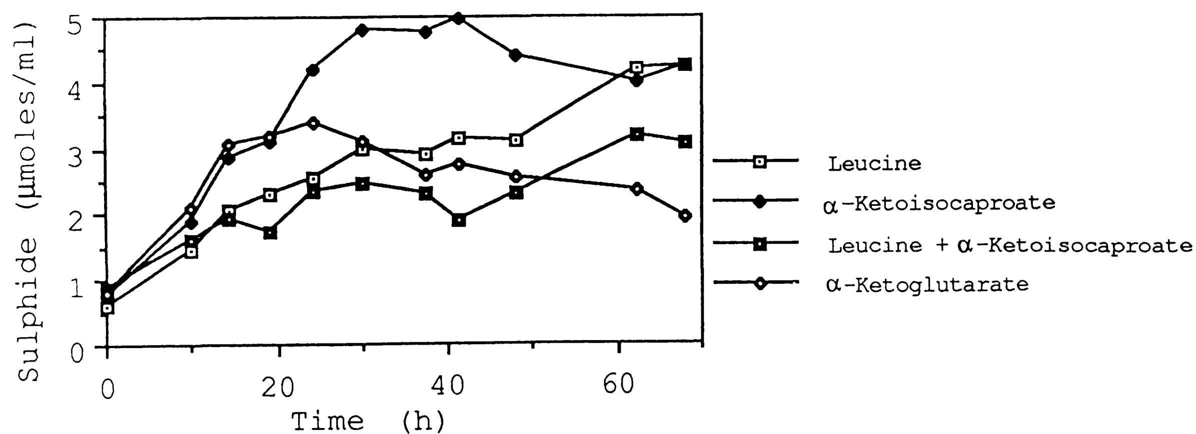
Fig.5.2. Growth and production of sulphide, NH_4, and volatile fatty acids on leucine and/or α-ketoisocaproate or α-ketoglutarate as control

a. OD(650nm)

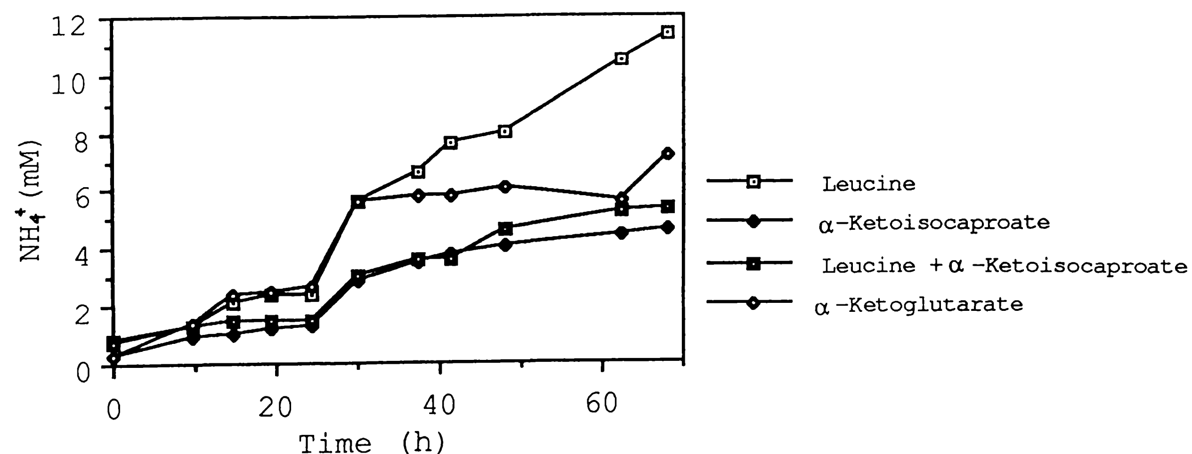
(after 48h in control of 0.05% peptone only the OD (650nm) was 0.05)



b. Production of sulphide



c. Production of NH_4



5.2.d. Production of fatty acids

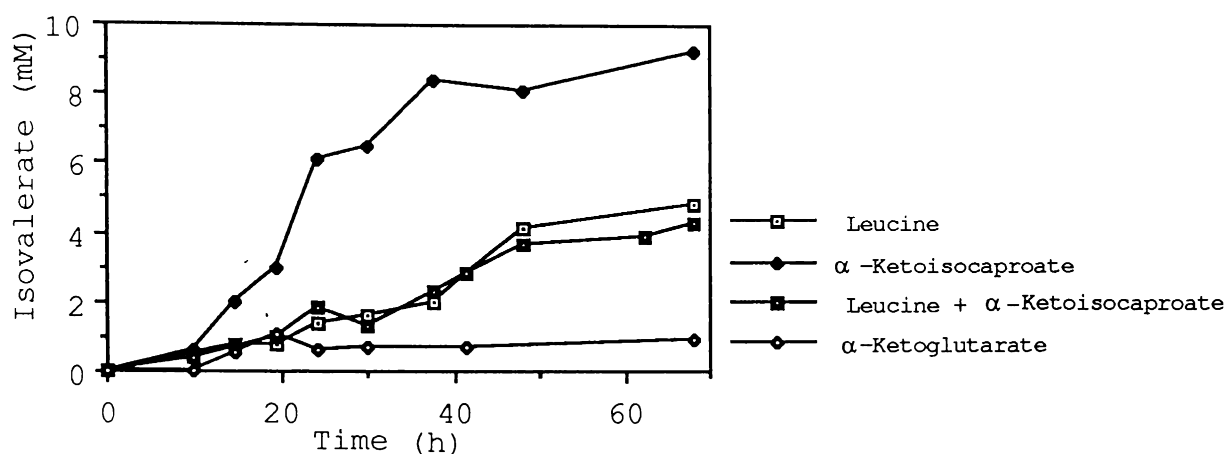


Table 5.1. Amount of of α-ketoisocaproate and α-ketoglutarate present in the medium after various hours growth

Incubation time (h.)	Culture	α-Keto acid (mM)
0	10 mM KIC[a]	9.2
"	10 mM KIC + 10mM leucine	8.7
"	10mM KG[b]	9.3
19:15	10mM KIC	2.0
24:15	10mM KIC	~0.1
48:00	10mM KIC	0.0
"	10mM KIC + 10mM leucine	5.2
"	10mM KG	7.8

[a]α-ketoisocaproate
[b]α-ketoglutarate

When examining production of sulphide and NH_4 (Fig.5.2.b,c) and disappearance of α-ketoisocaproate (Tale 5.1), it becomes clear that less turnover of α-ketoisocaproate and of leucine (less NH_4 produced than with leucine alone) took place in the presence of both substrates than when leucine alone was added.

Production of NH_4 was highest in cultures grown with leucine. The only source of NH_4 was peptone in samples with α-ketoisocaproate, thus the low levels of NH_4 produced. Samples with both leucine and α-ketoisocaproate present contained amounts of NH_4 similar to those with α-ketoisocaproate alone. This indicates further that leucine was metabolized only to a small extent in these cultures. Cultures grown

with α-ketoglutarate, which served as controls, showed similar growth to cultures with leucine or leucine and α-ketoisocaproate up to 41 hours incubation, after which time growth declined. For the first 20 hours, cultures with α-ketoglutarate showed similar levels of NH_4 and sulphide production as those with α-ketoisocaproate. However, less than 2mM α-ketoglutarate was taken up.

Possible inhibition by the high concentration of substrate in the medium with both leucine and α-ketoisocaproate was investigated. Cells were grown in 0.05% peptone medium with 10mM and 20mM α-ketoisocaproate. After 24h. at 75°C the OD_{650} of cultures with 10mM α-ketoisocaproate was 0.12 compared to 0.10 in those grown with 20mM. Increased substrate concentration could not have been the cause of repressed growth in the presence of α-ketoisocaproate and leucine.

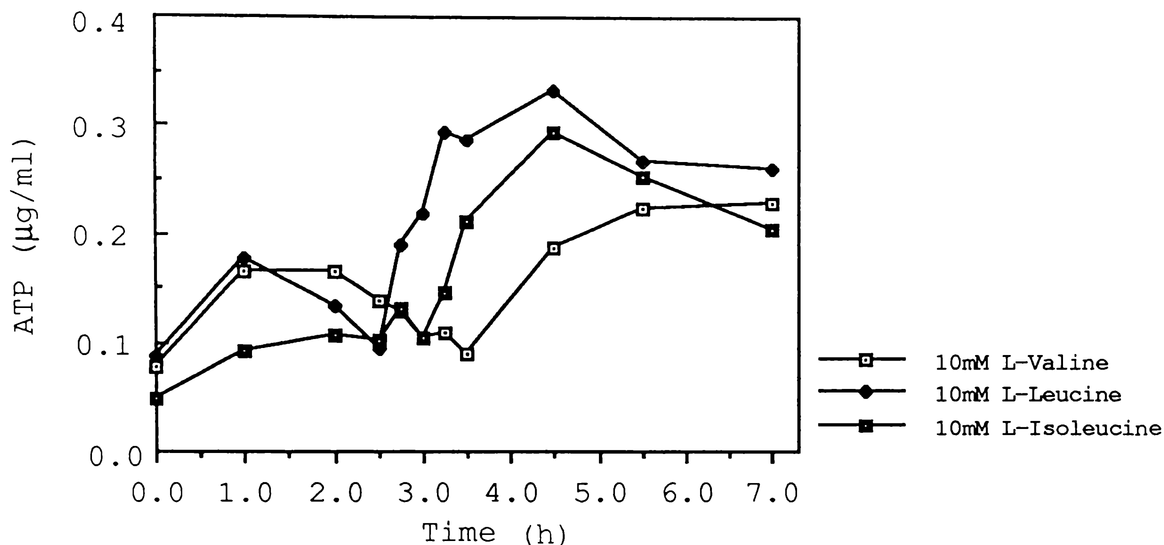
5.3.2. ATP production upon addition of amino acids and α-ketoisocaproate

In section 4.3.2.1 the production of ATP in cell suspensions of ANI was shown. Results showed that NaCl was required for ATP production, and that levels of intracellular ATP rose upon addition of leucine, valine, or trypticase. ATP production in cell suspensions upon addition of leucine, isoleucine, and valine was measured to see whether the effect was similar with each of them. Levels of cellular ATP were slowest to rise upon addition of valine, and fastest to respond upon addition of leucine (Fig.5.3.a). To test for utilization of the amino acid various amino acids were added to cell suspensions of 'starved' ANI. Amino acids added were D-leucine, L-threonine, L-tryptophan, L-alanine, and L-lysine. Threonine, alanine, and tryptophan were previously found to stimulate growth (3.3.5.), while lysine was only a rather poor stimulant of growth yield of ANI. As D-leucine stimulated growth yield of ANI it was used to see whether both isomers of the amino acid would have a similar effect on ATP production. In addition α-ketoisocaproate was tested. In Fig.5.3.b the effects of the various substrates on the cellular ATP level of ANI are shown. As pointed out before levels of cellular ATP cannot be compared on an absolute basis between different lots of cell suspensions (i.e. those shown in Fig.5.3.a and 5.3.b).

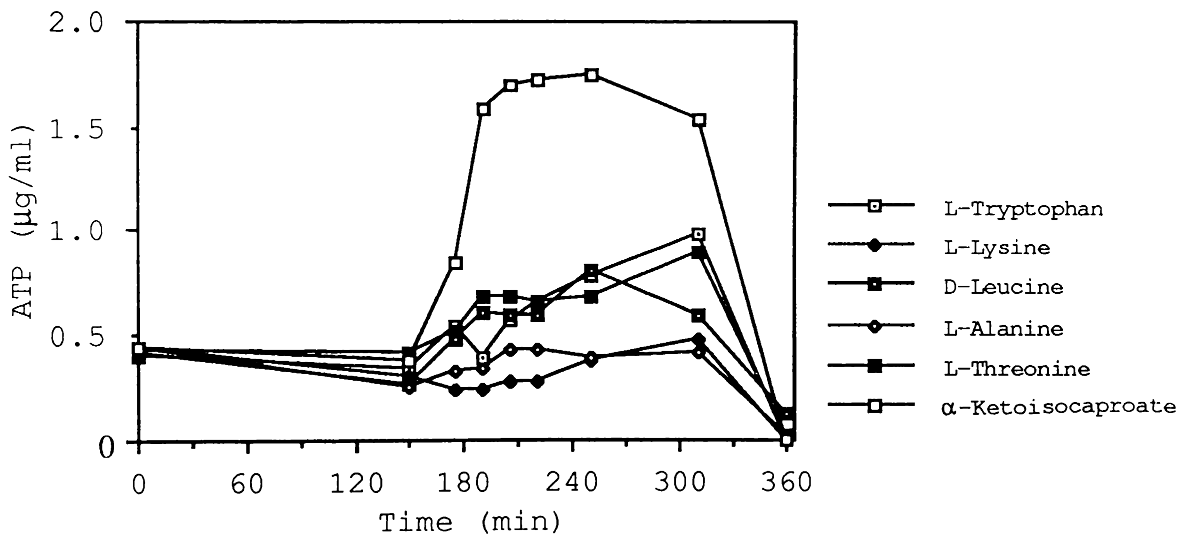
As in studies on growing cultures of ANI α-ketoisocaproate was the best substrate. Its addition resulted in a steep rise in cellular ATP. None of the amino acids added had a stimulatory effect to a similar degree.

Fig.5.3. Effect of various substrates (10mM each) added to cell suspensions starved for 2.5. hours at 75°C

a) Leucine, isoleucine, and valine



b)Other amino acids, α-ketoisocaproate



Tryptophan, D-leucine, and threonine produced an increase in ATP levels comparable to the L-forms of leucine, isoleucine, and valine. Alanine did not stimulate ATP production which is in contrast to its enhancing effect on growth of cultures of ANI. Addition of lysine to cell suspensions resulted in a slow increase in cellular ATP reflecting its lower stimulatory effect on growth of ANI.

