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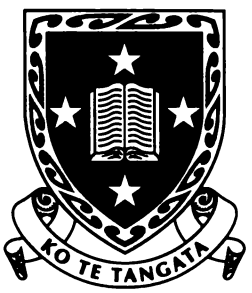
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The Characterisation of Three Hyperthermophilic Archaea from New Zealand Hot Springs



The University of Waikato
Te Whare Wānanga o Waikato

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

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Abstract

Archaea are a unique group of microorganisms that have been isolated from extreme environments such as geothermally active areas, highly saline habitats and extremely acidic sediments. Their capacity to withstand extreme conditions, and even thrive under them, makes them interesting objects of research.

The aim of this research was to isolate and characterise a variety of hyperthermophilic archaea from New Zealand hot springs. Three organisms were chosen for investigation.

The coccoid isolate Wai21.S1 is a novel strain of the genus *Thermococcus* that was isolated from a hot spring at Waimangu Volcanic Valley. It is only the second described fresh-water isolate of this genus. Wai21.S1 is strictly anaerobic and strictly heterotrophic. The preferred carbon sources are starch, amylose and amylopectin. Elemental sulfur is not required and its presence does not enhance growth. There is no dependence on sodium chloride. Wai21.S1 grows optimally at 89°C and at pH 5.9. The 16S rRNA gene sequence shows a close relationship to *T. zilligii*. Wai21.S1 is proposed to be the type strain of a new species *Thermococcus waimanguensis*.

The isolate Tok2 was found in a sample from a hangi pool at Tokaanu, Turangi and was obtained from an enrichment culture containing konjac glucomannan as carbon source. Tok2 belongs to the genus *Pyrobaculum*. It is a strictly anaerobic organism which growths mainly heterotrophically on yeast extract and tryptone. Autotrophic growth was observed on CO_2/H_2 with thiosulfate or elemental sulfur as electron acceptor. Tok2 is rod-shaped with spherical bodies frequently observed at one or both ends, and the centre of the cells. Branched cells were occasionally observed. Tok2 grows optimally at 93°C and can grow up to 101°C. The optimum pH is 6.7. Tok2 is capable of reducing nitrate. The 16S rRNA gene sequence shows the close relationship of Tok2 to two other *Pyrobaculum*-like isolates from Tikitere, New Zealand the preliminarily named "*Pyrobaculum neutrophilus*" and strain IsoX. Phylogenetic data suggests that the three New Zealand isolates are strains of *Pyrobaculum organotrophum* despite strong physiological evidence against it. Further studies will be carried out to clarify the taxonomy of the New Zealand *Pyrobaculum* strains.

Tok1, a second organism found in the same enrichments culture as Tok2, could not be isolated and was therefore maintained in a consortium together with Tok2. Tok1 is a regular coccus which grows heterotrophically on glucomannan as sole carbon source. It is microaerotolerant and grows optimally at 98°C. The optimum pH is 7.3. Strain Tok1 is highly sensitive to yeast extract and sensitive to elemental sulfur. The 16S rRNA gene sequence which was obtained by cloning, shows that Tok1 is a member of the kingdom Crenarchaeota and is a representative of a novel genus. Phylogenetically strain

Tok1 is positioned at the base of the order "Igneococcales". Since strain Tok1 could not be purified, the novel species had to be described as a candidatus. The name Candidatus "*Igneosphaera vetusta*" was proposed.

The enzyme responsible for the degradation of konjac glucomannan was characterised and partially purified. The β-endomannanase was produced by strain Tok1 and is extremely thermostable. It shows highest activity around 110°C and is still enzymatically active at 120°C. The optimum pH for activity was at 6. Besides the mannanolytic activity, it exhibits a low xylanase activity. The enzyme is cell wall bound and no activity was found in the culture supernatant.

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" We forget that there is no hope of joy except in human relations. If I summon up those memories that have left with me an enduring savour, if I draw up the balance sheet of the hours in my life that have truly counted, surely I find only those that no wealth could have procured me. True riches can not be bought."

Antoine de Saint-Exupery
from "Wind, Sand and Stars"

Living in a country half a world away from home makes you very aware of the truth in Saint-Exupery's words. I feel a deep gratitude to all those people who made me feel

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Chapter 1 - Introduction

1.1. Archaea and the three domain concept of life

Since the early seventies more and more microorganisms have been isolated and characterised which differ in some vital physiological aspects from the typical mesophilic bacteria. Most of these organisms were found in habitats distinguished by their extreme conditions, such as hot springs, high-salt lakes and deep-sea geothermal situations. Investigation of the 16S rRNA genes of these organisms led Carl Woese to hypothesise that they represent a distinct group among prokaryotes. He gave them the name "archaebacteria" and named the remaining bacteria "eubacteria" in order to clearly set them apart from each other. Further isolations of new archaebacteria, as well as phylogenetic and physiological data, strongly supported the introduction of a new classification for all living things. In 1990 Woese, Kandler and Wheelis suggested the establishment of three domains: Eucarya, Bacteria and Archaea, and the division of the domain Archaea into the kingdoms Crenarchaeota and Euryarchaeota (Woese et al. 1990) on grounds of 16S rRNA sequence analysis (Winker and Woese 1991) and physiological evidence. Figure 1.1. shows a recent phylogenetic tree based on 16S rRNA gene sequence data which includes representatives of the main branches of the three domains.

The main physiological differences between the domains Archaea and Bacteria are in the structure of the cell walls (Kandler 1994), in the lipid composition of the cell membranes (Langworthy et al. 1982; DeRosa and Gambacorta 1988) and in the structure and organisation of their transcription and translation machinery (Zillig and Stetter 1980a; Huet et al. 1983).

Ever since their discovery, archaea have been extensively studied because of their significance to the understanding of evolution and the origins of life.

In many aspects archaea resemble more the Eucarya than the Bacteria. The ATPase subunit type V is common to both Archaea and Eucarya whereas the F-type is found in the Bacteria (Gogarten et al. 1989). Another example for the relationship between Archaea and Eucarya is the structural similarity of the archaeal and the eukaryotic DNA-dependent RNA-polymerase (Zillig and Stetter 1980a). Despite having - like bacteria - only one RNA-polymerase, which is responsible for the synthesis of all the different types of RNA, the archaeal RNA polymerase is in its subunit structure and its gene sequences and organisation very similar to the RNA polymerases of the Eucarya.

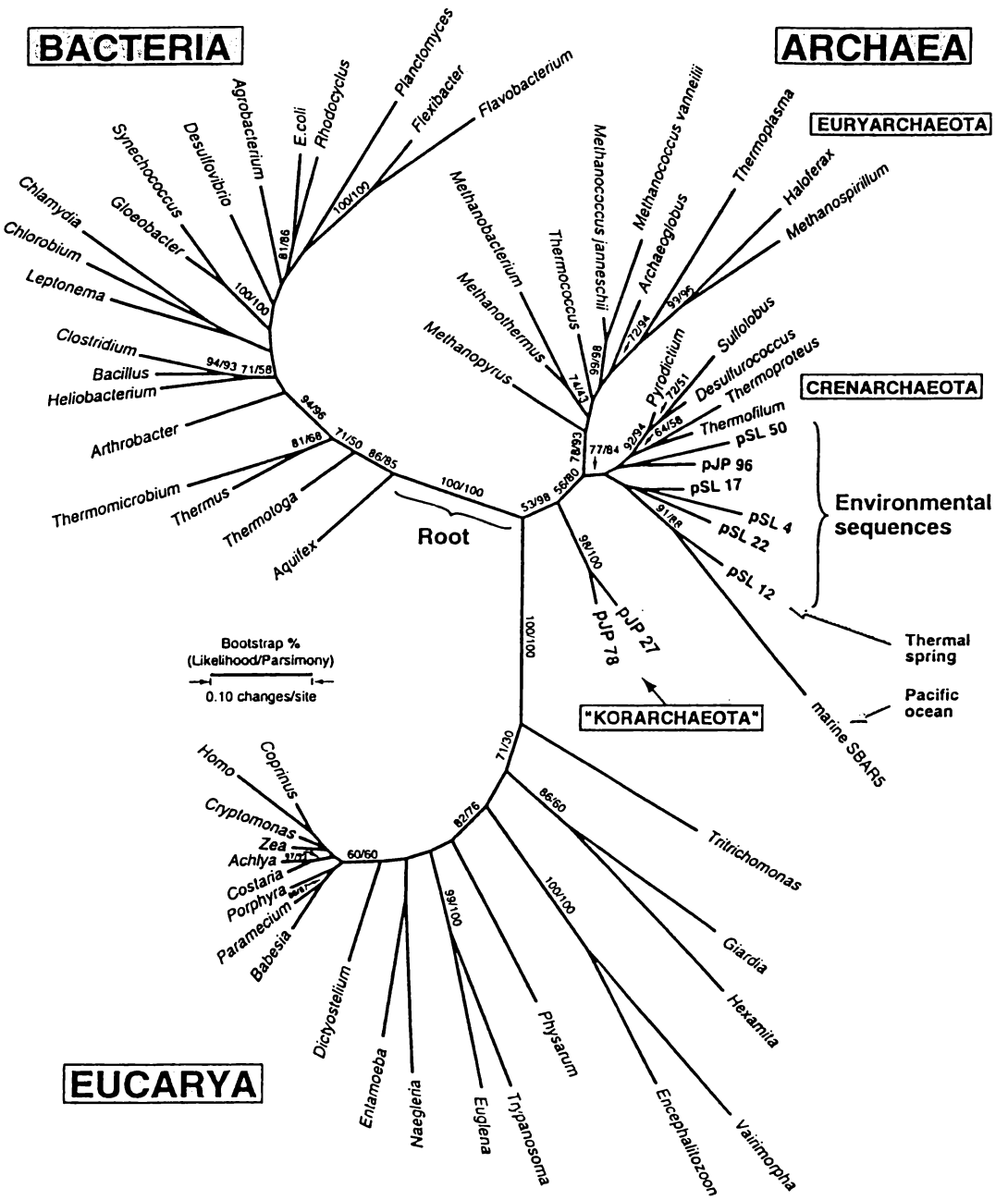


Fig. 1.1. Universal tree of life.
(reproduced from Barns et al. (19996)

Sequence data analysis indicates that the archaeal-eucaryal type of RNA polymerase is an early development whose complexity was lost in the bacteria (Langer 1994). On the other hand it appears that archaeal enzymes involved in metabolism are more closely related to their bacterial counterparts than to their eukaryotic ones (Olsen and Woese 1997).

Many hypotheses about the phylogenetic relationship of the three domains, the possible position for a root and the origins of the eukaryotic cells have been proposed. Phylogenetic analysis of 16S rRNA genes results in grouping of the Archaea and Eucarya as sister domains with the "Korarchaeota" as the first branch after the bifurcation of the two domains and puts the root into the bacterial branch (Woese et al. 1990; Barns et al. 1996). This model favours a common thermophilic ancestor for all three kingdoms since the prokaryotic branches closest to the proposed position for a root consist of thermophilic organisms. For the Archaea these are the "Korarchaeota", whose ribosomal RNAs were amplified from total DNA isolated from samples from Obsidian Pool in Yellowstone National Park (Barns et al. 1994). The earliest branch of the bacteria is represented by the Aquificales, isolated from hot marine sediment and growing up to 90°C (Huber et al. 1992; Burggraf et al. 1992) and the Thermatogales, which can also grow up to 90°C (Ravot et al. 1995).

Analysis of sequence data for the genes for the amino-acyl-tRNA synthetases (Brown and Doolittle 1995) however favours a polyphyletic origin of the three domains but maintains the close relation of Archaea and Eucarya.

For the origin of the Eucarya, Zillig proposed a chimeric model. Here two separate lineages of Archaea and Bacteria originated from a common ancestor, the "cenancestor" and a cellular fusion of one species of each lineage created the first eukaryotic cell (Zillig et al. 1991c). Recently Doolittle suggested that eukaryotes stem from symbiosis of a bacterium and an archaeal host (Doolittle 1998) challenging Lyn Margulis's endosymbiont hypothesis in which the host is a primitive eukaryotic cell (Margulis 1970).

1.2. The kingdom Euryarchaeota

The kingdom Euryarchaeota consists of three major groups which are physiologically quite distinct from each other: the extremely halophilic archaea, the methanogens and the thermophilic euryarchaeota.

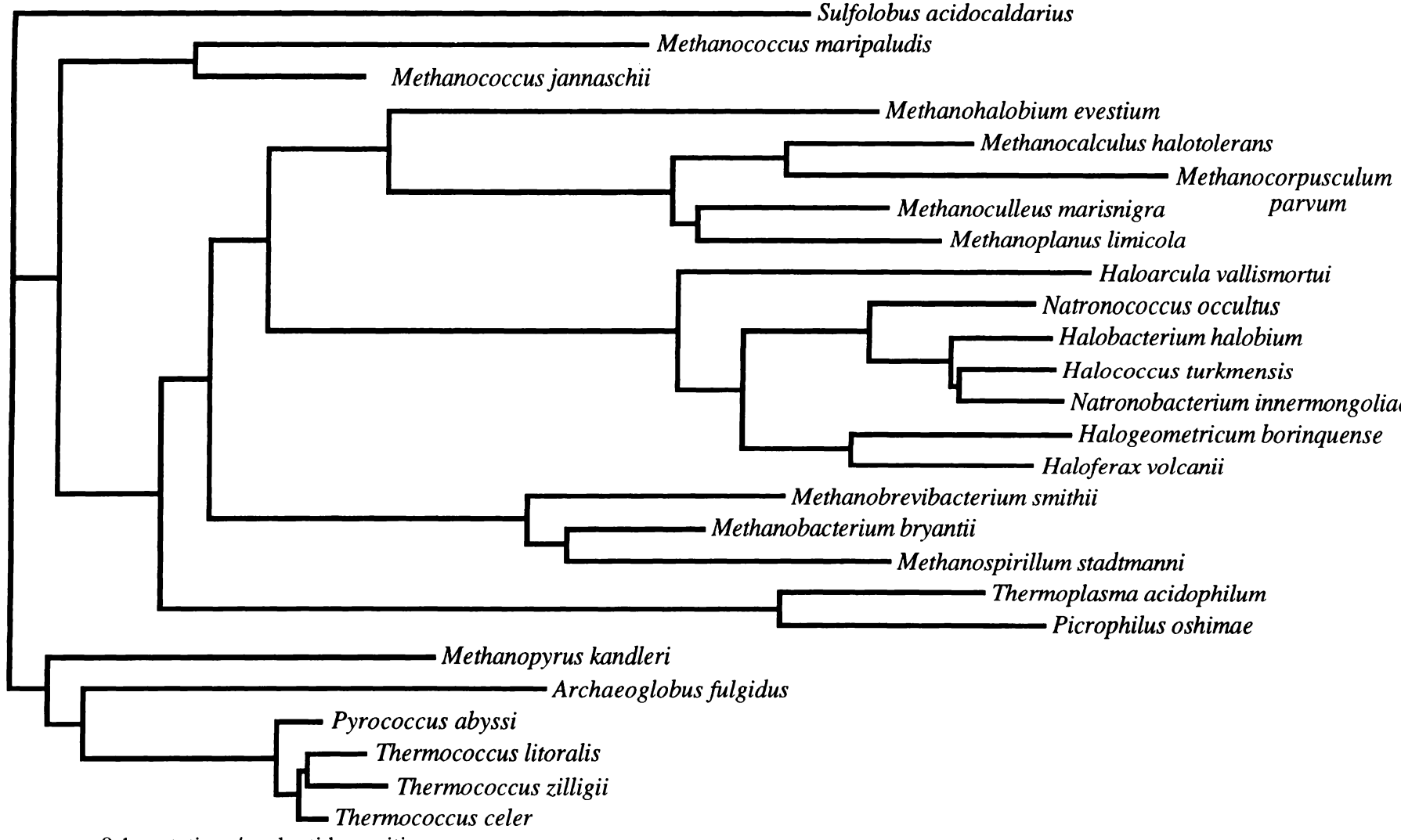


Fig.1.2. Phylogenetic tree of the kingdom Euryarchaeota
(tree made with Clustal V and Tree View based on 16S rRNA gene sequences, see appendix 1 for accession numbers)

1.2.1. The methanogens

The methanogens, which are found in habitats like anaerobic digesters, anoxic sediments, flooded soils, gastrointestinal tracts and geothermally heated sites (Whitman et al. 1991) are a diverse group identified by producing methane as the main product of their energy metabolism and by being strictly anaerobic. CO_2 usually serves as electron acceptor and the most commonly used electron donors are H_2, formate or some alcohols (Zellner and Winter 1987). Some methanogens, such as *Methanobacterium thermoautotrophicum*, are able to use CO as electron donor (Daniels et al. 1977). Other possible electron donors include methylated C1 compounds, such as dimethylamine or trimethylamine. Some species of the genera *Methanosarcina* and *Methanosaeta* can metabolise acetate to methane and CO_2. Methanogens contain a number of coenzymes essential for methane production which are unique to the euryarchaeota. Coenzyme M is required for the methyl transfer reaction (Taylor and Wolfe 1974) and has not been detected in any other taxonomic group (Gunsalus 1977). Coenzyme F_{420} acts as an electron carrier in a role usually occupied by ferredoxin (Tzeng et al. 1975) and is not found either in any other group than the methanogens (Eirich 1978) or the closely related genus *Archaeoglobus* (Möller-Zinkhan et al. 1989) and to a much lower extent in the genus *Thermoplasma* (Segerer and Stetter 1991b). Although most of the described species are mesophilic to moderately thermophilic, reflecting the conditions in the habitats in which they are commonly found, many truly thermophilic species have been isolated. Thermophilic species include isolates from submarine volcanic vents such as *Methanococcus jannaschii* (Jones et al. 1983) and from shallow marine, hydrothermally heated systems such as *Methanopyrus kandleri* (Kurr et al. 1991), from cattle pasture such as *Methanobacterium thermoaggregans* (Blotevogel and Fischer 1985), from geothermally heated sea sediments such as *Methanococcus thermolithotrophicus* (Huber et al. 1982) or from anaerobic digestors such as *Methanothrix* sp. (Zinder et al. 1984).

Phylogenetically methanogens are spread across the kingdom Euryarchaeota (Barns et al. 1996) with the hyperthermophilic *Methanopyrus kandleri* (Burggraf et al. 1991) representing the earliest branch of the Euryarchaeota (and the most thermophilic organism known so far) and the *Methanomicrobiales* representing the highest branch, showing a close phylogenetic relationship to the extreme halophiles (Burggraf et al. 1991).

	Methano-bacteriaceae	Methano-thermaceae	Methano-coccaceae	Methano-microbiaceae	Methano-corpuscul-aceae	Methano-sarcinaceae
Morphology	rods	rods	irregular cocci	rods, spirals, plates, cocci	small, irregular cocci	pseudosarcina or sheathed rods
Motility	non motile	non motile	motile	motile or non motile	motile or non motile	usually non motile
Substrates for methano-genesis	H_2/CO_2, formate, alcohols	H_2/CO_2	H_2/CO_2, formate	H_2/CO_2, formate, alcohols	H_2/CO_2, formate, alcohols	H_2/CO_2, acetate, methyl compounds
Gram stain	mostly Gram+	Gram +	Gram -	Gram -	Gram -	Gram - or +
GC content in mol%	23-61	33-34	29-34	39-61	48-52	36-52

Table 1.1. Some characteristics of the families of the methanogens

1.2.2. The Family Halobiaceae

Members of the Family Halobiaceae are characterised by the high amount of salt they require to maintain their viability (Dundas 1977). They are found in natural and artificial saline lakes, soda lakes (Tindall et al. 1984) and have been isolated from food, such as herring or meat which has been salted for preservation purposes (Tindall 1991). Salt concentrations of 4.5M are optimal for growth and cell lysis usually occurs below about 1M NaCl. The internal concentration of salt is about the same as the external, indicating that all the enzymes must be adapted to function at high salt concentration. Another important characteristic of the halobacteria is the presence of retinal proteins as integral parts in certain areas of the cell membrane, called "purple membrane" (Oesterhelt and Stoeckenius 1973). Bacteriorhodopsin serves as a light-driven proton pump enabling the cells to grow by anaerobic photophosphorylation (Krebs and Khorana 1993). Further retinal proteins present in halobacteria include halorhodopsin, a light-driven chloride pump (Oesterhelt and Tittor 1989) and two sensory rhodopsins responsible for phototactic response (Spudich 1993).

Remarkable as well is the genetic instability of some of the halobacteria which is due to the presence of unstable plasmids containing multiple insertion elements. These transposable genetic elements lead to phenotypic variants and are well investigated (Pfeifer 1988; Charlebois and Doolittle 1989).

Two genera of the six recognised genera of the halobacteria are distinguished by high pH optima. Species of the genera *Natronococcus* and *Natronobacterium* grow at a pH range of 8-11 and at low Mg^{2+} levels (Tindall et al. 1984), reflecting their original habitats like Kenyan soda lakes (Oren 1994). These two genera represent a distinct lineage within the halobacteria (McGenity and Grant 1993).

1.2.3. The thermophilic Euryarchaeota

The thermophilic Euryarchaeota consist of three major lineages: the genus *Thermoplasma* with the closely related genus *Picrophilus*, the related genera *Archaeoglobus* and *Ferroglobus*, and the order Thermococcales.

The main characteristic of the genus *Thermoplasma* is the lack of cell walls which led to its original classification in the bacterial genus *Mycoplasma* (Darland et al. 1970). *T. acidophilus* was first isolated from a coal refuse pile but *Thermoplasma* isolates have now been found in solfataric fields, in various geothermally active places as well in an acidic, tropical swamp in Java (Segerer and Stetter 1991b).

	Halo-bacterium	*Haloarcula*	*Haloferax*	*Halococcus*	*Natrono-bacterium*	*Natrono-coccus*
Morphology	rods	pleomorphic rods	pleomorphic rods, flattened discs	cocci, single or in pairs, tetrads, sarcinae	rods	cocci, often in pairs
Motility	motile	motile or nonmotile	may be motile	non motile	motile	non motile
Salt requirement	3.0-5.2 M	2.0-5.2 M	2.5-3.0 M	3.5-4.5 M	2.0-5.2 M	1.4-5.2 M
Mg^{2+} requirement	5 mM	5 mM	0.2 M, tolerates 1 M		< 10 mM	low
pH range	7	7	6.7-7		8.5-10	8.5-10
Temperature range in °C	15-50	30-55	25-55	30-45	25-50	25-50
Substrates	complex media only	complex media, or single carbon source	complex media, or single carbon source	complex media, single sugars	single carbon source	sugars, gelatin

Table. 1.2. Some characteristics of the halophilic genera

The extremely acidophilic members of the genus *Picrophilus* were both isolated from dry, acidic, heated soil from solfataric locations in Japan (Schleper et al. 1995a; Schleper et al. 1995b). Both species grow optimally at pH 0.7 but are capable of growing at pH 0 with an upper pH limit of 3.5. *Picrophilus* species are aerobic heterotrophs with an obligate requirement for yeast extract. Recently the isolation of a close relative of *Picrophilus* has been reported (Norris and Burton 1998). This isolate is moderately thermophilic, has a pH optimum of around 1.6 and its 16S rRNA gene puts it phylogenetically close to *Picrophilus* but into a new genus.

Members of the genus *Archaeoglobus* are strictly anaerobic sulfate- (Burggraf et al. 1990; Stetter 1988) or sulfite-reducers (Huber et al. 1997). They grow between 60-95°C and were isolated from submarine hydrothermal habitats (Stetter 1991) and have an obligate requirement for salt. As in methanogens coenzyme F_{420} was detected, but not coenzyme M (Huber et al. 1997).

The genus *Ferroglobus* is phylogenetically and physiologically closely related to *Archaeoglobus*. The only species so far described is *F. placidus* (Kurr et al. 1991). *F. placidus* oxidises Fe^{2+}, H_2 or sulfide using NO_3^- as an electron acceptor. As the members of the genus *Archaeoglobus*, they show a blue-green fluorescence at 420 nm but *F. placidus* is not able to reduce sulfate or sulfite.

	Thermo-plasma	*Picrophilus*	Archaeo-globaceae	*Pyrococcus*	*Thermo-coccus*
Morphology	highly variable filamentous, coccoid, rod-shaped	irregular cocci	irregular cocci	cocci	cocci
Aerobic growth	facultatively anaerobic	strictly aerobic	anaerobic	strictly anaerobic	strictly anaerobic
Temperature range in °C	35-67	40-67	64-95	90-105	60-98
pH range	0.8-4	0-4	4.5-8.5	5-8	5-9
NaCl requirement	no salt required	no salt required	0.1-0.8 M	0.25-0.6 M	0.05-0.7M
Substrates	peptides, carbo-hydrates	yeast extract	organic acids, glucose, starch, peptides	peptides, proteins, starch, maltose	peptides, proteins, amino acids, complex carbo hydrates
Autotrophic growth	-	-	+	-	-
S^0 respiration	+	-	-	+	+

Table 1.3. Some characteristics of the thermophilic euryarchaeota.

The Order Thermococcaceae comprises two genera which belong to the family Thermococcales (Zillig 1991a). The genus *Pyrococcus* is distinguished from the genus *Thermococcus* by higher temperature optima and by lower GC contents (Zillig 1991a). Furthermore all DNA polymerases of *Thermococcus* which have been investigated so far contain inteins which have not yet been found in DNA polymerases of *Pyrococcus* (Cambon et al. 1998) but have been shown for other proteins from *Pyrococcus* species
All known members of the Genus *Pyrococcus* were isolated from marine environments, they are strictly anaerobic heterotrophs and growth is stimulated by elemental sulfur without being dependent on it (Zillig et al. 1987; Gonzáles et al. 1998; Fiala and Stetter 1986a; Erauso et al. 1993).
All the species of the Genus *Thermococcus* were isolated from marine solfataric environments, with the exception of *T. zilligii*, which is an isolate from a terrestrial hot spring in New Zealand (Klages and Morgan 1994; Ronimus et al. 1997). *T. celer, T. stetteri, T. alcaliphilus, T. litoralis, T. acidaminovorans, T. gorgonarius* and *T pacificus* were isolated from shallow marine hot springs in Italy, the Kurile Islands and New Zealand (Zillig et al. 1983; Neuner et al. 1990; Miroshnichenko et al. 1989; Miroshnichenko et al. 1998; Keller et al. 1995; Dirmeier et al. 1998) whereas *T. fumicolans, T. hydrothermalis, T. chitonophagus, T. peptinophilus* and *T. barossii* were found in samples from deep-sea hydrothermal vents as deep as 2600 m (Duffaud et al. 1998; Godfroy et al. 1996; Godfroy et al. 1997; Gonzáles et al. 1995; Huber et al. 1995). All *Thermococcus* species grow on peptides, some of them are able to grow on simple carbohydrates, such as maltose (Godfroy et al. 1996; Godfroy et al. 1997), on maltooligosaccharides (Duffaud et al. 1998), on complex carbohydrates such as starch and pectin (Miroshnichenko et al. 1989), or chitin (Huber et al. 1995) and on amino acids (Dirmeier et al. 1998). Elemental sulfur usually stimulates growth or is even absolutely essential (Miroshnichenko et al. 1989), but can be replaced in some species by cysteine or polysulfide (Godfroy et al. 1996; Godfroy et al. 1997). Some *Thermococcus* species are able to grow without any electron acceptor at all, indicating some sort of fermentation takes place (Keller et al. 1995; Godfroy et al. 1997; Gonzáles et al. 1995). Physiological differences between the marine *Thermococcus* species and the fresh water isolate *T. zilligii* led to the suggestion that *T. zilligii* might represent a new genus of the Thermococcaceae (Zillig 1991a). *T. zilligii* has a considerably lower salt concentration optimum than the other *Thermococcus* species, possesses unusual membrane lipids, grows at lower temperature and shows differences in its sensitivities to antibiotics (Klages and Morgan 1994). Phylogenetic analysis of the 16S rRNA gene sequences nevertheless does not support the establishment of a new genus (Ronimus et al. 1997). *Thermococcus* species commonly show a high level of conservation of the 16S rRNA gene. Some isolates have high homologies of the 16S rRNA gene to the

genes of already characterised *Thermococcus* species despite obvious physiological differences. *T. barossii* for example differs in almost every physiological feature from *T. celer* but the 16S rRNA genes are 99.7% homologous (Duffaud et al. 1998).

1.3. The kingdom Crenarchaeota

Until the recent discovery of crenarchaeotal 16S rRNA in environmental samples from mesophilic and psychrophilic environments, the Kingdom Crenarchaota was considered to consist of thermophilic, sulfur-dependent organisms only (Woese et al. 1990). According to a novel classification the thermophilic Crenarchaeota comprise three orders, Sulfolobales, Thermoproteales and the newly proposed order "Igneococcales" (Burggraf et al. 1997). Subsequent to the discovery of organisms that either do not need sulfur or sulfur compounds for their metabolism or are even inhibited by sulfur, such as *Pyrobaculum aerophilum* (Völkl et al. 1993), *Pyrolobus fumarii* (Blöchl et al. 1997) and *Sulfophobococcus zilligii* (Hensel et al. 1997) the great physiological diversity of the crenarchaea has become obvious.

1.3.1. The Order Sulfolobales

The Order Sulfolobales contains four genera: *Sulfolobus, Metallosphaera, Acidianus* and *Stygiolobus* which are restricted to acidic environments.
The Genus *Sulfolobus* was first described in 1972 by Brock et al. Cells are irregular, lobed cocci, found in continental solfataric fields and hot soils. *Sulfolobus* species are strictly aerobic and thermophilic, facultatively heterotrophic and grow by oxidation of elemental sulfur to sulfate (Segerer and Stetter 1991a) with the exception of *S. acidocaldarius*, which lacks this ability. The members of the Genus *Metallosphaera* greatly resemble the *Sulfolobus* species, but differ in their ability to grow on sulfidic ores (Huber et al. 1989; Fuchs et al. 1995), which is - except for *S. metallicus* (Huber and Stetter 1991a) - not found among the *Sulfolobus* species. 16S rRNA sequence data analysis nevertheless does not group *S. metallicus* with the genus *Metallosphaera* (Fuchs et al. 1996). *Metallosphaera* strains were isolated from solfataric fields as well as from a smouldering slag heap of a uranium mine in Thüringen, Germany.
The Genus *Stygiolobus* contains hitherto only one species, *S. azoricus* isolated from a solfataric field on São Miguel Island, Azores (Segerer et al. 1991c). *S. azoricus* differs from all the other known Sulfolobales by being strictly anaerobic and by being unable to oxidise S^0. Phylogenetic analysis shows that *S. azoricus* is most closely related to *Sulfolobus* (Fuchs et al. 1996).

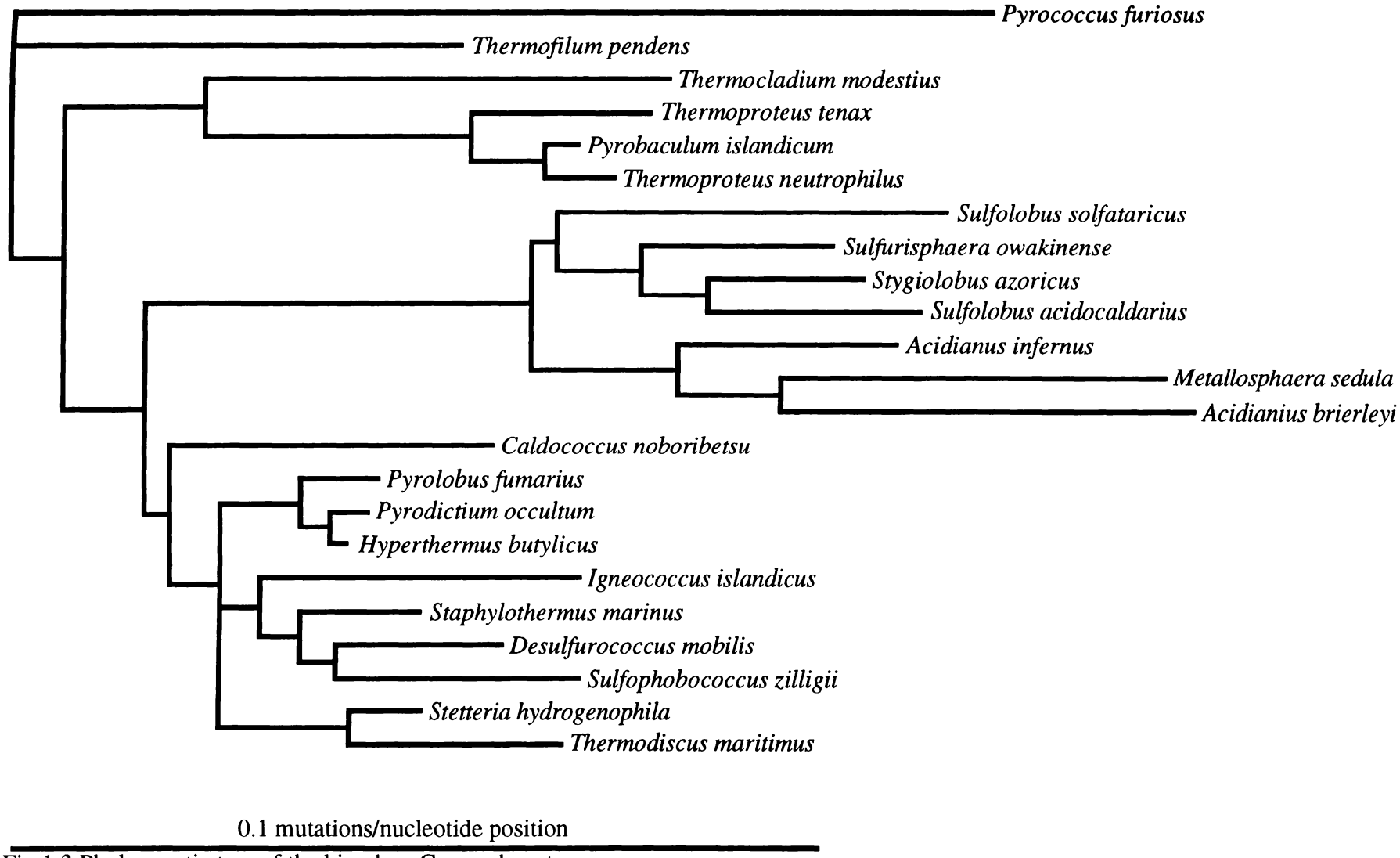


Fig.1.3 Phylogenetic tree of the kingdom Crenarchaeota..
(tree made with Clustal V and Tree View based on 16S rRNA gene sequences, see appendix 1 for accession numbers)

Three species of the Genus *Acidianus* are so far described, *A. infernus, A. brierleyi*, which used to be classified as *Sulfolobus brierleyi* (Zillig et al. 1980b), and *A. ambivalens* which used to be named *Desulfurolobus ambivalens* (Fuchs et al. 1996). *Acidianus* species have the ability to either oxidise or reduce S^0 depending on aerobic or anaerobic growth conditions. *Acidianus*-like strains have been isolated from both marine and terrestrial geothermal locations (Segerer et al. 1986) and is therefore the only genus in the order Sulfolobales to contain marine species.

	Sulfolobus	*Acidianus*	*Stygiolobus*	*Metallosphaera*
Morphology	lobed cocci	irregular cocci	lobed cocci	cocci
Aerobic growth	strictly aerobic	facultatively aerobic	strictly anaerobic	strictly aerobic
Temperature range in °C	50-87	45-95	57-89	50-80
pH range	1.5-4.5	1-5.5	1-3.5	1-4.5
S^0 oxidation	+	+	-	+
Chemolitho-autotrophy	heterotrophic or mixotrophic	facultatively lithoautotrophic on H_2/S^0	strictly lithoautotrophic H_2/S^0	facultatively lithoauto-trophically on sulfidic ores or S^0
Substrates	complex organic substrates, mono-, oligo-, polymeric sugars	peptides, casamino acids	no heterotrophic growth	complex organic compounds

Table 1.4. Some characteristics of the genera of the Order Sulfolobales.

1.3.2. The Order Thermoproteales

The Order Thermoproteales comprises two families, Thermoproteaceae and Thermofilaceae, which represent the only rod-shaped species known within the Crenarchaeota. They form a phylogenetically distinct cluster in the Kingdom Crenarchaeota.

The Family Thermofilaceae consists of only one genus containing one validly named species and one as yet undescribed isolate (Burggraf et al. 1997). *Thermofilum pendens* is an anaerobic sulfur respirer, which grows on peptides and is obligately dependent on a fraction of polar lipids from *Thermoproteus tenax*. *T. pendens* shows a great variability in length and frequently sharp bends of the cell appear. During exponential growth spherical bodies have been observed on one or both ends of the cells (Zillig et al. 1983a).

The Family Thermoproteales contains three genera, *Thermoproteus, Thermocladium* and *Pyrobaculum.*

The members of the genera *Thermoproteus* and *Pyrobaculum* are morphologically and physiologically quite similar (see Table 1.5.). The optimum growth temperatures of the *Thermoproteus* species are about 10°C lower (Bonch-Osmolovskaya et al. 1990). The recently isolated *Thermocladium modestius* has an upper temperature limit for growth of 82°C and an optimum temperature of 75°C (Itoh et al. 1998b). *T. modestius* grows optimally at pH 4 and in a range of 2.6-5.9 and is therefore the first acidophilic member of the Thermoproteales. All Thermoproteales species are facultative or obligate heterotrophs. *T. modestius* is stimulated by archaeal cell-extracts and CO_2 in the medium.

	Thermo-proteus	*Pyrobaculum*	*Thermocladium*	*Thermofilum*
Morphology	rods	rods	rods	long very thin rods
Aerobic growth	strictly anaerobic	strictly anaerobic to microaerophilic	anaerobic to microaerophilic	strictly anaerobic
Temperature range in °C	80-102	74-104	45-82	85-100
pH range	2.5-6.8	5-9	2.6-5.9	2.8-6.7
Chemolithoautotrophy	+	facultatively autotrophic	heterotrophic	-
S^0 respiration	+	+	+	+
Substrates	peptides, mono- and polymeric sugars, alcohols	peptides, archaeal cell homogenates	glycogen, starch, gelatin, proteinaceous substrates	peptides, requires lipid fraction of *T. tenax*

Table 1.5. Some characteristics of the genera of the Order Thermoproteales.

With the exception of *P. aerophilum* all known Thermoproteales strains were isolated from terrestrial, solfataric habitats (Völkl et al. 1993; Huber and Stetter 1991b). *P. aerophilum* differs as well in the ability for dissimilatory nitrate reduction and for aerobic respiration. Growth of *P. aerophilum* is inhibited by elemental sulfur whereas the other *Thermoproteus* species can use S^0 as electron acceptor for autotrophic growth with H_2 and CO_2. Furthermore *P. aerophilum* has a 713-bp intron within its 16S rRNA gene which was until recently the only reported intron in a prokaryotic 16S rRNA gene (Burggraf et al. 1993). Introns have now been discovered in the 16S rRNA genes of two *Thermoproteus* strains, strain IC-033 and strain IC 061 and in *T.*

neutrophilus strain JCM 9278 (Itoh et al. 1998a). The 16S rRNA genes of these three strains contain more than one intron. Strains IC-033, IC-061 and *T. neutrophilus* strain JCM 9278 have five, three and two introns respectively. For the genus *Pyrobaculum* only *P. aerophilum* has been reported to contain an intron in its 16S rRNA gene sequence. Phylogenetically nevertheless *P. aerophilum* shows a close relationship to *P. islandicum.*

All members of the family Thermoproteales exhibit like *T. pendens* spherical bodies at the ends of a small percentage of the cells during exponential growth. For *P. aerophilum* it is reported that the spheres enlarge with a simultaneous shortening of the rods and can over time convert entirely into coccoid cells (Völkl et al. 1993). No publication about purpose, structure and regulation of those spherical bodies is available to date.

1.3.3. The Order "Igneococcales"

It was recently suggested that the third order of the crenarchaeota be named "Igneococcales" (Burggraf et al. 1997). This name is not yet officially accepted.

The Order "Igneococcales" contains two families, the family Pyrodictiaceae and the family Desulfurococcaceae. All members of this order are coccoid or disc shaped, hyperthermophilic and with the exception of *Aeropyrum pernix* anaerobes, which prefer neutral pH. For all genera of the order "Igneococcales" very few species have been described so far. Seven of nine validly described genera contain only one species, the genus *Desulfurococcus* contains two and the genus *Pyrodictium* contains three species.

1.3.3.1. The Family Pyrodictiaceae

The Family Pyrodictiaceae is characterised by optimum growth temperatures above 100°C whereas no member of the family Desulfurococcaceae can grow above 100°C despite having optimum growth temperatures of more than 85°C. Phylogenetically the three genera of the Pyrodictiaceae are very closely related but there are significant physiological differences.

The genus *Pyrodictium* is distinguished from the other genera by the presence of a network of fibres connecting the disk- or dish-shaped cells. These fibres lead to the appearance of colony or cobweb-like aggregates in unshaken liquid cultures. *Pyrodictium* species are capable of growth by hydrogen-sulfur autotrophy and of autotrophic growth on H_2/CO_2 with sulfite as electron acceptor. Only *P. abyssi*

is strictly heterotrophic, fermenting proteins, carbohydrates, cell extracts, acetate and formate (Pley et al. 1991). All *Pyrodictium* isolates are isolates from marine solfataric situations in Italy, Iceland or off Guaymas, Mexico.

	Pyrodictium	*Hyperthermus butylicus*	*Pyrolobus fumarii*
Morphology	disc shaped, network of fibres	irregular cocci	lobed cocci
Temperature range and **optimum** in°C	8-110 **~105**	95-107 **100**	90-113 **106**
Metabolism	H_2/S^0 autotrophy * or fermentation**	fermentation of proteolysis products	obligate chemolitho-autotroph, oxidation of H_2
Oxygen sensitivity	strictly anaerobic	strictly anaerobic	aerobe

Table 1.6. Some characteristics of the genera of the order *Pyrodictiaceae*.
* *P. occultum* and *P. brockii*, ** *P. abyssi*

Hyperthermus butylicus the only representative of the genus *Hyperthermus*, was isolated from the sea floor on the coast of São Miguel island, Azores. *H. butylicus* is a strictly anaerobic and heterotrophic irregular coccus which grows up to 108°C and optimally at 100°C. *H. butylicus* ferments peptides to CO_2 and *n*-butanol but growth is enhanced by the addition of S^0 and H_2 (Zillig et al. 1990; Zillig et al. 1991b).
Pyrolobus fumarii is the organism with the highest growth temperature so far discovered. The irregular coccus grows between 90 and 113°C with an optimum at 106°C. Additionally it is capable of growing at pressures up to 25 000kPa reflecting the deep sea environment from which it was isolated. *P. fumarii* is able to use O_2 at low concentrations as an electron acceptor but usually gains energy by oxidation of H_2 with nitrate or thiosulfate (Blöchl et al. 1997). Growth is inhibited by elemental sulfur as well as by acetate, pyruvate, glucose and starch.

1.3.3.2. The Family Desulfurococcaceae

The Family Desulfurococcaceae comprises six validly described genera and some isolates which still have to be described but for which 16S rRNA gene sequence data are already available. Besides the genus *Desulfurococcus* which contains three species all the other genera of the family Desulfurococcaceae contain only one species.
All members of the family Desulfurococcaceae are heterotrophs, utilising preferentially proteins and peptides such as yeast extract, tryptone or peptone by fermentation like *Sulfophobococcus zilligii* (Hensel et al. 1997) and *Thermosphaera aggregans* (Huber

et al. 1998a) or by both fermentation and sulfur respiration such as the *Desulfurococcus* species (Zillig et al. 1982). Some of them are inhibited by sulfur (*T. aggregans, S. zilligii*) whereas others are obligately dependent on sulfur such as *Stetteria hydrogenophila* (Jochimsen et al. 1997).

	Temperature range and **optimum** in ℃	pH range and **optimum**	Oxygen sensitivity	Metabolism
Desulfuro-coccus species	75-93 **~85**	5-7 **6**	strictly anaerobic	heterotrophic, utilises proteins, peptides by fermentation or sulfur respiration
Thermosphaera aggregans	65-90 **85**	5-7 **6.5**	strictly anaerobic	heterotrophic on yeast extract, peptone, gelatin, xylan*
Sulfophobo-coccus zilligii	70-95 **87**	6.5-8.5 **7.6**	strictly anaerobic	obligate heterotroph, fermentative on yeast extract only
Stetteria hydrogenophila	68-102 **95**	4.5-7 **6**	strictly anaerobic	heterotrophic on yeast extract, peptone, tryptone**
Staphylo-thermus marinus	65-98 **92**	4.5-8.5	strictly anaerobic	heterotrophic on yeast extract, peptone, cell extracts
Thermodiscus maritimus	75-98	5-7	strictly anaerobic	heterotrophic
Aeropyrum pernix	70-100 **~90**	5-9 **7**	strictly aerobic	heterotroph, yeast extract, peptone, tryptone

Table 1.7. Some characteristics of members of the family Desulfurococcaceae.
* heat treated xylan
** in the presence of H_2 and S^0

The members of the family Desulfurococcaceae have a broad physiological range. The unifying factors are the lower temperature limit for growth in comparison to the Pyrodictiaceae and their exclusively heterotrophic metabolism with a strong preference for proteinaceous substrates. Phylogenetically the Order "Igneococcales" forms a distinct cluster separated from the acidophilic Sulfolobales and the rod-shaped Thermoproteales.

1.4. Archaea discovered in environmental studies

16S rRNA sequence data from environmental samples clearly indicate the existence of a vast variety of as yet unknown and uncultured organisms for all three domains of life and show the existence of archaea in places which until recently were not considered to be suitable habitats. Nucleic acid fragments belonging to Archaea and, surprisingly, to the Crenarchaeota could be found in samples from soils (Borneman et al. 1996; Jurgens et al. 1997), arctic and antarctic marine waters and picoplankton (DeLong et al. 1994; Giovannoni et al. 1990; Fuhrman et al. 1993), freshwater and lake sediments (MacGregor et al. 1997; Schleper et al. 1997a), in symbioses with a sponge (Preston et al. 1996) and even in the hindgut of a sea cucumber (McInerney et al. 1995). Furthermore, studies of "typical" crenarchaeotal environments, like submarine volcanic vents and terrestrial hot springs, revealed an abundance of still uncultured and undescribed Crenarchaeota (Barns et al. 1994). Only one species, *Thermosphaera aggregans*, whose 16S rRNA gene was isolated from an environmental sample, cloned and sequenced, could be cultured and characterised subsequently (Huber et al. 1995a). *Cenarchaeum symbiosum*, the symbiont of a marine sponge, is as yet uncultured but some aspects of its physiology, such as the lipid content (DeLong et al. 1998) and the characteristics of its DNA polymerase (Schleper et al. 1997b) have been investigated nevertheless.

These findings show an immense diversity of the Archaea and phylogenetic analysis of the 16S rRNA sequences indicate the possible existence of a third archaeal kingdom, preliminarily named "Korarchaeota" (Barns et al. 1996; Barns et al. 1994), besides the two recognised kingdoms Euryarchaeota and Crenarchaeota (Woese et al. 1990). Whether those sequences really represent members of a novel kingdom or low branches of the Crenarchaeota still has to be determined. The results of these studies also imply several novel groups which diverged early from the crenarchaeal line (Barns et al. 1996; Bintrim et al. 1997; McInerney et al. 1997). Quantification of 16S rRNA in environmental samples show that Archaea make up from 4% in American Pacific coast (DeLong 1992) and 8% in the north-east Atlantic ocean (McInerney et al. 1997) to up to 21-34% in Antarctic waters (DeLong et al. 1994) of the total prokaryotic SSU rRNA.

No physiological conclusions can be drawn from 16S rRNA sequence data. Sequence similarities with 16S rRNA genes from already cultured organisms can provide only vague presumptions about physiology and ecological significance of the organisms belonging to the newly found sequences. Isolation, purification and characterisation of these organisms or in situ assays with environmental samples (DeLong et al. 1998) are

necessary for the understanding of the physiology of these organisms and for further investigation of their phylogeny.

1.5. Carbohydrate degrading enzymes in Archaea

1.5.1. Amylolytic enzymes

Starch is a storage material of plants analogous to fat in animals and is one of the most abundant molecules on earth. Starch is a mixture of two polymers, amylose which is a linear (1-4)-α-D-glucan and amylopectin, which is a branched D-glucan with α-D-(1-6) linkages.

Enzymes involved into the first steps of the enzymatic degradation of starch are: α-amylases, β-amylases and pullulanases. α-amylases endohydrolyse 1,4-α-D-glycosidic linkages. All known α-amylases, except one bacterial enzyme, belong to the same α-amylase family (Jespersen et al. 1994). β-amylases are exoenzymes hydrolysing 1,4-α-D-glycosidic linkages from the non-reducing end. Pullulanases break the 1,6 a-D-glycosidic bonds in amylopectin, pullulan and glycogen.

In Archaea, starch degrading enzymes have been found mainly in species belonging to the family Thermococcaceae and the genera *Sulfolobus, Thermoproteus* and *Archaeoglobus*. Most of the heterotrophic archaea prefer proteinaceous substrates to monomeric and polymeric carbohydrates. In the family Thermococcaceae, about a third of the species are capable of metabolising complex carbohydrates, particularly starch, and one member of the genus *Thermococcus*, *T. chitonophagus,* even grows on chitin (Huber et al. 1995b).

Archaeal amylolytic enzymes have been isolated hitherto from *Pyrococcus furiosus* (Koch et al. 1991; Dong et al. 1997), *P. woesei* (Koch et al. 1991) and *Pyrococcus* sp. KOD1 (Tachibana et al. 1996), from *Thermococcus hydrothermalis* (Legin et al. 1998; Gantelet and Duchiron 1998a), from *T. profundus* (Chung et al. 1995) and from *T. litoralis* (Brown and Kelly 1993). All but one α-amylase of *P. furiosus* are extracellular enzymes, *P. furiosus* has both an extra- and an intracellular amylase (Dong et al. 1997).

In *P. furiosus*, *Thermococcus hydrothermalis* and *T. litoralis* amylopullulanases, which have both α-1,4 and α-1,6 hydrolytic activity have been found (Gantelet and Duchiron 1998b; Brown and Kelly 1993). All archaeal amylases exhibit high thermostability. For an α-amylase of *Pyrococcus woesei* enzymatic activity was still detected after five hours autoclaving at two bars and 120°C (Koch et al. 1991).

1.5.2. Mannanases

Mannan is a branched heteropolysaccharide which is found in softwood, hardwood, roots, seeds, coffee beans and fruit. Galactoglucomannan consists of a backbone of β-1,4-linked mannose and glucose residues with α-1,6-linked galactose or acetyl side chains (Stålbrand et al. 1995). The content of glucose is highly variable. Glucomannans which are mainly found in hardwood do not contain galactose.

The degradation of galactoglucomannan is performed by endo-acting β-endomannanases and exo-acting β-mannosidases. The side chain sugars are removed by enzymes such as α-galactosidases or β-glucosidases (Duffaud et al. 1997).

β-endomannanases (EC 3.2.1.78) with the formal name 1,4-β-D-Mannan mannanohydrolase randomly cleave the β-1,4-linked backbone of the galactoglucomannans (see Fig.1.4.). β-D-mannosidases (EC 3.2.1.25) with the formal name β-D-Mannoside mannohydrolase hydrolyse β-1,4-linked mannosides and release mannose from the non-reducing end of manno-oligosaccharides. Galactose side chains are cleaved by α-D-galactosidases (EC 3.2.1.22) with the formal name α-D-galactoside galactohydralase and acetyl groups are released by acetyl mannan esterases (EC 3.2.1.96).

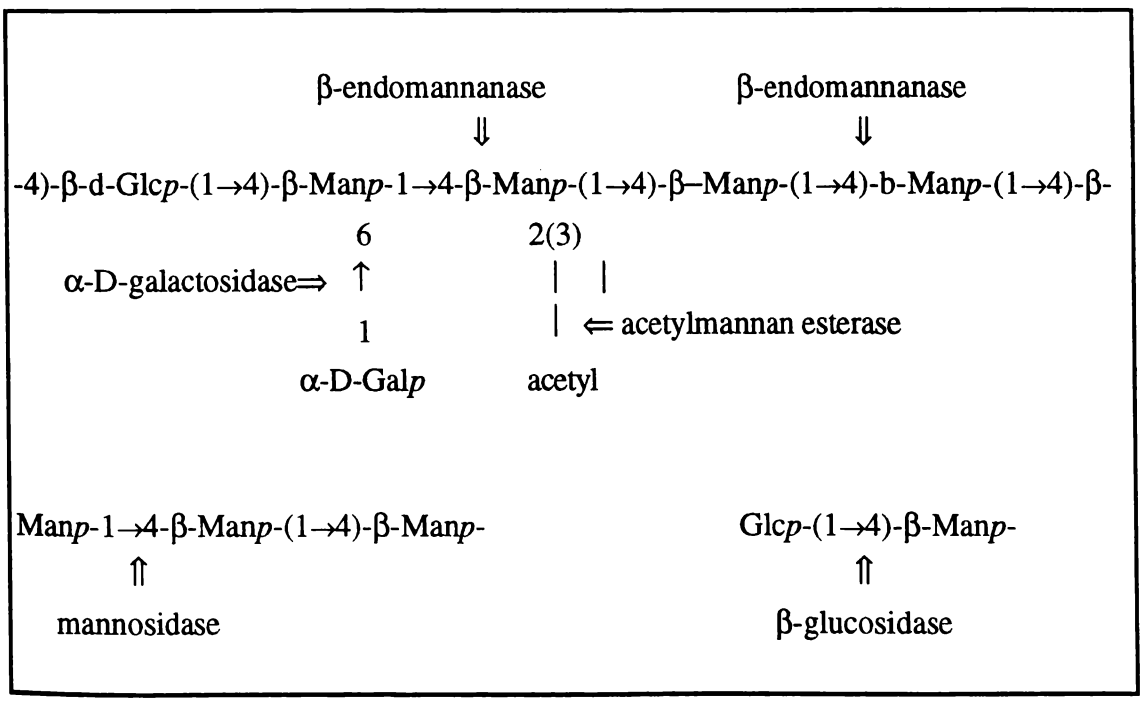


Fig. 1.4. Mode of action of enzymes involved in degradation of galactoglucomannan. Glc*p* : glucopyranose; Man*p*: mannopyranose; Gal*p*: galactopyranose. Diagram adapted from Puls and Schuseil (1993).

β-endomannanases and the other enzymes involved in the degradation of mannan are frequently found in fungi, bacteria and plants (Bicho et al. 1991). Fungal β-endomannanases, such as from the white-rot fungus *Ceriporiopsis subvermispora* (Sethuraman et al. 1998) or the filamentous fungus *Trichoderma reesei* (Stålbrand et al. 1995) are involved in the decay of wood. Bacterial β-endomannanases and α-D-mannosidases are found in soil bacteria (Kataoka and Tokiwa 1998) and in bacteria inhabiting the human intestinal tract (Nakajima and Matsuura 1997). In plants β-endomannanases play a major role in the germination of seeds (Welbaum et al. 1998; Bewley 1997) and in the ripening of some fruit, such as bananas (Prabha and Bhagyalakshmi 1998).

Whereas enzymes that degrade complex carbohydrates belonging to the hemicelluloses, such as xylanases, mannanases and pectinases are frequently found in bacteria such as *Bacillus subtilis* (Zakaria et al. 1998b), *Pseudomonas fluorescens* (Scott et al. 1998) and *Caldocellulosiruptor saccharolyticus* (Gibbs et al. 1996), few archaea are known to be capable of utilising cellulose, xylan, pectin or galactoglucomannan. Archaeal xylanases are reported only for the euryarchaeote*T. zilligii* (Andreas Uhl, Thermophile Research Unit, unpublished work) and the crenarchaeote *Thermosphaera aggregans* (Huber et al. 1998a). *T. aggregans* is able to grow on heat treated xylan but not on native xylan. β–mannanases have been found in some thermophilic bacteria, such as *Caldocellulosiruptor saccharolyticus* (Gibbs et al. 1996), *Thermotoga neapolitana* (Duffaud et al. 1997) or *Bacillus stearothermophilus* (Ethier et al. 1998) but have not been found in archaea so far.

Industrial applications for mannanolytic enzymes are in the food industry where β-endomannanases are employed for the treatment of coffee beans during the production of instant coffee (Zakaria et al. 1998a). Further application are in the treatment of *Amorphophallus konjac*, a tuber vegetable from the family Araceae which is sold as a dietary food in Japan (see Fig. 2.1.). More applications are possible in the oil and gas industries where mannanases are used for the in situ hydrolysis of galactomannan used for well stimulation (Duffaud et al. 1997) and in the pulp and paper industry for the bleaching of Kraft pulp.

Chapter 2 - Material and methods

2.1. Materials

Materials frequently used such as salts, carbohydrates, yeast extract, tryptone, buffers and chemicals for standard analytical purposes were obtained from Sigma, Difco, Gibco, BDH and Riedel-de-Haen. For analytical purposes chemicals were used at analytical grade.

SeaKem agarose	FMC, BioLab Scientific, NZ
Thermostable agarose	Industrial Research Ltd., Lower Hutt, NZ
Custom made primers	Life Technologies, Auckland, NZ
Taq Polymerase	Perkin Elmer, Australia
Desoxy ribonucleotides	Böhringer, Mannheim, Germany
RNAse	Sigma-Aldrich, NZ
ProteinaseK	Sigma-Aldrich, NZ
PronaseE	Sigma-Aldrich, NZ
Restriction enzymes	Böhringer, Mannheim, Germany
Gel Extraction Kit for DNA fragments	Qiagen, Düsseldorf, Germany
pGEM®-T Easy Cloning Kit	Promega, Auckland, NZ
Mini Plasmid Prep	Promega, Auckland, NZ
ZAP Express™ Predigested Vector Kit	Stratagene
T4 PNK	Böhringer, Mannheim, Germany
^{33}P-labeled ATP	Amersham, Amersham, England
Konjac glucomannan	Mr. M. Tsumuta, Kobe, Japan
Koniyaku	The Japan Store, Auckland
Slides for whole cell hybridization	Marienfeld, Bad Mergentheim, Germany
Phast protein gels and buffer	Pharmacia

2.2. Methods

2.2.1. Cultivation of strain Wai21.S1 and other *Thermococcus* strains.

Strain Wai21.S1 was grown anaerobically on a culture medium with the following composition:

Strain Wai21.S1:

Compound	grams per litre
KH_2PO_4	1.5
$Na_2HPO_4.7H_2O$	4.2
NH_4Cl	0.5
$MgCl_2.6H_2O$	0.38
$CaCl_2$	0.05
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.039
Na_2CO_3	1.0
Yeast extract	2.0
Starch soluble	5.0
Cysteine HCl	1.0
Resazurin	0.0002

Table 2.1. Composition of the medium for the cultivation of Wai21.S1.

Trace elements (stocksolution):

Compound	mg per litre
$CoCl_2.6H_2O$	2.9
$NaMoO_4.2H_2O$	2.4
Na_2SeO_3	0.17
$MnCl_2.4H_2O$	2.0
$ZnSO_4$	2.8

Table 2.2. Trace element solution for Wai21.S1 medium and consortium medium.

1ml of trace elements was added per litre of medium and the pH was adjusted to 7.0.

Strain Wai21.S1 was grown under anaerobic conditions in 10ml Hungate tubes or in Schott bottles of up to 2 litres volume if larger cell quantities were required. The medium was boiled, gassed with N_2 and dispensed into Hungate tubes according to Hungate et al. (1969). Larger volumes were boiled and gassed for approximately five minutes. The tubes were then autoclaved for 20min at 121°C. Tubes were inoculated with a syringe through the septum with a 1% inoculum and incubated at 80°C without shaking.
Pure cultures were obtained by two subsequent platings in roll tubes. For roll tubes 8g/l Gelrite was added to the medium. The medium was boiled and gassed with N_2 and 5ml were dispensed into Hungate tubes. The tubes were autoclaved and cooled down to ~90°C. 100µl of an exponentially growing culture at various dilutions were injected with a syringe through the septum and 50µl 1M $MgCl_2$ were added. The tubes were rolled on the bench until the solidification of the medium occurred. The tubes were placed into a rack at a 45° angle for incubation.
Stock cultures were stored at room temperature for up to 6 months. For long-term storage cultures were preserved at -70°C with 20% glycerol as cryoprotectant. Cultures were also freeze-dried and deposited in the Thermophile Research Unit culture collection.

Other species and strains of thermococci used in this study were cultured on the following media:
T. celer was grown on medium 266 as described in the DSM catalogue (1993).
Wt1 was grown on medium 377 as described in the DSM catalogue 1993.
T. zilligii was grown as described by Klages and Morgan (1994).

All media were prepared anaerobically as described above.

2.2.2. Medium and cultivation of the consortium culture and strain Tok2

The consortium culture and strain Tok2 were cultivated in the medium described in Table 2.3. 2ml of the trace elements solution (see Table 2.2.) were added per litre and the pH adjusted to 7.0.
Konjac glucomannan was kindly provided by Mr M. Tsumuka, Kobe, Japan. For some weeks Konjac glucomannan was replaced by koniyaku, a common food in Japan which was obtained from The Japan Store, Auckland (Fig 2.1).

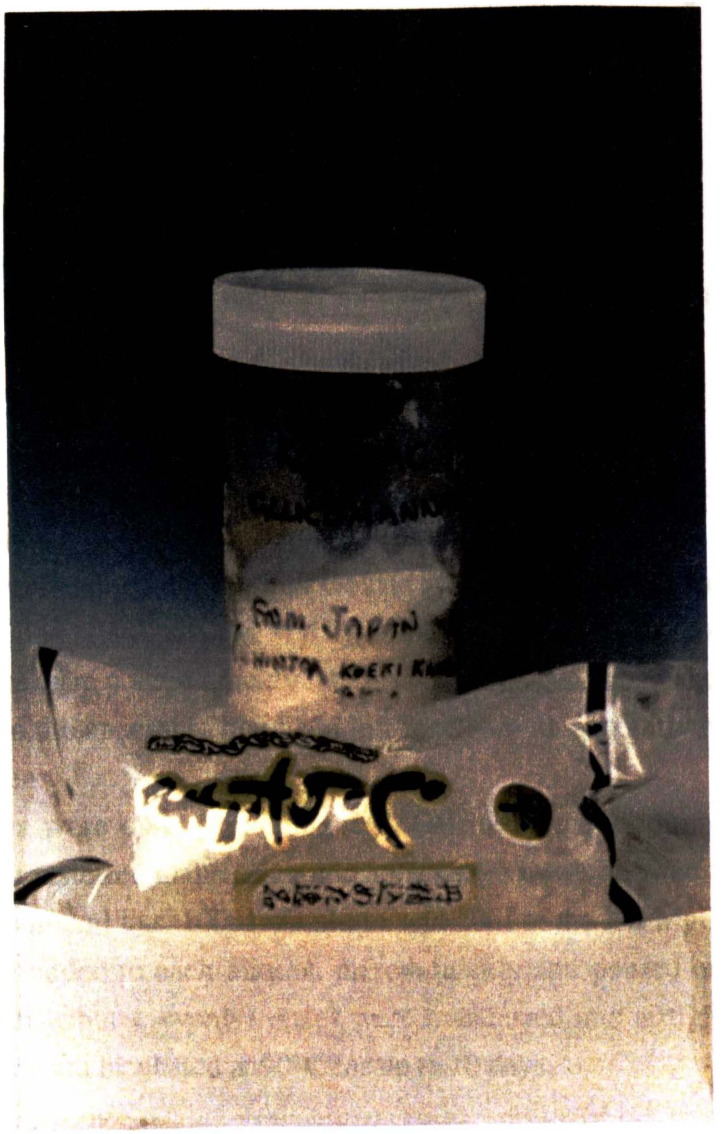


Fig. 2.1. Types of konjac glucomannan used in this study.

Compound	grams per litre
K_2HPO_4	0.2
$(NH_4)_2SO_4$	0.2
$MgSO_4.7H_2O$	0.1
$NaNO_3$	0.05
$Na_2S_2O_3.5H_2O$	0.96
Konjac glucomannan	2.0

Table 2.3. Composition of the medium for cultivation of the consortium culture.

For cultivation of strain Tok2, 2g of yeast extract were added in place of the Konjac glucomannan.

Hungate tubes containing liquid cultures were prepared as described in section 2.2.1. Cultures were incubated at 90°C without shaking.

Plates were prepared by adding 8g/l Gelrite and heating the medium in a microwave oven until boiling. 10ml of a 1M $MgCl_2$, 0.3M $Ca(NO_3)_2$ solution per litre were added and 25ml/plate were poured quickly into glass petri dishes. After setting the plates were transfered into the anaerobic hood and left for 24h with a slightly open lid in order to remove oxygen.

Plate overlays were done by adding 2g/l Gelrite to the medium and boiling. The medium was transferred to the anaerobic hood where it was allowed to cool to 85°C and then aliquoted in 2ml lots. 100µl of culture, and 20µl of the 1M $MgCl_2$/0.3M $Ca(NO_3)_2$ solution were added to each aliquot, mixed quickly and poured onto the plates. The overlay was distributed evenly. Plates were transferred into airtight containers in the anaerobic hood and incubated at 90°C for up to 10 days.

2.2.3. Carbon source tests

Mono- and di-saccharides, organic acids and amino acids were used at a concentration of 0.2% (w/v). Polymeric carbohydrates such as starch, glycogen, amylose and amylopectin were used at concentrations between 0.2-0.5% (w/v). Peptides and proteins were added to a final concentration of 0.2%, except for casamino acids which were used at 0.02%.

Growth was observed - when possible - by measuring the increase in optical density at 650nm. If this was not possible cells were counted in a Thoma chamber (0.02mm depth) under the phase contrast microscope.

2.2.4. Determination of growth parameters

Sodium requirement:

For the determination of optimum sodium concentration for growth, media were prepared with potassium salts instead of sodium salts, where possible. Growth was followed by measuring the optical density at 650 nm. Doubling times were calculated using the equation: $\ln(y_2) - \ln(y_1) / (x_2 - x_1) = z$; $dt = \ln 2 / z$
In this equation y stands for the OD_{650} values and x stands for the time elapsed since inocculation.
If growth could not be measured photospectrometrically, cells were counted in a Thoma chamber under the phase contrast microscope.

Temperature range and optimum:

Cultures for the determination of temperature range and optimum were incubated in a gradient heater (University of Waikato workshop). Growth measurement and calculation of doubling times were done as described above.

pH range and optimum:

Cultures for the determination of pH ranges and optima were buffered with Bis/Tris and the pH was adjusted at growth temperature using Eichpuffer solutions (Philips) as reference buffers. Growth measurement and calculation of doubling times was done as described above.

2.2.5. Electron microscopy

Electron microscopy was carried out by David Wild (MIRINZ, Hamilton, New Zealand). Preparations were shadowed with uranyl formate.

2.2.6. Isolation of DNA

Cultures were harvested during late exponential growth by centrifugation. The pellets were resuspended in lysis buffer (10mM Tris/HCl, 100mM EDTA, pH 7.5) to

approximately 1/100 of the original volume of the cultures. The cells were treated with 0.4 mg/ml ProteinaseK + 0.1% SDS for at least 3h but usually overnight at 65°C, or alternatively with 1mg/ml PronaseE at 37°C overnight. PronaseE was preincubated at 1mg/ml in Pronase buffer (10mM Tris pH 7.4, 10mM EDTA, 0.5% SDS, 1mg/ml PronaseE) for 60min at 37°C to prevent nuclease activity due to impurity of the Pronase before the resuspended cells were added.

Proteolysis was followed by three phenol extractions, two extractions with chloroform/ iso-amyl alcohol (24:1, vol:vol) and a single extraction with chloroform. The organic solvents were added in equal volumes to the aqueous phase. The yield of total DNA could be increased by back-extracting the first phenol phase with TE-buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

The DNA was precipitated with 0.3M Na-acetate (final concentration) and two volumes of absolute ethanol, or alternatively 0.7 volumes of isopropanol. The DNA was spooled, washed with 70% ethanol and dried under vacuum in a desiccator. The pellet was redissolved in a suitable volume of TE buffer and incubated for 60 min at 37°C with 0.2mg/ml RNAse. This was followed by another phenol extraction. The DNA was stored at -20°C.

To obtain very pure DNA for restriction assays and cloning, DNA was further purified in a CsCl-gradient. CsCl was added to 1g/ml (w/v), ethidium bromide was added to 5µg/ml and the gradient was run for 24h at 44000 rpm at 15°C in a fixed angle rotor. The band was extracted with a needle and syringe, the ethidium bromide was removed by three washes with iso-propanol and CsCl was removed by overnight dialysis against a large volume of TE-buffer.

2.2.7. Amplification of 16S rRNA genes

PCRs were carried out with the following reaction conditions:

Compound	Concentration
Primers	0.1µM each
Nucleotides	200µM each
Taq polymerase	1U
Buffer*	1/10 final volume
DNA	10-50ng

Table 2.4. Concentration of compounds in a standard PCR.
* Buffer provided by the supplier of Taq polymerase

For analytical purposes PCRs were assembled in 25µl volumes, but the volume of the reaction was increased to 100µl for direct sequencing and cloning.

Primers for 16S rRNA genes:

Name†	Direction	Sequence
af2-21	Forward	5'-TTC CGG TTG ATC CYG CCG GA-3'
af515	Forward	5'-GTG CCA GCM GCC GCG GTA A-3'
af1042-1060	Forward	5'-GAG AGG WGG TGC ATG GCC-3'
pA 8-28	Forward	5'-AGA GTT TGA TCC TGG CTC AG-3'
ar976-958	Reverse	5'-CCG GCG TTG AMT CCA ATT-3'
ar1510-1492	Reverse	5'-GGT TAC CTT GTT ACG ACT T-3'
pH*1542-1522	Reverse	5'-AAG GAG GTG ATG CAG CCG CA-3'

Table 2.5. Primers used for amplification of 16S rRNA genes.
† Numbers in the primer name indicate position in the 16S rRNA gene (*E. coli* numbering). Primer sequences from Achenbach and Woese, 1995

Amplification program for 16S rRNA genes:

	Function	Time (sec)	Temperature (°C)
Step 1 (1x)	Initial denaturation	225	94
Step 2 (35x)	Denaturation	30	94
	Annealing	30	55
	Extension	120	72
Step 3 (1x)	Final extension	120	72

Table 2.6. Program used for amplification of 16S rDNA.
The numbers in brackets in the first column indicate the number of cycles.

2.2.8. Primers and amplification program for 16S-23S intergenic region

PCRs were executed as described in section 2.2.7.

Primers were kindly provided by Dr. Joann Roskoski, national Science Foundation, Washington, USA.

Primers:

Direction	Name	Sequence
Forward	Rosk16SF-D1	5'-GTA GCC GTA GGG GAA CCT GC-3'
Reverse	Rosk23SR-D2	5'-CCC GAG CCG AGC CAT CCA CC-3'

Table 2.7. Primers used for amplification of 16S-23S intergenic region.

PCR program:

	Function	Time (sec)	Temperature (°C)
Step 1 (1x)	Initial denaturation	225	94
Step2 (35x)	Denaturation	30	94
	Annealing	30	52
	Extension	60	72
Step 3 (1x)	Final extension	120	72

Table 2.8. Program used for amplification of the intergenic region.
The numbers in brackets in the first column indicate the number of cycles.

2.2.9. Agarose gel electrophoreses

Agarose gels were usually prepared with 1% agarose (w/v) in 1x TAE buffer. For smaller fragment sizes the agarose concentration was increased to 1.5%. Gels were run at 30-100mV, stained with 0.3µg/ml aqueous ethidium bromide solution and destained with tap water. The stained gels were analyzed and documented using the Eagle Eye gel system.

TAE buffer: 2.0 M Tris/Base, 1.0 M Acetic acid, glacial, 50 mM EDTA; pH 8.0

Loading buffer: 50% Glycerol, 1mM EDTA, 0.25% Xylene cyanol, 0.25% Bromophenol Blue

2.2.10. Isolation of DNA fragments from agarose gels

DNA fragments were isolated from agarose gels using the QIAquick Gel Extraction Kit from Qiagen following the instructions provided by Qiagen.

2.2.11. DNA sequencing

Direct sequencing of purified PCR products was achieved using the Prism™ Ready Reaction Dichlororhodamine Terminator Cycle Sequencing Kit (Perkin Elmer) following the manufacturer's protocol. Sequences were analyzed on the Applied Biosystems Model 377 automated DNA sequencer from Perkin Elmer.

2.2.12. Cloning of 16S rRNA genes

PCR fragments were isolated and purified as described in section 2.2.10. Purified fragments were cloned into pGEM®-T Easy vector from Promega as recommended by the supplier.

2.2.13. DNA-DNA similarity assay

Quantitative dot-blot hybridization was carried out following the method described by Hiraishi (1991), but instead of hybridising the blotted DNA with biotinylated DNA, the probe DNA was end-labelled with ^{32}P.*

50ng DNA of each strain was dot-blotted on Hybond membrane and crosslinked to the membrane by exposing the membrane to UV light for 30 seconds. The membrane was then prehybridised for one hour at hybridization temperature (optimally 15°C below the melting temperature of the probe DNA) with 10ml of hybridization buffer. The labelled DNA* (50ng for each dot) was then added to the hybridization buffer and the membrane was hybridised overnight. The buffer was removed and the membrane was washed with hybridization buffer several times for 20 min each wash. The temperature was increased after every wash. The final wash was carried out at five degrees below melting temperature of the probe DNA. The membrane was dried and cut into equal pieces each containing one dot of DNA. Individual membrane pieces were placed at the bottom of scintillation vials containing 2ml of POPOP scintillation solution (0.2g/l POPOP in toluene) and radioactivity was measured in a liquid scintillator over a period of 40min.

*The DNA was ^{32}P end-labelled with T4 polynucleotide kinase following the instructions provided by the supplier.

Hybridization buffer: 70g SDS in 500ml H_2O, 1ml 0.5M EDTA, 500ml phosphate buffer (not autoclaved).

Phosphate buffer: 89g $Na_2HPO_4 \cdot 2H_20$ in 900ml H_2O, adjusted to pH 7.2 with ~ 4ml H_3PO_4, made up to 1L, sterilised by autoclaving.

2.2.14. Determination of mol% G+C

Mol% G+C was determined by following the isochromatic shift during the melting of the DNA at OD_{260nm}. The DNA was additionally purified in a 1g/ml CsCl gradient and dialysed overnight against 1xSSC buffer. The concentration of the DNA was adjusted to an OD_{260nm} of 0.8 (40µg/ml) and the DNA was placed into a cuvette in a temperature controlled spectrophotometer. The increase in temperature was 0.5°C/min. The %G+C content of the the DNA was calculated using the equation:

$$\text{Mol \% G+C} = \{[T_m + (T_ma - T_mref)] -69.3\} / 0.41$$

where T_m is the melting point of the investigated DNA; T_ma is the assumed melting point of the reference DNA calculated with equation: T_ma = [(mol% G+C) x 0.41] + 69.3; T_mref is the melting point of the *Thermococcus zilligii* reference DNA in the experiment (Marmur and Doty 1962).

2.2.15. Whole cell hybridization with fluorescent probes

Chromophore-labelled oligonucleotide probes were purchased custom-made and purified from LifeTechnologies, Auckland.
Cell fixation, preparation of slides, hybridization and microscopy were carried out according to the protocol described by Stahl et al. (1993) with the modifications of Burggraf et al. (1994).

1. Cell fixation:

Three volumes of the paraformaldehyde fixative solution were added to one volume of sample and kept at 4°C for 3 hours. The cells were pelleted, washed once in PBS and resuspended in PBS to a final concentration of 10^8-10^9 cells/ml. One volume of ice-

cold 98.8% ethanol was added and mixed. Special slides with teflon-rimmed wells (Marienfeld, Bad Mergentheim, Germany) were cleaned thoroughly and coated with gelatin by dipping into warm (70°C) gelatin solution. 3µl of the fixed cells were spread onto the slide and air dried. The cells were dehydrated by successive application of 50%, 80% and 100% ethanol (3min each).