5.3.3. Enzymatic studies

5.3.3.1. Branched-chain amino acid aminotransferase

Activity of branched-chain amino acid aminotransferase was assayed in extracts of cells grown on 0.8% peptone, on one or two amino acids or α-ketoisocaproate with reduced peptone to test for induction of the enzyme. Specificity of the enzyme was tested with several amino acids used as substrate.
Extracts of cells grown on 0.2% peptone with addition of either 10mM valine or 10mM leucine or 5mM alanine plus 5mM threonine were used in assays for aminotransferase in order to establish whether activity of the enzyme increased in cells growing on branched-chain amino acids. To see if there was more than one enzyme involved in deamination of branched-chain amino acid activity of each extract was assayed with leucine, isoleucine, and valine as substrate. Multiple enzymes would, however, only be detected if any were inducible. In Table 5.1 results of these assays are shown.

Table 5.2. Branched-chain amino acid aminotransferase activity in crude cell extracts

Growth medium	Specific activity[a] Assay substrate			Controls	
	Leucine	Valine	Isoleucine	No substrate	No α-KG[b]
Valine	1000	240	280	3	<1
Leucine	520	60	50	1	<1
Alanine +Threonine	760	160	90	1	<1
Leucine[c]	<0.1	0	0	0	0
No extract	3	0	0	0	0

[a]expressed as nmoles α-keto acid per min per mg protein
[b]α-ketoglutarate
[c]extract boiled for 10min prior to assay

The three cell extracts all showed highest activity with leucine as substrate. Activity on valine and isoleucine was similar and 5 to 10 times lower than on leucine. Virtually no activity was observed in the

absence of α-ketoglutarate, amino acid substrate, or cell extract. Dependence on α-ketoglutarate indicates that the activity assayed was an aminotransferase and not that of another deaminating enzyme.
Aminotransferase activity (α-ketoglutarate-dependent) with other amino acids as substrate was investigated. Extracts from cells grown with 10mM valine and 0.2% peptone were used in reaction mixtures with several single amino acids as substrates. Activities in these assays are shown in Table 5.3.

Table 5.3. Specificity of α-ketoglutarate-dependent amino acid aminotransferase for cell extracts grown on valine as major carbon source

Substrate	% Specific activity[a]
Leucine	100
Valine	26
Alanine	2
Threonine	0
Tryptophan	0
Lysine	~0.3
Proline	0
Arginine	0

[a]nmoles of α-keto acid produced per min per mg protein (100%=820nmoles)

In order to test whether this enzyme was constitutive in ANI extracts of cells grown on 0.8% peptone were compared for their α-ketoglutarate-dependent amino acid transferase activity with extracts from cells grown on 0.05% peptone with 10mM leucine. Also, extracts from cells grown on 0.05% peptone with 10mM α-ketoisocaproate were compared in their amino acid amino transferase activity to see whether the enzyme activity may be under negative control when cells grow on a α-keto acid. Activities of the different cell extracts on leucine as substrate are shown in Table 5.4.
Highest levels of activity were observed in extracts of cells grown with α-ketoisocaproate. Activities in extracts of cells grown with either leucine or 0.8% peptone were similar.

Table 5.4. Branched-chain amino acid amino transferase activity of extracts from cells grown on 0.8% peptone, leucine, or α-ketoisocaproate (leucine served as substrate)

Growth medium	Specific activity[a]	Controls	
		No α-ketoglutarate	No substrate
0.8% peptone	450	ND[c]	2
0.05% peptone +10mM leucine	370	21	1
0.05% peptone +10mM KIC[b]	820	30	30

[a]nmoles α-keto acid produced per min per mg protein
[b]α-ketoisocaproic acid
[c]not determined

The activity of the branched-chain amino acid aminotransferase was not reduced when cells were grown with α-ketoisocaproate as major carbon source.

5.3.3.2. α-Keto acid oxidoreductase

Crude extracts of cells grown on 0.8% peptone were assayed for α-keto acid oxidoreductase activity. Several keto acids were tested as substrates with several electron acceptors. The results are summarized in Table 5.5. The dependence on thiamine pyrophosphate was investigated by omitting it from some reaction mixtures. In the assays cell extracts were used which had either been centrifuged for 5min. in a benchtop centrifuge following sonication, or for 30min at 13529 x g which gave a clearer supernatant.
To confirm that the reaction measured in this assay was the oxidation of the the α-keto acid to the acyl-CoA with concomitant reduction of an electron acceptor, dependence on free CoA was demonstrated. Crude cell extract from cells grown on 0.8% peptone was treated with an anion-exchange resin to remove free CoA as described by Harwood and Canale-Parola (1981). Dowex-1 anion exchange beads were used. Binding of free CoA to these beads was first tested in a solution of CoA-SH in H_2O. Before addition to Dowex-1 the CoA solution had an A_{233} of 1.6. After treatment with Dowex-1, the A_{233} of the solution was 0.08. Thus, most of the CoA had been removed. Cells for these experiments were grown on 0.8% peptone medium.

Table 5.5. Activity of α-keto acid oxidoreductase of crude cell extract of isolate ANI (grown on 0.8% peptone)

Substrate	Electron acceptor	TPP[+a]	Specific activity[b]
α-ketoisocaproate	FAD	+	5.65[c]
"	"	+	11.30[d]
"	FMN	+	7.60[d]
"	"	-	6.00[d]
"	NADP	+	0.00[d]
α-ketoisovalerate	FAD	+	5.30[c]
"	"	-	6.00[c]
"	FMN	+	3.30[c]
"	"	-	4.00[c]
"	NAD	+	0.00[c]
α-ketobutyrate	FAD	+	4.60[c]
"	FMN	+	1.00[c]
Pyruvate	FAD	+	1.80[c]
α-ketoglutarate	FAD	+	1.30[c]
No substrate	FAD	+	0.19[c]
"	FMN	+	0.22
α-ketoisocaproate	No acceptor	+	0.00[c]
no dithiothreitol	FAD	+	0.08
α-ketoisocaproate	FAD	+	0.00[e]

[a] thiamine pyrophosphate
[b] nmoles of electron acceptor reduced per min per mg protein
[c] sonicate centrifuged in benchtop centrifuge
[d] sonicate centrifuged at 13529 x g/30min.
[e] no cell extract added

Removal of free CoA from cell extracts resulted in a sharp decline of enzyme activity when CoA-SH was omitted from the reaction mixture (Table 5.6). Enzyme activity was dependent on the addition of exogenous CoA.

Finally, α-keto acid oxidoreductase activity was examined with respect to induction and specificity. Cells were grown on 0.8% peptone, or 0.1% peptone with either 10mM α-ketoisocaproate or α-ketoisovalerate. Oxidoreductase activities detected in extracts of the different cultures are summarized in Table 5.7.

Table 5.6. Requirement for CoA-SH in reaction mixtures for α-keto acid oxidoreductase (with FAD as electron acceptor)

Cell extract	Substrate (KIC[b])	Exogenous CoA addition	Specific activity[a]
Untreated	+	+	4.75
"	+	-	0.53
"	-	+	0.22
Dowex-1 treated	+	+	6.25
"	+	-	0.15
"	-	+	0.03

[a]expressed in nmoles FAD reduced per min per mg protein
[b]α-ketoisocaproate

Table 5.7. Activity of α-keto acid oxidoreductase in extracts of cells grown either with a branched-chain α-keto acid or with 0.8% peptone

Growth medium	Substrate	Specific activity[a]
0.1% peptone + 10mm KIC[b]	KIC	7.6
"	KIV[c]	8.9
	KG[d]	0
0.1% peptone + 10mM KIV	KIC	8.9
"	KIV	8.7
0.8% peptone	KIC	1.8
"	KIV	2.2

[a]expressed as nmoles FAD reduced per min per mg protein

[b]α-ketoisocaproate

[c]α-ketoisovalerate

[d]α-ketoglutarate

Cells grown with either α-ketoisocaproate or α-ketoisovalerate displayed greater oxidoreductase activity than did cells grown on 0.8% peptone. Activity was possible on either branched-chain α-keto acid independent of which was the growth substrate. By contrast, α-ketoglutarate was not a substrate for enzyme activity under these assay conditions.

5.3.3.3 Phosphate acyltransferase

Phosphate acyltransferase was assayed in the forward direction (from isovaleryl-CoA and isobutyryl-CoA respectively to isovaleryl- and isobutyryl phosphate) by following the change in absorbance at 233nm (Bergmeyer *et al.* 1974). Free CoA absorbs less than bound CoA at that wavelength thus the reaction is followed by a decrease in absorbance. Phosphate acyltransferase was also assayed by measuring formation of acyl phosphate using hydroxylamine as reagent (which reacts with acyl phosphate to form acyl hydroxamate [Harwood and Canale-Parola 1981]). Measurements of absorbance at 233nm did not show a any activity of phosphate acyltransferase. No change in absorbance was detected (Table 5.8).
Phosphate acyltransferase activity was assayed in the forward direction using isovaleryl-CoA as substrate, by measuring increasing hydroxamate formation. Assays were performed at 55°C and 75°C. Very low activity was detected after incubation times between 5 and 30min (Table 5.8). Most hydroxamate was detected in samples without cell extract added.
Assays were carried out in reversed direction with acetyl phosphate as substrate where activity was measured as a decrease in hydroxamate. Since acetate is one of the major end products of ANI grown on 0.8% peptone presence of phosphate acyltransferase activity was a possibility. Activity was detected. Although hydroxamates (acetyl phosphate) did decrease over time in the absence of cell extract, there was a markedly greater (4.5 times) decrease when cell extract was present. Instability of acetyl phosphate increases with more alkaline pH (Dawson *et al.* 1986). The pH of the reaction mixture was therefore changed to 6.5. A reaction temperature of 55°C was chosen since at 75°C 100% of acetyl phosphate in Tris buffer, pH 8.0 disappeared within 10min as measured by hydroxamate formation. Only 35% was lost at 55°C

in these control experiments. Results of assays of the forward and back reaction catalyzed by phosphate acyltransferase are summarized in Table 5.8.

Phosphate acyltransferase was also assayed in coupled reactions to either fatty acid kinase, glucose-6-phosphate dehydrogenase, and hexokinase, or to α-keto acid oxidoreductase, or to fatty acid kinase. When coupled to hexokinase no activity was detected (no change in absorbance at 340nm).

Table 5.8. Phosphate acyltransferase activity with isovaleryl-CoA, isobutyryl-CoA, and acetyl phosphate respectively as substrate (assayed by change in absorbance at 233nm or formation/disappearance of hydroxamate-forming compounds)

Method	Substrate	Reaction mixture	Specific activity
ΔA_{233}	Isovaleryl-CoA	complete	0.00[a]
(Bergmeyer	Isobutyryl-CoA	"	0.00[a]
et al. 1974)			
Hydroxamate	Isovaleryl-CoA	"	10.3[b]
(formation)	"	no substrate	0.00[b]
"	"	no phosphate	11.8[b]
"	"	no cell extract	23.2[b]
Hydroxamate	Acetyl phosphate	complete pH 8.0	46.3[c]
(disappearance)	"	no substrate	<0.1
	"	complete pH 6.5	183.7[c]
"	"	no CoA "	115.0[c]
"	"	no arsenate "	139.0[c]
"	"	no substrate "	<0.1

[a]nmoles acyl-CoA disappearance per min per mg protein
[b]nmoles acyl hydroxamate produced per min
[c]nmoles acetyl phosphate disappearance per min per mg protein corrected for 'no cell extract' control

The hexokinase coupled reaction involved coupling the kinase reaction to hexokinase catalyzed production of glucose-6-phosphate from glucose and the glucose-6-phosphate dehydrogenase was the substrate for glucose-6-phosphate dehydrogenase and that reaction is coupled to reduction of NADP which can be monitored spectrophotometrically at 340nm. It was hoped that removal of the produced ATP would favour the phosphate acyltransferase catalyzed reaction to occur.
In assays coupling phosphate acyltransferase to oxidoreductase catalyzed reaction, α-ketoisocaproate was the substrate. Neutralized hydroxylamine and potassium dihydrogen orthophosphate (50mM) were added to the reaction mixture of the keto acid oxidoreductase assay. Reaction mixtures were incubated at 55°C for 5, 10, 15 and 30min but no hydroxamate was detected at any time. Samples with and without hydroxamate were also analyzed gas-chromatographically for detection of branched-chain fatty acid formation but none were detected.
Assays for phosphate acyltransferase were also coupled to fatty acid kinase assays (see below).

5.3.3.4. Fatty acid kinase

Fatty acid kinase was detected in crude cell extracts. There was little difference in activity whether the substrate was isovalerate or isobutyrate. Lower activity was observed with acetate as substrate. The reaction required ATP and hydroxamates were detected and activity measured was therefore that of an acyl kinase. Hydroxylamine was required in the reaction mixture from the start of the assay onwards to bind formed acyl phosphate and to ensure that the reaction was favoured to proceed in the direction of acyl phosphate.

Coupling of the fatty acid kinase catalyzed reaction to the conversion of the product acyl phosphate to acyl-CoA was attempted for demonstration of phosphate acyltransferase activity. Free CoA-SH was added to the fatty acid kinase reaction mixture and hydroxylamine was omitted. To favour the reaction to proceed ATP was added at ten times the concentration of previous assays (100mM instead of 10mM). Hydroxamate-forming compounds were detected by addition of hydroxylamine and allowing for hydroxamates to form.
Fatty acid kinase catalyzed production of acyl phosphate could not be coupled to the phosphate acyltransferase catalyzed reaction as

hydroxylamine was required in the assay mix to drive the reaction towards acyl phosphate removing it as it was produced. Presence of CoA-SH in the reaction mixture resulted in an increase in hydroxamate formation (Table 5.9).

Table 5.9. Activity of fatty acid kinase in cell extracts of ANI

Substrate	Changes in reaction mixture additions	hydroxylamine add.[b]	Specific activity[a]
Isovalerate	-	start	12.4
"	+ CoA[c]	start	23.8
"	no cell extract	start	<0.1
Isobutyrate	-	start	12.0
"	+ CoA[c]	start	26.2
"	+ CoA[c]	end	0.5
"	-	end	0.5
"	- ATP	start	0.1
"	+ 10 x ATP[c]	end	<0.1
Acetate	-		7.6

[a]nmoles acyl hydroxamate formed per min. per mg protein
[b]hydroxylamine addition at the beginning or the end of the assay period
[c]reactions carried out in an attempt to couple fatty acid kinase reaction to phosphate acyltransferase reaction

5.4. Discussion

5.4.1. Growth on leucine and α-ketoisocaproic acid

For growth α-ketoisocaproate was a superior substrate over leucine. Growth observed in the presence of α-ketoglutarate was mostly due to utilization of peptone in the medium until that source was exhausted. However, α-ketoglutarate had some stimulatory effect on growth as growth yield after 48h was higher than in the presence of 0.05% peptone alone.