Paraformaldehyde solution: 4g paraformaldehyde in 65ml H_2O were heated under stirring to 60°C. One drop of 2M NaOH was added and stirred until the solution clarified. After removing the solution from heat 33ml 3x PBS were added and the solution was quickly cooled to 4°C. The solution had to be used within 24h.

3x PBS: 390mM NaCl, 30mM sodium phosphate buffer (pH 7.2)

Gelatin solution: 0.1% gelatin, 0.01% $CrK(SO_4)_2$

2. Hybridization:

Hybridization was carried out in a sealed moisture chamber. A plastic container with a tightly sealing lid was used for this purpose.

A Whatman 3M paper was soaked in hybridization buffer and placed on the bottom of the container and the chamber was allowed to equilibrate for some minutes at hybridization temperature. For each spot 8µl hybridization buffer was mixed with 50ng probe and placed on the spot of fixed cells. The slides were put into the moisture chamber and hybridised at a suitable temperature for 2 hours. After hybridization the slides were rinsed with ~2ml of hybridization buffer each and submersed in 50ml hybridization buffer for 20min at a temperature 3 degrees higher than the hybridization temperature. The slides were rinsed briefly with dH_2O to remove salts and air-dried in the dark. Slides were observed under a Polyvar epifluorescent microscope (Reichert&Jung) using the filter blocks G1 (narrowband green, excitation 546/10, DS 580, LP>590) for rhodamine detection and B1(blue, excitation 450-495, DS 510, LP 520) for fluorescein detection.

Hybridization solution: 0.9M NaCl, 20mM Tris-HCl (pH 7.2), 0.01% SDS, various amounts of formaldehyde (0-70%).

2.2.16. Cloning of the glucomannanase gene into an expression vector

ZAP Express™ Predigested Vector Kit from Stratagene was used for the cloning of the glucomannanase gene into an expression vector. In this system DNA is partially digested with Sau3A and the DNA fragments obtained are cloned into a vector based on the λ phage.To obtain randomly cut DNA fragments of a suitable average size, 3μg DNA was digested with various amounts of the restriction enzyme Sau3A for 30 min at 37°C and the size distribution was analyzed on a 0.8% agarose gel.
40μg of DNA were digested with the appropriate amount of Sau3A for 30 min. Ligation and infection of *E. coli* host cells were performed following the instructions of the manual. The plaques from each plate were pooled and the inserts were excised and subcloned into a pBK-CMV phagemid vector according to the manufacturer's instructions. The cells were plated onto LB/ampicillin plates and incubated overnight at 37°C. Duplicate plates were obtained and screened for mannanase positive clones (see section 2.2.16.1.).

2.2.16.1. Screening for mannanase positive clones

with Congo Red:

E. coli cells containing the phagemid with the insert were plated on LB/ampicillin plates. Replica plates on LB plates containing 0.1% Konjac glucomannan were obtained and incubated until colonies appeared. The plates were then incubated overnight at 70°C. The plates were flooded with 1% Congo Red solution for 5min and then destained with 1% NaCl in aqueous solution. Colonies with glucomannanase activity are indicated by a zone of clearing.

with Remazol Brilliant Blue labelled substrate:

Konjac glucomannan was labelled with Remazol Brilliant Blue following the method described by Fülöp and Ponyi (1997). The labelled substrate was added to LB plates (0.1%) and the plates were used for replica plates of the plates with the subclones. After the appearance of colonies the plates were incubated at 70°C overnight and examined for colonies surrounded by zones of clearing.

2.2.17. Southern blotting of DNA onto Hybond membrane

Southern blots were carried out following the protocol in Maniatis (1989).

Agarose gels were run as described in section 2.2.9. To denature the DNA, the gel was soaked in several volumes of 1.5M NaCl and 0.5M NaOH for 1 hour at room temperature. The gel was then neutralized in several volumes of 1M Tris/HCl (pH 8.0) and 1.5M NaCl for an hour at room temperature. Transfer of the DNA to Hybond membrane was accomplished with 10xSSC as transfer buffer. The gel was placed on a piece of 3MM Whatman paper on a piece of Plexiglass. The membrane was carefully placed on the gel to avoid air bubbles. A stack of paper towels and a weight were put on top. The stack was placed in a tray and 10xSSC buffer was filled almost to the top of the plexiglass. Transfer proceeded overnight.
The membrane was dried and the transferred DNA was crosslinked to the membrane by exposure to UV light for 30sec.

2.2.18. Determination of metabolic endproducts and intermediates

2.2.18.1. Sulfide

Sulfide was determined qualitatively by adding 20µl of a saturated lead citrate solution to 1ml of culture. The presence of sulfide was indicated by the precipitation of brown PbS.

2.2.18.2. Elemental sulfur

Elemental sulfur was determined qualitatively by cyanolysis as described by Steinmetz and Fischer (1981).
The sulfur which pelleted below the cells was resuspended in water after removal of the cells, washed twice and dried at 60°C in an incubator. A small amount (~100µg) was put into a test tube containing 1ml H_2O, 0.5ml 1% NaCN and 50µl 1M Tris (pH>10). This was heated at 80°C for 15min. The volume was adjusted to 4.75ml with H_2O and 0.25ml of $Fe(NO_3)_3$-HNO_3 reagent (3g $Fe(NO_3)_3$ and 3.125ml HNO_3 in 10ml H_2O)

were added. Orange colour development after vigorous shaking indicated the presence of elemental sulfur.
Alternatively, cultures were filtered through a 0.2μm filter and the filter was put into a test tube containing 3ml of the sodium cyanide solution. The test was continued as described above.

2.2.18.3. Nitrate and nitrite

Nitrate and nitrite were determined as described by Holding and Collee (1971). Nitrite was determined by adding 1ml of culture supernatant to 0.1ml 1% sulphanilic acid in 5N acetic acid followed by 0.1ml 0.6% dimethyl-α-naphthylamine in 5N acetic acid. Red colour indicated the presence of nitrite. In case of a negative result, the presence of residual nitrate was shown by adding ~1g of zinc dust per ml of culture. Red colour of the supernatant after spinning down the zinc dust indicated the presence of nitrate.

2.2.18.4. End-products of carbon metabolism

Substances like mono-, di- and polysaccharides and organic acids were investigated by running samples on a HPLC.
For sugars and polysaccharides a BioRad HPX.42A column was used with desalted and degassed water as carrier and a refractive index detector for detection. Standards were run at 50mM. Organic acids, as well as single sugars were investigated using a BioRad HPX.87H column (carrier 0.01N H_2SO_4) and refractive index detection. Standards were applied at 50mM. Samples from culture supernatants were filtered through a 0.22μm filter to remove cells.
Intermediates and endproducts of starch degradation were investigated using thin layer chromatography. Substrates at a concentration of 1% in 0.1M acetate buffer (pH 6) were incubated with 200μl of cell extract and incubated at 80°C for 20 hours. For time curves of starch hydrolyses, the total volume was increased and samples were taken at regular time intervals throughout incubation and cooled quickly on ice. 2μl of each sample were run for 3h on silica gel membranes with n-butanol : ethanol : H_2O (4 : 1 : 2) as solvent. Development was carried out by dipping the membrane into $AgNO_3$ solution and spraying it with 0.5M NaOH in aqueous ethanol. Maltose, glucose, maltotriose and maltohexose were run as standards at concentrations of 2.5mg/ml. For concentrations below the detection limit samples were concentrated by freeze-drying and redissolving them in 1/10 of the original volume.

$AgNO_3$ solution: 0.1ml saturated aqueous $AgNO_3$ was diluted to 20ml with acetone and water was added dropwise until $AgNO_3$ redissolved.

2.2.19. Enzyme activity assays for amylase and mannanase

Mannanase assays: Assays to determine the activity of the mannanase from the consortium were carried out by incubating 3ml 0.2% konjac glucomannan in 20mM Mops buffer (pH 7) at 80°C with 50µl of a cell preperation. Alterations to this standard procedure are referred to in the corresponding section.

Enzyme preparations: Exponentially growing cultures were harvested, washed and resuspended in 1/5 volume 20mM Mops buffer. The resuspended cells were sonicated on ice for 5min at the highest energy setting and employed for enzyme assays.
For the investigation of the enzymatic activity in various cell fractions exponentially growing cultures were harvested, washed in 20mM Mops and resuspended in 1/5 volume. The culture supernatant was passed through a 0.22µm sterile filter. The cells were sonicated for 5min at the highest energy setting and centrifuged for 10min at 12000 rpm (Sigma 2-15). The supernatant was removed carefully and the pellet was resuspended in the same volume.

Amylase and pullulanase assays: The activity of amylolytic enzymes was investigated by incubating 0.2% substrate (soluble starch, amylose, amylopectin or pullulan) in 20mM Mops (pH 6.5) at 80°C with 50µl enzyme preparation.

Enzyme activity of the amylase as well as of the mannanase was calculated by measuring the increase of reducing sugars throughout incubation. Concentrations of reducing sugars were determined with PAHBAH assays.

PAHBAH-assay:

1ml of PAHBAH working solution was added to 0.5ml of sample, mixed and boiled for 10min. The tubes were centrifuged for 3min and the extinction was measured at 420nm. The blank consisted of water. Standards were prepared containing 0nmol, 4nmol and 16nmol of glucose.

Working solution:

To 50ml H_2O were added 10ml each of: 0.5M trisodium citrate, 1M Na_2SO_3, 0.2M $CaCl_2$ and 5M NaOH (in this order). 1.54g of p-hydroxybenzoic acid hydrazide was added and the volume was made up to 100ml with H_20.

2.2.20. Polyacrylamide gels for protein analysis and silver stain

Purity and molecular weights of proteins were investigated by running small samples of protein on a polyacrylamide gradient gel with a gradient from 10-15% bisacrylamide. Ready made gels were obtained from Pharmacia Biotech and run in a Pharmacia Phast Gel system.
For denaturing gels, denaturation buffer was added to a protein sample (1vol buffer/2 vol sample) and boiled for 10min. After a brief spin ~1µl of each sample was deposited on the gel using the Phast Gel applicator. The gels were run at 10mA for ~20min at 15°C with SDS in the running buffer. The gels were subsequently silver-stained.

Denaturation and loading buffer: 0.96g SDS, 2ml β-mercaptoethanol, 4.0g glycerol, 0.3g Tris base, 2ml bromophenol blue (0.1% [w/v] in aqueous solution, dissolved in 20ml H_2O (pH 6.8).

Silver stain:

>30min	40% methanol, 10% acetate	
2x15min	20% ethanol, 5% acetate	
5min	8.3% glutaraldehyde	Rinse thoroughly with water
20min	0.25% $AgNO_3$	Rinse thoroughly with water
~20min	0.04% formaldehyde in 2.5% Na_2CO_3	

To stop the staining 5% acetic acid were added. A drop of glycerol was added to prolong storage life. The gels were placed between acetate sheets and dried.

Molecular weight markers: trypsinogen (24kDa), pepsin (34.7kDa), bovine albumin (66kDa) at 10µg/ml each

Chapter 3 - Isolate strain Wai21.S1

3.1. Results

3.1.1. Isolation and purification

Samples were taken from a hot pool on a sunken terrace called Nga Puia o te Papa at Waimangu Volcanic Valley (Fig. 3.1.). The temperature of the original source varied slightly between 80-95°C and the pH was 6.5-7. The spring was quite small, with rocky edges and the water appeared to be clear. The samples were transported quickly to the laboratory without temperature control in Bellco bottles filled to the brim to maintain anaerobicity.

For enrichment 1ml of the sample was injected into Hungate tubes containing 10ml of *Dictyoglomus* medium (see section 2.2.1.), with 5% soluble starch as carbon source and N_2 in the headspace, and incubated at 80°C without shaking.

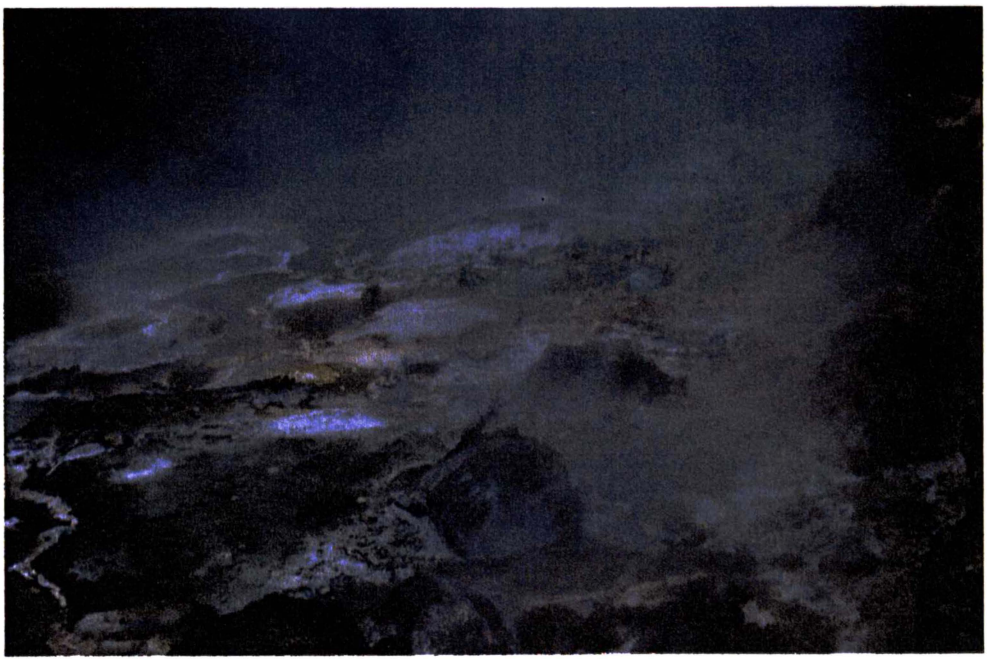


Fig. 3.1. Nga Puia o te Papa, Waimangu Volcanic Valley.

A pure culture was obtained by picking a single colony from roll tubes consisting of *Dictyoglomus* medium solidified with Gelrite (see section 2.2.1.) which were inoculated with 100µl of the enrichment culture. Colonies became visible after 26h - 30h of incubation at 80°C. A single colony was picked and resuspended in 1ml of medium and the procedure was repeated to ensure purification. Colonies were white to creamy coloured and watery (Fig. 3.2.). Four subsequent attempts to enrich for organisms resembling Wai21.S1 from that particular hot spring were successful whereas none of the samples taken from five similar springs at Waimangu Volcanic Valley contained this strain.

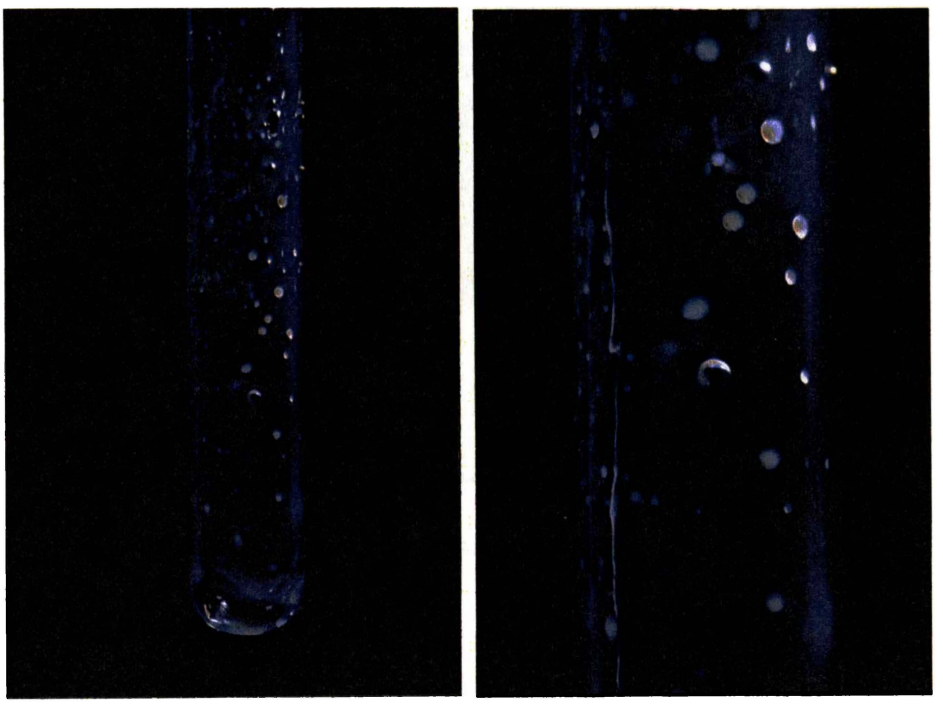


Fig. 3.2. Roll tubes with Wai21.S1 colonies after 30 hours incubation.

3.1.2. Morphology

Phase contrast microscopy and scanning electron micrographs (Figs. 3.3-3.4.) showed a regular coccus of 1-1.5μm in diameter. In growing cultures up to a third of the cells were diplococci indicating cell division by constriction.

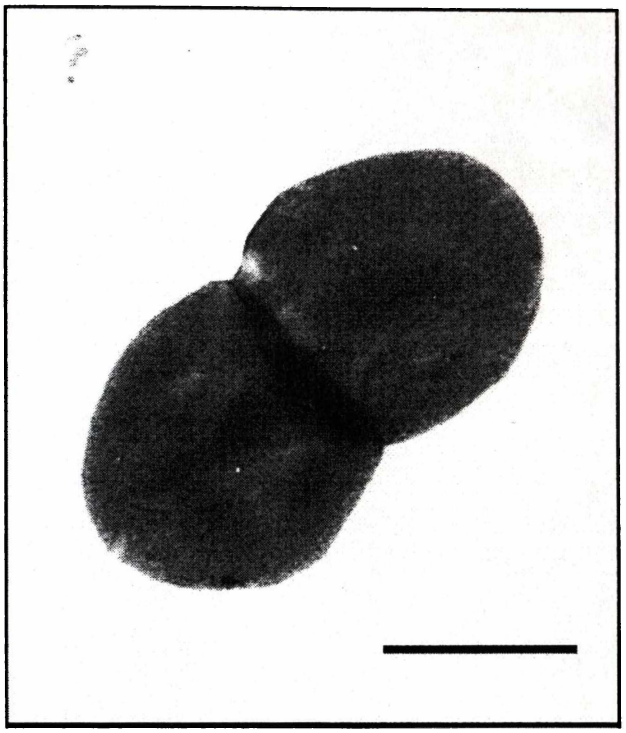


Fig. 3.3. Transmission electron micrograph of dividing Wai21.S1 cells. Bar=1μm. Magnification x43000.

In cultures growing on casein or glycogen, cells with a diameter of up to 5μm could be observed. Cells of cultures in stationary phase (after 20-24h at optimal growth conditions) very often appeared distorted, resembling deflated soccer balls. Flagella were not observed either in flagella stains or by transmission electron microscopy. Wai21.S1 stained Gram negative.

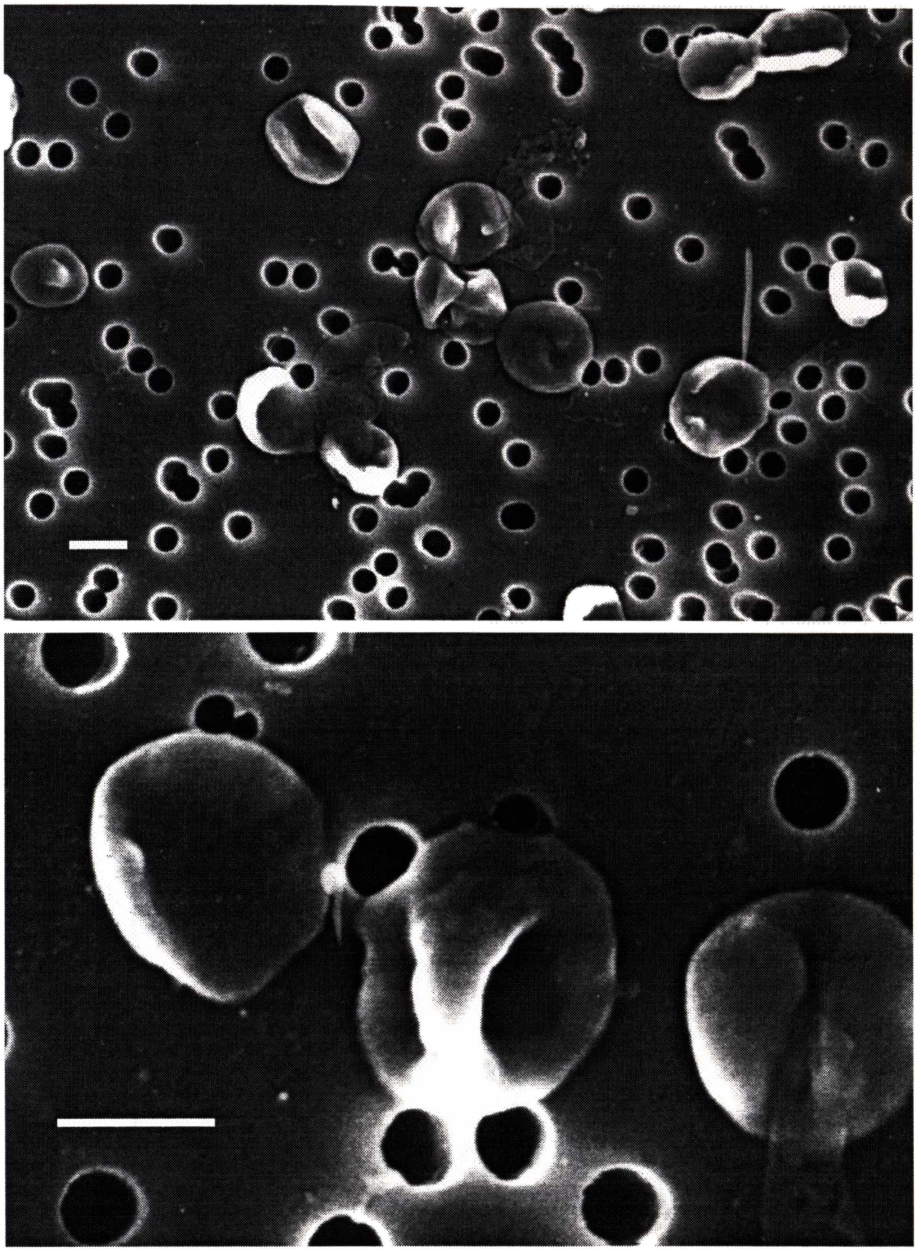


Fig. 3.4. Scanning electron micrographs of Wai21.S1 cells during stationary phase. Bar = 1µm. Magnification x10000 (top), x30000 (bottom).

3.1.3. Growth parameters

3.1.3.1 Oxygen sensitivity

Wai21.S1 was strictly anaerobic. Even traces of oxygen as indicated by resazurin inhibited growth totally. At room temperature nevertheless Wai21.S1 proved to be

less sensitive to oxygen. All inoculations from colonies into liquid medium could be done without working in an anaerobic hood as long as manipulations were performed at room temperature.

3.1.3.2. Temperature optimum and range

All growth experiments were carried out in duplicate. Cultures were grown in *Dictyoglomus* medium with 0.5% starch as carbon source and were incubated at 80°C without shaking. Growth was determined by measuring the optical density at 650nm. Readings were taken every hour for exponentially growing cultures and every two hours during lag phase and after the cultures reached their highest densities.
Growth occurred between 55-93°C with an optimum at 89°C (Fig. 3.5.).

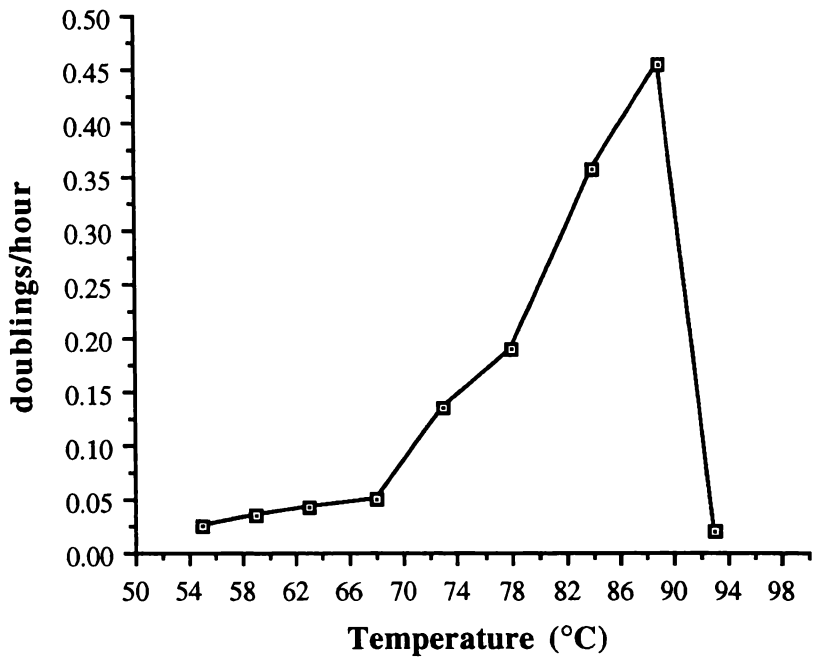


Fig. 3.5. Specific growth rates of strain Wai 21.S1 as a function of temperature.

When cultures reached stationary phase the cells lysed quickly. At higher growth temperature the decrease in cell density was more rapid (Fig. 3.6.). Under phase contrast microscopy the cells of these cultures looked phase transparent and disrupted. All attempts to inoculate new cultures with these cells failed.

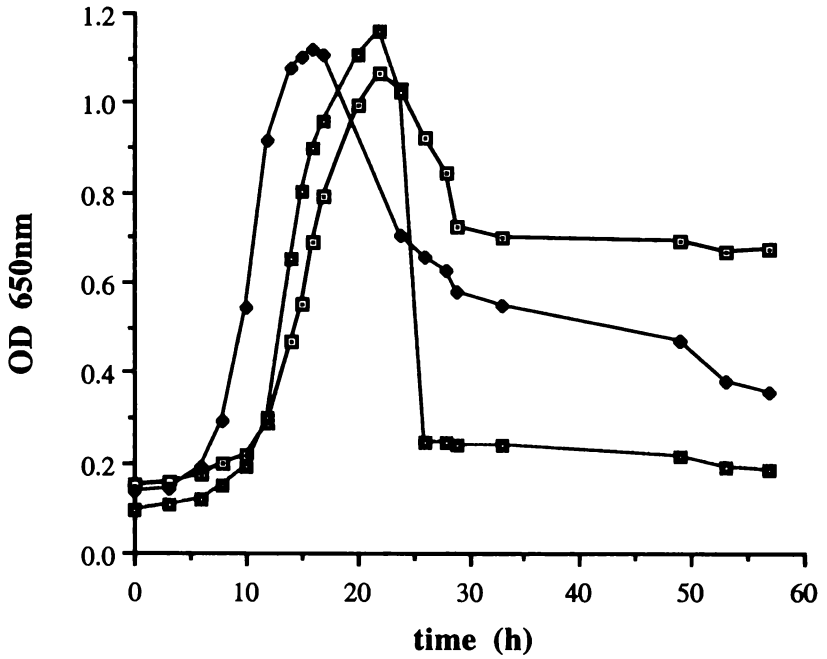


Fig. 3.6. Growth curves of Wai21.S1 cultures at three different temperatures. 93°C (red squares); 89°C (blue diamonds); 86°C (black squares).

3.1.3.3. pH tolerance and optimum

10mM Bis/Tris was used to buffer the medium for the determination of the pH optimum. The pH values of the medium were adjusted at 80°C before degassing and autoclaving.

Wai21.S1. grew in the pH range 4.75 - 7.6 and optimally at pH 5.9. In unbuffered growth medium an average drop of two pH units was observed during growth indicating the production of organic acids during fermentation.

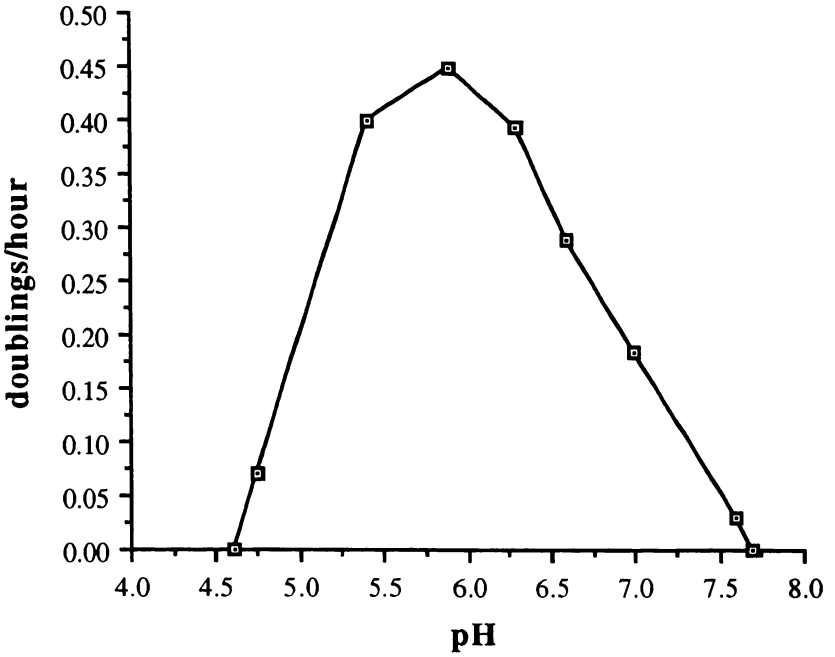


Fig. 3.7. Specific growth rate as a function of pH.

3.1.3.4. Salt requirements

For the determination of the optimum NaCl concentration medium was prepared in which all sodium salts were replaced by the corresponding potassium salts and NaCl was added in various concentrations.

Growth was observed from 0% to up to 1.3% NaCl (222mM Na^+) with an optimum at 0.1% (17mM Na^+) (Fig. 3.8.). Inoculation from the routinely used medium to medium without any sodium led to an extended lag phase after the first transfer but the culture grew up to the same cell density as cultures growing on the regular sodium containing medium.This extended lag phase was not observed after a second and a third transfer into sodium-deprived medium.

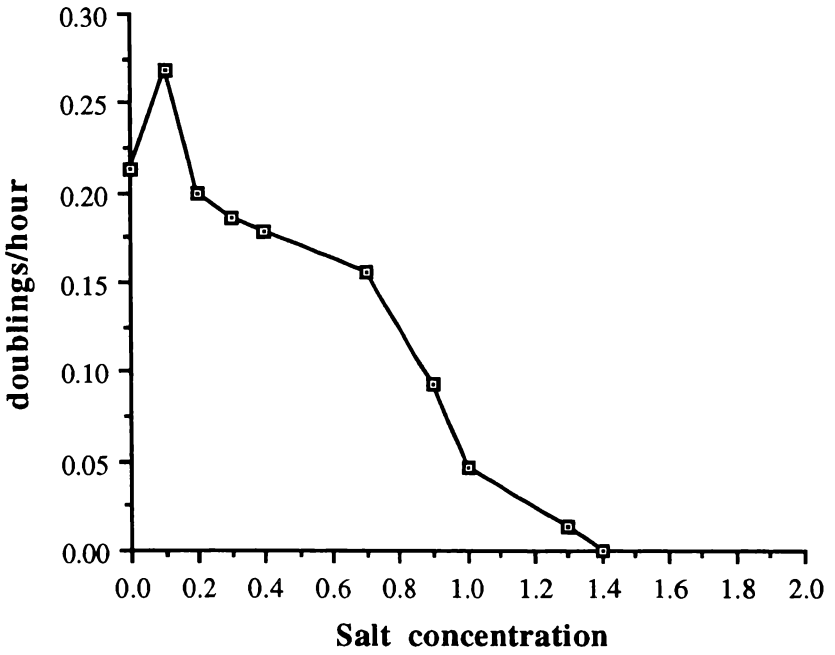


Fig. 3.8. Specific growth rate as a function of salt concentration.

3.1.4. Carbon sources

Carbon sources were tested at a concentration of 0.2% (w/v) with the exception of casamino acids, which were used at 0.02% (w/v) and polymer carbohydrates, which were used at a concentration of 0.5% (w/v). All cultures showing growth were transferred at least two more times to ensure no traces of starch from the original inoculum were left and could lead to false results.

Wai21.S1 showed a definite preference for starch as a source of carbon (Table 3.1.) but growth occurred only with the addition of 0.2% yeast extract and 0.2% tryptone to the medium (Fig.3.1.9.). No growth was observed on yeast extract and tryptone only. Amylose and amylopectin were utilised to a lesser extent than starch whereas neither maltose nor glucose could serve as carbon sources. No growth was observed on glucose, fructose, sucrose, lactose, maltose, mannose, arabinose, ethanol, methanol, pyruvate, formate, succinate, acetate, fumarate, propionate, lactate, L-tryptophan, L-valine, L-glutamine and L-glutamate.

No growth was observed on H_2/CO_2 with any of the tested electron acceptors.

Carbon source	Growth
Starch	++++
Amylose	+++
Amylopectin	+++
Glycogen	+
Maltose	-
Glucose	-
Casein	+
Casamino acids	+
H_2/CO_2	-

Table 3.1. Utilization of different carbon sources by strain Wai21.S1.

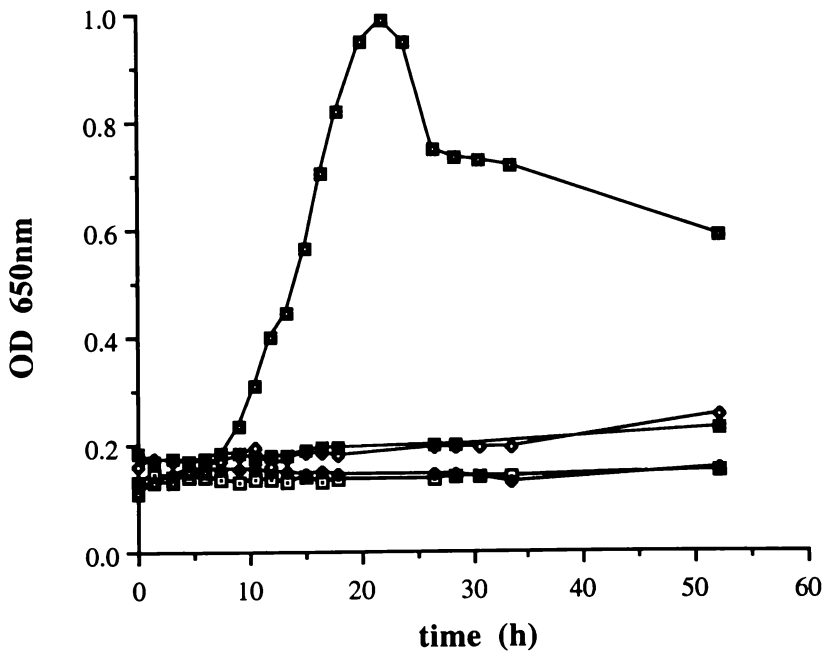


Fig. 3.9. Growth of Wai21.S1 on 0.2% yeast extract (pale blue squares), 0.2% tryptone (dark blue crosses), 0.5% starch (green diamonds) and 0.2% yeast extract, 0.2% tryptone and 0.5% starch (red squares).

3.1.5. Electron acceptors and sulfur sources

Wai21.S1 was able to use cysteine, sulfide and sulfate equally well as sulfur sources. Elemental sulfur did not enhance growth. Wai21.S1 was capable of growing without electron acceptor indicating the ability to carry out some kind of fermentation.

3.1.6 Metabolic endproducts

H_2S was determined by adding 20µl of a saturated lead citrate solution to 1ml of culture. A dense brown precipitate indicated the presence of a high amount of H_2S.

Metabolic endproducts, such as organic acids and volatile fatty acids were investigated by running 50µl samples of culture supernatant taken at regular intervals through an BioRad HPX.87H HPLC column with a refractive index detector. Standards at 50mM were run after every fifth run to ensure that the retention time did not shift. It was not possible to determine the concentrations precisely.
Glucose (t_R: 9.5min), iso-butyric acid (t_R: 19.7 min) and small amounts of acetic acid (t_R: 14.8 min) were detected as the main metabolic endproducts when Wai21.S1 was grown on starch. Concentrations of these substances could not be determined but data from PAHBAH assays which included supernatant from Wai21.S1 cultures suggest that the concentration of glucose in the supernatant is around 1.2mM. One compound with a retention time of 20.58min which was found in significant amounts could not be identified. This compound is not alanine (t_R=8.3min), maltose (8.1), formate (14.1), propionic acid (17.7), n-butyric acid (21.5), iso-valeric acid (24.8) or n-valeric acid (29.5). In unbuffered medium growth led to an acidification of the medium of two pH units.
To investigate starch degradation patterns, samples were run through a BioRad HPX.42A column which separates carbohydrate polymers.
In exponential cultures maltose hexamers, maltose trimers, maltose and glucose were found.

3.1.7. %G+C-contents

The %G+C content of Wai21.S1 and of another *Thermococcus* strain from New Zealand (Wt1) were determined by following the isochromatic change at 260nm

during thermal melting of pure DNA. The DNA used for this experiment was purified in a 1g/ml CsCl gradient and dialysed against water.

The melting temperature of DNA is highly dependend on its purity and the salt concentration of the DNA solution. For this reason the equation used to calculate the %G+C content (see section 2.2.14) corrects for variations in experimental conditions by including an "assumed melting point" based on the %G+C content of the reference DNA. The equation used to calculate the assumed melting point is (Marmur and Doty (1962):

$$T_{ma} = [(\text{mol\%G+C})x\ 0.41]$$

For this experiment DNA from *T. zilligii* was used as a reference. The %G+C content of *T. zilligii* is 46.2 % (Klages and Morgan 1994). The assumed melting point of the DNA at 1x SSC is 88.24°C.

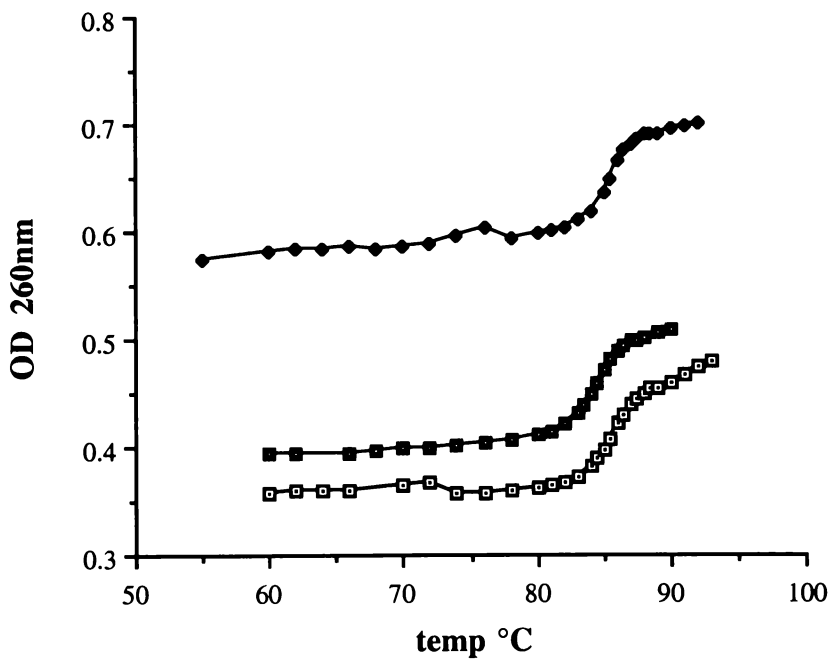


Fig. 3.10. DNA melting curves for *T. zilligii* (black squares), Wai21.S1 (blue diamonds) and isolate Wt1(red squares).

The DNA of Wai21.S1 melted at 86 °C which is the same temperature at which the reference DNA melted. The %G+C content is the same as for *T. zilligii* : 46.2%.

The DNA of strain Wt1 melted at 84.8°C. The %G+C content is 43.2%.

3.1.8. 16S rRNA gene sequence and 16S-23S rRNA genes intergenic region

The 16S rRNA gene was amplified with one universal primer (bf2-21) and an archaea-specific primer (ar1510-1492). The PCR product was subsequently sequenced in three reactions using the same primers and an internal, archaea-specific primer (af515). For sequences of the primers see section 2.2.7.
This sequence obtained (see Fig. 3.11) was deposited in the GenBank and is available under the accession number AF098975.

```
5'-GGGGGTCCGACTAAGCCATGCGAGTCATGGGGCGCCTCGCGCGCACCGGCGGACG
GCTCAGTAACACGTCGGTAACCTACCCTCGGGAGGGGGATAACCCCGGGAAACTGGGG
CTAATCCCCCATAGGTCTGAGGTACTGGAAGGTCCTCAGGCCGAAAGGGACTCTGTCC
GCCCGAGGATGGGCCGGCGGCCGATTAGGTAGTTGGTGGGGTAACGGCCCACCAAGCC
TAAGATCGGTACGGGCCATGAGAGTGGGAGCCCGGAGATGGACACTGAGACAAGGGTC
CAGGCCCTACGGGGCGCAGCAGGCGCGAAACCTCCGCAATGCGGGAAACCGCGACGGG
GGGACCCCCAGTGCCGTGGCATCGCCACGGCTTTTCCGGAGTGTAAGGAGCTCCGGGA
ATAAGGGCTGGGCAAGGCCGGTGGCAGCCGCCGCGGTAATACCGGCGGCCCGAGTGGT
GGCCACTATTATTGGGCCTAAAGCGTCCGTAGCCGGGCCCGTAAGTCCCTGGCGAAAT
CTCACGGCTCAACCGTGGGGCTCGCTGGGGATACTGCGGGCCTTGGGACCGGGAGAGG
CGGAGGGTACCCCTGGGGTAGGGGTGAAATCCTATAATCCCAGGGGGACCGCCAGTGG
CGAAGGCGCTCCGCTGGAACGGGTCCGACGGTGAGGGACGAAGGCCAGGGGAGCAAAC
CGGATTAGATACCCGGGTAGTCCTGGCTGTAAAGGATGCGGGCTAGGTGTCGGGTGAG
CTTCGGGCTCGCCCGGTGCCGGAGGGAAGCCGTTAAGCCCGCCGCCTGGGGAGTACGG
CCGCAAGGCTGAAACTTAAAGGAATTGGCGGGGGAGCACCACAAGGGGTGGAGCGTGC
GGTTTAATTGGATTCAACGCCGGGAACCTCACCGGGGGCGACGGCAGGATGAAGGCCA
GGCTGAAGGTCTTGCCGGACACGCCGAGAGGAGGTGCATGGCCGCCGTCAGCTCGTAC
CGTGAGGCGTCCACTTAAGTGTGGTAACGAGCGAGACCCGCGCCCCCAGTTGCCAGTC
CTCCCCGCTGGGGAGGAGGCACTCTGGGGGGACCGCCGGCGATAAGCCGGAGGAAGGA
GCGGGCGACGGTAGGTCAGTATGCCCCGAAACCCCCGGGCTACACGCGCGCTACAATG
GGCGGGACAATGGGATCCGACCCCGAAAGGGGAAGGGAATCCCCTAAACCCGCCCCCA
GTTCGGATTGCGGGCTGCAACTCGCCCGCATGAAGCTGGAATCCCTAGTACCCGCGTG
TCATCATCGCGCGGCGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCACTCCACC
CGAGCGGGGTCCGGGTGAGGCTTGGTCTCCCTTCGGGGAGGCCGGGTCGAGCCCGGGC
TCCTGAGGG-3'
```

Fig. 3.11. 16S rDNA gene sequence of Wai21.S1.

The intergenic region of all four investigated strains was about 300 nucleotides long. They were fully sequenced using primer Rosk16SF-D1. Comparison of the obtained sequences showed that the intergenic regions of *T. zilligii* and Wai21.S1 are identical, whereas Wt1 is 99.3% similar to these two strains. (see Appendix 3 for alignments).

```
5'-ACCTCCTATCGCCGGAAATCCCGTCCGGGGGGTTTAAGGGATGTCGGGCCTGCCA
CGGGTGGGCCGGTAGCTCAGCCTGGTATGAGCGCCGCCCTTGCAAGGCGGAGGCCCCG
GGTTCGAATCCCGGCCGGTCCACCACGAAGAGGTGCACATCCCGAGCCAGGCTCGGGG
TGGAAGGGCCTGAGACCCCGAACAGGGGTCACGGTGAGGGCCGTGCACAGGCGAGCTG
GTCCAGGAAACGTCCCGCCCGGTGGCAACTAAGCCACCTGGTGGATGGCTCGGCTCGG
GA-3'
```

Fig. 3.12. Sequence of the 16S-23S intergenic region of Wai21S and *T. zilligii*.

```
5'-ACCTCCTATCGCCGGAAATCCCGTCCGGGGGGTTTAAGGGATGTCGGGCCTGCCA
CGGGTGGGCCGGTAGCTCAGCCTGGTATGAGCGCCGCCCTTGCAAGGCGGAGGCCCCG
GGTTCGAATCCCGGCCGGTCCACCACGAAGAGGTGCACATCCCGAGCCAAGCTCGGGG
TGGAAGGGCCTGAGACCCCGAACAGGGGTCACGGTGAGGGCCGTGCATAGGCGAGCTG
GTCCAGGAAACGTCCCGCCCGGTGGCAACTAAGCCACCTGGTGGATGGCTCGGCTCGG
GA-3'
```

Fig. 3.13. Sequence of the 16-23S intergenic region of Wt1.

The sequences were compared with other available 16S rRNA gene sequences using BLAST search. The 16S rRNA gene of Wai21.S1 showed a very high homology - 99% - to that of *T. zilligii* and close homology with the 16S rRNA gene sequences of most of the other *Thermococcus* species.

	16S rRNA gene	16S-23S intergenic region
Strain RT3	98%	d.n.a.
Strain Wt1	d.n.a	99.3%
T. zilligii	99%	100%
T. celer	97%	84.6%
T. gorgonarius	96.8%	d.n.a.
T. pacificus	97.6%	d.n.a.
T. stetteri	97%	94%
T. hydrothermalis	97%	83.7%
T. litoralis	92.1%	d.n.a.
T. chitonophagus	96%	d.n.a.
T. profundus	98%	90.5%
T. fumiculans	95%	d.n.a.
T. barophilus	96%	d.n.a.
T. mexicalis	97%	85.8%
P. furiosus	95.8	85.5%

Table 3.2. Homologies of strain Wai21.S1 gene sequences with other Thermococcaceae species. (d.n.a.: data not available)

3.1.9. DNA-DNA hybridization

DNA from all the tested organisms was blotted on Hybond membranes and each membrane carrying DNA from each strain was hybridized with the DNA of one of the organisms. The amount of hybridization was measured by counting radioactivity by liquid scintillation (see section 2.2.13.). The radioactive decay of each sample was counted for 40min. For each hybridization a background (a piece of membrane of the same size as the membrane containing the DNA blots) was determined and this value was subtracted from the number of counts of a DNA sample.

	Wai21.S1		*T. zilligii*		Wt1		*T. celer*	
	C	% S	C	% S	C	% S	C	%S
Wai21.S1	1171	100	34	2.3	645	19.3	98	7.5
T. zilligii	62	5.2	1465	100	757	22.6	77	5.9
Wt1	496	42.3	975	66.5	3335	100	83	6.4
T. celer	70	5.9	161	10.9	760	22.7	1296	100
E. coli	16	1.3	159	10.85	366	10.9	43	3.3

Table 3.3. Data for the DNA/DNA hybridization WAi21.S1 with various *Thermococcus* strains and *E. coli*.
C: detected scintillation events; %S: % similarity.

The hybridization results indicated that Wai21.S1 has a high similarity with strain Wt1 which is an isolate from Champagne Pool, Waiotapu. The similarity of 42.3% in the assay with Wai21.S1 DNA as probe DNA put Wt1 close the species limit of Wai21.S1 whereas the similarity in the assay with Wt1 DNA as probe DNA put it outside the species threshold of 70% similarity. *T. zilligii* was well outside the species limit of Wai21.S1 but shows high similarity with Wt1. The control hybridization with DNA of the *Thermococcus* type showed the expected result. Differences in similarity values occur when probe and target DNA roles are reversed. This phenomenon is due to slight differences in hybridization and washing temperatures, which are based on the %G+C content of the probe DNA.

3.1.10. The amylolytic activities of Wai21.S1

3.1.10.1. Location of amylolytic activities

The amylolytic activity of Wai21.S1 was investigated by incubating appropriate substrates in 50mM Mops buffer (pH 6.6) with various cell fractions and measuring the increase in reducing sugars with a PAHBAH assay (see section 2.2.19)
Several culture fraction were checked for various amylolytic activities. The investigated fractions were: culture supernatant, cell wash buffer, cytoplasmic fraction (supernatant of cells after sonication) and cell wall fraction (pellet of the sonicated cells).
The various amylolytic activities were tested with the following substrates:
Amylose: α-amylase activity (1,4-α-D-glucanglucanohydrolase);
Pullulan: pullulanase activity (1,6-α-glucanohydrolase).
Enzymatic assays were incubated at 80°C and samples were taken at regular intervals and their content of reducing sugars was determined. Samples containing the substrate and culture fraction in the same concentrations as the assays were determined without being incubated to determine the background sugar concentration of the assay. Substrate without enzyme was incubated in order to check for thermal degradation.

In a first attempt to locate the amylolytic activity in Wai21.S1 cells, enzymatic assays were incubated for only 30 minutes. Amylolytic activity was found mainly in the cell wall fraction and consisted primarily of α-1,6-glucosidase activity. About 10% of the total activity of this fraction was α-1,4-glycosidic (see Fig. 3.14). Some α-1,6-glucosidase activity was also found in the cytoplasmic fraction. Like the cell wall fraction, this fraction exhibited some α-1,4-glycosidic activity. Little activity was found in the culture supernatant and this consisted mainly of α-1,4-glycosidase activity. The cell wash buffer showed no activity.

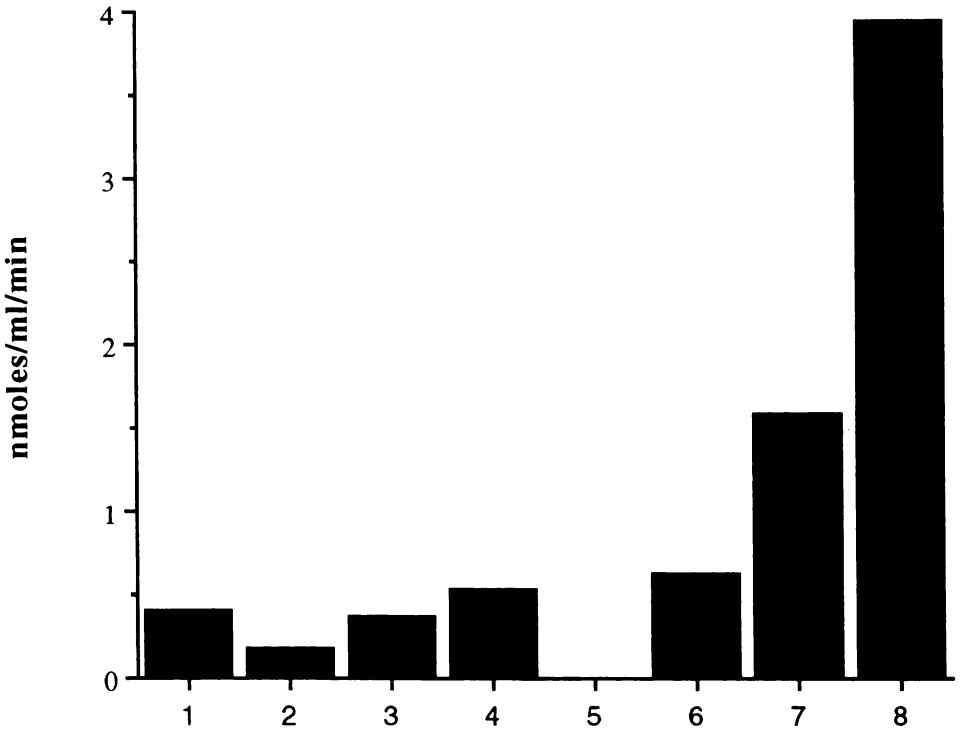


Fig. 3.14. Activity of various culture fractions of Wai21.S1 on amylose and on pullulan.
1. Culture supernatant on amylose; 2. Cell wash on amylose; 3. Cytoplasmic fraction on amylose; 4. Cell pellets after sonification on amylose; 5. Culture supernatant on pullulan; 6. Cell wash on pullulan; 7. Cytoplasmic fraction on pullulan; 8. Cell pellets after sonification on pullulan;

When the assays were incubated for longer however, it became obvious that the cytoplasmic fraction contained an enzyme that cleaved amylase and pullulan equally well, whereas the cell wall fraction contained an enzyme which preferentially cleaved α-1,6-glycosidic bonds (see Fig.3.15). The supernatant was filtered through a 0.2μm filter and concentrated 5-fold with Centricon filters (10000Da cut off) but still no significant activity other than a little α-1,4-glycosidase activity could be detected. The filter paper used to filter the supernatant was soaked in Mops buffer and tested for activity in case extracellular enzyme was bound to starch and removed from the supernatant by filtering. No activity was found in this fraction either.
On starch the cytoplasmic fraction showed a high activity whereas the activity of the cell wall fraction against starch was only about 40% of the activity found in the cytoplasmic fraction.

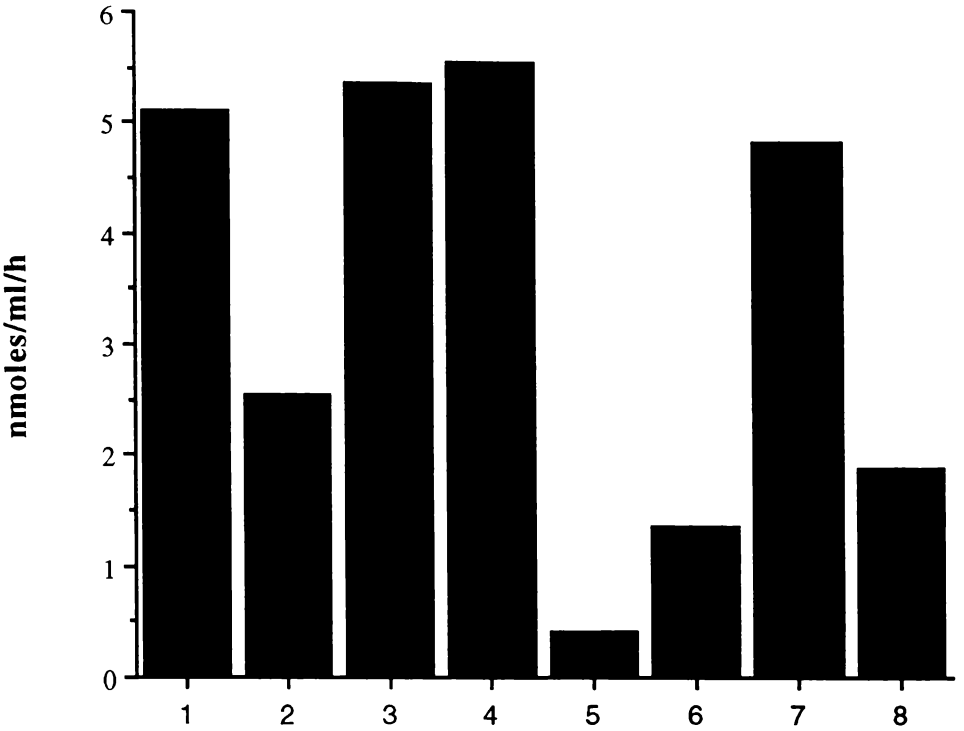


Fig. 3.15. Activity of various cell fractions on various substrates during a 20h incubation.
1. Cytoplasmic fraction on amylose; 2. Cell wall fraction on amylose; 3. Cytoplasmic fraction on pullulan; 4. Cell wall fraction on pullulan; 5. Culture supernatant on pullulan; 6 Culture supernatant on amylose; 7. Cytoplasmic fraction on starch; 8. Cell wall fraction on starch.

3.1.10.2. Identification of endproducts and intermediates

The mode of action of the amylolytic enzymes was investigated by separating maltose oligomers from samples taken throughout enzymatic assays on amylose and pullulan by thin layer chromatography. Reducing sugars were stained with the $AgNO_3$/NaOH method as described in section 2.2.18.4.

Due to the low concentration of the reducing sugars in the sample taken it was difficult to distinguish stains from the background especially in early samples.

For both the cytoplasmic fraction and the cell wall fraction on pullulan it was impossible to follow the degradation of the pullulan due to insufficient detection of intermediates. It can nevertheless be shown that glucose, maltose and small maltose oligomers accumulate in later samples (Fig. 3.16.). In the sample taken after 90 minutes of incubation, a faint spot indicates the presence of maltose hexamers, which is still present after 180 minutes incubation. In this sample maltose and glucose can

also be identified. After 225 minutes the concentration of maltose and glucose increases. After 20h the main products are glucose and maltose but maltose oligomers up to hexamers are still present.

The TLC with samples from the incubation of cell wall fraction on pullulan did not stain well. In the latest sample (20h) only, glucose and maltose oligomers up to hexamers could be identified (Fig. 3.17.).

The cytoplasmic fraction on amylose produces increasingly short chains of maltoseoligosaccharides throughout incubation. Maltose oligomers longer than hexamers are present in the samples at 45, 90 and 135 minutes (Fig. 3.18.). In the sample taken at 180 minutes maltose hexose, maltose triose and maltose can be found. After 225 minutes glucose is present in addition and after 20 hours of incubation a variety of maltose oligomers, maltose and glucose are present.

For the samples of amylose incubated with cell wall fraction the TLC was barely readable (Fig. 3.19.). An increase in glucose, maltose and maltotriose during the late samples can be seen.

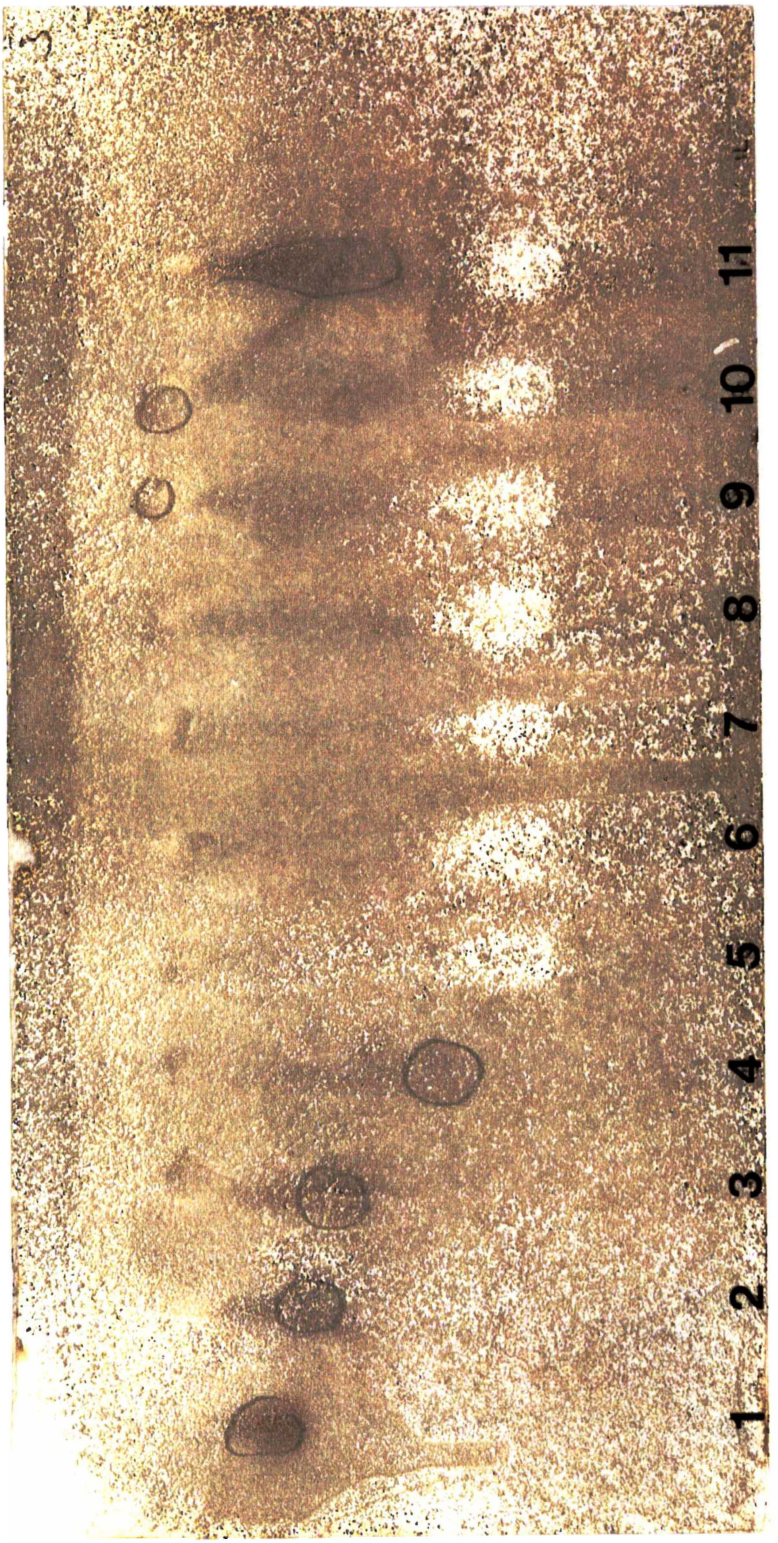


Fig.3.16 Separation of breakdown products of pullulan degradation after incubation with a cytoplasmic fraction of strain Wai21.S1.
Lane 1: glucose ; lane 2: maltose; lane 3: maltotriose; lane 4: maltohexose; lane5: sample 0min, lane 6: sample 45min; lane7: sample 90min; lane 8: sample 135min, lane 9: sample 180min; lane10: sample 225min; lane 11: sample 20h.

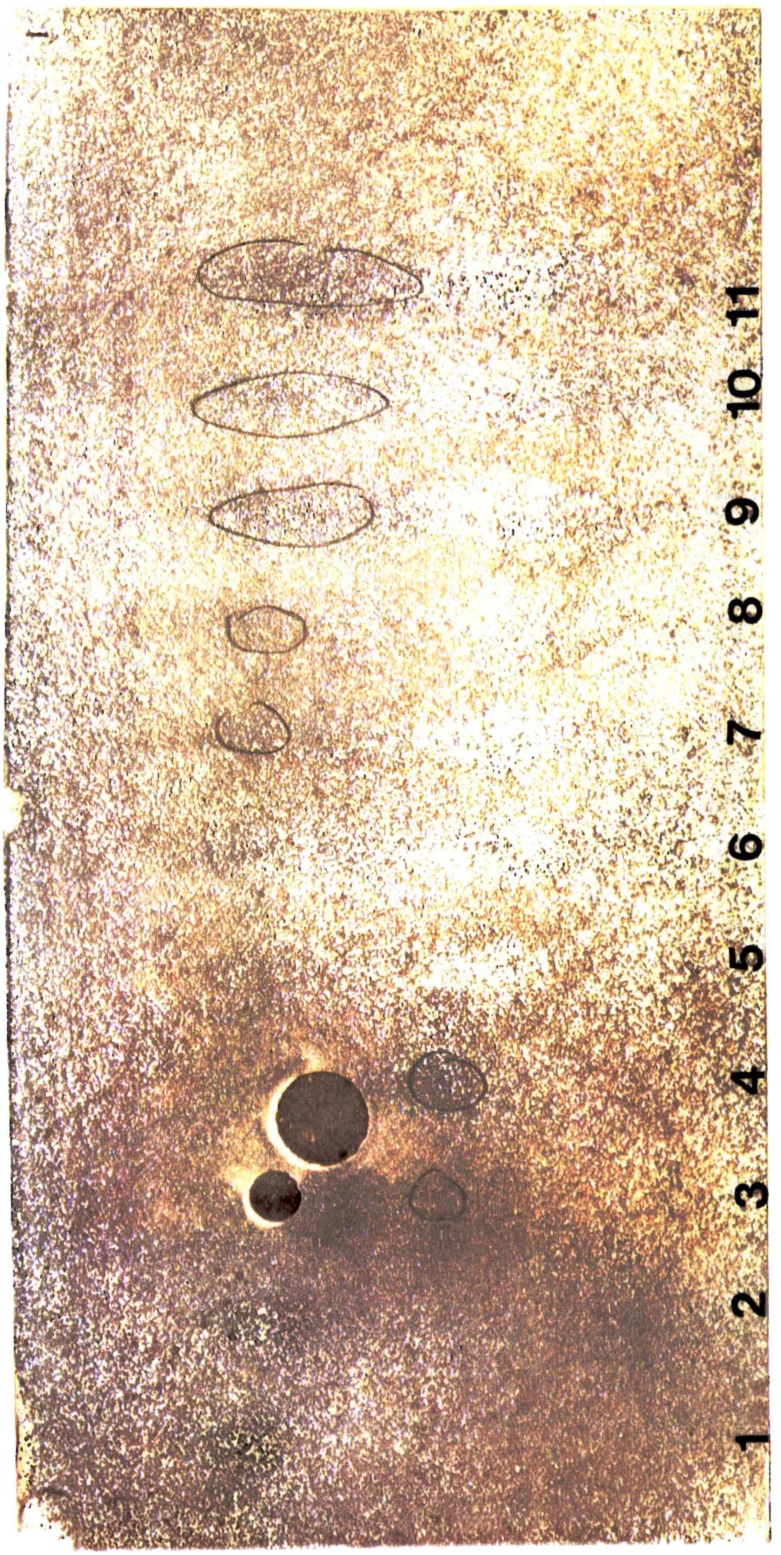


Fig.3.17. Separation of breakdown products of pullulan degradation after incubation with a cell wall fraction of strain Wai21.S1.
Lane 1: glucose ; lane 2: maltose; lane 3: maltotriose; lane 4: maltohexose; lane5: sample 0min, lane 6: sample 45min; lane7: sample 90min; lane 8: sample 135min, lane 9: sample 180min; lane10: sample 225min; lane 11: sample 20h.

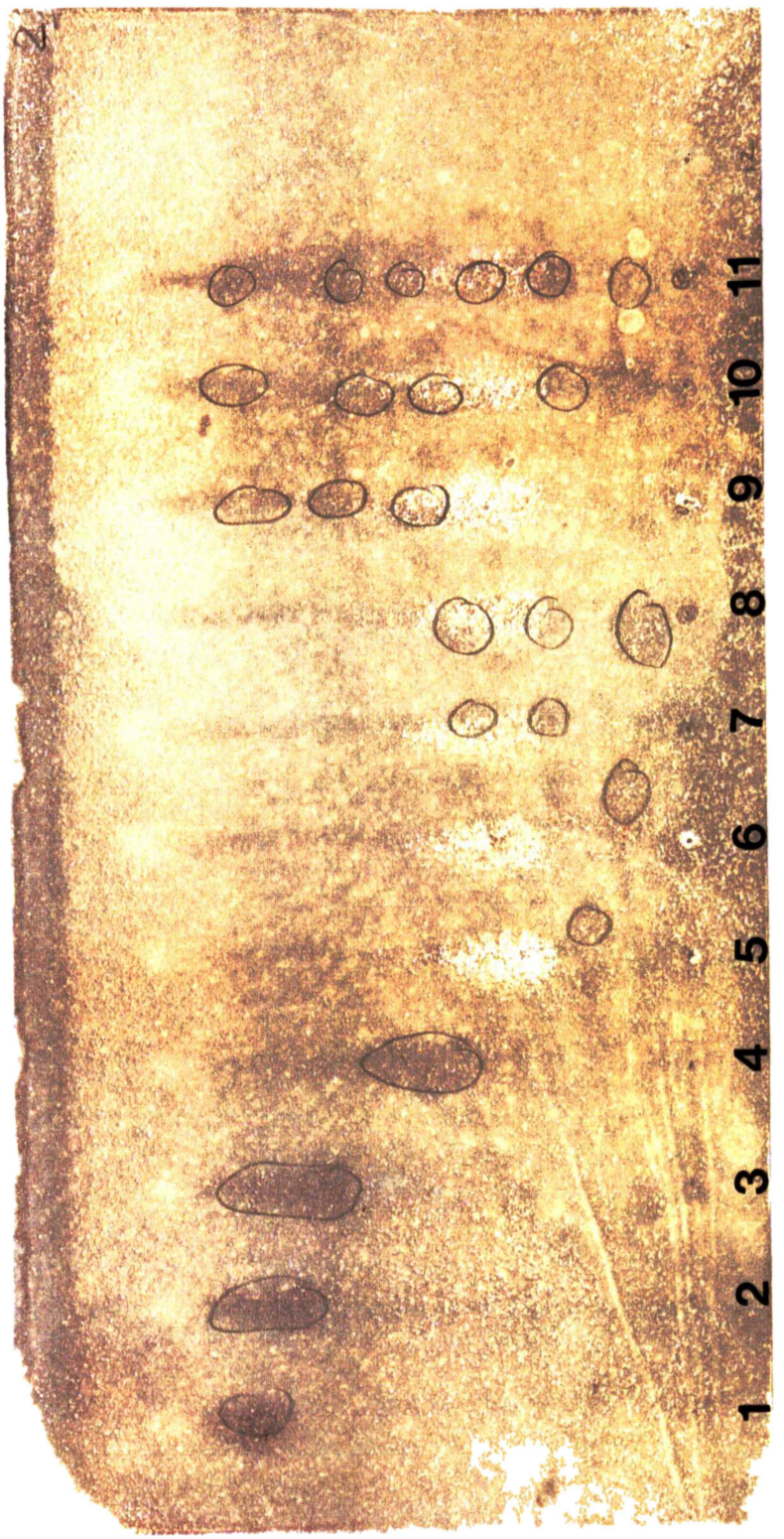


Fig.3.18. Separation of breakdown products of amylose degradation after incubation with a cytoplasmic fraction of strain Wai21.S1.
Lane 1: glucose ; lane 2: maltose; lane 3: maltotriose; lane 4: maltohexose; lane5: sample 0min, lane 6: sample 45min; lane7: sample 90min; lane 8: sample 135min, lane 9: sample 180min; lane10: sample 225min; lane 11: sample 20h.

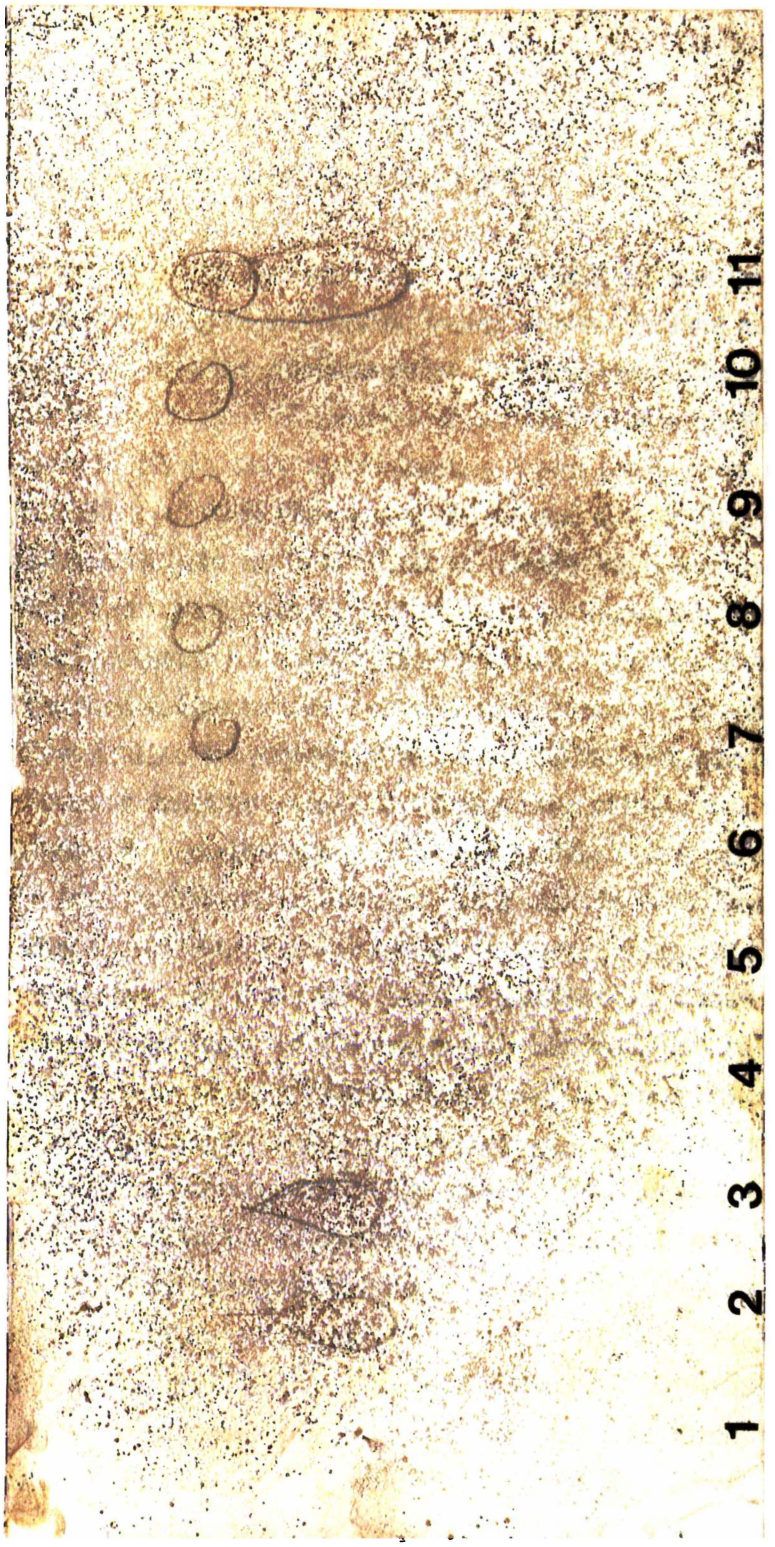


Fig.3.19. Separation of breakdown products of amylose degradation after incubation with a cell wall fraction of strain Wai21.S1.
Lane 1: glucose ; lane 2: maltose; lane 3: maltotriose; lane 4: maltohexose; lane5: sample 0min, lane 6: sample 45min; lane7: sample 90min; lane 8: sample 135min, lane 9: sample 180min; lane10: sample 225min; lane 11: sample 20h.

3.2. Discussion

3.2.1. The ecology of strain Wai21.S1

The novel hyperthermophilic *Thermococcus* strain Wai21.S1 was isolated from a terrestrial hot spring in Waimangu Volcanic Valley in New Zealand and is only the second fresh water isolate of the abundant genus *Thermococcus* to be described. The only other fresh water isolate - *T. zilligii* - was found in Kuirau Park, Rotorua (Klages and Morgan 1994), only approximately 30km away from the place of isolation of Wai21.S1. Two more *Thermococcus*-like strains have since been isolated from terrestrial hot springs in New Zealand. Strain Wt1 was isolated from Champagne Pool, Waiotapu (Frank Robb, University of Maryland, Baltimore, MD, USA, pers. comm.) and strain RT3 was isolated from a hot spring in Rotorua (Bharat Patel, Griffith University, Queensland, Australia, pers. comm.). The four fresh water isolates from New Zealand are physiologically very similar and genetically closely related. This discussion therefore focuses on a comparison of these strains.