In cultures grown in the presence of both leucine and α-ketoisocaproate it appeared that the presence of one (leucine) inhibited utilization of the other (ketoisocaproate). Among possible explanations for this phenomenon may be that both substrates were taken

up by the same uptake system which may have a higher affinity for leucine, or that leucine repressed the utilization of α-ketoisocaproate.

Growth inhibition due to antagonistic effects between branched-chain amino acids has been observed in several organisms and studied in detail in *Escherichia coli* (de Felice *et al.* 1979). It has been found that some organisms show growth inhibition by L-valine (or L-leucine) alone which can be alleviated when both, or L-isoleucine, are added. The major cause for such inhibition is that one amino acid represses synthesis of the acetohydroxy precursors of all branched-chain amino acids. This may explain the better growth on ketoisocaproate than on leucine assuming the amino acid and not the α-keto acid is repressing synthesis of related amino acids. Such suggestion would have been supported by greater stimulation of growth when both leucine and valine were added to the medium than when only one of these was added (section 3.3.5). However, when both leucine and valine were added together metabolism of either appeared repressed compared to when they were added singly. Repression of common metabolic routes by one amino acid would not explain why α-ketoisocaproate was not utilized in the presence of L-leucine.

The rate of peptone utilization also declined in the presence of leucine or α-ketoisocaproate. That some peptone was still metabolized is evidenced by the production of NH_4 upon addition of α-ketoisocaproate, and the spectrum of fatty acid end products with both substrates (Fig.5.2; Table 5.1).

5.4.2. ATP production in cell suspensions upon addition of amino acids and α-ketoisocaproate

Most of the amino acids added, and α-ketoisocaproate had a stimulatory effect on ATP levels which reflected their stimulation of growth of ANI (section 3.3.5). The stimulation of ATP synthesis upon addition of α-ketoisocaproate was markedly above that observed for any of the amino acids. The stimulation by leucine was greater than by isoleucien or valine.

The results obtained with alanine were not in accordance with observations on growth stimulation in cultures (section 3.3.5). However, the uptake mechanism of alanine may differ from that of other

amino acids used here in that it may require ATP or a related compound directly. Cells were starved and the level of intracellular ATP was low thus the cells may not have been capable of uptake via ATP dependent systems. Uptake of alanine may also require some medium component which was not present in the cell suspension experiments.

5.4.3. Enzymes involved in degradation of branched-chain amino acids

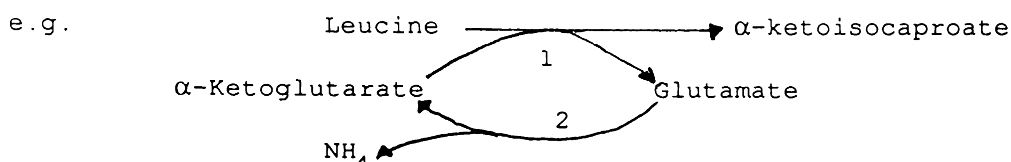
5.4.3.1. Branched-chain amino acid amino transferase

Branched-chain amino acid amino transferase catalyzes the first step in the metabolic degradation of branched-chain amino acids. This enzyme has been purified from several eubacteria (Massey *et al.* 1976). Amongst archaebacteria the only amino acid aminotransferase studied so far is aspartate amino transferase of *Sulfolobus acidocaldarius* (Marino *et al.* 1988; Sannia and Marino 1990).

In the reaction catalyzed by this enzyme, the amino group of the branched-chain amino acid is transferred to α-ketoglutarate to form glutamate. In this respect findings on glutamate dehydrogenase in ANI are of importance. Glutamate dehydrogenase constitutes about 5% of all soluble protein in ANI (R. Hudson pers. comm.). As glutamate is not a major amino acid utilized by the organism such high levels of the enzyme seemed inexplicable on the grounds of glutamate catabolism. However, ANI utilizes amino acids as its major carbon, nitrogen, (and energy) source in laboratory cultures at least. Not all amino acids utilized may be broken down via a transaminase in ANI, but as the major fatty acid end products on peptone medium are largely from degradation of branched-chain amino acids, these are presumed to be the favoured substrate components of peptone. Glutamate dehydrogenase may thus be required largely for the transfer of the amino group of other amino acids to α-ketoglutarate (Fig.5.4) rather than for catabolism of glutamate.

Fig. 5.4. Proposed transfer of amino groups from branched-chain amino acids to ketoglutarate and the importance of glutamate dehydrogenase

e.g.



1-(branched-chain) amino acid aminotransferase
2-glutamate dehydrogenase

Glutamate dehydrogenase concentration in *Sulfolobus solfataricus* is about ten times the level of other enzymes in that organism (Consalvi *et al.* 1990), suggesting an important role for this enzyme in the metabolism of diverse archaebacteria.

Since trace peptone was required for growth and since branched-chain amino acids are always utilized (deduced from fatty acid end products) induction experiments were not possible because cells could not be grown in the absence of branched-chain amino acids. This could be overcome if a totally synthetic medium (i.e. in the absence of peptone) devoid of branched-chain amino acids could be devised.

Pseudomonas cepacia has been found to possess two branched-chain amino acid amino transferase isoenzymes (Wong and Lessie 1979). One of these was a constitutive enzyme, probably involved in biosynthesis of amino acids, while the other was induced by growth on branched-chain amino acids and thus probably involved in their catabolism. The presence of two enzymes for biosynthetic and catabolic functions respectively has also been suggested in *Ps. putida* (Martin *et al.* 1973).

Branched-chain amino acid aminotransferase appeared to be constitutive in ANI. Some results (Table 5.2) indicated a possible induction of the enzyme. Comparison of extracts from cells grown with valine versus alanine/threonine would point to 'induction' of the enzyme; increase of enzyme activity in the presence of a suitable substrate. However, low levels of activity in extracts from cells grown with leucine are not in accordance with this. As cells grow to a higher final yield and since leucine is the preferred substrate for branched-chain amino acid aminotransferase activity lower enzyme levels may be required when

grown on leucine, activity is higher with that substrate. Since no increase in activity was found in a second experiment (Table 5.4) with extracts from cells grown with leucine compared to extracts from cells grown on 0.8% peptone or α-ketoisocaproate a constitutive nature of the enzyme is likely. Increased activity of branched-chain amino acid aminotransferase was observed when α-ketoisocaproate was the major growth substrate (Table 5.4). However more non-specific activity was present in the controls where either α-ketoglutarate or leucine were omitted from the reaction mixture. Amino acid aminotransferase activity appeared to be specific for branched-chain amino acids under assay conditions used. For further characterization of the enzyme activity, substrates like norleucine and norvaline (which were both utilized by ANI) may be tried as potential substrates. It would be of interest to assay extracts of ANI for D-amino acid dehydrogenase, since the organism grows well on D-isomers of branched-chain amino acids as well.

The greater activity on leucine than on isoleucine or valine as substrate may be due to the presence of two enzymes: one specific for leucine, the other specific for branched-chain amino acids. Another explanation is that only one enzyme may be present with greatest activity on leucine. These possibilities could not be distinguished by the method used here.

5.4.3. 2. α-Keto acid oxidoreductase (dehydrogenase)

Oxidation of branched-chain keto acids in mammalian systems has been found by most investigators to be by a single high molecular weight dehydrogenase distinct from pyruvate oxidoreductase (Pettit *et al.* 1978). Branched-chain keto acid dehydrogenase from bovine kidney was active with α-ketoisovalerate, α-ketoisocaproate, α-ketoglutarate, α-keto-β-methylvalerate, α-ketobutyrate, and pyruvate. NAD served as electron acceptor. The enzyme is organized in a dehydrogenase-dihydrolipoyl transacetylase complex (see also Fig.1.2).

Amongst aerobic eubacteria one common enzyme for all branched-chain keto acids has been reported in *Ps. putida* (Martin *et al.* 1973) and in *Bacillus subtilis*, where most activity was on α-ketoisovalerate and least on α-ketoisocaproate (Massey *et al.* 1976). NAD was the electron acceptor.

An α-keto acid dehydrogenase complex is absent in anaerobic eubacteria

and archaebacteria (Kerscher *et al.* 1982). Instead, oxidative decarboxylation of α-keto acids is catalyzed by a less complex enzyme. NAD would not be a suitable electron acceptor, since its redox potential is not negative enough for removal of redox equivalents by the hydrogenase reaction, and thus NAD would tend to act as electron trap (Kerscher and Oesterhelt 1982). Therefore, ferredoxin, or F_{420} in methanogens, serves as the electron acceptor.

In species of *Clostridium* studied (Gottschalk 1986) and in the extremely thermophilic eubacterium *Thermotoga maritima* pyruvate oxidoreductase is dependent on thiamine pyrophosphate. This cofactor is not required for activity of this enzyme in *Pyrococcus furiosus* (Adams 1990b). The catalytic cycle for pyruvate oxidoreductase involving thiamine pyrophosphate as proposed by Kerscher and Oesterhelt (1982) may thus not be universal among anaerobic prokaryotes.

In isolate ANI α-keto acid oxidoreductase activity was detected in crude cell extracts. Specific activity was lower than that of aminotransferase. In assays for α-keto acid oxidoreductase FAD and FMN served as electron acceptors, while no activity was observed with α-ketoisocaproate or α-ketoisovalerate as substrate and NADP or NAD as electron acceptor. Activity with FAD or FMN was reduced when α-ketobutyrate was used as substrate, and only very low with pyruvate or α-ketoglutarate as substrate. FAD was the preferred electron acceptor since higher activities were obtained with FAD over FMN (Table 5.5). In other organisms mentioned above the natural electron acceptor is (or is proposed to be) a ferredoxin which may be the case also in ANI.

Increased activity was detected when cell sonicates were centrifuged at greater force 13529 x g versus 6500 x g). Possibly some constituents of the cell sonicate interfered with or inhibited the reaction but were removed at higher g-forces. Addition of thiamine pyrophosphate did not seem to be required for activity of the α-keto acid oxidoreductase. However, the enzyme was assayed in crude cell extract and sufficient thiamine pyrophosphate may have been contained in the extract to meet the requirement for the reaction to proceed. Assays for the α-keto acid oxidoreductase never proceeded linearly for more than two or three minutes. Anaerobic conditions were required for activity as shown by a control without dithiothreitol (Table 5.5). Although all reaction mixture constituents were prepared anaerobically reactions were carried

out in the presence of air. Addition of either substrate or electron acceptor in greater amounts did not increase either the reaction rate or duration of the linear phase. Use of an electron acceptor which was probably different from the natural one in this reaction and performance of assays at a temperature 20°C below the growth temperature of the organism may have reduced the specific activity. Dependence of the reaction on free CoA implies that the product formed is the acyl-CoA. In contrast to the branched-chain amino acid aminotransferase which was most active with leucine (α-aminoisocaproic acid) as substrate, the α-keto acid oxidoreductase showed about equal activity on α-ketoisocaproate and α-ketoisovalerate, the keto acids of leucine and valine respectively. The growth yield of ANI, however, was greater on α-ketoisocaproate than on α-ketoisovalerate. Further investigations on the specificity of the α-keto acid oxidoreductase, for example with α-ketobutyrate, would be of interest. Induction of the enzyme is suggested by increased specific activity in extracts grown primarily with α-ketoisocaproate or α-ketoisovalerate as substrate compared to extracts of cells grown in 0.8% peptone medium (Table 5.7). Again, induction would be difficult to investigate, due to the presence of peptone in all media, and thus presence of branched-chain amino acids.

5.4.3.3. Phosphate acyltransferase

Presence of phosphate acyltransferase could not be demonstrated conclusively with isovaleryl-CoA or isobutyryl-CoA as substrate. However, several results obtained indicate the presence of such enzyme:
1) Activity of α-keto acid oxidoreductase activity was CoA-dependent, indicating that acyl-CoA was the product.
2) Addition of free CoA to fatty acid kinase assay reaction mixtures resulted in formation of more hydroxamate than when CoA was omitted. Although hydroxylamine primarily reacts with acyl phosphate, it does react also with the acyl-CoA to form hydroxamates (Dawson *et al.* 1986). Increased hydroxamate production may, therefore, have been due to formation of some acyl-CoA from acyl phosphate before it reacted with hydroxylamine and became unavailable. The amount of hydroxamates produced from hydroxylamine and acyl-CoA were barely above control blanks in these assays (where hydroxamates were measured at the onset of assays involving acyl-CoA and the acyl-CoA was at a concentration of 10mM).

Presence of cell extract in the forward reaction of phosphate acyltransferase assays with hydroxylamine may have had a stabilizing effect on the substrate (isovaleryl-CoA) (Table 5.8, measuring hydroxamate formation). The increased hydroxamate formation in absence of cell extract was possibly due to abiotic degradation of the acyl-CoA. Low enzymatic activity in the cell extract would not have been apparent above the instability of the substrate and the presumed stabilizing effect by the cell extract or components thereof.
As for α-keto acid oxidoreductase, assays for phosphate acyltransferase had to be carried out at 20°C below the growth temperature of ANI most certainly reducing the specific activity of crude cell extracts.
Presence of arsenate which was added to prevent the reaction of acyl phosphate to the fatty acid increased activity only by 20% (Table 5.8), indicating that the reaction from acetyl phosphate to acetate was not a major cause for low activity in the assay for phosphate acyltransferase.
Recently it has been observed that activity of phosphate acyltransferase in extracts of a marine spirochete apparently increased the more dilute samples of crude extract were used in the spectrophotometric assay (P.Janssen, pers. comm.). Possibly some component(s) in the crude cell extract are inhibiting the reaction or bind reaction mixture components in other reactions. In more dilute extract samples these inhibitory constituents were apparently too low in concentration to interfere with the reaction sufficiently to completely mask any phosphate acyltransferase activity.
An alternative possibility is that the pathway in ANI does not involve a phosphate acyltransferase-catalyzed step. Instead, the fatty acid may be produced directly from the acyl-CoA analogous to acetate from acetyl-CoA and ADP (catalyzed by acetyl-CoA synthetase [E.C.6.2.1.13]) or AMP (acetyl-CoA synthetase [E.C.6.2.1.1]). Such a pathway has been suggested as a possibility in *Thermoplasma acidophilum* although the former enzyme has only ever been found in a human eukaryotic parasite (Danson 1989). Similar enzymes for branched-chain acyl-CoA have not been reported. Presence of such enzymes involved in this pathway is not likely since hydroxamates were detected which are formed primarily with acyl phosphates. Cell extracts of ANI showed activity of phosphate acyltransferase with acetate as substrate.