No other fresh water isolates belonging to the family Thermococcaceae have been reported so far from terrestrial geothermal areas outside New Zealand despite extensive investigation of some terrestrial geothermal areas (Barns et al. 1994). It is nevertheless impossible to say if the New Zealand geothermal situations are the only places where freshwater strains of *Thermococcus* can be found since extensive geothermal fields in eastern parts of Africa, in Asia and in South America have not been studied to any great extent to date and we can not discount the possibility that more concerted efforts in locations such as Yellowstone Park, Japan or Iceland would not be positive. It is reported for many marine geothermal situations, hydrothermal vents as well as shallow solfataric habitats, that the most frequent isolates are members of the family Thermococcaceae (Wolfram Zillig, Max-Planck-Institut für Biochemie, Martinsried, pers. comm.; Francesco Canganella, University of Tuscia, Viterbo, Italy, pers. comm.). Recently seven new *Thermococcus* strains were isolated from the subseafloor of the North Gorda Ridge, North East Pacific which were released into the sea water during an eruption (Summit and Baross 1998). In marine geothermal environments, Thermococcaceae are ubiquitous whereas fresh water isolates seem to be strictly limited to New Zealand hot springs.

3.2.2. The morphology of Wai21.S1

The morphological characteristics of strain Wai21.S1 are consistent with the characteristics of the genus. Like all known *Thermococcus* species, Wai21.S1 is a regular to slightly irregular coccus with a high frequency of diploforms, which occur especially during the log phase of growth of a culture, indicating duplication by restriction. In an actively growing culture all stages of this restriction division can be observed. Very occasionally two cells still attached by a confined string can be seen. Wai21.S1 has no flagella and is not motile, like the other fresh water *Thermococcus* isolate from New Zealand, *T. zilligii* (Klages and Morgan 1994; Ronimus et al. 1997). Refractile granular bodies were reported for *T. zilligii* cells grown with S^0 in the medium. This was not observed for Wai21.S1 when it was grown with S^0 nor is this reported for any other *Thermococcus* species. In cells of the closely related species *Pyrococcus furiosus* crystalline structures, which might be similar to the granular bodies in *T. zilligii*, were occasionally found (Erauso et al. 1993).
As reported for many species of this genus, the phase-dense cells of Wai21.S1 become increasingly phase-transparent throughout late log phase and the stationary phase. Under the phase contrast microscope a high percentage of these cells clearly appeared to have lysed. Cultures at this stage of growth are rapidly losing optical density and can not be used as inoculum for fresh cultures. The velocity with which the cells lose their viability and the percentage of cells that appear to be damaged is highly dependent on the temperature at which the cultures are grown. Cell death occurs faster at higher temperatures (see Fig. 3.6.), even if the temperature is close to the optimum temperature for growth. This indicates that either only metabolising cells are capable of withstanding the high temperatures which are nevertheless optimal for rapid growth, or that the high amount of H_2S which accumulates as a metabolic endproduct might be toxic for the cells. The concentration of H_2S should nevertheless be the same for cultures grown above 80°C since they grow up to about the same cell density with the production of similar amounts of H_2S which should have the same damaging effect. Probably slower growing cultures are capable of some kind of detoxification which faster growing cultures cannot perform in the same way. This phenomenon of rapid cell lysis at high temperature and the high proportion of unculturable cells under non-optimal conditions was reported for *T. peptonophilus* as well. The unculturable phase is here interpreted as a physiologically dormant phase to resist unfavourable conditions such as too low or too high temperatures, the presence of oxygen, change of pH etc. (Gonzáles et al. 1996). The original hot spring from which Wai21.S1 was isolated is an at some times violently bubbling spring with a little overflow to lower lying hot pools which have in general different characteristics

that would make a dormant phase useful to ensure a continuous habitation of this rapidly changing environment.

3.2.3. The physiological characteristics of Wai21.S1

Wai21.S1 is a strictly anaerobic and heterotrophic organism. Both characteristics are typical for all known members of the order Thermococcales. Even traces of oxygen inhibit growth totally. As reported for all *Thermococcus* species and for some of the other thermophilic archaea, Wai21.S1 is far less sensitive to oxygen at low temperatures. It was possible to perform all transfers from plates to Hungate tubes under aerobic conditions as long as the plates were kept at room temperature. This ability to withstand oxygen under conditions which are not suitable for growth might provide thermophilic organisms with the possibility to survive sudden changes in their habitats and is discussed for marine thermophilic archaea as a mode to travel long distances in cold, oxygenic sea water and populate isolated hot springs.

Wai21.S1 grows on starch and to a lesser extent on amylose, amylopectin, glycogen and casamino acids in the presence of yeast extract and tryptone. Proteinaceous compounds alone do not support growth whereas *T. zilligii* grows preferentially on yeast extract or tryptone and is not capable of utilizing starch (Klages and Morgan 1994). Maltose and glucose are not used as carbon sources by Wai21.S1. It is nevertheless difficult to say if that is due to a lack of the ablity to utilise these compounds or to their high thermal instability. Attempts to grow Wai21.S1 on single sugars by adding the sugars at low concentrations at regular short time intervals failed, indicating that Wai21.S1 might not be capable of utilizing glucose and maltose. Most *Thermococcus* species grow exclusively on peptides; a few are able to grow on complex carbohydrates. In contrast to Wai21.S1, two of the other amylolytic *Thermoccus* species, *T. litoralis* and *T. profundus*, are capable of growing on maltose as well (Kobayashi et al. 1994; Neuner et al. 1990). Many *Thermococcus* species have a requirement for one or more amino acids or are able to grow exclusively on amino acids (Dirmeier et al. 1998). Amino acid requirements for Wai21.S1 have not been determined but the dependency on traces of yeast extract indicate the need for one or more compounds from yeast extract which might easily be amino acids.

Wai21.S1 is clearly distinguished from the other *Thermococcus* species by having no need for NaCl. In medium where all the sodium salts had been replaced by the equivalent potassium salts, growth appeared not to be affected beyond a slightly extended lag phase. A second transfer into sodium free medium produced cultures growing almost as rapidly to the same high cell densities as cultures in medium containing small amounts of sodium chloride. The salt concentration for optimum

growth lies between 0-0.1% (see Fig. 3.8.) which is slightly lower than the optimum salt concentration for *T. zilligii* and much lower than for all the marine *Thermococcus* species (Zillig 1991). *T. zilligii* is not able to grow without at least 0.05% NaCl (Klages and Morgan 1994).

For many members of the genus *Thermococcus* elemental sulfur is either obligately required or at least enhances growth considerably by between 4-10 fold. For *T. zilligii* it is reported that S^0 can be replaced by various compounds such as L-cysteine or oxidized glutathione (Klages and Morgan 1994). Wai21.S1 does not require any elemental sulfur and even seems to be slightly inhibited by it. This has not been reported for any other *Thermococcus* species to date.

The optimum growth temperature of Wa21.S1 of 89°C is one of the highest among the *Thermococcus* species and about ten degrees higher than the optimum temperature of *T. zilligii*. The optimum pH of 5.9 is one of the lowest in the genus. Only *T. celer* has a similar low pH optimum (Zillig et al. 1983). The optimum pH for growth of *T. zillligii* at 7.4 is about 1.5 units higher than that of Wai21.S1.

Wai21.S1 produces large quantities of H_2S during growth. Since H_2S is toxic for many organisms at certain concentrations this might be one reason for the rapid decline in viable cells in aging cultures. Besides sulfide, Wai21.S1 produces acetic acid, n-butyric acid and high quantities of an as yet unidentified substance. This unknown substance is not one of the organic acids which are frequently produced by other *Thermococcus* species. All *Thermococcus* species are known to ferment proteinaceous substrates or carbohydrates to organic acids (Zillig 1991), especially acetate, propionate, iso-butyrate and isovalerate, and H_2S, and sometimes also CO_2 or H_2. The production of organic acids leads to an acidification of the medium if it is unbuffered. During growth, maltose hexamers, maltose trimers, maltose and glucose could be detected using the BioRad HPX.42A HPLC column.

3.2.4. The amylolytic activity of Wai21.S1

The enzymatic degradation of starch is accomplished by the concerted action of a variety of enzymes. The most important of these are α-amylases which are endoacting, the exoacting β-amylases and pullulanases which cleave the α-1,6-glycosidic bonds of the branches of amylopectin. Amylolytic enzymes are frequently found in members of the genus *Thermococcus* and have been extensively studied. Many of the enzymes involved with the initial steps of the degradation of complex carbohydrates are extracellular since it is easier to transport smaller fragments into the cells than a large molecule. Extracellular α-amylases are reported for all of the amylolytic members of the order*Thermococcales* such as *Pyrococus woesei* (Koch et

al. 1991), *P. furiosus* (Brown et al. 1990) or *Thermococcus profundus* (Chung et al. 1995). These enzymes are generally extremely thermostable and highly active. For *P. furiosus* several amylases have been reported, including extracellular as well as intracellular ones. It is surprising that only a low amylolytic activity could be found in the culture supernatant of Wai21.S1 despite a five fold concentration of the supernatant and the removal of sugars from the supernatant which could inhibit an amylolytic enzyme. All assays were done aerobically which might cause inactivation of oxygen sensitive enzymes. None of the reported amylolytic enzymes from members of the family Thermococcaceae is known to be sensitive to oxygen. It is possible that the low α-1,4-glycosidic activity which was found in the culture supernatant sufficiently degrades starch to transport the degradation products into the cell.

The cell wall fraction harboured an enzyme which predominantly cleaved α-1,6-bonds but about 40% of its total activity was an α-1,4-glucosidase activity. The pullulan used in the enzyme assays consists of α-1,4-linked maltotriose units which are α-1,6-glycosidic linked. As was shown by thin layer chromatography pullulan gets degraded by the cell wall fraction into mainly glucose and maltose which further demonstrates the presence of two different enzymatic activities in this fraction since pullulan is only capable of cleaving β-1,6 glycosidic bonds. Pullulanase activity alone would not produce maltose and glucose as the only degradation products.

The cytoplasmic fraction contained an enzyme with both pullulanase and amylase activity. The enzyme in the cytoplasmic fraction shows the same ratio of pullulanase to α-amylase activity. There are many reports about amylopullulanases found in archaea and bacteria which have α-1,4-glucosidase activity as well as α-1,6-glucosidase activity (Brown and Kelly 1993; Ganghofner et al. 1998).

3.2.5. Genetic analysis of the isolate Wai21.S1

The %G+C content of Wai21.S1 is about 46.2 which is the same as the %G+C of *T. zilligii* (Klages and Morgan 1994). For another terrestrial *Thermococcus* isolate from New Zealand, Wt1, the %G+C was slightly lower, about 43.2%. In general the %G+C in this genus lies higher. The recently isolated *T. barossii* has a %G+C of 60 (Duffaud et al. 1998) and most of the other *Thermococcus* spp. have %G+C contents above 50. The *Pyrococcus* species *P. abyssi* and *P. horikoshii* have similarly low %G+C like the terrestrial isolates and *T. litoralis, T. alcaliphilus* and *T. chitonophagus* (Huber et al. 1995b; Keller et al. 1995; Neuner et al. 1990) showing that %G+C contents might not be a good characteristic by which to separate the genera *Thermococcus* and *Pyrococcus*.

The comparison of the 16S rRNA gene sequences of Wai21.S1, *T. zilligii* and Wt1 revealed high homologies - around 99% - between the 3 strains. Additionally the 16S-23S intergenic regions of Wai21.S1, *T. zilligii* and Wt1 were sequenced and analysed. This spacer region between the genes for the 16S rRNA and the 23S rRNA should have accumulated more mutations over time since it is not a functional gene in which mutations are restricted by the need to produce a functioning gene product. The obtained sequences however are not sufficiently different from each other to prove on a sequence level that these three strains are different species. The intergenic region of Wai21.S1 is identical to that of *T. zilligii.* The intergenic region of Wt1 is 99% identical to those of Wai21.S1 and *T. zilligii.*

DNA/DNA hybridization studies nevertheless indicate that Wai21.S1 and *T. zilligii* are separate species. Their similarity is about 5% and well below the 70% threshold which is conventionally considered to be the lower hybridization limit for identical species. Between Wai21.S1 and Wt1 the DNA similarity is around 20-42% and therefore close to the species limit threshold (Table 3.3.). Wt1 is physiologically very similar to Wai21.S1 (Table 3.5.) and might represent a second strain of the same species. A fourth terrestrial, amylolytic *Thermococcus*-like isolate from New Zealand, RT3 (Bharat Patel, Griffith University, Queensland, Australia, pers. comm.) could not be obtained and therefore was not included in the hybridization studies. The 16S rRNA gene sequence which is available in the gene data bases (accession number: AF017455) shows 98% homology to the 16S rRNA gene of strain Wai21.S1.

	T. zilligii	Wt1	RT3
Homology with the 16S rRNA gene sequence of Wai21.S1 in %	99.2	?	98
Homology of the 16S-23S inter spacer region with Wai21.S1	100%	99%	?
DNA/DNA Hybridization with Wai21.S1	5%	20-42%	?

Table 3.4. Genetic comparison of the four terrestrial *Thermococcus* isolates from New Zealand.

It is known that most of the species of the genus *Thermococcus* have very high similarities of their 16S rRNA genes. The 16S rRNA gene of the recently decribed marine isolate *T. barossii* is 99.7% identical to the 16S rRNA gene from *T. celer.* DNA/DNA hybridization results as well as physiological properties nevertheless

	Wai21.S1	*T. zilligii* *	Wt1†	RT3‡	*T. celer*	*T. fumicolans*	*Pyrococcus furious*
Place of isolation	Waimangu	Kuirau Park, Rotorua	Champagne Pool, Waiotapu	Rotorua	Vulcano Island, Italy	hydrothermal vent , North Fiji basin	Vulcano Island, Itally
Temperature range and **optimum** in °C	55-93 **89**	55-85 **75**	65-90 **85**	?	**88**	73-103 **85-90**	70-103 **100**
pH range and **optimum**	4.75-7.6 **5.9**	5.4-9.2 **7.4**	5-8 **7**	?	**5.8**	4.5-9.5 **8**	5-9 **7**
Salt range and **optimum** in%	0-1.3 **0.1**	0.05-1.2 **0.29**	0.01-0.4 **0.1**	?	3.5-6.0 **4**	1-8 **2-4**	0.5-5 **2**
Effect of S^0	no effect, sulfur source: cysteine	can be replaced by cysteine or oxidized glutathione	can be replaced by cysteine	?	enhances growth 10 fold	enhances growth	enhances growth
mol% G+C	46.2	46.2	51.3	?	56.6	55	38
Carbon sources	starch, amylose, amylopectin, glycogen	yeast extract, tryptone, casein	peptone, yeast extract, tryptone	starch	peptides	proteolysis products, amino acids	yeast extract, peptone, tryptone, cell extracts, casein, starch

Table 3.5. Comparison of the physiological properties of four terrestrial *Thermococcus* isolates from New Zealand.
* Klages and Morgan, 1994
† Physiological data of Wt1: Gonzales, J.M., pers. comm.
‡ Physiological data of RT3: Patel, B., pers. comm.

support the establishment of *T. barossii* as a novel species (Duffaud et al. 1998). As more *Thermococcus* like isolates are reported which exhibit high homologies of the 16S rRNA gene with previously described species, either additional sequence data or a careful physiological comparison is necessary to distinguish new *Thermococcus* species. It was recently suggested that sequences of DNA polymerases rather than 16S rRNA gene sequences be used as taxonomic tools (Cambon et al. 1998). The sequences of DNA polymerases from various *Thermococcus* species are already available and might provide a good taxonomic tool to distinguish the species of this genus.

3.2.6. Is the establishment of Wai21.S1 as a novel species of the genus *Thermococcus* justified?

The very high sequence homologies of both the 16S rRNA genes as well as the 16S-23S intergenic regions of the fresh water *Thermococcus* isolates from New Zealand lead inevitably to the question of whether each represents a novel species or if at least some are just strains of the same species. Since no additional sequence data are available in the case of the novel isolates Wai21.S1, Wt1 and RT3, substantial physiological differences are needed to support the establishment of Wai21.S1 as a new species of *Thermococcus*.

As shown in Table 3.5., there are significant physiological differences between Wai21.S1 and *T. zilligii* and less significant differences between Wai21.S1 and Wt1. Wai21.S1 has a significantly higher optimum growth temperature and a lower pH optimum than *T. zilligii* . There are no reports that S^0 is inhibitory for *T. zilligii* as it is for Wai21.S1 and oxidized gluthathione cannot replace cysteine as a sulfur source. Additionally the optimal requirement for NaCl of Wai21.S1 is only a third of that of *T. zilligii*. Wai 21.S1 is capable of growing without the presence of NaCl, whereas 0.05% Na^+ or Li^+ are essential for *T. zilligii*. *T. zilligii* is not able to utilize starch, amylose or amylopectin which are the favored carbon sources of Wai21.S1.

Wt1 has a similar optimum growth temperature to Wai21.S1 but its optimum pH lies about one unit higher than that of Wai21.S1. The optimum salt concentration of Wt1 is about the same as that of Wai21.S1 but the upper limit of salt concentration is much lower. No inhibition by S^0 is reported for Wt1 either. Wt1 does not utilize carbohydrates.

All three isolates seem to be sufficiently different to justify the establishment of three separate species. In the case of Wai21.S1 and *T. zilligii* this is strongly supported by DNA/DNA hybridization results. For Wai21.S1 and Wt1 this is not as strongly but

still sufficiently supported. Unfortunately there is no more data available for the amylolytic isolate RT3.
Based on the results of the DNA/DNA hybridization results and the significant differences of Wai21.S1 in comparison to *T. zilligii* and to Wt1 the establishment of isolate Wai21.S1 as a novel species of the genus *Thermoccoccus* is suggested. In reference to the place of origin - Waimangu Volcanic Valley - the name *Thermococcus waimanguensis* is proposed.

3.2.7. The phylogenetic position of Wai21.S1

The phylogenetic analysis of the genus *Thermococcus* shows that the terrestrial isolates from New Zealand form a separate cluster in the genus *Thermococcus* (Fig. 3.20.). Furthermore it appears that the four isolates are phylogenetically among the youngest organisms of this genus. Both marine isolates from New Zealand, *T. gorgonarius* and *T. pacificus* are on the same main branch but phylogenetically rather distant whereas *T. pacificus* seems to be more closely related to the terrestrial species than *T. gorgonarius*.
Bootstrap values do not support that branching order strongly. Bootstrapping provides a statistical measure for the probability of a certain branching order. In this case bootstrap value of 31/100 for the branching point between the *T. litoralis* group and the terrestrial isolates and 32/100 for the branching point between *T. pacificus* and the branch which includes the *T. litoralis* group and the terrestrial isolates are very low. The bootstrap values obtained for the branching order within the terrestrial New Zealand isolates (100/100 and 86/100) nevertheless strongly support this branching order.
In the cluster of terrestrial species "*T. waimanguensis*" is placed phylogenetically between Wt1 and *T. zilligii*. "*T. waimanguensis*" appears to be close to the branching point from which the various fresh water isolates separated into different species. Whether the clustering of the fresh water isolates is due to a geographic separation or due to a physiological condition cannot be answered for sure until more fresh water *Thermococcus* isolates from other parts of the world have been found and analysed.

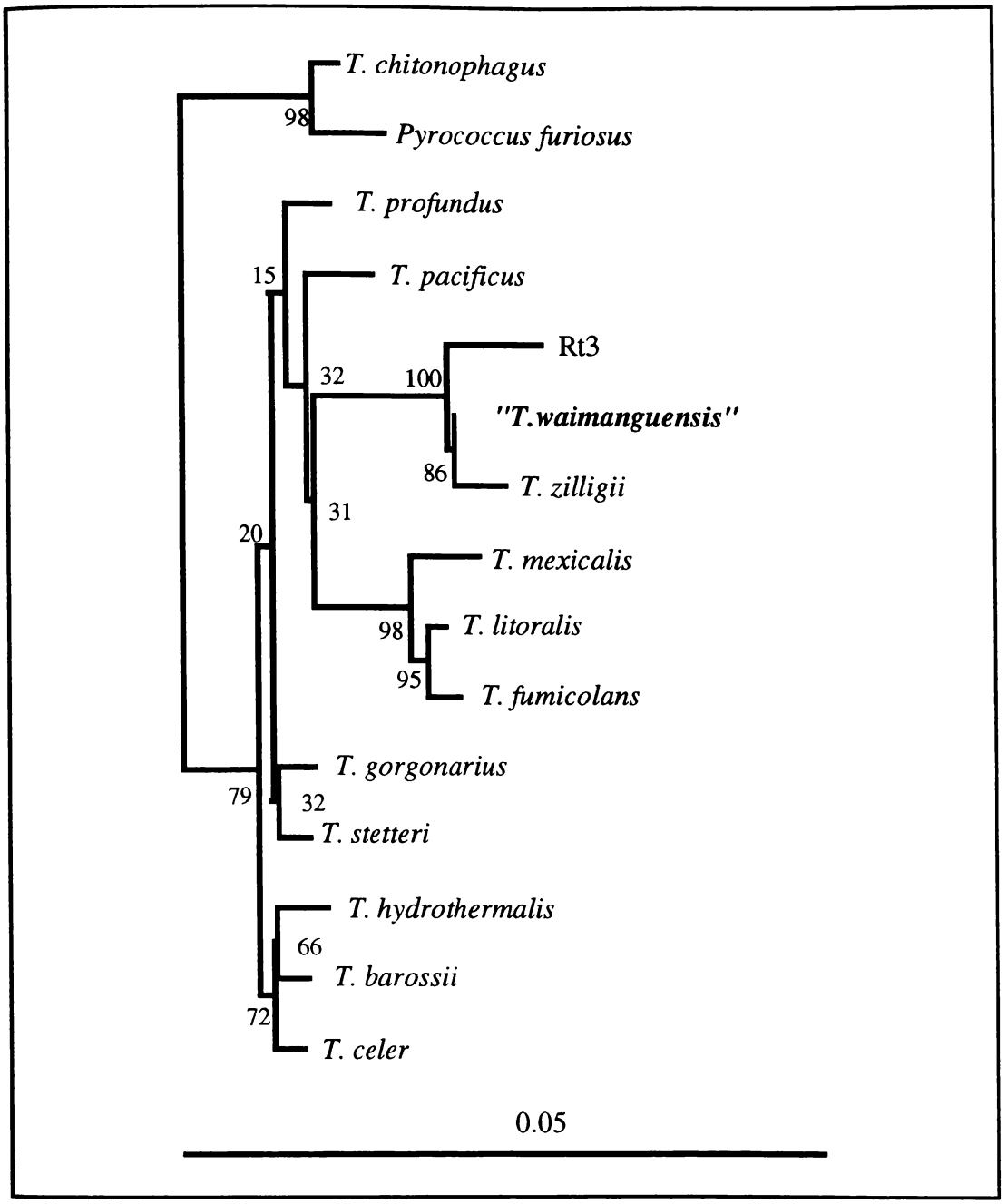


Fig. 3.20. Phylogenetic tree of the genus *Thermococcus* .
Tree is based on 800 bp fragment of 16S rRNA genes with *Pyrococcus furiosus* as an outgroup. Numbers at branching points are bootstrap values. Bootstrap values in %. For sequence alignment see Appendix 2.

The geographic isolation of the New Zealand hot springs which cannot be easily overcome by floating through cold sea waters and inhabiting new territories makes it probable nevertheless that the development of the fresh water isolates is a locally restricted event.

The most parsimonious assumption is that the fresh water isolates stem from marine ancestors (see Fig. 3.20.). This leads to the question how these ancestors found their way into terrestrial hot springs. One explanation might be the geological history of New Zealand. Some 60000 years ago large parts of the North Island were submerged due to an elevated sea level (Prof. Roger Briggs, Earth Science Department, Univerity of Waikato, per. comm.) and a huge lake covered the area from Rotorua to Taupo. Ancestral *Thermococcus* strains could have been left behind in hot springs and terrestrial water systems when formerly submerged land fell dry and they might have evolved into the present fresh-water strains. It is otherwise hardly imaginable how marine microorganisms entered terrestrial hot springs at a time when there were no scientists around to transfer organisms from sampling site to sampling site.

3.2.8. Description of *Thermococcus waimanguensis* sp. nov.

Thermococcus waimanguensis sp. nov. Götz and Morgan. wai.mang.u.en'.sis mod. L. adj. f. Maori wai mangu black water, referring to its place of isolation, Waimangu Volcanic Valley.

Cells are Gram-negative, regular cocci of 1-1.5μm in diameter. Flagella have not been observed. Cell division by constriction. Growth occurs between 55-93°C with an optimum at 89°C. pH optimum is 5.9 with a range from 4.75-7.4. Sodium chloride is not obligately required. Optimal salt concentration is around 0.1%. Grows up to 1.3% NaCl. Cells are highly sensitive to oxygen.

Obligate heterotroph, fermenting starch, amylose, amylopectin, glycogen, casein and casamino acids. Elemental sulfur not obligately required but slightly inhibitory. Suitable sulfur sources are cysteine, sulfide and sulfate. Metabolic endproducts are sulfide, n-butyric acid, acetic acid and some as yet unidentified compounds.

The G+C content is 46.2 %. 16S rRNA sequence comparison affiliates *T. waimanguensis* with the genus *Thermococcus*. GenBank accession number for the 16S rRNA sequence is AF098975. Isolated from a hot spring at Nga Puia o te Papa at Waimangu Volcanic Valley, New Zealand.

Type strain is *T. waimanguensis*, Wai21.S1.

Chapter 4 - The consortium

4.1. Introduction

The consortium was obtained from an enrichment culture of a sample which was taken from a spring at Tokaanu, a small geothermal area south of Lake Taupo. The temperature of the spring was around 90°C with some boiling areas and the pH was neutral. The pool was brown and muddy-looking and was frequently used for hangi.



Fig. 4.1. Hangi pool in Tokaanu geothermal area.

An anaerobic enrichment culture with the hemicellulose konjac glucomannan as sole carbon source yielded a culture with coccoid cells as well as rod-shaped cells (Fig. 4.2.). One rod-shaped organism was isolated by plating and named Tok2. The morphologically-conspicuous Tok1 is further discussed in sections 4.4. and 4.5. This coccoid organism has resisted all isolation attempts so far and had to be characterised as a member of the consortium.

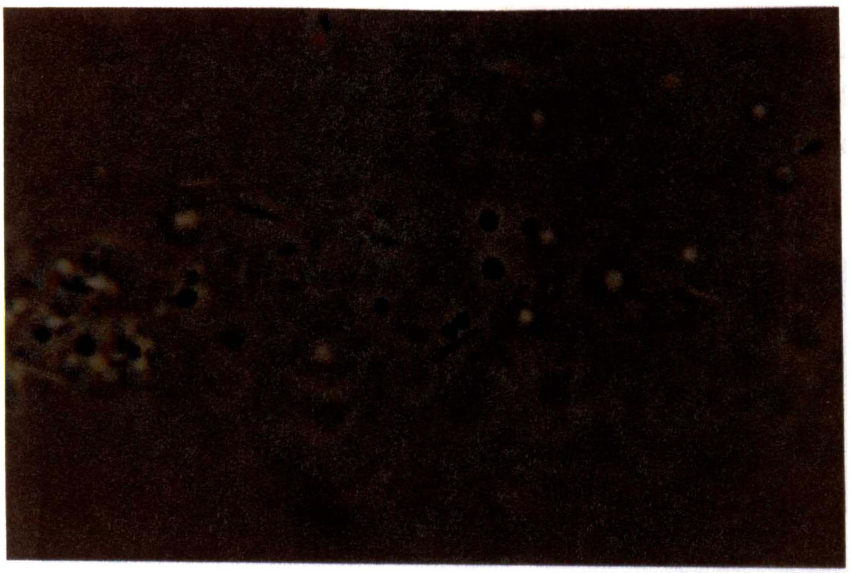


Fig 4.2. Phase contrast micrograph of the consortium. Magnification x1000.

4.2. Isolate strain Tok2 - Results

4.2.1. Sampling and purification

The consortium was grown on a simple medium containing sodium thiosulfate as electron acceptor and konjac glucomannan as carbon source (see section 2.2.2). Incubation was carried out at 90°C without agitation. The rods appeared after 30h of inoculation whereas coccoid cells became apparent after 4-5 days.

To obtain pure isolates, a well grown enrichment culture was plated on medium containing 0.2% glucomannan and 0.05% yeast extract which was solidified with 0.8% Gel Rite (see section 2.2.2.). The plates were incubated in an airtight container for up to seven days at 90°C. The plates were kept in the anaerobic hood for 24h prior to plating in order to remove oxygen. Plating and transfer of plates to the airtight container was done in the anaerobic hood. Two different types of colonies were observed on the plates: tiny clear colonies and greyish-green colonies of 0.5-1.0 mm in diameter (Fig.4.3.3.). Inoculation of liquid cultures with the green colonies was easily possible even without working under strict anaerobic conditions. The pure culture obtained from a green colony was named Tok2. Replating of green colonies resulted in the formation of white colonies after 4-5 days of incubation which turned greenish/brown after another 2 days of incubation.

Every attempt to obtain liquid cultures from the small white colonies was unsuccessful. Phase contrast microscopy revealed that these colonies also consisted of rod-shaped organisms. These rods were on average 4-10 μm long and about 30% of them exhibited terminal spherical bodies.

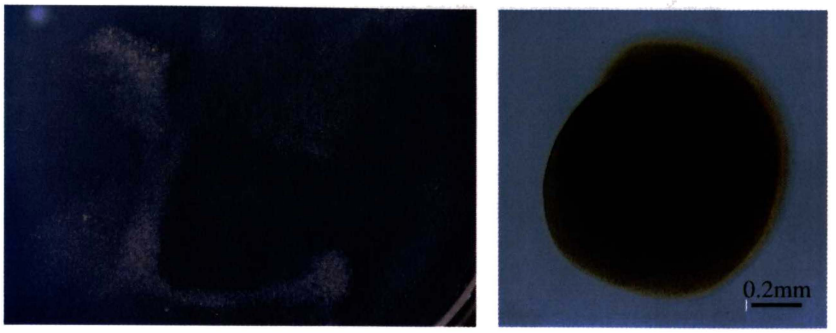


Fig. 4.3. Colonies of Tok2 on Gelrite plates after five days incubation (left); brownish-green colony after seven days of incubation (right).

Liquid cultures of Tok2 could be stored for up to three months at room temperature. For longer storage cultures were concentrated tenfold and kept at -70°C with 20% glycerol added as cryoprotectant

4.2.2. Morphology

Scanning electron microscopy and phase contrast microscopy of strain Tok2 showed rod-shaped cells 4-8μm long and 1μm wide. Approximately 20% of the cells of a culture appeared to be branched. Cultures grown on 0.2% yeast extract however often contained cells up to 40μm long many of which had spherical bodies on one or both ends of the cells (Fig. 4.4.). These spherical bodies appeared less frequently after subsequent transfers. Cultures which went through more than 30 cycles of transfers no longer produced spherical bodies. No directional motility was observed but some rotation around the centre of axis was observed frequently.

Clusters of cells, from three to several hundred cells could frequently be observed and these were more prevalent in media containing konjac glucomannan than in medium containing only yeast extract.

Tok2 stained Gram negative.

Scanning electron micrographs (Fig. 4.5.) revealed a hexagonal pattern of the cell wall. Flagella were not observed.

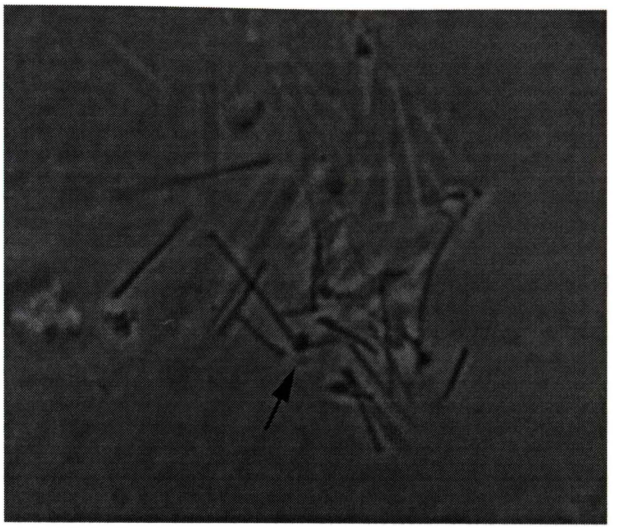


Fig. 4.4. Phase contrast micrograph of stack of strain Tok2 cells growing on yeast extract.
Magnification x1000. Arrow indicates spherical body.

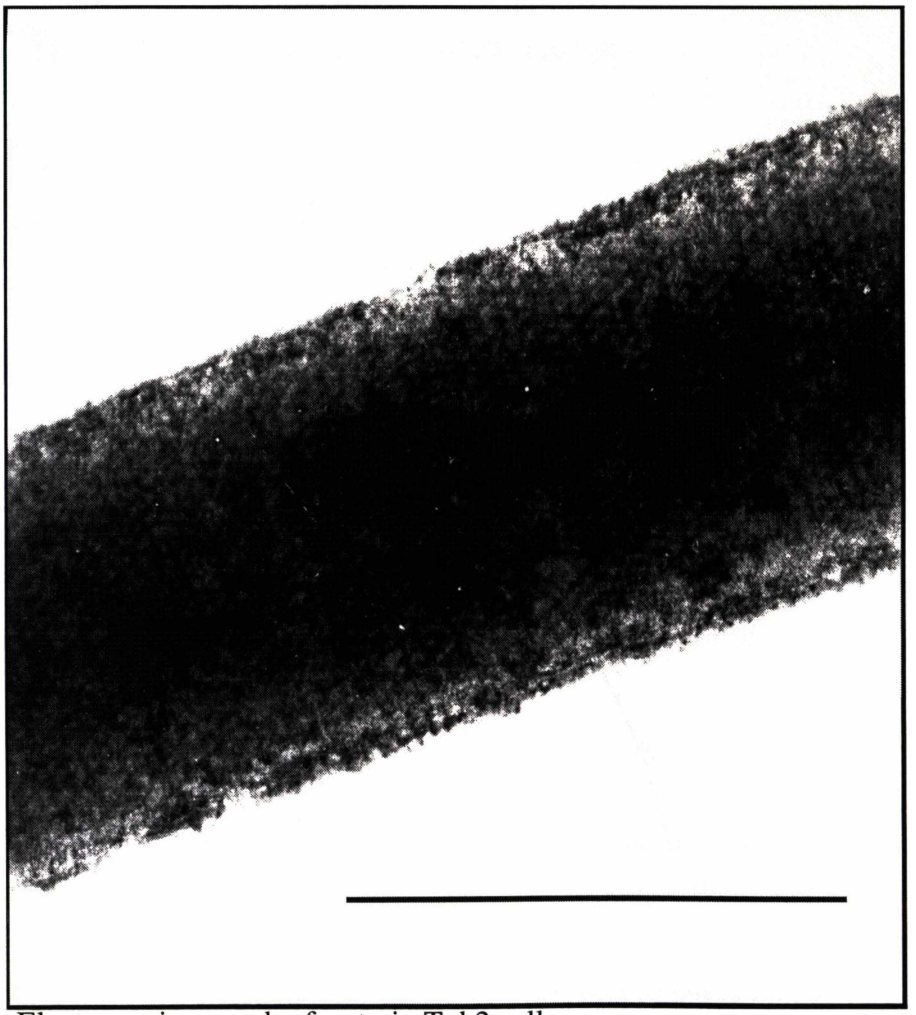


Fig. 4.5. Electron micrograph of a strain Tok2 cell
Bar=0.1μm. Magnification x154000.

4.2.3. Growth parameters

4.2.3.1. Oxygen sensitivity

The sensitivity to oxygen was tested by injecting various amounts of air (contsining ~20% oxygen) through a sterile filter into the Hungate tubes prior to inoculation.
Strain Tok2 was sensitive to oxygen. No growth was observed when medium and headspace contained 0.1% oxygen or more. It was nevertheless observed in consortium cultures containing up to 3.3% oxygen initially that strain Tok2 was able to grow after the removal of the oxygen by the action of strain Tok1 (section 4.4.3.1.), which indicates that strain Tok2 is able to tolerate small amounts of oxygen at high temperatures for a short time even if it can not grow under those conditions.

4.2.3.2 Temperature optimum and range

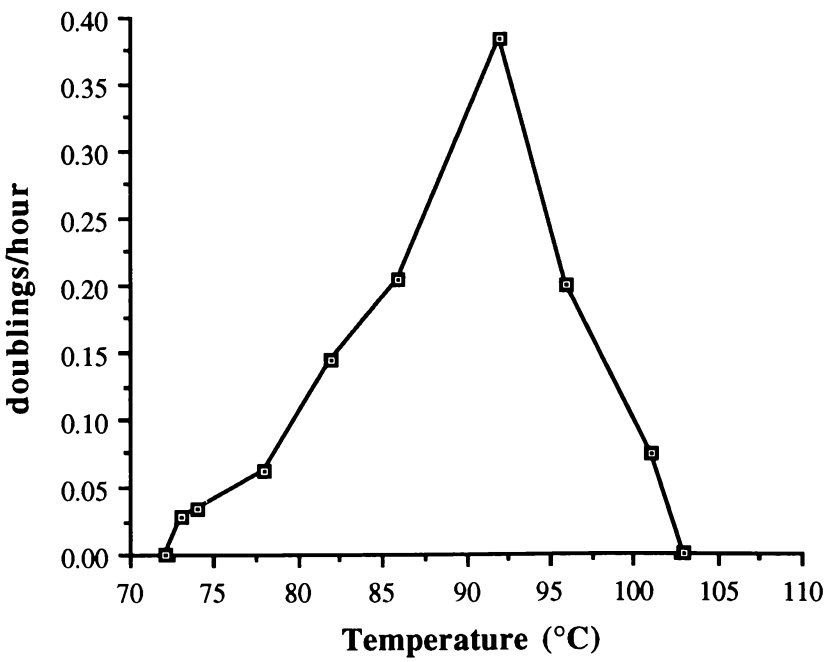


Fig. 4.6. Specific growth rate of strain Tok2 as a function of temperature.