5.4.3.4. Fatty acid kinase

Activity of fatty acid kinase was detected with isovalerate, isobutyrate, and acetate as substrate although activity on the latter was lower.
In this study intermediates of the metabolic breakdown of isoleucine have not been used but it is assumed that metabolism of isoleucine is analogous to that of leucine and valine involving common enzymes. The existence of a pathway for branched-chain amino acid degradation coupled to synthesis of ATP from ADP and P_i as proposed in section 5.1 is strongly supported by results of studies on the enzymes catalyzing the reactions involved. Findings of this study are shown in Fig. 5.5.

5.4.4. Production of acetate

In chapter 3 acetate production from alanine and cystine was shown. It was proposed that degradation of both substrates proceeds via pyruvate (section 3.4). Presence of most enzymes involved in a catabolic pathway of cystine and alanine leading to acetate (shown in Fig.5.5) was demonstrated in results in this chapter. Activity of α-keto acid oxidoreductase and fatty acid kinase on pyruvate and acetate respectively was lower than on branched-chain substrates. No activity was detected with alanine as substrate for α-ketoglutarate-dependent amino acid aminotransferase. The first step in metabolism of alanine may involve a reaction catalyzed by a different type of enzyme e.g. a dehydrogenase.

Fig.5.5. Proposed pathway for branched-chain amino acid fermentation in isolate ANI

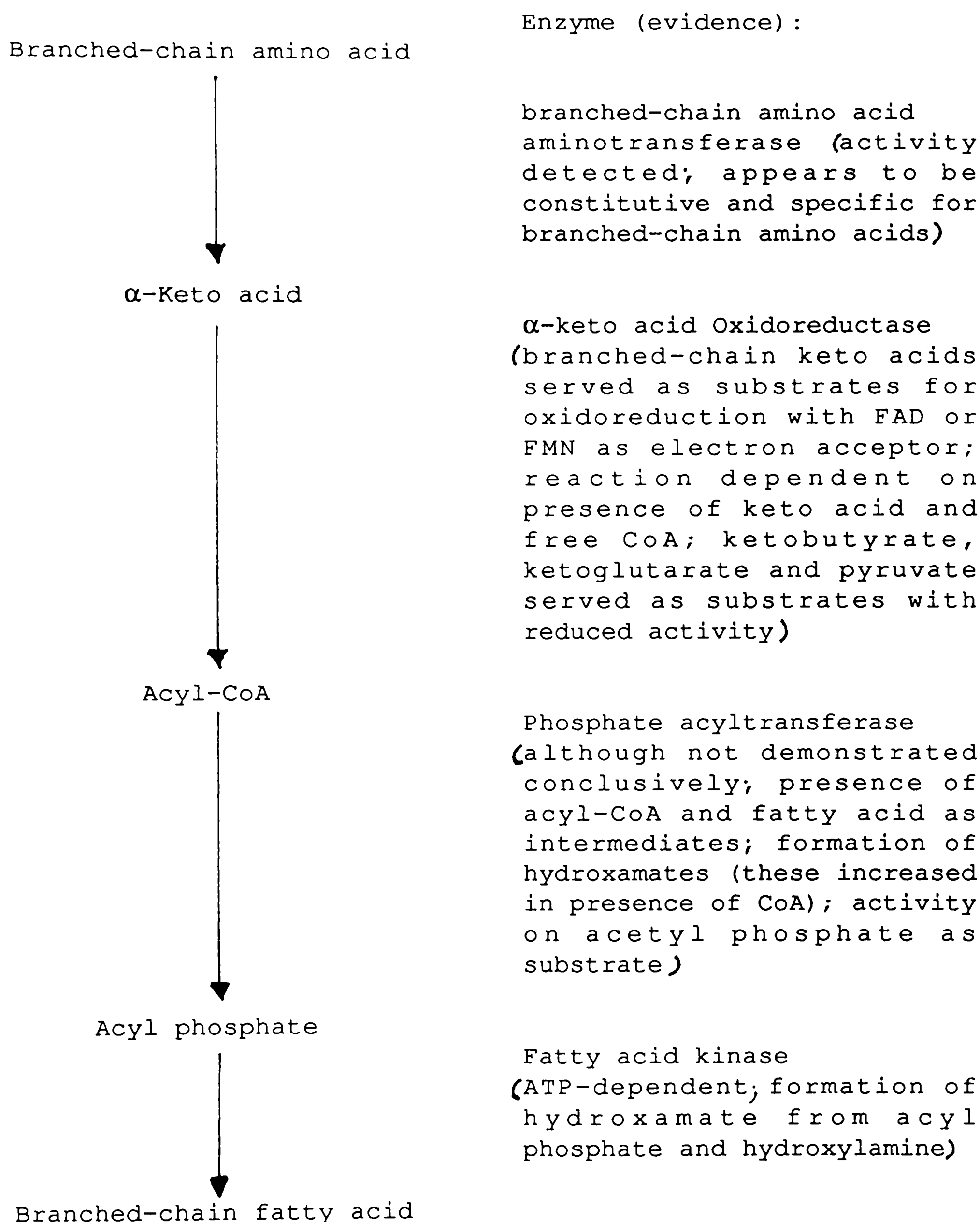
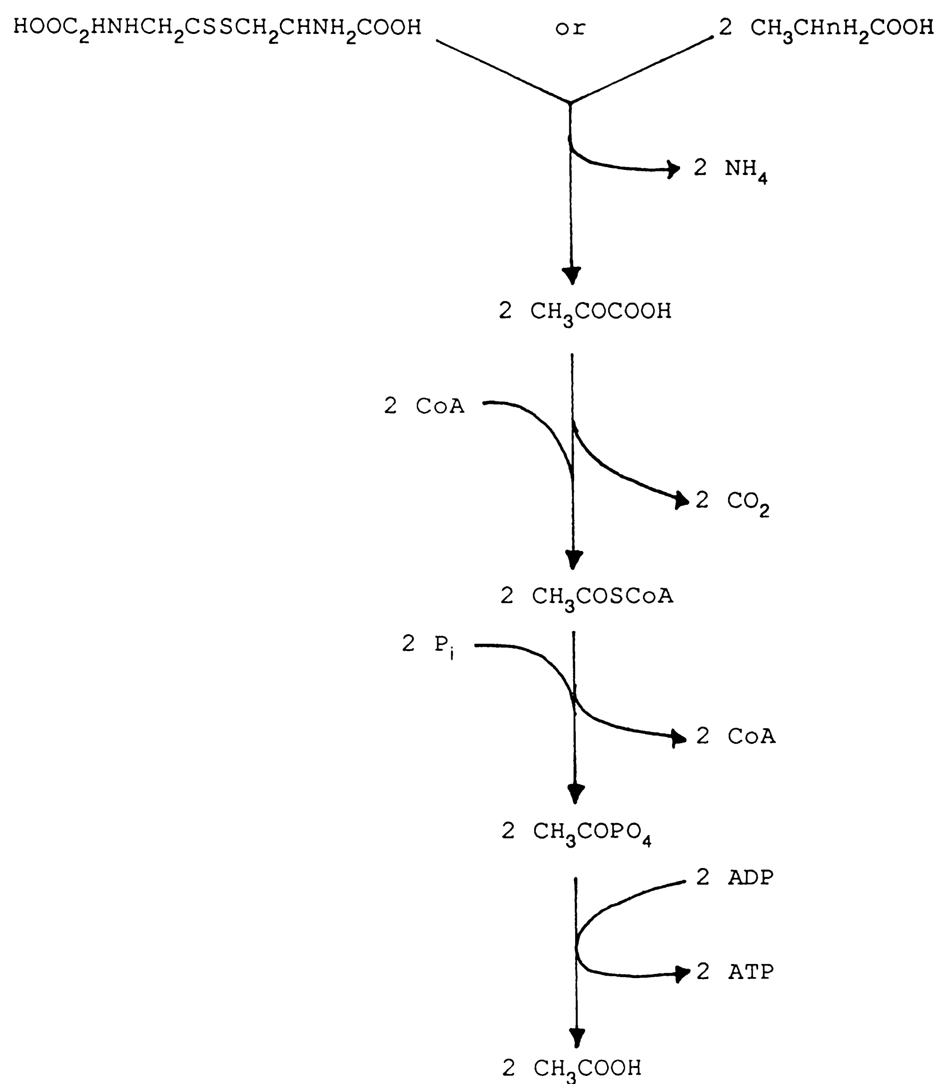


Fig.5.6. Possible degradative pathways for acetate production from cystine and alanine in ANI



Chapter 6: Production of sulphide and hydrogen

6.1. Introduction

Isolate ANI was found to have an obligate requirement for elemental sulphur (or cystine or glutathione) for growth (section 3.3.6). It was shown earlier that concentrations of 30mM of sulphide had an inhibitory effect on growth. It was therefore of interest to investigate why the organism reduced sulphur forming an inhibitory product when it was apparently capable of disposing of excess reducing equivalents (electrons) by producing H_2.

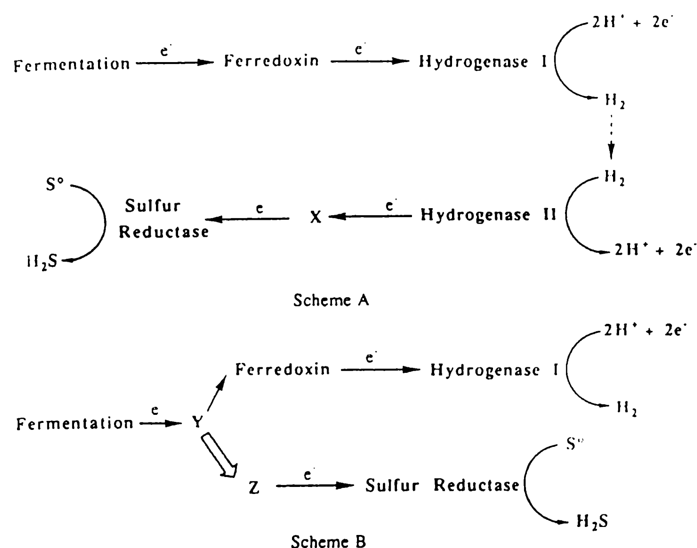
To date *Pyrococcus furiosus* is the only archaebacterium reported to produce H_2 during its primary mode of growth (Adams 1990). Sulphide production is thought to be a method of detoxification as H_2 inhibits growth (Fiala and Stetter 1986) and no energy is thought to be gained from the reduction of sulphur. This distinguishes it from other sulphur-metabolizing archaebacteria which are thought to generate H_2S in an energy-yielding reaction (see section 1.5).

A model for relief of H_2 inhibition by S^o has been presented (Adams 1990a), and is shown in Fig.6.1.

Fig.6.1. <u>Possible schemes for the relief by S^0 of the inhibition of growth by H_2</u>

(X, Y, and Z are hypothetical electron carriers)

(reproduced from Adams 1990a)



Reductant for S^0 reduction in *P. furiosus* could either be generated directly from fermentation diverting electrons prior to proton reduction by the hydrogenase (Fig.6.1, scheme B). Alternatively, induction of a second 'uptake' hydrogenase which would recycle H_2 produced specifically for S^0 reduction is possible (Fig.6.1, scheme A). The presence of an 'uptake' hydrogenase is well-established in eubacterial sulphate-reducers (Gottschalk 1986). However, evidence indicates that in *P. furiosus* scheme B is the more likely possibility. Only one hydrogenase could be detected which was in the cytoplasmic fraction of cell extracts of *P. furiosus* (Bryant and Adams 1989; Adams 1990a). In contrast to ANI *P. furiosus* can grow in the absence of S^0 under a N_2 atmosphere (Fiala and Stetter 1986) although final growth yield is reduced by at least 5 x, if the culture is not flushed with N^2 continuously. Under an H_2/CO_2 atmosphere virtually no growth was observed in the absence of S^0.

ANI was grown under a N_2 and H_2 atmosphere to determine whether the presence of H_2 would influence production of sulphide and H_2 by the organism.

6.2. Materials and methods

Media used were prepared as stated under section 2.2.3. The amount of peptone added varied, and is stated separately for each experiment. When specified, 10mM leucine was added to the medium prior to autoclaving. Cultures were grown in 50ml medium in 120ml or 150ml serum bottles unless otherwise stated. Media always contained 2g/ℓ S^0. All cultures were incubated on a shaking tray at 75°C. (Cultures were cooled to room temperature prior to sampling.)

Hydrogen, sulphide, and volatile fatty acids were measured as described under section 3.2.5.

Crude cell extract was prepared as for the cytochrome scan and an absorbance scan was performed as described (section 3.2.2).