Strain Tok2 grew between 73-101°C and optimally at 92°C. No growth occurred at 72°C or at 103°C (Fig. 4.6.). At 101°C strain Tok2 grew only to half or slightly less then half the density of a culture grown at lower temperatures.

4.2.3.3. pH optimum and range

For the determination of pH range and optimum the medium was buffered with 10mM Bis/Tris and the pH was adjusted at 90°C.
Tok2 grew optimally at pH 6.7 and in the range 5.8 to 8.5 (Fig. 4.7.). No growth was observed at 5.6 and at 8.9.

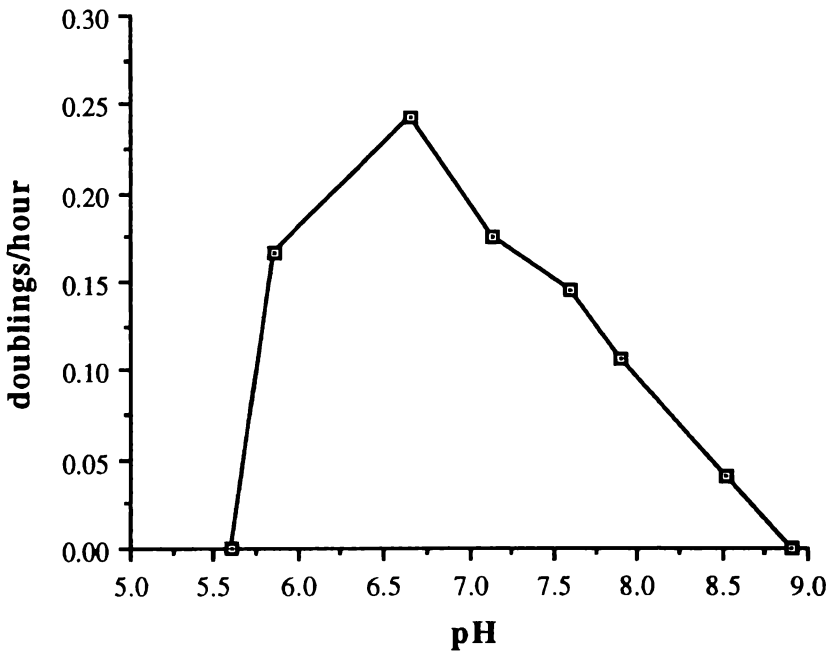


Fig. 4.7. Specific growth rate of strain Tok2 as a function of pH.

4.2.3.4 Sodium requirement

For the determination of sodium optimum and range all the sodium salts were substituted with the equivalent potassium salts with the exception of sodium thiosulfate which could not be replaced. The lowest salt concentration that could be tested was 0.017% (w/v) which equals 2.9mM Na^+.

Optimal growth was obtained at 0.1% (w/v) NaCl which equals 17mM (Fig. 4.8.). No growth was observed at 1.8% (308mM). Growth was very poor at concentrations higher than 0.5% (86mM).

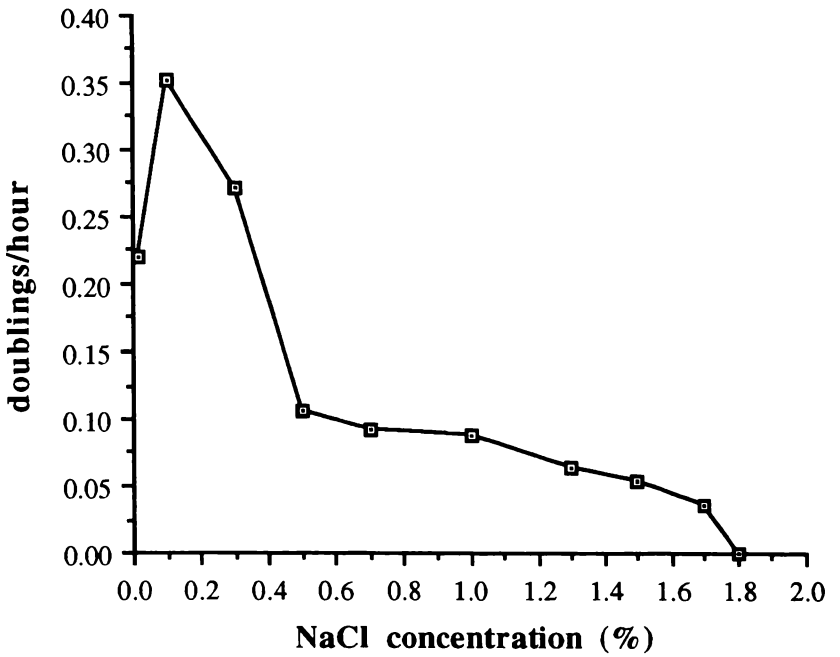


Fig. 4.8. Specific growth rate of strain Tok2 as a function of sodium concentration.

4.2.4. Carbon sources

Best growth of strain Tok2 was obtained on yeast extract and tryptone; some growth was also observed on glycogen, starch, amylose and pyruvate (Table 4.1.). On glycogen, starch, amylose and pyruvate, growth was so poor that it was not detectable by spectrophotometric measurement. Growth was indicated by the production of H_2S and by counting cells under the phase contrast microscope. No growth was observed on glucose, fructose, lactose, lactate, sucrose, maltose, galactose, fumarate, acetate, propionate, formate, ethanol, methanol, casein, casamino acids, xylan or cellulose.
Growth on konjac glucomannan was very poor but was greatly enhanced when Tok2 was grown in a mixed culture with the coccoid-shaped isolate Tok1. On mannose some growth was observed early after inoculation but ceased during further incubation as browning of the medium occurred.
Growth was also observed on CO_2/H_2 and sodium thiosulfate, sulfite and S^0 as electron acceptor.

Carbon source	Growth in OD_{650}*
Yeast extract	++++
Tryptone	+++
Glycogen	++
Starch	+
Amylose	+
Pyruvate	++
Konjac glucomannan	+/-
Mannose	+/-
CO_2/H_2	+
Glucose	-

Table 4.1 Carbon sources utilised by strain Tok2
++++ equals OD_{650} between 0.3-0.35, +++ equals OD_{650} between 0.3-0.15, ++ equals OD_{650} between 0.1-0.15, + equals OD_{650} between 0.1-0.02, +/- indicates that growth had to be determined microscopically.

4.2.5. Electron acceptors

Possible electron acceptors were tested at a concentration of 10mM with yeast extract as substrate.
The regularly used electron acceptor thiosulfate could be replaced with sulfite and S^0. Sulfite supported growth better than thiosulfate or S^0 but could not serve as a sulfur source. With sulfite as electron acceptor and sulfate as sulfur source strain Tok2 grew up to cell densities of an OD_{650} >0.4 whereas cultures growing with thiosulfate as electron acceptor reached a maximal OD_{650} of 0.35. Strain Tok2 was therefore routinely grown with thiosulfate which could serve as both sulfur source and electron acceptor. Nitrate could not support growth as sole electron acceptor.

4.2.6. Nitrate reduction

Nitrate and nitrite were determined using the qualitative sulfanilic acid/dimethyl-α-naphthylamine method (see section 2.2.18.3.).
Tok2's regular growth medium contained 0.6mM $NaNO_3$.
After 24h of growth neither nitrate nor nitrite could be detected in the medium. Uninoculated medium tested the same way as a positive control exhibited red colour, proving that the test was sensitive enough to detect the low concentration of nitrate

present in the medium. Gas bubbles in Durham tubes were observed. The nature of this gas was not investigated. Growth of strain Tok2 with nitrate as an electron acceptor was not observed.

4.2.7. Metabolic endproducts

Metabolic endproducts were investigated by separating them on a BioRad HPX.87H column on a Varian R1-4 HPLC system at 0.6ml/min flow rate with refractive index detection. Standards were used at 50µM to determine the retention times of compounds such as organic acids and single sugars. It was not possible to determine concentrations of standards with great accuracy. Standards run at different concentration did not result in peak areas which correlated precisely with the concentrations of the standards but did not show variations of the retention times. Retention times of one standard were checked every five runs to ensure reproducibility.
Metabolic end products produced by Tok2 when growing on 2g/l yeast extract and 1g/l konjac glucomannan were H_2S, iso-valeric acid, traces of acetic acid and propionic acid and several unidentified compounds.

Retention time (min)	Compound	Approximate concentration in mM
9.10	?	17
12.0	?	2
14.8	acetic acid	0.003
17.5	propionic acid	0.009
23.7	?	1
24.5	iso-valeric acid	8
34.66	?	1.3

Table 4.2. Metabolic endproducts of strain Tok2 growing on yeast extract/glucomannan medium analysed with an HPX.87H column.

The end product (t_R 9.10min) which is present in the highest concentration (~17µM) could not be identified, nor could three further compounds which are present at lower concentrations. The unidentified compounds are not alanine (8.3min) citrate (8.7min), glucose (9.5min), mannose (10min), pyruvate (10.34), lactate (13.1min), formate (14.1), iso-butyric acid (19.8min), n-butyric acid (21.5min) or n-valeric acid (29.45min).

H_2S was another metabolic end product which was detected qualitatively by adding 100µl of a saturated lead citrate solution into 0.5ml of a well grown culture. A brown precipitate indicated the presence of H_2S.

4.2.8. Elemental sulfur

In cell pellets of cultures of the consortium grown in large volumes, a yellow precipitate was found beneath the layers of pelleted cells. In a qualitative assay (see section 2.2.18.2.) this precipitate was shown to be elemental sulfur. Pure cultures of Tok2 did also produce elemental sulfur indicating that Tok2 is responsible for the production of S^0.

4.2.9. Antibiotic sensitivities

Tok2 was sensitive to erythromycin (50µg/ml), colistin (50µg/ml) and nalidixic acid at a concentration of 75µg/ml but not at 50µg/ml. No inhibition of growth was caused by nitrofurantoin (20µg/ml), chloramphenicol (50µg/ml), rifampin (100µg/ml), vancomycin (50µg/ml) or streptomycin (200µg/ml).

4.2.10 %GC content of Tok2

The %GC of strain Tok2 was determined by following the isochromatic shift spectrophotometrically during thermal denaturation of the DNA of Tok2 (Fig. 4.9.). For this purpose the DNA was further purified in a CsCl gradient additionally to the routinely performed DNA extraction (section 2.2.14.) and dialysed against 0.2 times SSC buffer.

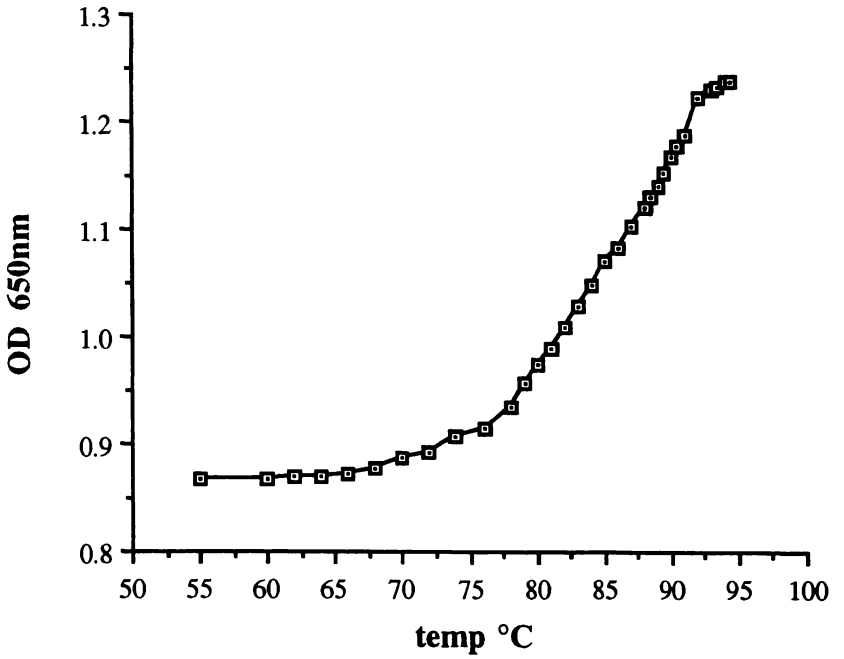


Fig. 4.9. DNA melting curve of Tok2.

The % GC was calculated as described in section 3.1.7.
DNA from *T. zilligii* was used as reference. *T. zilligii* has a % GC content of 46.2 (Klages and Morgan 1994). The DNA of *T. zilligii* melted at 86°C.
The DNA of Tok2 melted at 86°C. The %G+C of Tok2 is therefore around 46.2%.

4.2.11. 16S rRNA gene sequence

The 16S rRNA gene was amplified using the primers af2-21 and ar1510-1492 (see section 2.2.7. for sequence). The PCR reaction gave one DNA fragment of approximately 1500 bp. Prior to sequencing, the PCR product was purified using Wizard PCR Preps, Promega, according to the manufacturer's instructions.
The product was sequenced (section 2.2.11.) using the two primers used for amplification and an additional internal forward primer, af515. A sequence of 1466 nucleotides could be obtained (Fig. 4.10.).

5'-GGACCCGACCGCTATCGGGGTAGGGCTAAGCCATGCGAGTCGCGCGCCCGGGGGCG
CCCGGGAGCGGCGCACGGCTCAGTAACACGTGCCTAACCTAACCTCGGGAGGGGGACAC
CCCCGGGAAACTGGGGTCAATCCCCCATAGGGGAAGGGCGCTGGAAGGCCCCTTCCCCG
AAAGGGCCGGCGGCCGATCCGCCGCCAGCCGCCCGAGGGTGGGGGCACGGCCCATCAGG
TAGTTGGCGGGTTAAAGGCCCGCCAAGCCGAAGACGGGTAGGGGCGGTGAGAGCCGCGA
GCCCCGAGATGGGCACTGAGACAAGGGCCCAGGCCCTACGGGGTGCAGCAGGCGCGAAT
ACTCCGCAATGCGGGCAACCGCGACGGGGCTACCCCGAGTGCCGGGCGAAGAGCCCGGC
TTTTGCCCGGTCTAAAAAGCCGGGAGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCC
GCGGTAATACCAGCTCCGCGAGTGGTCGGGGTGTTTACTGGGCTTAAAGCGCCCGTAGC
CGGCCCGGCAAGTCGCTCCTGAAATCCCCGGGCTCAACCCGGGGGCCGGGGGCGATACT
GCCGGGCTAGGGGGCGGGAGAGGCCGCCGGTACTCCGGGGGTAGGGGCGAAATCCGTTA
ATCCCCGGAGGACCACCAGTGGCGAAAGCGGGCGGCCAGAACGCGCCCGACGGTGAGGG
GCGAAAGCCGGGGGAGCAAAGGGGATTAGATACCCCTGTAGTCCCGGCCGTAAACGATG
CGGGCTAGCTGTCGGTCGGGCTTAGGGCCCGGCCGGTGGCGAAGGGAAACCGTTAAGCC
CGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCGGGGGGGCACC
ACAAGGGGTGAAGCTTGCGGCTTAATTGGAGTCAACGCCGGAAACCTCACCCGGGGCGA
CAGCAGGATGAAGGCCAGGCTAACGACCTTGCCGGACGAGCTGAGAGGAGGTGCATGGC
CGTCGTCAGCTCGTGCCGTGAGGTGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCGC
CCCTAGTTGCTACCCCTCCCTACGGGGAGGGGGGCACACTAGGGGGACTGCCGGCGTAA
GCCGGAGGAAGGAGGGGGCCACGGCAGGTCAGTATGCCCCGAAACCCCGGGGCTGCACG
CGAGCTGCAATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGCAATCCCGTAA
ACCCCGCCCCAGTAGGGATCGAGGGCTGCAACTCGCCCTCGTGAACGTGGAATCCCTAG
TAACCGCGTGTCACCAACGCGCGGTGAATACGTCCCTGCCCCTTGCACACACCGCCCGT
CGCACCACCCGAGGGAGCCCTCTGCGAGGCCCCTCGCCGACAAGGTGGGGGGACGAGCA
GGGGGCTCCCAAGGGGGGTG-3'

Fig. 4.10. 1432 nucleotides (*E. coli* numbering 19-1492) of the 16S rRNA gene sequence of strain Tok2.

The sequence was compared with the 16S rRNA sequences which are available in the GenBank database by doing a Blast search (Altschul et al. 1997) and comparison with the 16S rRNA gene of "Pyrobaculum neutrophilum" which was supplied by Reinhard Hensel (Gesamthochschule Universität, Essen) was carried out by aligning the sequences with the ClustalV program (see Appendix 4 for alignments).
The 16S rRNA gene sequence of Tok2 showed the highest homologies with the 16S rRNA genes from members of the family Thermoproteales.

Species	% homology of the 16S rRNA gene
"Pyrobaculum neutrophilum"	99.2
Pyrobaculum islandicum	97.5
Pyrobaculum organotrophum	>99
Pyrobaculum aerophilus	98
Thermoptoteus tenax	94.5
Sulfolobus acidocaldarius	86
Pyrococcus furiosus	85

Table 4.3. 16S rRNA gene homologies (in %) between Tok2 and members of the family Thermoproteales and selected members of the archaea.

4.3 Isolate strain Tok2 - Discussion

4.3.1. Does isolate Tok2 represent a novel species of the genus *Pyrobaculum* ?

The 16S rRNA gene sequences of strain Tok2 shows high homologies with the equivalent gene (see Table 4.3.) from two other *Pyrobaculum*-like isolates from Tikitere, New Zealand, the preliminarily-named strain "Pyrobaculum neutrophilum" and strain IsoX (Reinhard Hensel, Gesamthochschule Universität, Essen, Germany, pers. comm.) which were isolated and characterised by Klaus Michalke and Reinhard Hensel. The three New Zealand isolates are according to the 16S rRNA gene sequences closely related to *Pyrobaculum organotrophum.* In a phylogenetic analysis executed with ARB, a phylogenetic program designed especially for 16S rRNA gene sequence analysis (see Appendix 6), they appear to be strains of *P. organotrophum* (Klaus Michalke, Reinhard Hensel, pers. comm.). Physiologically the New Zealand isolates have obvious differences from *P. organotrophum* and there are less marked differences among them. *P. organotrophum* has a higher optimum growth temperature and a lower pH optimum than the New Zealand isolates and growth is dependent on elemental sulfur, whereas neither Tok2 nor the two isolates from Tikitere have an obligate requirement for S^0. Tok2 is able to grow autotrophically which is not the case with either the New Zealand isolates or *P. organotrophum.* DNA/DNA hybridization experiments are necessary in order to decide whether Tok2, "P. neutrophilum" and IsoX are strains of one species and if so, whether they are strains of *P. organotrophum* or whether the placement of Tok2 as a novel species of the genus *Pyrobaculum* would be justified.

Among the order Thermoproteales the three New Zealand strains are clearly placed on the *Pyrobaculum* branch (Fig. 4.10.) well separated from the recently discovered acidophilic organism *Thermocladium modestius* (Itoh et al. 1998) and the family Thermofilaceae. In phylogenetic analyses executed with ARB (Appendix 6) and with the Clustal programs alike (Fig. 4.11.), it seems that the evolutionary distance between the genera *Pyrobaculum* and *Thermoproteus* is very small but the separation between the *Pyrobaculum* species and *Themoproteus tenax* is nevertheless supported by a high bootstrap value . The main physiological difference between the two genera used to be the lower optimal growth temperature of *Thermoproteus tenax* (Huber and Stetter 1991b). The New Zealand *Pyrobaculum* isolates' optimum growth temperatures are as low and even lower than those of *T. tenax.* According to the phylogenetic analysis done with the Clustal phylogenetic programs, *Thermoproteus neutrophilus* seems to be a

member of the genus *Pyrobaculum* rather than of the genus *Thermoproteus*, and bootstrap values support this. It appears that there is a need for a thorough phylogenetic analysis of the family Thermoproteaceae including all available 16S rRNA gene sequences of members of this family which might lead to a reclassification of the two genera as one, or to the transfer of some species into a new genus.

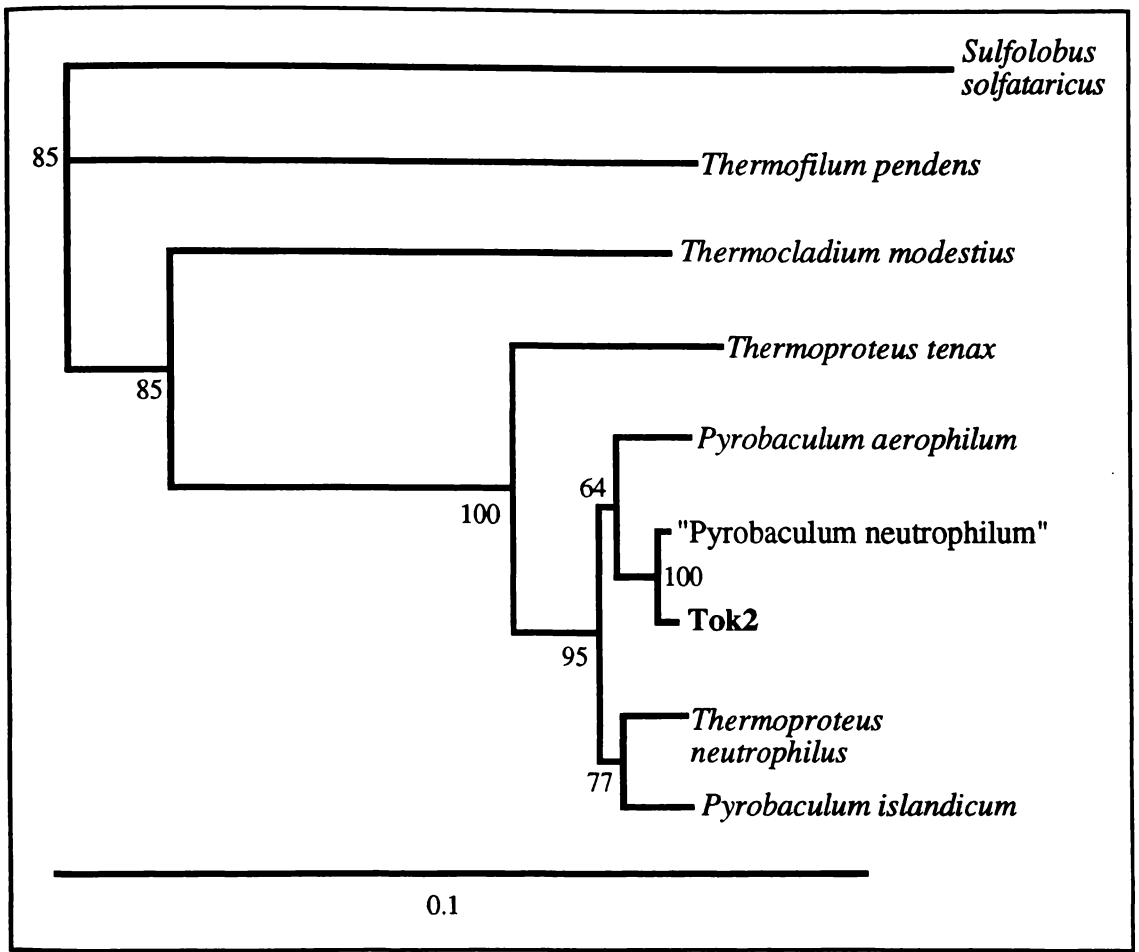


Fig. 4.11. Phylogenetic tree based on the 16S rRNA genes of the order Thermoproteales with *Sulfolobus solfataricus* as outgroup.
Bootstrap values in %.

4.3.2. The ecology and morphology of Tok2

Strain Tok2 was isolated from an enrichment culture with the hemicellulose konjac glucomannan as sole carbon source. The original sample was taken from a hot spring in Tokaanu at the southern end of Lake Taupo. This pool is frequently used for hangi, a traditional Maori steam cooking method in hot pools, and might therefore have a regular input of complex nutrients, like fat, carbohydrates and protein. The water of the pool is medium brown and muddy, with a lot of particulate matter and manuka shrubs (*Leptospermum scoparium*) grow at some places around its shore. The temperature of

the pool is around 90°C but there are some localised boiling hot spots as well. Some dead scorched looking manuka trees close by might be an indicator of occasional heavy geothermal activity of this spring. Apart from *P. aerophilum*, which is an isolate from a shallow marine hot spring on Vulcano Island, Italy (Völkl et al. 1993), all members of the order Thermoproteales described to date are from terrestrial hot springs. All anaerobic enrichments with yeast extract as sole carbon source from four similar hot pools in Tokaanu yielded *Pyrobaculum*-like cells. The geothermal field in Tokaanu is rather small. The various pools are close together and some of them are visibly connected, which might lead to distribution of organisms throughout the pools of this area.

Under the phase contrast microscope Tok2 exhibited the phenotypic characteristics of the order Thermoproteales. It is a stiff rod from 6-10µm long and about 0.8-1µm wide. Tok2 shares with *P. aerophilum* the average length of the cells and is roughly twice as long as *P. organotrophum* and *P. islandicum*. Occasionally much longer rods - with lengths of up to 40µm - can be observed. This variability in size has been observed in other members of the Thermoproteales, such as *Pyrobaculum aerophilum* as well (Völkl et al. 1993). A small percentage of the cells were bent at a 130-150° angle which has been reported for most members of the Thermoproteales, like *Thermoproteus tenax* and *T. uzoniensis* and all the described *Pyrobaculum* species (Bonch-Osmolovskaya et al. 1990; Huber et al. 1987; Völkl et al. 1993; Zillig et al. 1981). Frequently sharper bends at a 30-45° angle are observed. These are generally considered to be dividing cells prior to separation (Huber and Stetter 1991b).

A further recurrent morphological characteristic of this group which is also present in Tok2 is the appearance of spherical bodies at one, or sometimes both ends, or in the middle of the cell. For *P. aerophilum* it is reported that these spherical bodies get larger during growth while the rods shorten, and that sometimes in stationary phase the culture consists of coccoid cells with no rod-shaped cells present (Völkl et al. 1993). This was never observed for Tok2. The highest frequency of rods with spherical bodies attached was about 25% of the total culture. The frequency of rods with spherical bodies reduced with serial transfer. No investigation of role, function and regulation of the spherical bodies has been published so far. It is not known what purpose the spherical bodies serve for the cell or if they serve a purpose at all. Since their frequency is reduced during serial transfer where conditions of incubation and substrate supply are constant it might indicate that the spherical bodies are an adaptation to adverse or changing conditions. Thin sections of spherical bodies did not reveal any particular recognisable structures under the electron microscope, but it was observed that spherical bodies are usually free of the typical hexagonal proteinaceous surface layer (S-layer) which is typical for Thermoproteales (Davorin Janekovic, Wolfram Zillig, Max-Planck-Institute, Martinsried, pers. comm.).

4.3.3. The physiological properties of Tok2

Tok2 is a strictly anaerobic extremely thermophilic organism. Only one member of the order Thermoproteales known so far is microaerophilic, *Pyrobaculum aerophilum* (Völkl et al. 1993), with all the others being highly sensitive to oxygen (Bonch-Osmolovskaya et al. 1990; Huber et al. 1987). Like *P. aerophilum,* Tok2 is able to reduce nitrate which is used as an electron acceptor by *P. aerophilum* under strictly anaerobic conditions. Tok2 seems not to be able to use nitrate as an electron acceptor since no growth is observed with nitrate as electron acceptor and sulfate as sulfur source. *P. aerophilum* accumulates nitrite in the medium while growing on nitrate. *P. aerophilum* reduces nitrite further when culture is continuously gassed with N_2/CO_2 and an organic substrate is present. No nitrite was detected after growth of Tok2 indicating that Tok2 is able to further reduce nitrite without gassing the culture with N_2/CO_2. The identity of the nitrate reduction product was not investigated, as there are no simple tests for nitrogen and nitrous oxide gases.

The physiological properties of growth of Tok2 are very much like the characteristics of the other members of this genus and of the order Thermoproteales (Table 4.4.). The pH range and optimum of strain Tok2 is about the same as the pH range of *P. aerophilum.* Like the other members of *Pyrobaculum,* and only few other terrestrial Archaea, strain Tok2 can grow above 100°C but its optimum temperature of 92°C is 8-10°C lower than that of the other *Pyrobaculum* species. The optimum growth temperature of the two other *Pyrobaculum* isolates from New Zealand "P. neutrophilum" and strain IsoX is 85°C and therefore much lower than that of the other *Pyrobaculum species* and even lower than that of *T. tenax.* "P. neutrophilum" and strain IsoX are still capable of growing above 100°C. As described for many thermophilic organisms strain Tok2 was able to withstand oxygen at temperatures below the minimum temperature for growth. This ability might be crucial to survival in rapidly changing environments where temperature and the amount of oxygen dissolved in the water can change rapidly. Electron acceptors of strain Tok2 are thiosulfate and sulfite. Tok2 was not able to use cysteine as an electron acceptor which can serve as an electron acceptor for *P. islandicum* and *P. organotrophum.*

Strain Tok2, "P. neutrophilum" and strain IsoX are the first *Pyrobaculum* isolates which are able to utilise carbohydrates as a source of carbon, but glycogen, starch and amylose supported growth to a much lower extent than the preferred substrates, yeast extract and tryptone. The other described *Pyrobaculum* species grow heterotrophically on proteinaceous substrates or autotrophically with the exception of *P. organotrophum.* Strain Tok2 is capable of growing like *P. islandicum* with H_2/CO_2 and thiosulfate or elemental sulfur autotrophically. Autotrophic growth yields only about a tenth of the cell

	*P. aerophilum	†P. islandicum	†P. organotrophum	Tok2	‡P. neutrophilum IsoX	¤T. tenax
Temperature range and optimum in °C	75-104 **100**	74-102 **100**	78-102 **102**	73-101 **92**	73-101 **85**	78-96 **88**
pH range and optimum	5.8-9 **7**	5-7 **6**	5-7 **6**	5.8-8.5 **6.7**	? **7**	2.5-6 **5**
Length (µm)	3-8	~2.5	3-5.5	4-8	?	1-80
Flagella	+	+	+	-	-	-
G+C content mol%	52	44-48	44-48	46	46	55.5
S^0 dependency	-	-	+	-	-	+
Oxygen relations	microaerophilic	obligate anaerobe	obligate anaerobe	obligate anaerobe	obligate anaerobe	obligate anaerobe
Electron acceptors	oxygen, nitrate, nitrite	S^0, thiosulfate, sulfite, L(-) cysteine	S^0, L(-)cysteine, oxidised glutathione	thiosulfate, sulfite, S^0	?	S^0
Metabolism	facultatively autotrophic	facultatively autotrophic	strictly organotrophic	facultatively autotrophic	strictly organotrophic	strictly organotrophic
Carbon sources	yeast extract, meat extract, tryptone, gelatin, casein, casamino acids, propionate, acetate	yeast extract, meat extract, peptone, cell homogenates	yeast extract, meat extract, peptone, cell homogenates	yeast extract, tryptone, glycogen, starch, amylose	yeast extract, tryptone, starch, amylose, casamino acids	glucose, starch, amylose, glycogen, ethanol, formamide, malate, fumarate

Table 4.4. Some characteristics of the members of the genus *Pyrobaculum* and *Thermoproteus tenax*.
*Völkl et al 1993; † Huber et al. 1987; ‡Reinhard Hensel, pers. comm.; ¤Zillig et al. 1981.

numbers Tok2 cultures can yield when growing heterotrophically on yeast extract or tryptone. During heterotrophic growth S^0 is a possible electron acceptor but thiosulfate and sulfite were preferred. This sets Tok2 apart from *P. organotrophum* and *P. islandicum* which have an obligate requirement for S^0.

Tok2 differs widely from *P. organotrophum* in the production of iso-valeric acid. The production of volatile fatty acids or organic acids by members of the order Thermoproteales has previously not been reported (Bonch-Osmolovskaya et al. 1990; Selig and Schönheit 1994; Zillig et al. 1981). The other *Pyrobaculum* species produce H_2S and CO_2 exclusively. The amount of iso-valeric acid produced by strain Tok2 during growth on yeast extract medium or on yeast extract/konjac glucomannan medium is substantial. Some more substances which were present and could not be identified are none of the common organic acids. Like the other known *Pyrobaculum* species Tok2 produces high quantities of H_2S.

The accumulation of elemental sulfur in cultures of strain Tok2 (section 4.2.8.) and in consortium cultures is something that has not been reported for any other Thermoproteales species. In cell pellets from consortium cultures which were grown in large volumes a layer of elemental sulfur on top of the cells was visible after centrifugation. It was suggested that in *Desulfovibrio desulfuricans* S^0 is an intermediate of sulfur compound disproportionation during thiosulfate reduction (Cypionka et al. 1998). In this scheme S^0 gets reduced to H_2S which is found in substantial quantities in pure strain Tok2 and in consortium cultures (section 4.2.7.).

Physiological data supports the establishment of strain Tok2 as a new species of the genus *Pyrobaculum* most likely with the incorporation of "Pyrobaculum neutrophilum" and strain IsoX as strains of this species. The differences from *Pyrobaculum organotrophum* are substantial despite their minimal phylogenetic distance. DNA/DNA hybridizations including all the available species of this order will be carried out as soon as they can be obtained from the culture collections. In the event that the result of the hybridization supports the establishment of a new species containing the three New Zealand *Pyrobaculum* isolates, they will be named *Pyrobaculum neutrophilum.*

4.3.4. Description of strain Tok2

Rod-shaped organism with spherical bodies occuring at the ends or in the middle. Length 4-10µm, width 0.8-1.2µm. Anaerobic, probably microaerophilic. Grows optimally at 92°C (range 73-101°C) and at pH 6.7 (range 5.8-8.5). Optimal sodium ion concentration is 17mM (range 3-280mM). Facultatively autotrophic on CO_2/H_2 with thiosulfate as electron acceptor. Heterotrophic growth on yeast extract, tryptone, glycogen, starch and pyruvate. Possible electron acceptors are thiosulfate, sulfite and S^0. Reduces nitrate.

Isolated from hot spring in Tokaanu, New Zealand.

Close phylogenetic affiliation with *Pyrobaculum organotrophum*.

4.4. Strain Tok1 - Results

4.4.1. Sampling, purification attempts and maintenance

Tok1 was found in the same enrichment culture as Tok2 but was only evident in the culture 2-3 days after the rod shaped strain Tok2 had grown. It finally outnumbered Tok2 in a ratio 1:4 approximately.
All attempts to purify Tok1 were unsuccessful.
Attempts to purify strain Tok1 included:

Plating:

On konjac glucomannan/yeast extract plates solidified with 0.8% GelRite two different colony types could be obtained but neither of them consisted of coccoid cells. One type, small greenish-brown colonies, could be grown easily in liquid culture and the isolate was named Tok2 (section 4.4.2. and 4.4.3.), the other type, smaller whitish colonies could not be transferred successfully into liquid culture. These colonies consisted of long, often branched or bent rods. Subsequently, these colonies could not be obtained using cultures for plating which had undergone many transfers, implying that this organism had been lost. No colonies at all were obtained on plates with konjac glucomannan as sole carbon source.
Cell-free culture supernatant from strain Tok2 was incorporated into the solidified medium containing konjac glucomannan as a carbon source in order to enhance growth of strain Tok1 in case of a dependence of strain Tok1 on a metabolite of Tok2. This did not result in any other colony type than those containing rod-shaped organisms.
Sonicated cell fragments of strain Tok2 were incorporated into the medium in case of a dependence of strain Tok1 on a cell constituent of strain Tok2. This was equally unsuccessful.
Some archaea, such as many species of the Sulfolobales, are known to dislike growing on surfaces and are successfully grown on plates by using the overlay technique (Götz 1994) in which a small volume (<2ml) of medium containing 0.2% Gelrite and 100µl inoculum is poured on top of a plate which is otherwise identical to a common plate (section 2.2.2.). No colonies were obtained.
A novel highly thermostable agar, provided by the Crown Research Institute in Lower Hutt, New Zealand was tested as a solidifying agent in case the Mg^{2+} concentration of Gelrite plates was too high for the successful plating of the cocci. No colonies containing coccoid cells could be obtained using this agar.

Further attempts to plate strain Tok1 included plating on media with different pH (in a range from 5-8.5) and incubation at various temperatures (in a range from 75°C to 90°). Above 90°C both Gelrite and and the thermostable agar mentioned above lost stability which is probably due to a combination of high temperature, elevated pressure in the air-tight container and a high humidity. It was therefore not possible to raise the incubation temperature above 90°C.

Serial dilution:

Serial dilutions were carried out according to standard protocols. All cultures that could be obtained consisted of rod-shaped cells. Some cultures contained both rods and cocci. It was observed throughout the work with the consortium that strain Tok1 did not grow from a small inoculum whereas it was possible to grow strain Tok2 from a statistical inoculum of one cell. The high viscosity of the substrate konjac glucomannan led to clumps of cells that could hardly be separated without harm to the cells.

Antibiotics:

Various antibiotics were tested for their ability to selectively remove strain Tok2 from the culture. Strain Tok1 showed resistance to a number of antibiotics (section 4.4.6.) but strain Tok2 was resistant to each of these as well (section 4.2.9.).

Carbon sources:

The use of different carbon sources to selectively enhance growth of strain Tok1 in liquid culture as well as on plates was limited to the only other substrate which strain Tok1 could metabolize other than konjac glucomannan, locust bean gum (section 4.4.4.). Growth of strain Tok1 on locust bean gum was considerably lower than on konjac glucomannan. Growth of strain Tok2 was on the contrary enhanced, producing the opposite effect to that desired.

Selective conditions:

The incubation of consortium cultures under various selective conditions was not successful. Most of the growth parameters of strain Tok2 have a wider range than those of strain Tok1. Strain Tok1 grows at higher pHs than strain Tok2 but growth is so feeble at high pHs that consortium cultures could not be transferred more than twice successfully. The presence of 3% (v/v) oxygen in the medium resulted in cultures which contained only cocci in early stages due to the higher oxygen tolerance of strain

Tok1. Strain Tok2 appeared in the culture as soon as the oxygen was removed which was indicated by the decolouration of the redox indicator resazurin and might be due to the production of H_2S by strain Tok1.

Density gradient:

It was attempted to separate strain Tok1 from the rod-shaped organism by centrifugation through a density gradient of Percoll (kindly provided by Jacek Kryzsosiak, Lifestock Improvement, Hamilton). The gradient is self-forming at 14000g. Two different bands could be observed and one was highly enriched in coccoid cells but contained rods as well. The cells looked distorted and inoculation of konjac glucomannan medium and of yeast extract medium with either band did not yield cultures.

Since all attempts to purify strain Tok1 failed, it was maintained as a member of a consortium. It was grown anaerobically in Hungate tubes on a simple medium (see section 2.2.2.) containing konjac glucomannan as carbon source and sodium thiosulfate as electron acceptor. Large volumes of the consortium were grown in 1l or 2l Schott bottles with rubber stoppers. Medium was inoculated with a 1% inoculum from an exponentially growing culture and incubated at 90°C. Under these conditions both strains grew slowly but to approximately equal numbers for the first 24 h. Later Tok1 dominated the culture and grew to 3-4 times the density of Tok2.

4.4.2. Morphology of the strain Tok1

By phase contrast microscopy Tok1 is a regular coccus 1-1.5µm in diameter (Fig. 4.12.). Diplococci could be observed frequently and in exponential cultures large cell aggregates could be found. These aggregates were rarely seen in old cultures. They usually contained rods as well as cocci. No cell motion was observed.
Tok1 stained Gram negative.

Under the transmission electron microscope cells appeared fuzzy and covered in a mucous substance (Fig. 4.13.). No distinctive surface pattern could be seen. No flagella were observed.

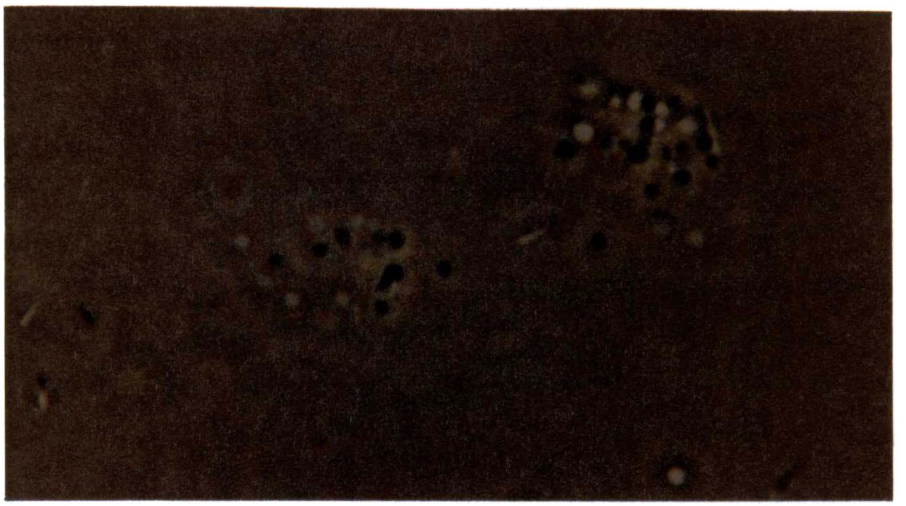


Fig. 4.12. Phase contrast micrograph of cells from the consortium during late exponential growth.
Magnification x1000.

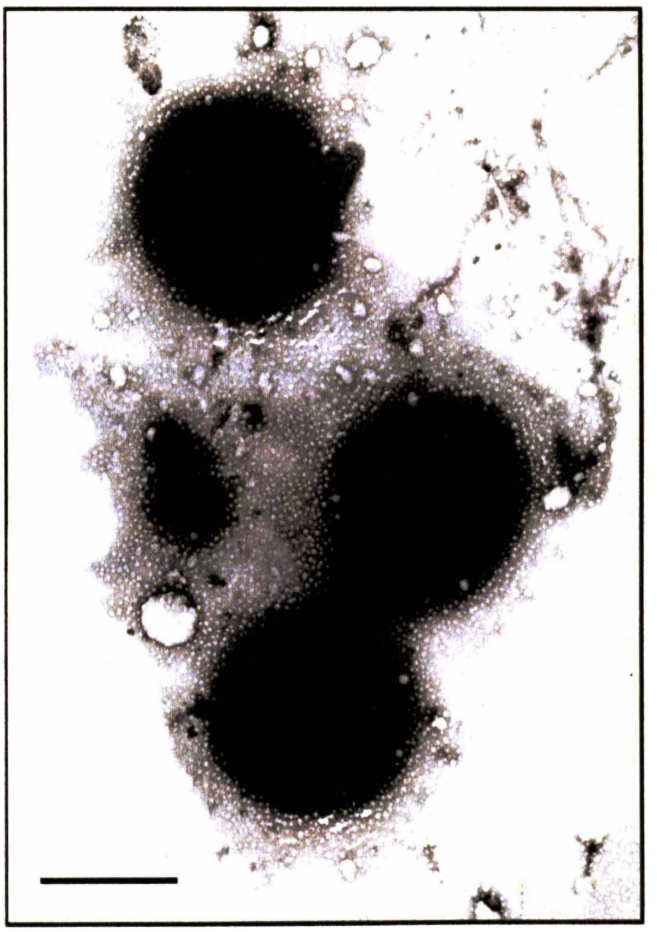


Fig. 4.13. Electron micrograph of Tok1 cells.
Bar=1mm. Magnification x20000

4.4.3. Growth parameters

The consortium did not reach cell densities which would have made it possible to follow growth by measuring the optical densities of cultures. It would have been difficult moreover to decide what amount of absorption is due to growth of Tok1 and which is due to growth of Tok2. Determination of growth parameters had to be done therefore by counting cells under the phase contrast microscope using a Thoma chamber. Due to the tendency of Tok1 to form clusters which contained hundreds of cells this could not be done very precisely. The medium containing 0.2% konjac glucomannan solidified when incubated at greater than 70°C and organisms seemed to get stuck in the gel that was formed by the substrate. Attempts to separate the clusters by gentle sonication were not successful.
Final growth yields were also dependent on the kind of glucomannan which was used. Konyaku, the food grade glucomannan (section 2.2.2.) supported growth less well. Optimum values for the various growth parameters are therefore estimates rather than precise measurements.

4.4.3.1. Oxygen sensitivity

Tok1 was able to tolerate 3.33% (v/v) oxygen in the medium. After 30h the oxygen disappeared as indicated by the colour change of the redox indicator resazurin. At this time Tok2, which is inhibited by 0.1% oxygen, had also started to grow.

4.4.3.2. Temperature optimum and range

Tok1 grew in a temperature range from 75-99°C and optimally at 98°C. Even under optimal conditions, growth was slow in the first 24h, after which rapid growth occurred which coincided with liquefaction of the medium. No growth was observed at 73°C or at 100°C. The carbon source konjac glucomannan was very stable over the entire temperature range tested (see Fig. 5.4.). Browning of the medium occurred at temperatures above 90°C in old cultures due to a caramelisation of the released sugars.

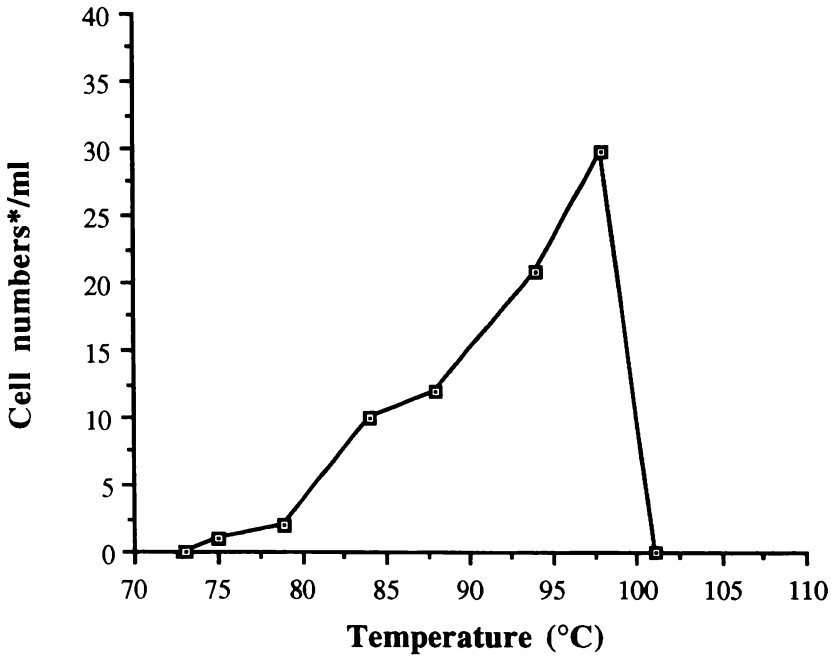


Fig. 4.14. Growth of strain Tok1 as a function of temperature.
* Cell count x 10^6

4.4.3.3. pH optimum and range

For the determination of the pH optimum and range the medium was buffered with 10mM Tris/Bis.

The optimum pH for growth was around 7.3 and Tok1 grew in a range from 5.5 to 10.2. No growth was observed at pH 3.5 or at pH 11.

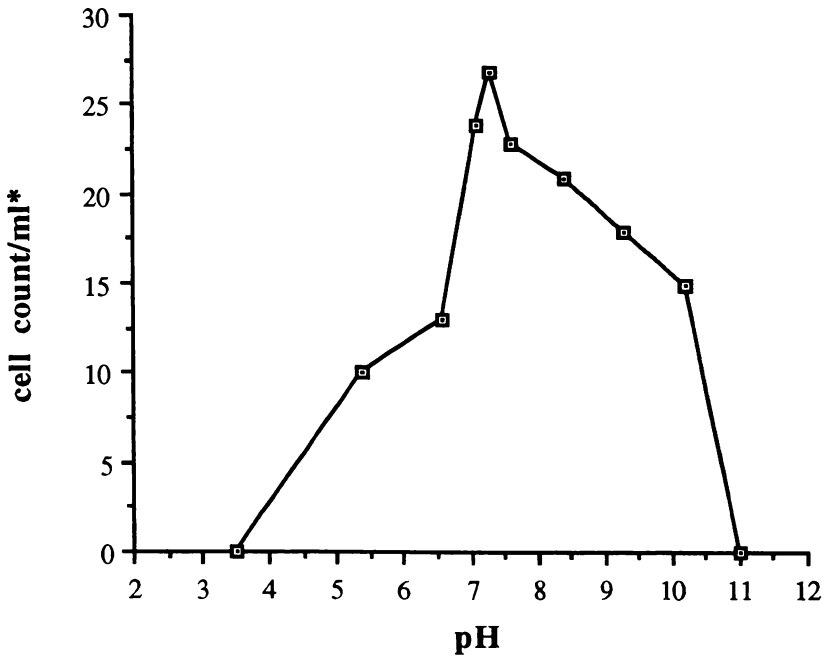


Fig. 4.15. Growth of strain Tok1 as a function of pH.
* Cell count x 10^6

4.4.3.4. Sodium concentration

To determine the optimal sodium concentration for growth NaCl was added to growth medium at various concentrations.

Optimal grwoth was obtained when no additional sodium ions were added to the medium. This corresponded to a sodium concentration of 2.9mM which was contributed by the nitrate and thiosulfate components of the medium. No growth was observed at 0.9% NaCl (154mM Na^+).

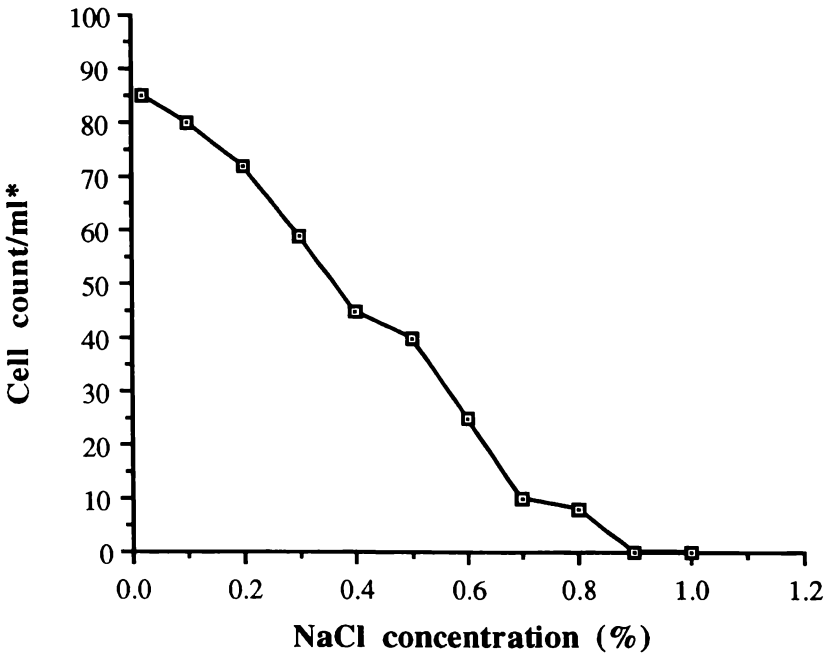


Fig. 4.16. Growth of strain Tok1 as a function of NaCl concentration.
* Cell count x 10^6

4.4.4. Carbon sources

Strain Tok1 grew well on konjac glucomannan and less well on locust bean gum. The final cell number on locust bean gum reached only 25% of the yields obtained on konjac glucomannan. The cell numbers of the second organism in the consortium, Strain Tok2 reached only a quarter of the number of strain Tok1 but grew slightly better on locust bean gum. Strain Tok1was able to grow autotrophically on CO_2/H_2 with sodium thiosulfate as electron acceptor but growth was very poor and very slow and reached only a tenth of the density of cultures grown heterotrophically. No growth was observed on yeast extract, tryptone, casein, casamino acids, glycogen, starch, amylose, amylopectin, glucose, fructose, sucrose, mannose, lactose, maltose, galactose, ethanol, methanol, pyruvate, propionate, fumarate, formate, acetate or lactate.
Tok1 was not only incapable of utilising yeast extract it was also inhibited by yeast extract (Fig.4.17.). The same was observed for tryptone.

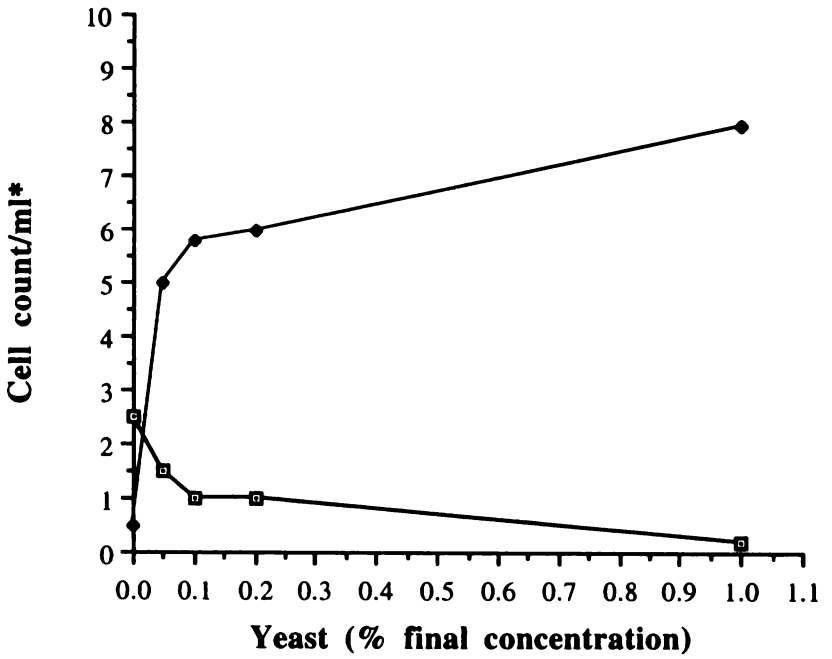


Fig. 4.17 Inhibitory effect of yeast extract on the growth of Tok1
Cell count of strain Tok1 (black squares), cell count of strain Tok2 (blue diamonds).
Cell count x 10^7

4.4.5. Electron acceptors

Electron acceptors were tested at a concentration of 10mM.

An electron acceptor was not absolutely necessary. Tok1 was able to grow without electron acceptor but growth was reduced to approximately one fifth of the usual cell density. Elemental sulfur not only failed to serve as electron acceptor but proved to be inhibitory for strain Tok1.

Sodium thiosulfate, the routinely used electron acceptor, could be replaced by various other compounds (Table 4.5.) of which sulfite was the most preferred. Growth on sulfite was about 125% of the growth obtained on thiosulfate. Tok1 was to a much smaller extent able to use nitrate and ferric sulfate as electron acceptors. Only a third to a quarter of growth yield was obtained on these electron acceptors. Even though some electron acceptors supported growth which did not support growth of strain Tok2 the growth was in general so poor that only two, sometimes three, transfers could be performed successfully which was not enough to remove all cells of Tok2 from the inoculum.

Electron acceptor	Growth
no electron acceptor	+
Na-thiosulfate	+++
O_2	+
Nitrate	++
Nitrite	+
Sulfite	++++
$Fe_2(SO_4)_3$	++
Na-fumarate	+
Thioglycollate	+
Cysteine	+
Malic acid	+
Maleic acid	+

Table 4.5. Electron acceptors tested for use by strain Tok1.
Cell counts: ++++ ~$8x10^7$ cells/ml; +++ ~$5x10^7$; ++ ~ $3x10^7$, + $1x10^7$

4.4.6. Antibiotic sensitivities

Strain Tok1 was sensitive to erythromycin (50µg/ml), gentamycin (50µg/ml), nitrofurantoin (50µg/ml), chloramphenicol (25µg/ml), tetracycline (50µg/ml) and colistin (50µg/ml). It was not inhibited by rifampin (100µg/ml), kanamycin (50µg/ml), netilmycin (50µg/ml), vancomycin (50µg/ml) and streptomycin (50µg/ml). Strain Tok2 was not sensitive to any of the antibiotics which did not inhibit growth of strain Tok1.

4.4.7. Metabolic endproducts and intermediates

Only metabolic endproducts that are different from compounds produced by Tok2 could be positively identified. Furthermore it is possible that some products could serve as carbon sources for Tok2 and disappeared from the medium before they could be identified or are further metabolised by Tok2 into another compound.

Presence of H_2S was shown by the precipitation of PbS when lead citrate solution was added. Pure cultures of Tok2 produce H_2S as well but more brown PbS precipitate was observed in consortium cultures when lead citrate was added to a sample from an exponentially growing culture than in Tok2 cultures.

Intermediates of konjac glucomannan degradation such as glucose, mannose and mannose polymers were investigated by running samples of cell free supernatant through a BioRad HPX.42A HPLC column (section 2.2.18.4.). For organic acids and simple sugars a BioRad HPX.87H HPLC column was used instead.

Throughout growth of the consortium a steady increase of glucose and mannose could be observed (see Fig.4.18.). Mannose and glucose are not observed in the culture supernatant of pure cultures of strain Tok2 growing on konjac glucomannan/yeast extract medium. The final concentration of glucose and mannose were around 19mM and 7mM respectively. The average ratio of glucose to mannose in konjac glucomannan is 5:8 (Shimahara 1975). The circumstance that less mannose than glucose is found in the culture supernatant despite its greater abundance in konjac glucomannan indicates that strain Tok1 preferentially metabolised mannose rather than glucose.

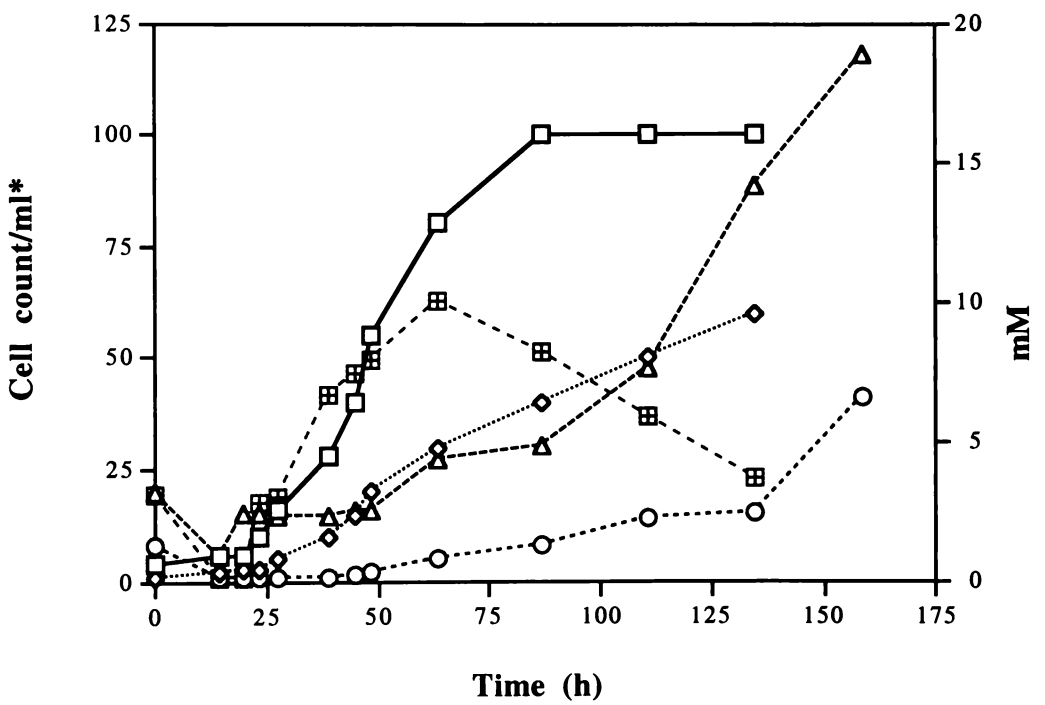


Fig 4.18. Increase of glucose, mannose and alanine throughout growth of the consortium.
Strain Tok1 cell count x10^6 (black squares); rods (red diamonds); alanine (turquoise crossed square); glucose (blue triangles); mannose (green circles).

Alanine, which is not found in the culture supernatant of Tok2, was detected in the supernatant of the consortium culture at concentrations up to 10mM. The final concentration could not be determined with certainty due to the unreliability of the quantifications of this HPLC system but a steady increase could be observed during

exponential growth and a slow decline in concentration during late exponential growth to a third of the concentration at exponental growth. In a culture which was seven days old very high concentrations of alanine were detected.
Some more compounds which could not be identified were present in the culture supernatant. One of them, with a retention time of 23.6min is present in the culture supernatant of Tok2 as well and might therefore not be a product of Tok1's metabolism. A compound with a retention time 27.7min could not be identified. Isovaleric acid which is one of the main metabolic products of strain Tok2 was not found in the culture supernatant of the consortium.

No clear mannan degradation pattern could be observed by running samples from culture supernatants through the HPX.42A column which separates oligosaccharides. In late samples (>50h) a peak is observed at a retention time of 17.3 min which is close to the expected retention time of sugar monomers (17.6). konjac glucomannan did not give a peak and was probably too big to enter the column.

4.4.8. 16S rRNA gene sequence

In order to sequence the 16S rRNA gene of Tok1 it was necessary to clone the PCR products obtained by amplification of the 16S rRNA gene from the total DNA isolated from the consortium (section 2.2.6.).
The gene was amplified using the primers af2-21 and ar1510-1492 (section 2.2.7.). The products were cloned into pGEM®-T which is a vector suitable for cloning PCR products with single 3' adenine overhangs which are produced by Taq polymerase. Positive clones were detected by blue/white screening.
Twenty positive colonies were investigated. A portion of each isolated plasmid was double digested with EcoRI in order to separate the insert from the vector and with EcoO109, an enzyme that possesses a slightly variable restriction site and cuts therefore frequently. The digested samples were run on a 1% agarose gel, stained with ethidium bromide and visualised on a UV transilluminator (see Fig.4.19.).
Of the 20 clones, 19 appeared to be the same; only one, clone 8, (Fig.4.19. lane 2) was different and showed a banding pattern as predicted for the 16S rRNA gene of Tok2 whose 16S rRNA gene sequence was known already (section 4.2.11.). All the bands of clone 8 were very weak and fuzzy. The clone was partially sequenced using the primer site T7. The obtained sequence contained many unidentifiable nucleotides. In a BLAST search executed with this sequence the matches with the highest similarity were the 16S rRNA genes from *Pyrobaculum aerophilum* and *Thermoproteus tenax*.

Two of the remaining 19 clones, M55 (Fig.4.19. lane 5) and M56 (Fig.4.19. lane 6), with the predominant banding pattern were chosen for sequencing. The clones were sequenced using the primers T7 and SP6 which flank the cloning site of the vector and two archaea specific internal 16S rDNA primers, af515 and af1042-1060 (section 2.2.7. for sequence)

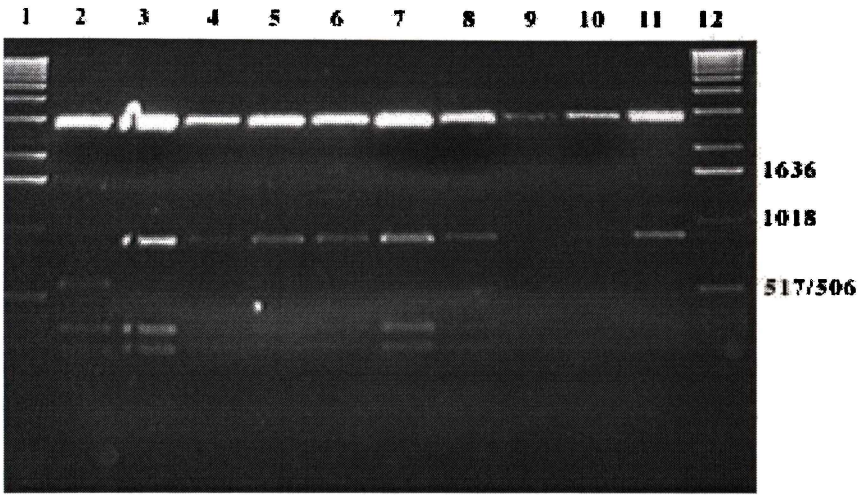


Fig. 4.19. EcoO109/EcoRI double restricted pGEM®-T plasmids containing 16S rDNA inserts amplified from DNA from the consortium.

The sequencing reactions with T7 and af515 worked well and about 600 nucleotides could be identified with each primer but the reactions with SP6 and af1042-1060 were both terminated prematurely. To overcome problems caused by a possible secondary structure of the insert all sequencing reactions were rerun with 10% DMSO in the reaction mix. This was not successful; nor was the attempt to use a different primer site on the vector. The reaction stopped abruptly at the same place, about 20 nucleotides away from the 3'-end of the gene.

Both clones had identical sequences and had the same orientation in the vector. None of the clones could be sequenced beyond 1200 nucleotides.



Fig. 4.20. Sequence of clone M56 containing the putative 16S rRNA gene sequence of consortium strain Tok1.
Only 1200bp were sequenced due to termination of sequencing reaction.

4.4.9. RFLP analysis of the PCR products obtained by amplification of the 16S rRNA gene portions from consortium DNA

In order to determine the number of organisms present in the consortium, an RFLP analysis of amplified consortium 16S rDNA was carried out.

Using the program Sequencher™ 3.0.1 Demo (Gene Codes Corp., Ann Arbor, MI, USA), the predicted restriction patterns of various restriction enzyme digests of M56 and Tok2 16S rDNA amplification products were determined. Restriction enzymes producing no more than 5 fragments were tested. The Sequencher™ analysis led to the selection of a portion of the gene in order to keep the restriction pattern simple and to exclude a more conserved region which gave identical restriction fragment sizes for both sequences. The length of this portion was 926 and 911bp for the M56 and Tok2 sequences respectively.

The predicted fragments produced by four restriction enzymes from the M56 and Tok2 amplification products are shown in Table 4.6.

Restriction enzymes	Fragments (bp) Tok1	Fragments (bp) Tok2
AluI	926	17, 123, 290, 481
ApaI	22, 316, 588	119, 320, 472
EcoO109	7,165, 169, 244, 341	7, 123, 155, 161, 465
HaeII	157, 235, 534	56, 99, 365, 391

Table 4.6. Predicted sizes of restriction fragments of the partial 16S rRNA gene of M56 and Tok2.

The amplification of 16S rDNA from the consortium was carried out with the forward primer af2-21 and an internal reverse primer, ar976-958 (section 2.2.7.). Due to the low melting temperature (44°C) of the reverse primer, the annealing temperature had to be lowered to 41.5°C. The PCR was otherwise carried out as outlined in section 2.2.7. A control PCR reaction with universal bacterial primers pA and pH* was run in parallel to check for the presence of bacterial members of the consortium. The consortium DNA was used at two different concentrations, 38ng and 60ng per 100µl reaction. The PCR gave fragments of the expected size for both reactions with the consortium DNA (see Fig.4.21.). The consortium DNA amplified with bacteria-specific primers did not result in any fragments. The control without DNA was also negative.
Amplification of DNA from strain Tok2 failed. The DNA however had an $OD_{260/280}$ ratio of 1.6 which means that the DNA preparation still contained some protein. This might have inhibited the PCR reaction.

The obtained DNA fragments were purified using a Wizard Prep (Promega) and concentrated two fold. Restriction digestion of the purified fragment was carried out with 8µl DNA and 1U restriction enzyme. The assays were incubated for 1.5 hours to ensure a full cleavage of the DNA at the appropriate temperature for each enzyme. The restriction digests were run on a 2% agarose gel at low voltage (50mV) for good separation of the fragments. The gel was stained with ethidium bromide and photographed using the Eagle Eye Gel system.

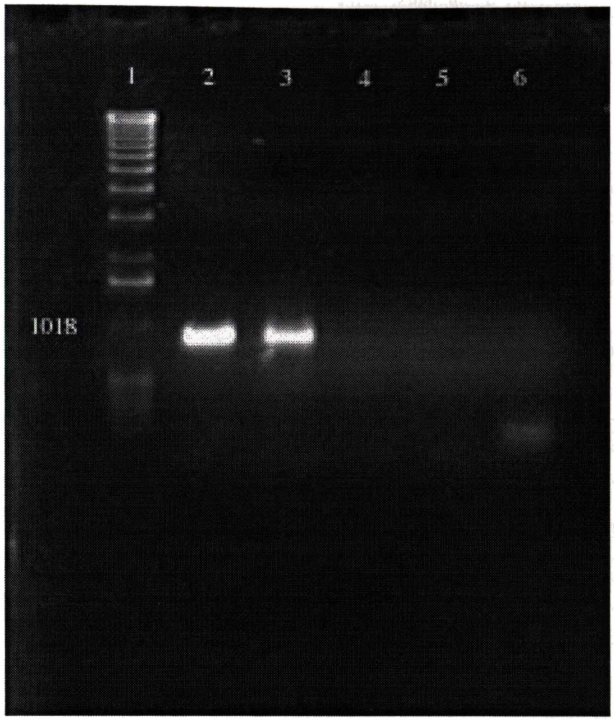


Fig. 4.21. Partial 16S rRNA gene amplification products on a 1% agarose gel.

All the restriction fragments obtained were as expected for the M56 fragment (see Fig.4.22.). No fragments were found in any of the four restriction digests which would correlate with any fragments predicted for the strain Tok2 16S rRNA gene, and no additional unaccounted fragment were observed. The PCR product seemed to consist solely of amplification products obtained from DNA of the M56 sequence.

4.4.10. Whole cell hybridization with fluorescently labelled oligonucleotide probes

To ascertain that the 16S rRNA gene sequence which was obtained by cloning the PCR products from the consortium derived from cells of strain Tok1, oligonucleotide probes were constructed which specifically hybridize with either the ribosomal RNA from Tok1 or Tok2. The probes had 6 nucleotide differences in their 15 base length (see below) and were labelled with two different fluorescent chromophores, fluorescein for the M56 probe and rhodamine for the Tok2 probe.

M56	AGTAAGTCCCCACAG	Fluorescein labelled
Tok2	GGCAAGTCGCTCCTG	Rhodamine labelled

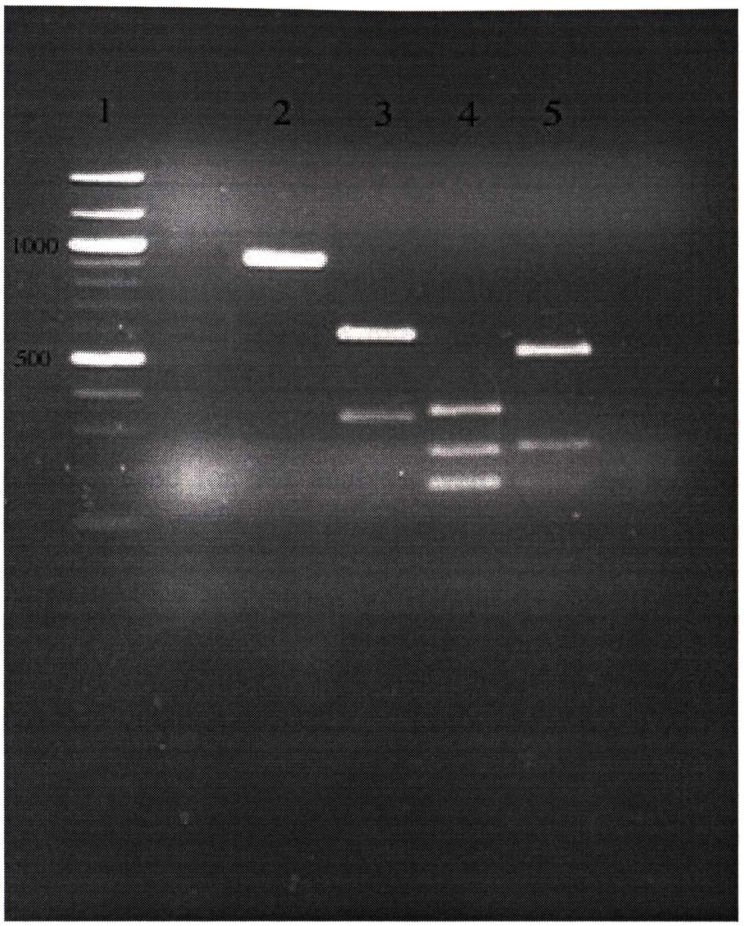


Fig. 4.22. RFLP of the 970bp PCR product obtained from consortium DNA with 16S rRNA primers.

Fixation and hybridization of the cells was carried out as described in section 2.2.15. Hybridization was carried out at two different temperatures (45°C and 50°C) and three different formaldehyde concentrations (0%, 20%, 40%) of the hybridization buffer, which contained both probes at 50ng per hybridization.

Coccoid objects were observed emitting weak green fluorescence, confirming that the M56 clone sequence was derived from the coccoid organism. Fluorescence however could not be captured on film. No rods emitting green fluorescence were observed. Adjustment of hybridization conditions is necessary and will be carried out in future.

4.4.11. Phylogenetic analysis of the 16S rRNA gene sequence from Tok1

The 16S rRNA gene sequence from strain Tok1 was compared using BLAST search (Altschul et al. 1997) with sequences available in the GenBank database. Closely related sequences were subsequently used to generate phylogenetic trees. Sequences were aligned with Clustal V and Clustal W 68K was used to calculate branching order, phylogenetic distances and for generating bootstrapping values. TreeView was used to draw phylogenetic trees using the data obtained with ClustalV and Clustal W 68K.
The 16S rRNA genes used for this analysis were truncated to 1200bp since the remaining 300bp of the 16S rRNA gene from strain Tok1 could not be sequenced.

The highest homologies of the 16S rRNA gene of Tok1 are with the 16S rRNA genes of members of the kingdom Crenarchaeota (Table 4.5.) and within that kingdom with members of the Order "Igneococcales" (Burggraf et al. 1997).

Species	% homology of the 16S rRNA gene
Caldococcus noboribetsu	92.4
Staphylothermus marinus	91.4%
Thermosphaera aggregans	91.4%
Stetteria hydrogenophila	90.6%
Sulfophobococcus zilligii	90.4%
Pyrodictium occultum	89.2%
Hyperthermus butylicus	88.4%
Desulfurococcus mobilis	88.3%
Thermodiscus maritimus	88.2%
Thermofilum pendens	87.6%
Thermoproteus tenax	84.8%

Table 4.7. % similarities between the 16S rRNA gene of Tok1 and close relatives.

In a further phylogenetic analysis, these sequences and sequences of some representatives of the other crenarchaeotal orders were used to generate a phylogenetic tree (see Fig.4.23) using the neighbour-joining method. The sequence of the euryarchaeote *Pyrococcus furiosus* was used as an outgroup.