6.3. Results

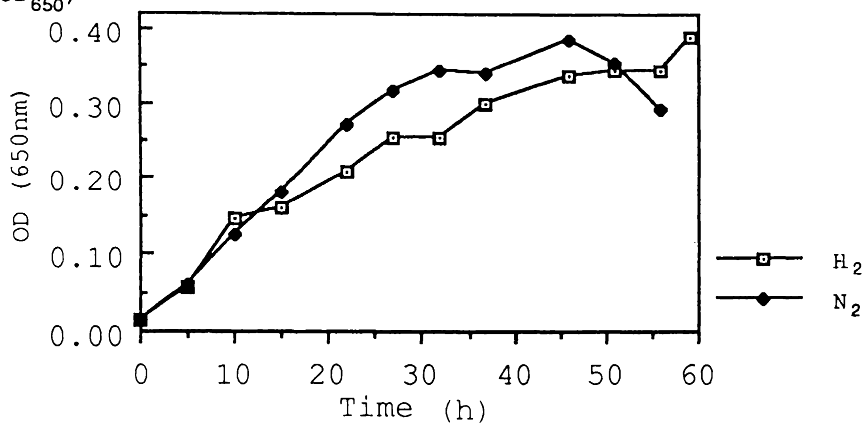
6.3.1. Growth studies

Growth and metabolic end products were compared from cultures grown under a N_2 or a H_2 atmosphere of 1 atmosphere pressure at room temperature (with medium containing 0.2% peptone plus 10mM leucine). Cultures were sampled periodically and at each sampling time 4ml of culture were removed. Results are shown in Fig.6.2.a-d and Table 6.1.

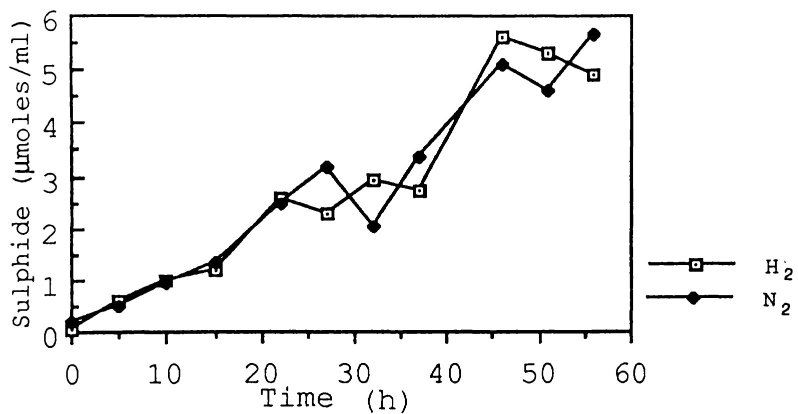
Growth and sulphide production of cultures under a nitrogen and hydrogen atmosphere were similar. Between 22h and 46h incubation time cultures under nitrogen had a slightly higher $OD_{(650)}$ (~0.05) and stationary phase was reached under nitrogen by 46h. Hydrogen was produced under N_2 at a steady rate throughout growth and production did not slow down towards stationary phase. Under hydrogen it was difficult to determine whether or not H_2 was produced because of the high background. When results were expressed as per ml of headspace no increase in H_2 was apparent. Contrary to this, the increase was dramatic when expressed per ml of culture. Similar amounts of sulphide were produced under N_2 and H_2. This discrepancy was due to the changing ratio of headspace:culture volume at every sampling time.
At the beginning of the experiment there was 50ml medium and 90ml headspace, while at the next sample there were 46ml medium and 94ml headspace and so on. The apparent greatest increase in H_2 production per ml of culture occurred towards the later stages of growth (Fig.6.2.c) when the proportional change of removing 4ml of culture changed the ratio more dramatically. Therefore, the apparent increase observed over time in cultures under H_2 when expressed per ml of culture is partly due to removal of relatively large amounts of sample in relation to the total culture volume. The creation of more headspace as sample was removed might also have allowed some further H_2 production. This error in H_2 concentration was not as apparent in cultures under N_2 since any H_2 produced was not being measured against a very high background.
Fatty acids production was similar in cultures under N_2 and H_2, although more isobutyrate and less acetate relative to growth yield was produced under H_2 than N_2.

Fig.6.2. Growth and production of H_2 and sulphide under a H_2 and N_2 atmosphere

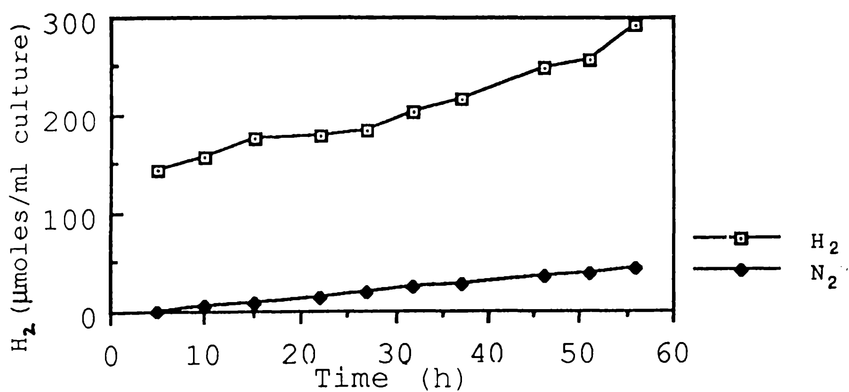
a) Growth (OD_{650})



b) Sulphide



c) H_2 (µmoles/ml culture)



d) H_2 (µmoles/ml headspace)

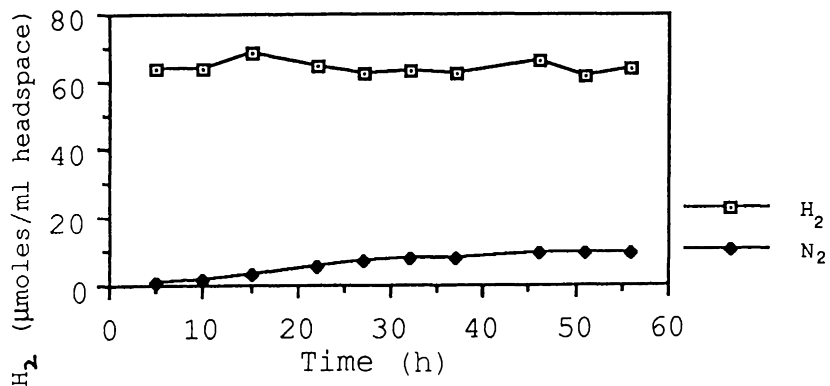


Table 6.1. Production of volatile fatty acids by ANI grown under a N_2 or H_2 headspace at 75°C

Time (h.)	Fatty acid (mM) Acetate		Isobutyrate		Isovalerate[a]	
	N_2	H_2	N_2	H_2	N_2	H_2
0	0.24	0.35	0.00	0.00	0.00	0.00
5	0.47	0.43	0.03	0.00	0.34	0.20
15	0.37	0.45	0.05	0.19	1.07	1.21
27	0.56	0.64	0.73	0.74	6.00	3.95
37	0.85	1.30	0.61	1.48	10.20	7.70
51	3.37	1.62	0.80	2.27	10.71	8.58

[a]isovalerate/2-methylbutyrate

In order to overcome the problem of measuring small increases in H_2 against a high background hydrogen overpressure and with changes in headspace volume due to sampling an alternative approach was taken. Cultures were incubated under either a nitrogen or hydrogen overpressure (in 0.2% peptone plus 10mM leucine medium) and a single analysis of growth, production of H_2, sulphide and fatty acid accumulation was carried out after 48h. Although not allowing a comparison of the effect of H_2 on growth rate this approach would allow assessment of any H_2 inhibition of growth and indicate whether H_2 inhibition could be alleviated by the use of sulphur as a repository for inhibitory H_2. Sulphide and H_2 were also measured at the beginning of the 48hr incubation period. Results are shown in Table 6.2.

While H_2 was produced under N_2, no production of H_2 was detected under a H_2 atmosphere. The amount of H_2 actually decreased in cultures under H_2 and uninoculated H_2 controls. More sulphide was produced under a H_2 overpressure than under a N_2 overpressure but the increase in the amount of sulphide produced under H_2 was not as much as the amount of H_2 detected in cultures under N_2. As in the previous experiment production of fatty acids was similar in relation to growth with relatively slightly less acetate and more isobutyrate produced.

Table 6.2. Growth and production of H_2, sulphide, and volatile fatty acids after 48h under N_2 and H_2 (0.2% peptone plus 10mM leucine medium)

	OD ($_{650}$)	H_2[b]	Sulphide[b]	Fatty acids (mM)		
				Ac[c]	IB[d]	IV/2-MB[e]
H_2 T_0	0.00	44.20	ND[f]	ND	ND	ND
H_2 T_{48hrs}	0.38	39.65	15.8	1.32	0.92	8.82
N_2 T_0	0.00	0.012	ND	ND	ND	ND
N_2 T_{48hrs}	0.40	8.24	12.5	2.42	0.45	9.90
H_2[a] T_0	0.00	48.65	ND	ND	ND	ND
H_2[a] T_{48hrs}	0.00	39.80	0.33	0.28	0.0	0.0

[a]uninoculated control
[b]expressed as µmoles/ml culture
[c]acetate
[d]isobutyrate
[e]isovalerate/2-methylbutyrate
[f]not determined

If ANI produced H_2 until inhibitory levels were reached and then switched to sulphide production as a means of discarding excess reducing equivalents, then H_2 production would be relatively high at early stages in growth while at later stages no further increase in H_2 would occur and sulphide would be produced. To see whether this was the case, cultures (in 0.8% peptone medium) under a N_2 atmosphere were incubated for different lengths of time: one duplicate of cultures was incubated for 4h, one for 6h, one for 7.5h, one for 10h, and one for 23h. At the end of their respective incubation times cultures were analyzed for growth yield and production of H_2 and sulphide. Results are shown in Table 6.3.

Sulphide and H_2 were produced concomitantly throughout growth. The hypothesis of H_2 production preceeding production of sulphide until toxic concentrations of H_2 accumulate is therefore not correct for ANI. Instead, sulphide was produced at a higher rate (µmoles/ml culture/h) during early stages of growth.

Table 6.3. Growth and production of H_2 and sulphide by ANI under a N_2 atmosphere over various incubation periods at 75°C (0.8% peptone medium)

Time (h)	OD $(_{650})$	H_2 (µmoles/ml culture)	Sulphide (µmoles/ml culture)
4	0.04	0.16	1.43
6	0.10	0.95	2.90
7.5	0.15	4.90	5.0
10	0.31	14.70	11.7
23	0.42	29.80	14.0

The samples taken at 10h and 23h show that over that time 15.1µmoles/ml of H_2 compared to 2.7µmoles/ml of sulphide were produced. Production of growth does not appear to occur early on in growth but towards late log phase.

The effect of available headspace volume was tested next. If H_2 was only produced to a certain concentration, then the headspace size may influence the amount of H_2 produced. Cultures were grown under various volumes of headspace under N_2 in 30ml of 0.15% peptone plus 10mM leucine medium in serum bottles of different sizes giving a headspace of between 10 and 125ml. Cultures were incubated at 75°C for 24h after which time the growth yield (OD_{650}), H_2, and sulphide produced were determined (Table 6.4).

The volume of headspace in cultures did not affect the final growth yield, production of sulphide or H_2. In this experiment the H_2 detected in all cultures was above the amount detected in cultures after 48h on similar medium (Table 6.2). This indicates that the production of H_2 between 24h and 48h incubation periods is reduced compared to earlier on during incubation.

Table 6.4. Effect of the headspace volume on growth and production of H_2 and sulphide

Headspace volume (ml)	Growth yield (OD_{650}nm)	H_2[a]	H_2[b]	Sulphide[b]
10	0.22	34.89	11.63	6.6
29	0.22	14.00	13.55	6.6
91	0.22	3.25	9.85	6.9
125	0.28	3.33	13.75	6.3

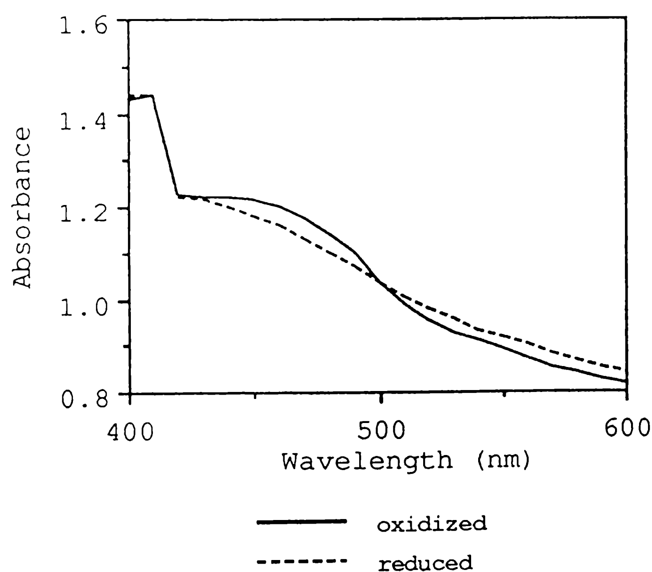
[a]µmoles/ml headspace
[b]µmoles/ml culture

6.3.2. Ferredoxin scan

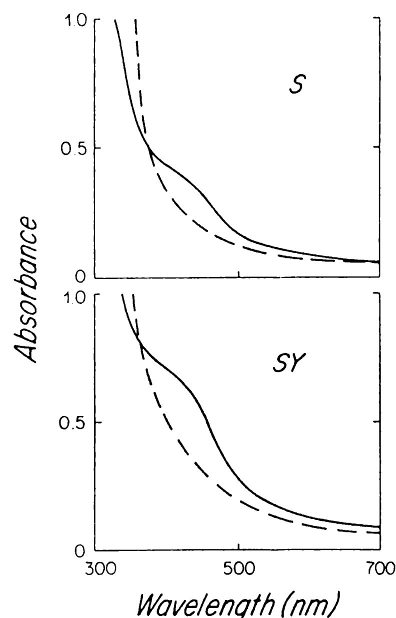
Crude cell extract from ANI grown for 36h at 75°C on 0.8% peptone was used. The scan is shown in Fig.6.3.

Fig.6.3. Optical absorbance scan of oxidized and reduced crude cell extract between 400 and 600nm
(optical absorbance spectra of soluble fractions of *Desulfurococcus* sp. strains S and SY are shown for comparison [Jannasch *et al.* 1988])

a) ANI



b) *Desulfurococcus* strains S and SY



Optical absorption spectra of soluble fractions from *Desulfurococcus* sp. strains S and SY in air oxidized as prepared (——) or reduced with dithionite (- - - - - -). The cuvettes contained 71 and 203 µg of protein per ml for S and SY, respectively.

6.4. Discussion

Growth of ANI under N_2 and H_2 reached a similar final growth yield. Similar amounts of volatile fatty acids were produced. The effect of H_2 on production of isobutyrate and acetate resulting in a slight increase and decrease respectively was not investigated. Hydrogen did not seem to be produced under a H_2 atmosphere in detectable amounts. However, frequent sampling of cultures resulting in availability of more headspace apparently permitted H_2 production. The production of H_2 **exceeded** production of sulphide: when increased headspace was available throughout growth (when periodic samples were taken) sulphide production was lower both under N_2 and H_2 (Fig.6.2.b) than when cultures were left untouched for 24 or 48h (Tables 6.2-6.4). However, when a large (125ml) versus a small (10ml) volume of headspace (N_2) was available at the onset of growth this did not result in proportionally more H_2 being produced (Table 6.4). This experiment was done only under N_2. Cultures grown under H_2 (48h) produced more sulphide than cultures grown under N_2 (Table 6.2), although the amount of sulphide and H_2 produced under N_2 exceeded the amount of sulphide produced under H_2.

Under N_2, sulphide is produced even when the level of H_2 is very low. It seems possible that H_2 was produced until the elemental sulphur was 'solubilized' to be used as electron acceptor. Observations of delayed growth under H_2 (Fig.6.2) are in support of this idea. However, H_2 production did not cease at later stages in growth and results from later experiments (Table 6.3) indicate that sulphide was produced in early stages in growth at a rate equal or greater than that of H_2 production. The overall cell yield under N_2 and H_2 was comparable indicating that presence of neither N_2 or H_2 had a stimulatory or inhibitory (overall) effect over the other on cell cultures.

In the natural hot pool environment both H_2 and sulphide may be removed rapidly upon production. Growth of the organism would probably not be at as high a rate as in the laboratory. The equilibrium of H_2 and sulphide production may also be affected by removal of e.g. H_2 by methanogens. It would be of interest to grow ANI in the laboratory in co-culture with a methanogenic organism to scavenge H_2 produced, and monitor sulphide production by ANI under these conditions.

Crude cell extract of ANI scanned at wavelengths between 400 and 600nm gave some indication of the presence of ferredoxin, by comparison with other ferredoxin scans published (Kerscher *et al.* 1982; Jannasch *et al.* 1988; Aono *et al.*1989).

Absence of cytochromes in ANI (section 3.3.2) indicates that the organism does not carry out sulphur respiration via a complex electron transport chain. No menaquinones, ubiquinone, *Caldariella* or *Sulfolobus* quinone were found in *Thermococcus celer* (Thurl *et al.* 1986).
Increased production of sulphide was not equalled by growth yield (section 3.3.6.b), which does not support direct energy gain from sulphur reduction.

In contrast to the proposed model for *P. furiosus* where sulphide production serves as a means for H_2 detoxification, in ANI this does not seem to occur. A hydrogen overpressure did not inhibit growth and H_2 was not the predominant product (compared to sulphide) during early stages of growth. Production of sulphide and H_2 continued throughout growth; rates slowing down towards later stages in growth. Results do not show preference of sulphide production over H_2 production (or vice versa), further indicating that no energy generation is involved in reduction of sulphur.

Chapter 7: Preliminary Studies on a Restriction Endonuclease of ANI

7.1. Introduction

Restriction endonucleases are enzymes present in many bacteria which act to restrict the expression of foreign DNA introduced through phage infection, conjugation, or transformation. There are three types of endonucleases (Endlich and Linn 1981). Type I systems consist of large complex molecules which require Mg^{2+}, S-adenosylmethionine, and ATP (which is hydrolyzed) as cofactors. While the recognition sequence is directly acted upon by the modification methylase, the site of cleavage of DNA by the enzyme is random, often 1-5 kilobase pairs (kb) from the recognition site. Type II endonucleases are less complex and require only Mg^{2+} for nuclease activity and S-adenosylmethionine for modification activity. In contrast to type I endonucleases, these enzymes cleave DNA within the recognition sequence. Type III endonucleases are similar to type I enzymes but do not show any measurable ATP hydrolysis. The DNA cleavage site is approximately 10-20 nucleotides from the recognition sequence and has a degeneracy of 1-2 bases.

Type II endonucleases are very useful and common tools in DNA cloning and mapping studies (Old and Primrose 1981; Glover 1984). They recognize and cut sequences of four or more nucleotides which have an axis of rotational symmetry. The DNA fragments created may have either blunt ends where both strands are cut at the nucleotide in the same position, or sticky ends, where the two DNA strands are cut at the same point in the recognition sequence but not at pairing nucleotides.

Restriction endonucleases have been found in many prokaryotes but none have yet been reported in eukaryotes.

Several type II endonucleases have been reported from archaebacteria. An endonuclease, Tha I, has been isolated from *Thermoplasma acidophilum* which cleaves the sequence CGCG in the centre (McConnell *et al.* 1978). A highly thermostable type II restricition endonuclease, Sua I, was isolated from *Sulfolobus acidocaldarius* which cuts the sequence GGCC in the centre producing blunt-ended fragments (Prangishvili *et al.* 1985). This enzyme is highly thermostable. Three type II endonucleases have been isolated from *Methanococcus aeolicus*, and one from *Methanobacterium* ***voltei*** (Brown *et al.* 1989).

While type II endonucleases are widely used and relatively easily purified to a sufficient level to be utilized as tools in molecular biology, they are relatively poorly characterized in biochemical terms. Endonuclease activity was detected in crude extracts of isolate ANI during a screening program for reverse gyrase activity (Collin 1990). The plasmid pBR322 was digested into several fragments, and activity did not require ATP. A partial purification and determination of the recognition sequence of this thermostable endonuclease are reported.

7.2. Materials and methods

7.2.1. Determination of restriction endonuclease activity

7.2.1.1. Restriction enzymes

Eco R1: A stock solution of Eco R1 (Bethesda Research Laboratories, USA) (10 units/μl) was diluted 10 fold in storage buffer (50mM Tris-HCl, pH 7.2; 0.3M NaCl; 0.5mM EDTA; 5mM mercaptoethanol; 5mM EGTA; 0.2% (v/v) TritonX-100; 500μg/ml BSA; 50% glycerol). The reaction buffer (10x concentration) contained: 500mM Tris-HCl (pH 8.0); 100mM $MgCl_2$; 1M NaCl. For restriction digestions, reaction mixtures contained 1μl of Eco R1 (1 unit/μl) and 1.5μl 10x reaction buffer, 1μg pBR322 DNA (1μl) (kindly supplied by PJ Murray) and 10.5μl milli-Q water. The reaction mixture was incubated for 1hr. at 37°C.

Hae III: A stock solution of Hae III (Bethesda Research Laboratories, USA) in storage buffer (50mM Tris-HCl [pH 7.5]; 0.1M KCl; 0.5mM dithiothreitol; 0.5mg/ml BSA; 50% [v/v] glycerol) diluted to a concentration of 1 unit/μl was used in restriction digests. The reaction buffer contained (10x concentration): 500mM Tris-HCl (pH 8.0); 100mM $MgCl_2$; 500mM NaCl. Reaction mixtures contained reaction buffer, endonuclease, DNA, and H_2O as for Eco R1.

ANi I': The reaction mixtures were the same as for Eco R1 with substitution of Eco R1 by ANi I preparations. When the amount of ANI extract added exceeded 1μl the amount of H_2O added was reduced accordingly. Reaction mixtures were incubated for 30min at 75°C.

For measurement of endonuclease activity restriction digests were electrophoresed on agarose gels unless otherwise stated. Agarose gel electrophoresis was carried out as described in section 3.2.3.

7.2.2. Partial purification of 'ANi I'

7.2.2.1. Growth of ANI

Medium used to grow up ANI was prepared as described in section 2.2.3 with 0.8% peptone. The organism was grown in 800ml volumes of medium in 1ℓ glass bottles (see section 3.2.1). Cultures of a total of 3.2ℓ were grown for 24h at 75°C, harvested by centrifugation (13100 x g/20min/4°C) and pellets washed twice in 10mM Tris-HCl (pH7.0) plus 10mM mercaptoethanol. The cell pellet (6.9g wet weight) was resuspended in 15ml of Tris buffer and cells were disrupted by sonication for 18min in 30 second bursts as described earlier (see section 3.2.) but using an intermediate sized sonication tip. The sonicate was centrifuged at 100 000 x g for 1hr at 4°C (Beckman TL-10 ultracentrifuge).

For the following purification steps the method described by Morris and Parish (1976) was followed. To the supernatant of the centrifuged sonicate steptomycin sulphate was added to 2.5% (w/v). The precipitate was removed by centrifugation (13 529 x g/30min/4°C). To the supernatant $(NH_4)_2SO_4$ was added to 40% saturation (w/v). The addition was made slowly in fractions over 12h while the solution was kept at 4°C. The $(NH_4)_2SO_4$ precipitate was recovered by centrifugation (13 529 x g/30min/4°C), dissolved in phosphate buffer (10mM potassium phosphate (pH 7.4); 10mM mercaptoethanol; 50mM KCl; 1mM EDTA; 10% glycerol) and was dialysed in 2 x 2ℓ phosphate buffer overnight. The supernatant of the $(NH_4)_2SO4$ precipitation was further precipitated to 60% saturation (w/v) with $(NH_4)_2SO_4$ and the precipitate recovered and treated as the 40% precipitate. The dialysates of 40% and 41-60% $(NH_4)_2SO_4$ precipitations were tested for activity. All endonuclease activity was found in the 40% $(NH_4)_2SO_4$ precipitate and the 41-60% precipitate was discarded.

The dialysate of 40% saturation $(NH_4)_2SO_4$ precipitation was run through a phosphocellulose cation exchange column (Whatman P11; Whatman Ltd., England). Prior to use the phosphocellulose had to be activated: 22g per 100ml were gently stirred into 25 column volumes of 0.5M NaOH and left for 5min. The supernatant was filtered off and the matrix washed on the filter until the filtrate pH was 11.0 or below. The matrix was gently stirred into 25 column volumes of 0.5M HCl and left for 5min. The supernatant was filtered off and the matrix washed until the filtrate was above pH 3.0. The matrix was then transferred into 20 column volumes of 5 x strength phosphate buffer and the pH corrected to

pH 7.4 with NaOH. The supernatant was filtered off and the matrix was Astirred into 20 column volumes of phosphate buffer, left for 5min, and the supernatant removed by filtration. This was repeated until the filtrate was pH 7.4. The matrix was dispersed into a measuring cylinder and allowed to settle. The volume was then adjusted to the volume of the matrix plus 20%. The slurry was poured into a 10ml column (Amicon) and phosphate buffer was pumped through it at 30ml/h (using a Pharmacia peristaltic pump P-1; Pharmacia Fine Chemicals, Sweden) until the column was packed. The ANI dialysate was pumped onto the column at a flowrate of 10ml/h. The dialysate was eluted with two column volumes of phosphate buffer and a 100ml gradient of 0.05-1M KCl. Fractions were collected using a Pharmacia FRAC-100 fraction collector (Pharmacia, Sweden). Protein content of the column eluate was monitored at 280nm with an ISCO Model UA-5 Absorbance-Fluorescence monitor (ISCO Instrumentation Specialities Co., USA). Every second sample was tested for endonuclease activity on plasmid pBR322 (kindly supplied by PJ Murray), where 10µl of the fraction collected was used in the assay.

7.2.3. Polyacrylamide gel electrophoresis

7.2.3.1. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

A denaturing PAGE gel was run of the phosphocellulose column fraction with most endonuclease activity (in the pBR322 assay system) according to the Laemmli buffer system (Laemmli 1970). Samples were prepared by adding $^{1}/_{4}$ volume of 4 x Laemmli sample buffer (63mM Tris-HCl (pH 6.8); 10% glycerol; 2% SDS; 5% mercaptoethanol; 0.0013% bromophenol blue) and boiling for 3min in a water bath, followed by centrifugation (6500 x g/5min) to separate any precipitate. The 7.5% discontinuous Laemmli gels contained:

	Separating gel	Stacking gel
milli-Q water	48.5ml	12.2ml
1.5M Tris-HCl (pH 8.8)	25.0ml	-
0.5M Tris-HCl (pH 6.8)	-	5.0ml
10% SDS (w/v)	1.0ml	0.2ml
Acrylamide/Bis (30% stock) (degassed for 15min/20°C)	25.0ml	2.6ml
10% Ammonium persulphate (freshly prepared)	0.5ml	0.1ml
TEMED[a]	0.1ml	0.02ml
Total volume	100.0ml	20.0ml

[a]*N,N,N',N'*-tetramethylethylenediamine

For preparation of separating gels all ingredients except for ammonium persulphate and TEMED were mixed and degassed. The latter two were added and the separating gel was poured, overlaid with isobutyl alcohol and allowed to set. The top of the separating gel was rinsed well with milli-Q water to remove the alcohol overlay and any unpolymerized acrylamide. All excess water was drained off. The stacking gel, prepared as for the separating gel, was poured on top and a comb was inserted in the stacking gel. The stacking gel was left to set. The comb was removed and the wells were rinsed with water and drained. The gel was then placed into the electrophoresis tank. The gel was 0.75mm thick and run on a Protean II Slab Cell system (BioRad) at ~15°C with a current of 13mA during stacking and 18mA for protein separation. The run time was 4-5h. The acrylamide gel was pre-run for 2h prior to loading the sample. The electrophoresis buffer was prepared in 5 x concentration which contained: Tris-base (15g/ℓ; glycine (72g/ℓ); SDS (5g/ℓ).

For staining of protein bands the silver stain procedure of Merril *et al.* (1982) was used. The gel was shaken gently in a glass dish at all staining steps. At each step 250ml of the respective reagent was added. The reagents and times of each step are summarized in Table 7.1.

Table 7.1. Silver staining procedure of SDS-PAGE gels

Step	Reagent	Time
1	Fixative A[a]	1hr or overnight
2	Fixative B[b]	30min
3	Fixative B	30min
4	Oxidizer[c]	10min
5	Milli-Q-water	10min
6	Milli-Q-water	10min
7	Milli-Q-water	10min
8	Silver nitrate[d]	30min
9	Milli-Q-water	1min
10	Developer[e]	30-60 seconds
11	Developer	~5min or until it turns yellow or brown 'smokey precipitate appears
12	Developer	~5min, until protein bands are developed sufficiently

[a]40% methanol, 10% acetic acid (v/v)
[b]10% ethanol, 5% acetic acid (v/v)
[c]3.4mM K_2CrO_7, 3.2mM HNO_3
[d]12mM silver nitrate; this step requires bright illumination
[e]280mM sodium carbonate, 0.05% formaldehyde solution

As molecular weight markers, mid-range rainbow markers (Amersham, England) were run on the gel. These standards contain (molecular weight in brackets): lysozyme (14,300); trypsin inhibitor (21,500); carbonic anhydrase (30,000); ovalbumin (46,000); bovine serum albumin (69,000); phosphorylase B (92,500); myosin (200,000). The molecular weight of protein bands observed on the SDS-PAGE gel was calculated by determining their R_f values (R_f=distance protein migrated/distance of solvent front) and plotting the R_f values of standards against their log molecular weight as a standard curve. The log molecular weight of sample protein bands was read off the standard curve.

7.2.3.2

Polyacrylamide gel electrophoresis of DNA

For separation of small fragments of DNA a 5% polyacrylamide gel was used. The ingredients were (per 100ml): 40% acrylamide, 12.5ml; 2% bisacrylamide, 12.5ml; 0.9M Tris-borate, 25mM EDTA (pH 8.3), 10ml; TEMED, 0.5ml; 10% ammonium persulphate, 1ml; milli-Q-water, 63.95ml. Preparation of the gel was as described for SDS-PAGE, only that this was one continuous gel and the comb was inserted into it. The gel was run at constant voltage of 30V for 18h, and detection of DNA bands was as described for agarose gels (section 3.2.3).

7.2.4. Determination of the cut sequence of 'ANi I'

The plasmid pBR322 was digested with 'ANi I' in single digests and in double digests with 'ANi I' and Eco R1. pBR322 and Eco R1 were chosen as the complete nucleotide sequence of pBR322 is known and Eco R1 cuts pBR322 once only (Maniatis *et al.* 1982). A Hae III digest of pBR322 was used for DNA fragment size standards and base pair (bp) sizes are given in Fig.7.3. A standard curve plotting the distance migrated on the gel against the DNA fragment size on log scale was obtained. The cut sequence of 'ANi I' was determined from the size fragments obtained. One of the five fragments obtained from single digestion was cut by Eco R1 (Fig.7.2). Since the Eco R1 site is known, the nucleotide sequence of pBR322 was searched for identical sequences of four or more base pairs on either side of the Eco R1 site at distances corresponding to the two fragments obtained upon double digestion.

The pattern of DNA restriction obtained with 'ANi I' on pBR322 was compared to the patterns obtained with other restriction endonucleases

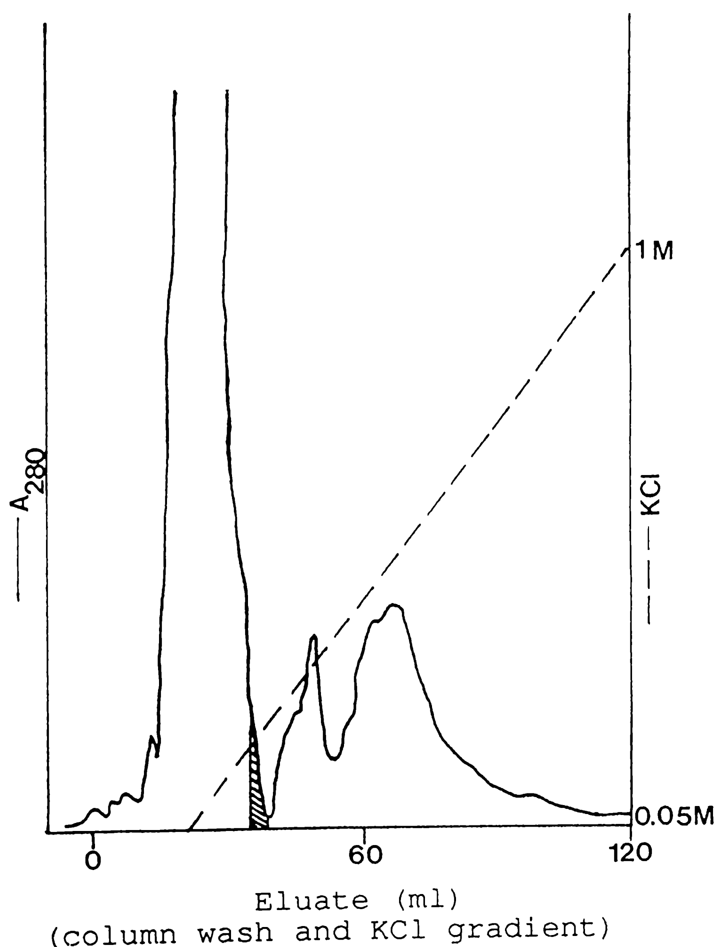
on pBR322 (Boeringer Mannheim Molecular Biology Products Catalogue (1988).

7.3. Results

7.3.1. Partial purification of 'ANi I'

A partial purification of the restriction endonuclease 'ANi I' of isolate ANI was carried out as described in section 7.2.2. The elution profile from the phosphocellulose column is shown in fig.7.1. The protein content of fractions with endonuclease activity (indicated by the shaded area in Fig.7.1) was 0.6mg protein/ml eluate.

Fig.7.1. Elution profile of protein from phosphocellulose column (shaded area indicates fractions with endonuclease activity)



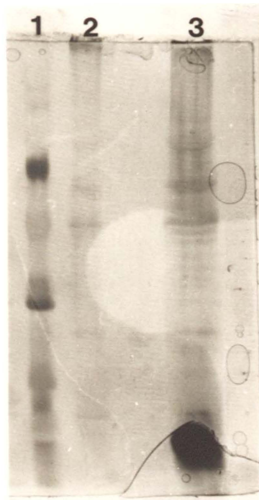
The fraction containing highest endonuclease activity (2.4ml) was used for analysis of purity. Samples of 10μl and 100μl were run on a SDS-PAGE gel (Fig.7.2). Major bands visible were of molecular weights of 56,200, 47,900, and 27,500. It is not known to which of these, if any, the endonuclease corresponds to.

7.3.3 . Determination of the recognition sequence of 'ANi I'

The plasmid pBR322 was digested with 'ANi I' only or with 'ANi I' and Eco R1 in double digestions. The restriction patterns obtained are shown in Fig.7.3. From single digestions with 'ANi I' five fragments were obtained; three long and two short fragments were observed. In 'ANi I'/Eco R1 double digests, the shortest of the long fragments was cut (Fig.7.3.a,b lanes 1-3). The three short fragments of double digests were approximately 320 bp, 260 bp, and 225 bp, the shortest fragment being the product of the digestion with Eco R1 and 'ANi I'. The smallest of the three big fragments in a double digest was approximately 800bp in size. Therefore, nucleotide sequences in approximate distance of 800 and 225 bp either side from the Eco R1 cut site were recognized by 'ANi I'. The nucleotide sequence of pBR322 was searched for common sequences at these distances on either side of the Eco R1 site. The nucleotides of pBR322 are numbered in a clockwise direction from the Eco R1 site and the position of each recognition site mentioned is given in brackets referring to the number nucleotide. The sequence 'CTAG' was found 803 (position 3142) and 232 (position 230) bp on respective sides of the Eco R1 site. The third small fragment in 'ANi I' digests was about 258 bp, and a sequence of CTAG is present in pBR322 253 bp (position 2969) upstream from the cut site yielding the 803 bp fragment.

The two biggest restriction fragments were approximately 1150 and 1300 bp in size, estimated from the agarose gel (Fig.7.3.a). However, determination of the large-size fragments was less accurate as the gel had been run under conditions primarily for good separation of smaller fragments and the standard (Hae III) had been chosen accordingly. The sequence CTAG occurs in pBR322 once more with fragments of 1481 (position 1488) and 1258 bp (position 231) respectively. The restriction pattern of pBR322 with the endonuclease Mae I (*Methanococcus aeolicus*) is the same as with 'ANi I' (Boeringer Mannheim Molecular Biology Products Catalogue 1988). The endonuclease cuts the sequence 5'-C↓TAG-3', producing sticky ends.

Fig.7.2. SDS-Polyacrylamide gel of the most active fraction of the phosphocellulose column



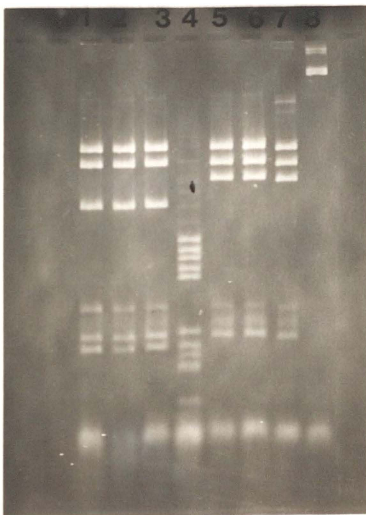
Lane 1:
molecular weight standards

Lane 2:
phosphocellulose fraction (10μl)

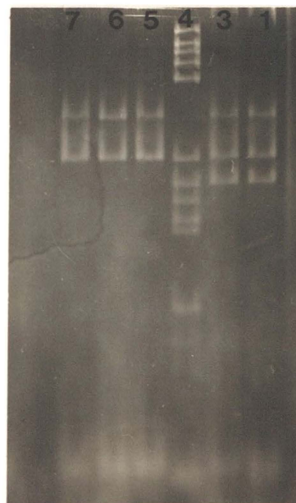
Lane 3:
phosphocellulose fraction (100μl)

Fig.7.3. Restriction digests of pBR322 with 'ANi 1' or 'ANi 1' and Eco R1

a) Agarose gel
(2%; run 340V/hrs)



b) Polyacrylamide gel
(5%; run 330V/hrs)

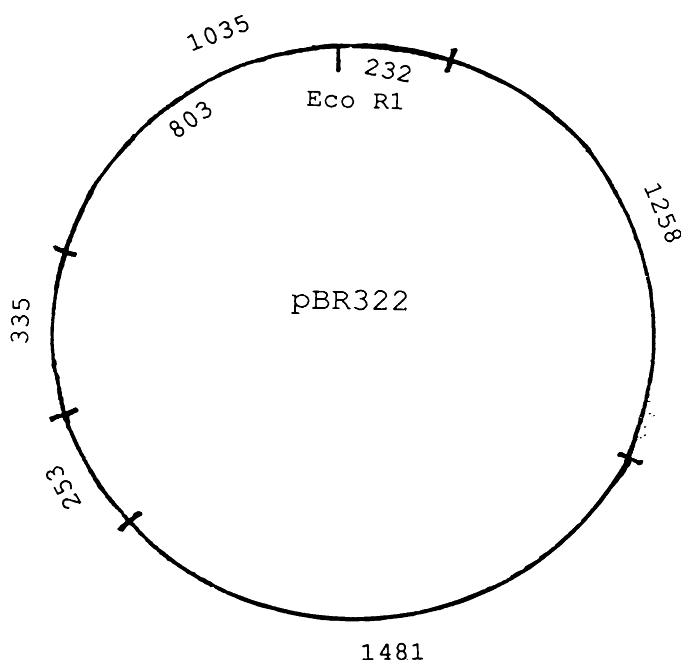


pBR322/Hae III fragments (bp):
587
540
504
458
434
267
234
213
192
184
124

in a) and b): Lane 1, 2, 3: 'ANi 1' / Eco R1 double digest;
Lane 4: Hae III standard;
Lane 5, 6, 7: ANi I digest
Lane 8: uncut pBR322

The restriction fragments obtained with 'ANi I' and Eco R1 are illustrated in Fig.7.4.

Fig.7.4. Restriction of pBR322 with 'ANI I' and Eco R1 and DNA fragments obtained (in bp)



7.4. Discussion

These preliminary studies on the restriction endonuclease have shown the presence of an active enzyme partially purified by streptomycin sulphate and ammonium sulphate precipitation steps followed by phosphocellulose column chromatography. More purification steps will be required to determine the molecular weight of the endonuclease.
No ATP was required for endonuclease activity and DNA was cut in a predictable and repeatable manner which indicate that 'ANi I' is a type II restriction endonuclease.
The recognition sequence consists of the four nucleotides CTAG; the recognition sequence is not longer as the nucleotides on either end of this sequence varied between cut sequences. If 'ANi I' represented an endonuclease which would cut the sequence of only three of the four

nucleotides (i.e. CTA or TAG) then a greater number of restriction fragments of pBR322 would have been generated.

To verify the recognition sequence of 'ANi I' and to test for contaminating exonuclease ligation experiments are required. These would also show whether any bases are lost upon cutting by 'ANi I'. If DNA is cut, ligated, and re-cut by the enzyme then it is assumed that no nucleotides from the ends are missing. Cutting DNA with 'ANi I' and Mae I and ligating fragments cut with one endonuclease with fragments cut with the other would show a common cut site for the two enzymes. Double digestion of DNA with Mae I and 'ANi I' would yield no further DNA fragments than single digestion with either if they shared a common sequence.

Although 'ANi I' showed activity under assay conditions used, determination of optima for temperature, pH, and NaCl are required to optimize activity.

The restriction endonuclease 'ANI I' may be useful to the molecular biologist. It is active at 75°C, and activity was easily detected in foucrude cell extracts. Mae I is the only available endonuclease cutting 'CTAG'. This enzyme is expensive and appeared not very active (R Ronimus pers. comm.).

Chapter 8: Conclusion

In the natural environment of hot pools from the Rotorua, Tokaanu, Waimangu, and Waitapu thermal regions of New Zealand organisms closely resembling isolate ANI were isolated from pools within a temperature and pH range of 55-91°C and pH 5.5-9.0 respectively. The presence of these organisms was shown to be dependent on the sodium content of the pool (above 0.5g/ℓ). Analysis of more pools within New Zealand and overseas for ANI-like organisms would be valuable for determination of the geographical distribution of ANI-like organisms. DNA-DNA hybridization or other genetic techniques would be required to show close relationship between isolates.

Isolate ANI was a neutrophilic, anaerobic, extreme thermophile. It was an obligate heterotroph with a requirement for peptone. Several amino acids and keto acids were utilized for growth, while of the carbohydrates tested only starch, glucose, and lactose stimulated growth. Isolate ANI had an obligate requirement for NaCl and sulphur. These requirements and the metabolism of branched-chain amino acids were investigated in more detail.

All validly named *Thermococcales* have been isolated from marine habitats. As a possible indication of its close relationship to these marine organisms, ANI required Na^+ for growth and cell viability, although at lower concentration than present in sea water, perhaps reflecting its terrestial habitat. Sodium plays a central role in the physiology of ANI. ATP synthesis upon addition of branched-chain amino acids was dependent on the presence of Na^+ (above 5mM). While use of labelled amino acids and Na^+ would allow verification of the presence of a Na^+/solute symport system for uptake of these amino acids, results obtained in this study strongly suggest such a mechanism.

Experiments performed on growing cultures and cell suspensions with ionophores and inhibitors of ATPases and H^+/Na^+ antiporters further supported the concept of Na^+ involvement in central physiological processes of the organism. The use of growing cultures however produced many variables. The necessary presence of peptone made results more difficult to interpret since a variety of amino acids (and low concentrations of salts) were added. While ionophores and other compounds used are not reported to be heat-labile, their potency as inhibitors may be reduced after 24 or 48h at 75°C. The effect of

ionophores on suspensions in shorter term experiments was more easily interpreted. Results obtained with various compounds added indicate that not only an external Na^+ concentration above 5mM (or 10mM as determined from growth studies) is required but also a Na^+ gradient across the cytoplasmic membrane. Additionally, either a H^+ gradient or a membrane potential is required for ATP generation in the presence of amino acids and also for growth. Dissipation of either the H^+ gradient or the membrane potential had only a temporary inhibitory effect. The generation of a Na^+ gradient is most likely to involve hydrolysis of ATP directly. Since the protonophore CCCP had only a slight temporary inhibitory effect on ATP synthesis, generation of a secondary Na^+ gradient linked to a primary H^+ gradient via a H^+/Na^+ antiporter is less likely, although an H^+/Na^+ antiport system may be present for regulation of gradients. The use of ionophores and inhibitors gives an indication of the nature of involvement of Na^+ in ANI metabolism. For detailed studies, membrane vesicles and labelled Na^+ would be preferable. Direct determination of amino acid utilization and refinement of measuring metabolic activity (sulphide and fatty acid production) would be useful. Further experiments with, for example, a diffusable charge counter ion of Na^+ or determination of the membrane potential would provide more information on membrane processes and their regulation.

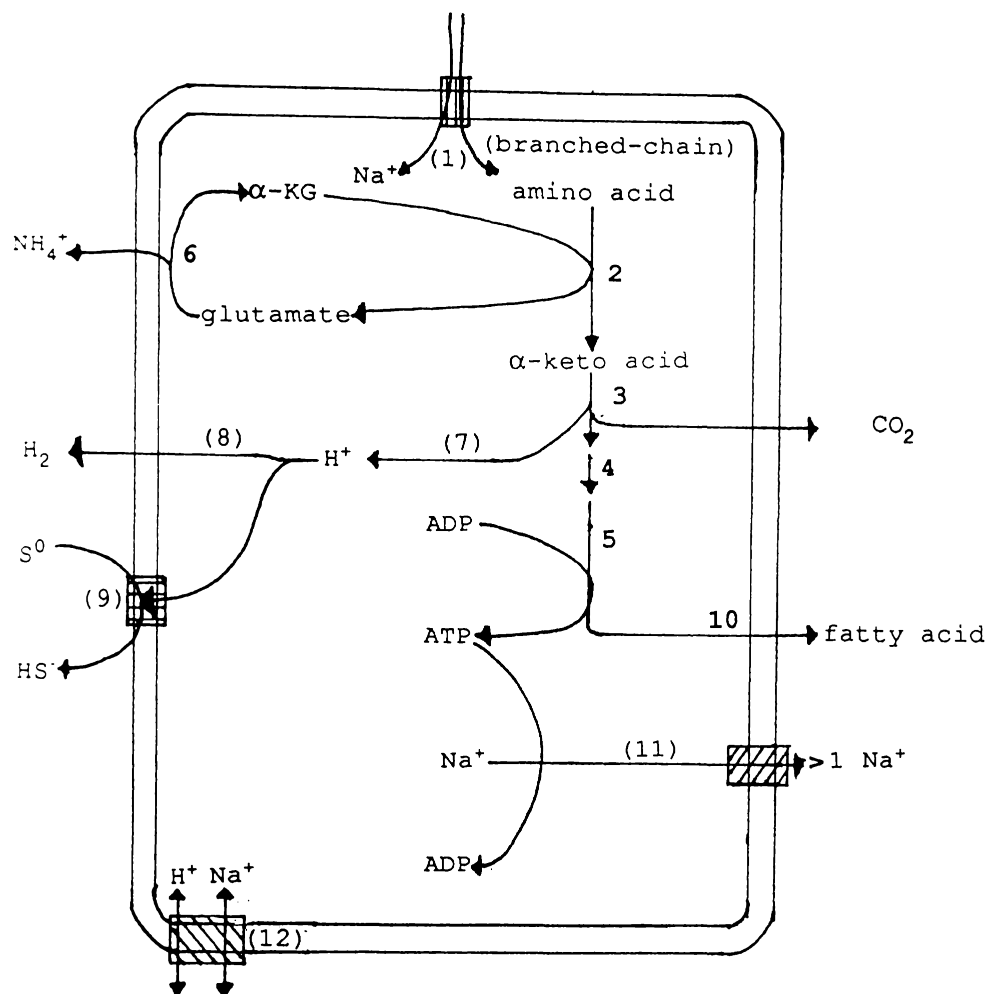
Generation of ATP in ANI is most likely to occur via substrate-level phosphorylation only. Studies on the catabolism of branched-chain amino acids have shown the existence of an ATP-yielding step catalyzed by fatty acid kinase. While the presence of three of the four enzymes of the proposed pathway of branched-chain amino acids to branched-chain fatty acids was demonstrated the presence of the fourth enzyme, phosphate acyltransferase, can only be inferred. Enzyme activities on other substrates would be of interest, and for the oxidoreductase-catalyzed reaction the use of a thermostable ferredoxin as electron acceptor might have increased enzyme activity. Availability of acyl phoshate substrates (i.e. isobutyryl phosphate and isovaleryl phosphate) for assay of phosphate acyltransferase activity via hydroxamate detection would conclusively demonstrate that such reactions occur in ANI.

The reducing equivalent produced during amino acid degradation by ANI are transferred via carriers ultimately to hydrogenase and sulphur reductase. Neither of these enzymes or carriers have been studied in

ANI and their presence can only be inferred from the observed production of H_2 and sulphide. No direct energy gain appeared to be associated with sulphide production and increased production was not equalled by an increase in growth yield. However, since ANI also produces H_2, and since sulphide is toxic to the organism, production of sulphide may serve a purpose other than as an acceptor of reducing equivalents. Conditions in the natural hot pool environment may be of importance in determining the flow of electrons leading to either the production of H_2 or sulphide. Cytochromes were not detected in ANI. A simple electron transport system may be present, coupling export of H^+ (or Na^+) to reduction of sulphur. Again, more detailed studies using membrane fractions and of the postulated enzymes involved, hydrogenase and sulphur reductase, would be required for support of such idea.
In this study an attempt was made to produce a general understanding of the ecology and physiology of isolate ANI: its natural habitat, its growth requirements, physiological characteristics, and some of its metabolic activities. A model based on studies of branched-chain amino acid metabolism and requirement for sulphur and sodium is presented in Fig. 8.1.

In Fig. 8.1 processes shown to occur are labelled with a number while processes which are suggested from results obtained are labelled with a number in brackets. Not indicated in this scheme are: (a) possible coupling of sulphur reduction to cation export as there is no indication of such process from the results obtained in this study; (b) possible other amino acid uptake systems e.g. via H^+/solute symport or ATP-driven (see chapters 4,5); (c) gradients of cations involved in pH homeostasis as there is nothing known about these.

Fig.8.1. Model of membrane-linked and cytological processes for substrate utilization and energy generation in isolate ANI



The numbers refer to the following processes:

1: Na^+/amino acid symport (chapter 5)
2: amino acid aminotransferase (chapter 5)
3: α-keto acid oxidoreductase (chapter 5)
4: phosphate acyltransferase(chapter 5)
5: fatty acid kinase (chapter 5)
6: glutamate dehydrogenase (R.Hudson pers. comm.)
7: ferredoxin as electron carrier (chaptors 5, 6)
8: hydrogenase (chapter 6)
9: sulphur reductase (chapter 6)
10: fatty acid export (not linked to cation export as no end product inhibition observed) (chapter 3)
11: Na^+-translocating ATPase (Na^+ exported per ATP would have to be higher than Na^+ symported with amino acids [process 1] to allow net ATP gain from [branched-chain] amino acid utilization) (chapter 4)
12: H^+/Na^+ antiporter (the direction it would function is not known and may depend on requirement for regulation of either gradient) (chapter 4)

The type II restriction endonuclease 'ANi I' recognizes the nucleotide sequence CTAG. While the endoclease Mae I recognizes the same sequence these two enzymes may differ in their cut site. 'ANi I' has the advantage of showing activity in a commonly used assay buffer and activity was superior to that observed with commercially available preparations of Mae I.

Appendix I.

Table I. Other organisms observed in hot pool enrichments

Pool	Db medium	YE medium	ANI(-NaCl) medium
Rt	thin rods cocci, oval rods large fat rods	many cocci, few oval rods	many rods
Rt 1A	few cocci	many cocci and small cocci	many rods
Rt 2	thin rods	few cocci	-
Rt 8	large, fat rods with spores	-	-
Rachel Spring	few thin rods	small cocci	rods, in pairs
RtOH 1	-	many large cocci	few short fat rods
RtOH 2	-	oval rods,	rods
RtOH 3	small cocci thin rods	many cocci, some egg-shaped	rods
RtOH 4	few thin rods	small cocci	rods
Wai 1	many thin rods small cocci	-	-
Wai 2	few thin rods	-	few rods
Wai 3	small cocci	-	some rods
Wai 4	small cocci	-	some rods
Wai 5	thin rods	-	some rods, small cocci
Wai 6	thin rods cocci	-	some rods,some short rounded rods
Wai 7	small cocci, few short rods	-	-
Wtp 1	many thin rods	-	short rounded rods
Wtp 2	short rounded rods many small cocci	few small cocci	small cocci, few short rods
Wtp 3	-	-	small cocci, few short rods
Wtp 4	few round fat rods	-	small cocci, few short rods
Wkt 1	-	-	round-ended rods with spores
Wkt 2	-	-	round-ended rods with spores
Wkt3	-	-	round-ended rods with spores
Wkt4	-	-	large rods
Wkt5	-	-	round-ended rods with spores
Wkt6	-	-	round-ended rods with spores

Pool	Db medium	YE medium	ANI(-NaCl) medium
Mok 1	-	-	-
Mok 2	-	-	-
Mok 4	thin rods, small rounded rods with spores	-	-
Steam Vent	-	-	-
Mok 7	-	-	-
Mok 20	-	-	-
Mok 21	-	-	-
Mok 28A	-	many wide rods, small thin rods	-
Mok 28D	-	many wide rods, small thin rods	-
Mok 28E	small cocci, large rods, big wide rods	-	-
Mok 28F	-	short fat rods	-
Mok 38	short, fat rods	short, fat rods	large rods with spores
Mok 39	-	many short fat rods	large rods with spores
Mok 40	-	large rods cocci, in chains	-
Mok 41	small cocci	cocci, short fat rods	-
Mok 42	-	short fat rods cocci, small cocci	-
Tok 1	long filamentous rods, fat rods thin rods	cocci	-
Tok 2	thin rods few small cocci	-	-
Tok 3	thin rods small cocci short rods	cocci	-
Tok 4A	thin rods cocci sporulating rods	few cocci	few big rods
Tok 4B	thin rods cocci	few short rods rods	few short fat rods
Tok 5	many thin rods	few thin rods	
Tok 7	-	-	-
Tok 8	short rods cocci	few cocci, few thin rods	-
Tok A	short rods, thin rods	-	rods
Tok B	thin rods	cocci	-
Tok C	short rods thin rods	-	-
Ket 2	very few thin rods	-	-
Ket 4	thin rods small and big cocci	very few rods and cocci	-
Ket 8	thin rods small and big cocci	few short rods	-
Ket 10	thin rods	-	-

The media used gave rise to a variety of organisms detected in the enrichments. Db medium is usually used for growing *Thermoproteus*and *Desulfurococcus*species. As it contains no NaCl, lower levels of organic nutrients and is of lower pH than ANI medium, it is selective for different organisms. In contrast to Db medium, YE medium contains 7.0g/ℓ NaCl, 0.5% yeast extract, is buffered with MOPS, and pH 7.0. This medium was used here to enrich for organisms with a higher salt tolerance in a medium rich in organics. ANi(-NaCl) medium contains a large amount of organic nutrient (0.8%peptone) at pH 7.4. It was used in order to determine if there were any organisms resembling ANI in pools of low Na^+ content which did not display its Na^+ requirement. Also, any other organisms might be enriched in this medium which could not tolerate the NaCl content of ANI medium.

Obviously the media will by no means show the full variety of organisms in any of the pools. However, these enrichments give some indication of the presence of organisms capable of survival at the incubation temperatures of 80° and 88°C.

Long thin rods in many Db medium enrichments resembled *Thermoproteus*, and 'golf-club' structures were observed.

Overall, these enrichments provided some insight into what can be found in hot pools. It was encouraging to see an abundance of organisms in enrichments on different media where no ANI-like organisms could be detected. It showed that the sample had not been mistreated and that cells had kept viable.

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