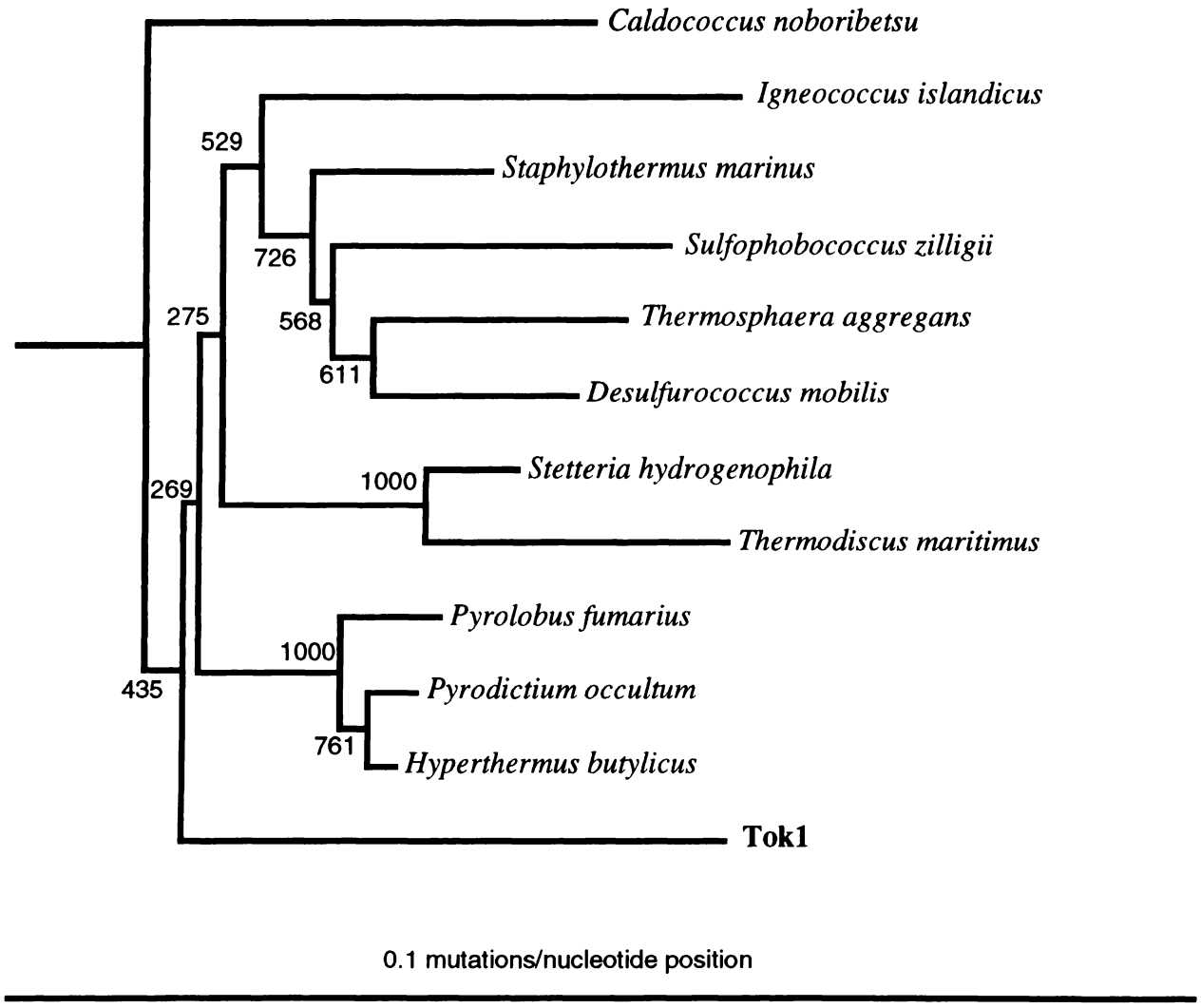


Fig. 4.23. Phylogenetic tree of the order "Igneococcales" rooted with *Sulfolobus acidocaldarius* as outgroup.

In the phylogenetic analysis, strain Tok1 was placed as a phylogenetically deeply-branching member of the order "Igneococcales". The branching order is not very well supported by the low bootstrap values obtained.
Only the isolate "*Caldococcus noboribetsu*" branches off lower than strain Tok1. "*Caldococcus noboribetsu*" is an isolate from Noboribetsu, Japan and has not been validly described and named yet. Aoshima et al. 1996 described the isolate as an acidophilic, moderately thermophilic crenarchaeote which is sulfur dependent.

4.5. Strain Tok1 - Discussion

4.5.1. Working with a consortium

Strain Tok1 was found in an enrichment culture growing on konjac glucomannan. Coccoid cells could be clearly visually distinguished from the rod-shaped organisms in the enrichment culture. One organism could be isolated by plating and was named Tok2 (see section 4.1. and 4.2.) Exhaustive attempts to purify the coccoid member of the consortium, named strain Tok1, included: plating on various media and solidifying agents; dilution series; growing at different pHs, temperature or with different electron acceptors and equilibration centrifugation (section 4.4.1.). Also, various cell fractions of the purified strain Tok2, such as cell free supernatant from a Tok2-culture or sonicated Tok2 cells, were added to the medium in order to enhance growth of strain Tok1 in case Tok1 was dependent on a cell compound or a metabolic product from strain Tok2. A dependence like this is known for some archaea such as *Thermofilum pendens* (Zillig et al. 1981) which needs a polar lipid fraction of *Thermoproteus tenax* for growth. All these attempts to isolate strain Tok1 failed. There was a possibility that the rods and cocci are just varied morphological shapes of the same organism. This hypothesis can clearly be dismissed. Strain Tok2 did not produce free coccoid-shaped cells if it was grown under the same conditions as the consortium and hardly grew at all with konjac glucomannan as sole carbon source. The spherical bodies frequently seen in cultures of Tok2 were always attached to the rod-shaped cells and were in general bigger and less regular than the cocci. For *Pyrobaculum aerophilum* (Völkl et al. 1993) it was reported that cells can shorten in ageing cultures until the culture consists mainly of coccoid cells. This was not observed for Tok2.

A dependence of strain Tok1 on the presence of strain Tok2 can not be absolutely excluded even though attempts to stimulate growth of Tok1 with cell extracts from Tok2 failed. It is possible that Tok1 needs a compound which only living cells of Tok2 can provide, such as a gaseous end product for example, or that Tok2 removes a compound from the medium which might be toxic to Tok1.

The isolation of Tok1 was made even more difficult by the resistance of Tok2 to most antibiotics and by its ability to withstand unfavourable conditions. An additional difficulty proved to be the inability of Tok1 to grow from a small inoculum. Only cultures containing more than ~$5x10^6$ cells/ml could successfully be used as inocula which made it impossible to isolate Tok1 by serial dilution. It was not possible to selectively remove Tok2 from an inoculum and all the ranges for the various growth parameters of Tok2 were slightly wider than for Tok1. Therefore it was not feasible to

isolate Tok1 with standard microbiological methods. The most promising difference between the two organism is the ability of Tok1 to tolerate oxygen up to 3.3% (vol/vol) whereas Tok2 is strictly anaerobic. Unfortunately growth of Tok1 is extremely poor and slow under these conditions and Tok2 started growing before an appropriate cell density of Tok1 was reached. By the time Tok2 started to grow the redox indicator resazurin had turned colourless indicating the removal of oxygen from the medium and therefore enabling Tok2 to grow as well. The disappearance of oxygen is likely to be due to the production of H_2S by Tok1. It might be possible to isolate Tok1 by setting up a culture where the oxygen concentration is held at a constant low level but this would be difficult given the gel-like properties of the substrate and this approach was not attempted.

The characterisation of strain Tok1 had to be done with a mixed culture which means some physiological properties can not be described with certainty until a successful isolation of the strain. A possible way to isolate Tok1 might be the use of "optical tweezers". Optical tweezers have recently been applied successfully to isolate the crenarchaeote *Thermosphaera aggregans* (Huber et al. 1995a; Huber et al. 1998a) and might be a useful tool to separate Tok1 from Tok2, but the gel-like consistency of the konjac glucomannan and the inability of strain Tok1 to grow from a small inoculum make this doubtful.

4.5.2. How many organisms are in the consortium?

It was possible to distinguish two morphologically different organisms in the consortium which defines a lower limit to the number of organisms present. The possibility of the presence of a second rod-shaped organism could not be excluded, nor could in fact the presence of more than one coccus. Several attempts were undertaken to clarify the number of species in the consortium.

The 16S rRNA genes were amplified with archaea-specific primers from total DNA of the consortium, cloned into *E.coli* and sequenced (section 4.4.9.). Two different inserts were found verifying clearly the presence of at least two different organisms. One type of insert represented only 5% of all the analysed clones. The amount of plasmid isolated from this clone was very small even though all clones were grown to similar cell densities and under conditions selective for plasmid carrying cells. Additionally this clone did not give a clear banding pattern in a restriction analysis and the bands did not add up to a fully sized 16S rRNA gene. The short nucleotide sequence obtained from this clone showed high similarity to the 16S rRNA gene sequence which was obtained by amplifying the 16S rRNA gene from DNA of a pure Tok2 culture.

In a RFLP analysis of a 970bp PCR product which was obtained by amplifying the 5' end of the 16S rRNA genes from consortium DNA using archaea-specific primers, no DNA fragments were found which could be predicted for the Tok2 16S rRNA gene based on the sequence (see section 4.4.11.). All banding patterns were those predicted for the Tok1 16S rRNA gene. This is consistent with the low cloning efficiency of the 16S rRNA gene from Tok2. The circumstance that one out of 20 clones contained the Tok2 gene shows that at least a small percentage of the PCR products are derived from Tok2. The amplification of the 16S rRNA gene from pure Tok2 DNA was easily possible with the primers used to amplify the 16S rRNA gene from the consortium DNA (see section 4.1.11.) and using the same PCR conditions. In a PCR with DNA from the consortium there seems to be a tremendous bias against amplification of the Tok2 gene. Another possibility might be that Tok2 yields less DNA in a DNA isolation from a consortium. Difficulties in the isolation of DNA were not encountered when DNA was isolated from a Tok2 culture but in a well grown consortium culture Tok1 outnumbers Tok2 by a factor of 3-4 which is presumably reflected in the DNA isolated. The second and most abundant type of clone obtained from amplified consortium DNA, was sequenced and the 16S rRNA gene sequence which was obtained showed the highest similarity to members of the order "Igneococcales" (Burggraf et al. 1997). It was not possible to sequence the entire 16S rRNA gene which might be due to premature termination of the sequencing reaction at a secondary structure at the 3'-end of the 16S rDNA insert. A repetition of the sequencing reaction with other clones or under slightly denaturing conditions failed to procure the missing piece of the sequence. Such sequencing problems are occasionally encountered (Stacey Farmer, Waikato Sequencing Unit) and are not easily overcome.

It was necessary to prove that this 16S rRNA sequence was indeed obtained from the coccoid member of the consortium. It was therefore attempted to hybridize whole cells with specifically designed oligonucleotide probes which were fluorescently labelled for the two 16S rRNA gene sequences. It was possible to construct oligonucleotides of 15 nucleotides length which differed by 6 nucleotides. The hybridization resulted in a positive result for Tok2 which hybridised with the probe designed for its ribosomal RNA without any cross reactions with the second probe. Very few coccoid cells could be seen at all on the slides after fixation under the phase contrast microscope. It was observed that the coccoid cells did not pellet well after paraformaldehyde fixation and should be filtered in future attempts to hybridise the cells of the consortium. Some cocci were observed which emitted light at the expected wavelength for the probe which was designed for the M56 sequence. No coccoid cells which cross-reacted with the Tok2 probe were ever found even under low stringency conditions.

There are also some physiological factors which support the hypothesis that only one coccoid strain is present. The cocci have an extremely narrow carbon source spectrum.

Konjac glucomannan and to a smaller extent locust bean gum are the only carbon sources that support growth. It seems rather unlikely to find two different organisms in one sample which grow exclusively on a carbon source which is not widely used by other related species. Inhibition by traces of yeast extract is another distinctive mark which is not very usual. The inhibitory effect of S^0 is slightly more common among the "Igneococcales". *Sulfophobococcus zilligii* (Hensel et al. 1997) and *Thermosphaera aggregans* (Huber et al. 1998a) are both known to be inhibited by elemental sulfur. Taking all this into account, it seems highly probable that there is only one coccoid strain - named Tok1 - in this consortium and that the 16S rRNA gene sequence obtained from clone M56 belongs to this coccoid organism. It would be too great a coincidence to find two morphologically indistinguishable organisms which have all these unusual characteristics in common unless they are two strains of the same species.
There is still a small probability that there is more than one rod-shaped organism in the consortium. The fact that two different colony-types which both consisted of rod-shaped organisms were obtained by plating on yeast extract/konjac glucomannan medium (see section 4.1.1.) supports this possibility. Inoculation of liquid medium with the brownish-green type of colony resulted in the isolation of Tok2. The tiny (<0.5mm in diameter) white colonies could not be transferred into liquid culture successfully. In later platings this type of colony was no longer observed. It is conceivable that in the early enrichment cultures a second rod-shaped organism was present which was lost after many transfers of the consortium on konjac glucomannan medium. No indication of a third organism was found in the cloning experiment (see section 4.4.9.) and the RFLP experiment described in section 4.4.10.

4.5.3. The morphology and physiology of strain Tok1

Strain Tok1 is morphologically and in many physiological aspects a typical member of the order "Igneococcales". All members of this order are coccoid or disc-shaped organisms. Cells of Tok1 exhibit a fuzzy surface under the electron microscope. It is unclear if this is due to a mucous layer around the cell or if this is an artefact caused by the very viscous substrate adhering to the cell walls. Konjac glucomannan has a marked ability to swell and forms gels at elevated temperatures. The growth medium which contained 0.2% (w/v) konjac glucomannan formed a solid gel at 90°C which could not be softened by shaking the tube. Only after growth of Tok1 started did the medium liquefy again. The large cell clusters which are found in exponential cultures might be a result of this property of the konjac glucomannan. These clusters were not found in late exponential cultures when the viscosity of the medium was much reduced. The clusters might therefore be caused by the konjac glucomannan gel holding cells together. There

are however a number of members of the order "Igneococcales" known to form aggregates. *Staphylothermus marinus* (Fiala et al. 1986) and *Thermosphaera aggregans* (Huber et al. 1998a) both form big clusters of cells.

All of the described "Igneococcales" species are hyperthermophiles which grow around neutral pH with the exception of the recently reported isolate 1904 (Prokofeva et al. 1998) from a hot spring on Kamchatka peninsula which grows optimally around pH 3.8 and 80°C and the as yet not fully described "Caldococcus noboribetsu" (Aoshima et al. 1996), which grows optimally at pH 3. Like *Sulfophobococcus zilligii* (Hensel et al. 1997) and *Thermosphaera aggregans* (Huber et al. 1998a), Tok1 is not capable of reducing elemental sulfur which all other "Igneococcales" species are. Like *S. zilligii* and *T. aggregans* Tok1 is even inhibited by elemental sulfur. Tok2 produces some S^0 but in very small amounts which seem not to inhibit growth of Tok1. Like these two sulfur-inhibited organisms Tok1 grows optimally at neutral pH (section 4.3.3.3.) but is also able to grow at much higher pHs. Growth at pH 10.2 is however very feeble. The optimal growth temperature of strain Tok1 is at 98°C (section 4.3.3.2) far higher than that of *S. zilligii* and *T. aggregans* and is among the highest of the order. Of the kingdom Crenarchaeota only members of the family Pyrodictiales have higher optimum growth temperatures (see Table 4.6.).

Strain Tok1 is set apart from all the other described members of the "Igneococcales" by the fact that growth is inhibited by even small amounts of yeast extract and tryptone. It is reported for *Thermoproteus tenax* (Zillig et al. 1981) that yeast extract partially inhibits growth at 0.005% and totally at 0.01%, but that growth is stimulated at concentrations between 0.001% and 0.002%. This was not observed for Tok1. Yeast extract and proteinaceous compounds are suitable and highly preferred substrates for all the other heterotrophic members of the "Igneococcales" and the mixotrophic *Stetteria hydrogenophila* (Jochimsen et al. 1997). *Sulfophobococcus zilligii* (Hensel et al. 1997) even grows exclusively on yeast extract.

Carbohydrates are rarely utilised by members of this order. *Thermosphaera aggregans* (Huber et al. 1998a) is able to grow on heat-treated xylan and *Pyrodictium abyssi* (Pley et al. 1991) can grow on starch. It is therefore surprising that a member of the "Igneococcales" grows exclusively on a complex carbohydrate such as konjac glucomannan, even though hemicelluloses might be found in many hot springs which often contain dead plant material. No other archaea are reported to be capable of degrading glucomannan or galactoglucomannan. Tok1 grew preferentially on konjac glucomannan. Konjac glucomannan consists of a backbone of β-1,4-linked mannose and glucose units and has no side-branches other than acetyl units on every sixtieth mannose unit, and does not contain galactose. The mannose to glucose ratio is 5:8 (Shimahara 1975). Locust bean gum which contains β-1,6-linked galactose side branches was utilised to a much lesser extent. Throughout growth of a consortium

culture, the concentration of glucose increased faster than the concentration of mannose which might indicate that Tok1 metabolises mannose more readily and that it is either not able to utilise glucose or that it has a preference for mannose. No breakdown pattern of the konjac glucomannan could be perceived. The rapidity of the decrease in viscosity of the medium suggests an endo-acting mannanolytic enzyme rather than an exo-acting one. In tests for substrate specificity a cell wall fraction which was highly active against konjac glucomannan exhibited some activity against xylan especially oat bran xylan (section 5.1.4.). Tok1 was nevertheless not able to grow on xylan as sole carbon source which might show that the enzyme is capable of cleaving the β-1,4 glycosidic bonds in xylan but that the organism can not utilise the break down products. One of the major metabolic endproducts is alanine which is frequently found as a metabolic product in archaea (Kengen and Stams 1994).

4.5.4. The phylogenetic position of strain Tok1

In a phylogenetic analysis executed with the Clustal programs Tok1 is clearly located within the order "Igneococcales". Using the 1200 nucleotide fragment which was obtained by sequencing clone M56 and truncating all the 16S rRNA sequences which were included in this analysis, it was shown that Tok1 branches off very early in the "Igneococcales" division of the Crenarchaeota (see Fig. 4.23.). Only "*Caldococcus noboribetsu*" branches off even earlier then strain Tok1. "*Caldococcus noboribetsu*" has not been properly described yet and information about it is meagre. It is a hyperthermophilic and acidophilic organism whose growth is enhanced by elemental sulfur.

The closest neighbours based on 16S rRNA gene sequence homology are *Thermosphaera aggregans* and *Sulfophobococcus zilligii* with which Tok1 shares the physiological characteristic of inhibition by elemental sulfur.

The phylogenetic analysis clearly shows that Tok1 is not only a novel species but represents a novel genus as well. It is astonishing that most genera of the "Igneococcales" contain only one species with the exception of the genera *Pyrodictium* and *Desulfurococcus*. One would expect that more representatives of the various genera remain to be discovered.

	Morphology	Temperature range and **optimum** in °C	pH range and **optimum**	Metabolism	Oxygen relations	Carbon sources
Candidatus "*Igneosphaera vetusta*" (Tok1)	regular coccus, forms aggregates	75-99 **98**	5.5-10.2 **7.3**	heterotrophic. facultatively autotrophic	microaero-tolerant	glucomannan
Thermosphaera aggregans	regular coccus, forms aggregates	65-90 **85**	5-7 **6.5**	heterotroph	strictly anaerobic	yeast extract, peptone, gelatin, xylan
Sulfophobococcus zilligii	regular to irregular coccus	70-95 **87**	6.5-8.5 **7.6**	obligate heterotroph	strictly anaerobic	yeast extract
Staphylothermus marinus	coccus, forms chains and clusters	65-98 **92**	4.5-8.5 **6.5**	heterotroph	strictly anaerobic	yeast extract, peptone, cell extracts
Desulfurococcus species	regular coccus	75-93 **85**	5-7 **6**	heterotroph	strictly anaerobic	proteins, peptides
Hyperthermus butylicus	irregular coccus	95-107 **100**	**6**	fermentative	obligate anaerobic	tryptone, digest of casein
Pyrodictium species	disc shaped with a network of fibres	80-110 **98-105**	5-7 **5.5**	H_2/S^0 autotrophy or fermentation	strictly anaerobic	yeast extract, meat extract, tryptone, starch
Aeropyrum pernix	irregular coccus with sharp edges	70-100 **90**	5-9 **7**	heterotroph	strictly aerobic	yeast extract, peptone, tryptone
Pyrolobus fumarii	lobed coccus	90-113 **110**		obligate chemolitho-autotroph	aerobic	CO_2
Stetteria hydrogenophila	irregular coccus, disc shaped	68-102 **95**	4.5-7 **6**	mixotrophic, S^0 and H_2 dependent	strictly anaerobic	yeast extract, peptone, tryptone, casamino acids

Table 4.8 Physiological characteristics of members of the order "Igneococcales"

4.5.5. Proposal to establish Tok1 as a novel species of the Crenarchaeota

Since strain Tok1 has not yet been purified, it is impossible to properly name and describe it. For such cases, the status "Candidatus" has been established to enable the description of novel organisms which have not been isolated and hence do not satisfy the requirements of the International Committee on Systematic Bacteriology for description as a formal taxon (Murray and Schleifer 1994).
On these grounds, the name Candidatus *Igneosphaera vetusta* is proposed for the strain Tok1. The prefix "Igneo-", from Latin *ignis*, fire or flame, reflects the hyperthermophily of Tok1; and "sphaera" from the Greek noun σφαιρα (*sphaira*) indicates its morphology. The Latin word "vetusta" means old in the sense "of long standing" (H.G. Trüper, pers. comm.) and indicates the phylogenetically ancient position of Tok1.

4.5.6. Candidatus *Igneosphaera vetusta*

Igneosphaera ig.neo.sphae'.ra *igneo* L. comb. form, f. *ignis*, fire; *sphaera* L. n.fem. f. Gk. σφαιρα (*sphaira*), sphere; vetusta ve.tus'.ta. L. adj. old, of long standing, referring to the phylogenetically ancient position of Tok1.

Regular coccus which forms aggregates maintained in a consortium with strain Tok2, a *Pyrobaculum* isolate. 1-1.2μm in diameter. Anaerobic to microaerotolerant. Can withstand 3.3% (v/v) oxygen. Grows optimally at 98°C (range 75-99°C) and at pH 7.3 (range 5.5-10.2) and grows up to 137mM Na^+ (optimally around 2.7mM). Electron acceptor thiosulfate, sulfite, to a lesser extent nitrate and ferrous sulfate. Growth is inhibited by elemental sulfur and yeast extract. Sole carbon sources are konjac glucomannan and to a much lesser extent locust bean gum.
Isolated from a hot spring at Tokaanu, New Zealand. Phylogenetically closely affiliated with the order "Igneococcales".

Chapter 5 - A mannanase from the consortium

5.1. Results

5.1.1. Determination of the producer strain of the mannanase

Mannanase activity was tested by following the increase of reducing sugars during incubation of konjac glucomannan with an enzyme preparation. The concentration of sugars was determined by PAHBAH assay (section 2.2.19.).

To determine which member of the consortium produced the mannanase, a culture of the consortium was grown on konjac glucomannan medium and a culture of strain Tok2 was grown separately on konjac glucomannan with 0.02% yeast extract to stimulate growth of strain Tok2. The activity present in culture supernatant, resuspended cells and sonicated cells of both cultures was determined. Since this work was done with crude fractions and not with a purified enzyme, it was impossible to relate activities to protein concentrations. For that reason increases in reducing sugars are related to 1ml of the assay. To correct for the five-fold concentration of the cells in comparison to the supernatant throughout the preparation process, values for cell preparations were divided by five to make them congruous with the values for the supernatants.

Tested fraction	Increase of reducing sugars during 30min (nmoles*)	Average increase per min (nmoles)
Negative control	0.2	0.006
Supernatant of the consortium	-8.7	-0.29
Cell pellet of the consortium	12	0.4
Supernatant of strain Tok2 culture	-2	-0.06
Cell pellet of strain Tok2	-1.06	-0.03

Table 5.1. Mannanolytic activities of supernatant and cell fraction of the culture and of strain Tok2.
*nmoles/ml of enzymatic assay

The mannanolytic activity was clearly present in the cell fragments of the mixed culture. There was no activity detected in the supernatant or in the cell fragments of Tok2. No activity was found in the supernatant of the mixed culture which had a very high concentration of reducing sugars already. No significant increase in reducing sugars was found in the negative control which contained substrate but no enzyme, indicating that konjac glucomannan is stable under the assay conditions.

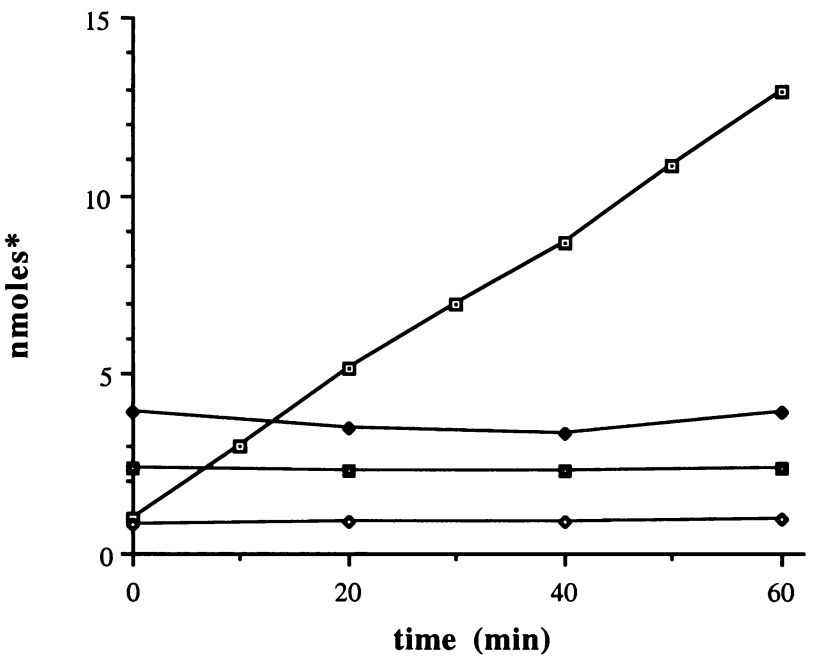


Fig. 5.1. Mannanase activities of consortium cells and supernatant and of strain Tok2 cells and cultures.
The values for the assay of the culture supernatant of the consortium were not plotted here because the high sugar background would overshadow the remaining curves - no increase in reducing sugars was observed (Table 5.1.). Consortium cell preparation (black squares), strain Tok2 supernatant (blue diamonds), strain Tok2 cell preparation (red squares), negative control (green diamonds). * nmoles per ml of assay

5.1.2. Determination of the location of the enzyme

In order to determine if the enzyme was secreted or cell-associated, and in the case that it was located in the cell, to determine its cellular location, several different cell fraction were assayed.

The tested fractions were:

- Cell free culture supernatant, which was obtained by filtering the culture supernatant through a 0.22μm filter.
- Washed cells supernatant, which was obtained by resuspending the cell pellet in 20mM Mops. The cells were collected by centrifugation and the supernatant was filtered through a 0.22μm filter.
- Cytoplasmic fraction (supernatant of the sonicated and pelleted cells)
- Cell wall fraction: suspension of the pellet of the sonicated cells in 10mM Mops.

The continuous increase of reducing sugars in the assay with the cell wall fraction during incubation (Fig. 5.2. and Table 5.2.) shows clearly that the enzyme is attached to the cell walls.

Tested fraction	Increase of reducing sugars during 30min (nmoles*)	Average increase per min (nmoles)
Negative control	-2	-0.066
Supernatant	2	0.066
Cell wash	-4	-0.133
Cytoplasmic fraction	-0.2	-0.006
Cell walls	33.6	1.12

Table 5.2. Mannanolytic activity of various cell fractions of the consortium.

The data show that reducing sugars were released in the assay using the resuspended pellet of the sonicated cells but not in the assay with the cytoplasmic fraction (Table 5.2). No significant activity was found in the culture supernatant indicating that the mannanase is not secreted. A large amount of reducing sugars is present in the assay with the supernatant from the start, which means that a lot of reducing sugars are present in the medium already. The supernatant data was therefore not plotted in Fig. 5.2. since it would distort the scale of the other curves.

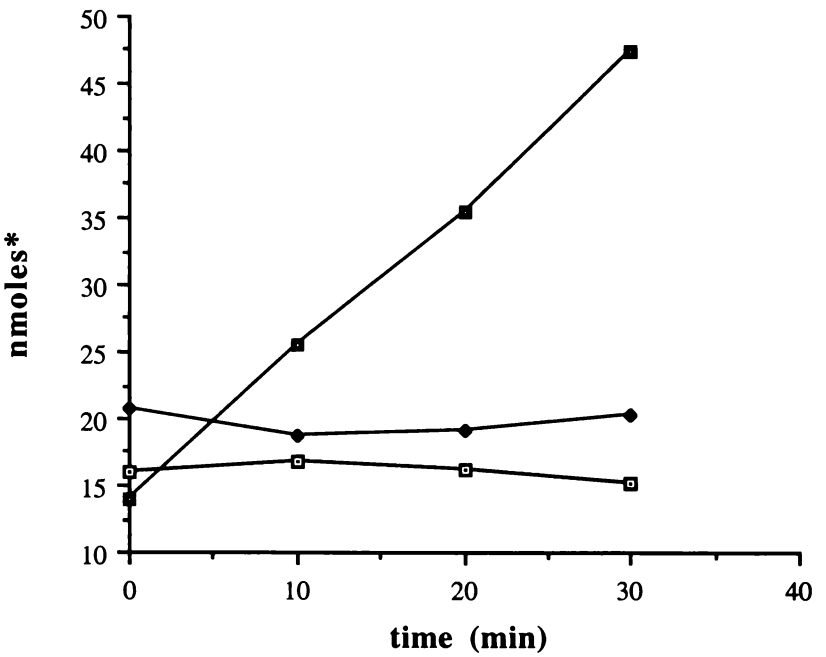


Fig. 5.2. Activities in various culture fractions of the consortium.
*nmoles/ml of enzymatic assay. Cell wall fraction (red squares); cell wash (black squares); cytoplasmic fraction (blue diamonds).

5.1.3. Substrate spectrum of the Tok1 mannanase

Substrate specificity was investigated by incubating various hemicelluloses with a fresh enzyme preparation. Exponentially grown cultures were harvested, washed with 20mM Mops and sonicated for five minutes at the highest energy setting. 50μl of this preparation were added to 3ml of 1% solutions of the tested carbohydrate polymer. For each substrate a negative control containing no enzyme was assayed in parallel to check for thermal degradation of the substrate.

The mannanase exhibited some activity against ivory nut mannan and against three different xylans, larchwood, birchwood and oatspelt xylan (Table 5.3) and against the crystalline cellulose Avicell. No activity or very low activity could be detected against starch, amylose and amylopectin, CMC or the galactoglucomannan-containing substances locust bean gum and guar gum. It was observed that ivory nut mannan and locust bean gum were highly insoluble.

Substrate	Increase of reducing sugars (nmol*) negative control	Increase of reducing sugars (nmol*) + enzyme	% Activity ‡
Konjac glucomannan	4.34	24.5	100
Ivory nut mannan	4	8.31	21.3
Locust bean gum	2.15	3.18	5.1
Guar gum	0.5	0.716	0.9
Starch	0.77	2.62	9.17
Amylose	3.1	2.46	-
Amylopectin	1	2.1	5.4
Birchwood xylan	1.7	5.18	17.2
Larchwood xylan	2.45	5.22	13.7
Oat spelt xylan	1.16	7.76	32.7
CMC	1.5	3.4	9.42
Avicell cellulose	1.2	5.0	18.8

Table 5.3. Increase in reducing sugars throughout incubation of various substrates with cell preparation from a consortium culture grown on 0.2% konjac glucomannan. *nmoles per ml of enzymatic assay. ‡ % activity is corrected for the increase of reducing sugars in the negative control.

Despite the clear indication of activity against other substrates than konjac glucomannan, especially of xylanase and cellulase activity, the main activity of the enzyme seems to be the mannan degrading activity. The activity against oat spelt xylan reaches 32.7% of the activity against konjac glucomannan and is therefore higher than the activity against ivory nut mannan.

5.1.4. Influence of temperature on the enzymatic activity

To determine the temperature at which the enzyme exhibited the highest activity, enzyme assays were run at different temperatures. Above 100°C an oil bath was used and the enzyme preparation was injected through a septum into the substrate. The substrate was prewarmed to the tested temperature. Substrate without enzyme was incubated at each temperature simultaneously to test for non-enzymatic degradation at the incubation temperature.

Temperature (°C)	Reducing sugars (nmoles) negative control	Reducing sugars (nmoles) with enzyme	Increase in reducing sugars corrected*	% activity‡
40	2.2	4.6	2.4	6.55
50	1.6	4.5	2.9	7.9
60	1.7	5.1	3.4	9.2
70	2.0	4.9	2.9	7.9
80	1.2	8.8	7.6	21.1
90	0.9	18.1	17.2	46.9
100	1.3	20.8	19.5	53.2
105	2.1	21.6	19.5	53.2
110	0.8	37.4	36.6	100
115	4.5	29.9	25.4	69.3
120	4.1	24.8	20.7	56.5

Table 5.4. Activity of the mannanase from the consortium at various temperatures.
* corrected for apparent thermal degradation. ‡ highest activity (110°C) set as 100%.

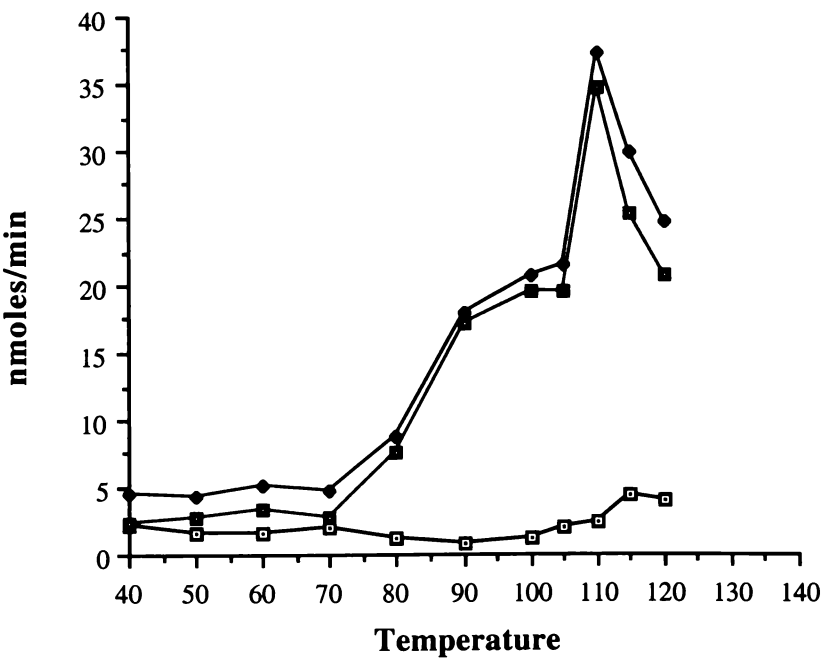


Fig. 5.3. Activity of the mannanase as a function of temperature.
Negative control (black squares), uncorrected data (blue diamonds), data corrected for thermal degradation of the substrate (red squares).

The enzyme was most active at 110°C and exhibited detectable activity from about 70°C onwards (Table 5.4.). The mannanase showed a high activity at 120°C, the highest temperature tested. Konjac glucomannan seemed to be stable up to 110°C. At 115°C some thermal degradation was observed.

5.1.5. Influence of pH on the enzymatic activity

To examine the dependence of the activity on pH the substrate was dissolved in buffers suitable for the tested pH. The following buffers were used: for pH 11 and pH 10 20mM CAPS, for pH 9 and pH 8 20mM TAPS, for pH 7 20mM MOPS, for pH 6 20mM MES and 10mM citrate buffer for pH 5 and pH 4 . The enzyme preparation was not especially buffered since only 50µl were added to 3ml of buffered substrate, thus contributing only 1.6% of the total volume. Substrate without enzyme was incubated at each pH value simultaneously to test for non-enzymatic degradation. Assays were incubated at 80°C.

pH	Reducing sugars (nmoles*) negative control	Reducing sugars (nmoles*) + enzyme	Reducing sugars corrected‡	% activity
4	5.0	12.5	7.5	72.8
5	6.1	15.2	9.1	88.3
6	6.2	16.5	10.3	100
7	6.3	14.5	8.2	79.6
8	8.8	14.1	5.3	51.4
9	10.4	10.8	0.4	3.8
10	3.8	6.9	3.1	30.0
11	4.1	8	3.9	37.8

Table 5.5. Activity of the mannanase from the consortium against konjac glucomannan at various pHs.
* nmoles per ml of assay. ‡ values corrected for pH dependent degradation

The mannanase seems to be most active around pH 6 but shows activity in a broad range from pH 4 to pH 8. At higher pH the konjac glucomannan is less stable and alkaline degradation occurred (Table 5.5.). At pH 10 and higher the response of the PAHBAH assay is not reliable (Colin Monk, Thermophile Research Unit, University of Waikato, pers. comm.) and should be disregarded.

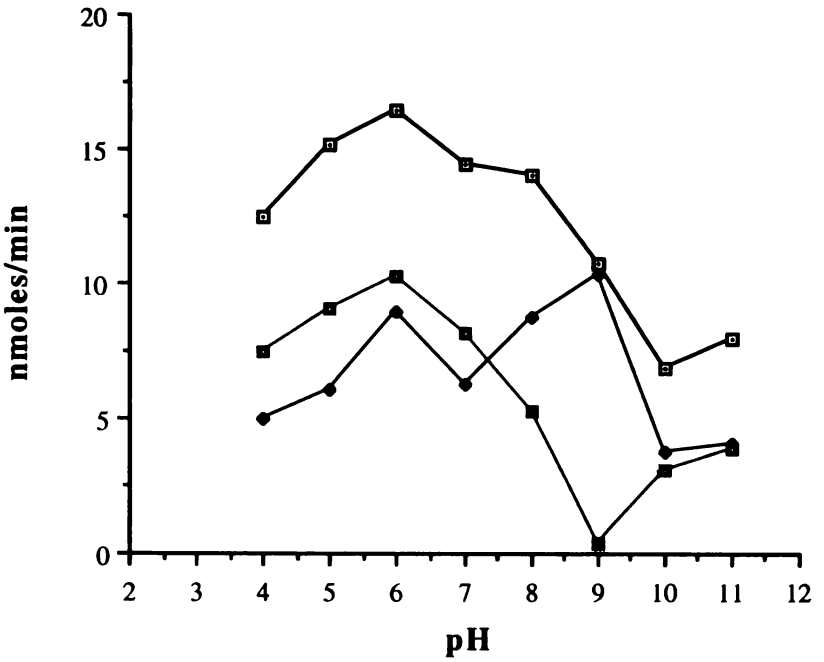


Fig. 5.4. Activity of the mannanase as a function of pH.
Negative control (blue diamonds), uncorrected data (black squares), data corrected for pH dependent degradation of the substrate (red squares).

5.1.6. Enzyme inhibitors

Several possible enzyme inhibitors were tested for inhibition of the mannanase activity. They were used at concentrations of 10mM, 1mM and 0.5mM. Heavy metals were supplied as the chloride salt. The inhibitors were added to the substrate/enzyme preparation and incubated at 80°C for 30 minutes. The amount of reducing sugars was determined with a PAHBAH assay.

Only some heavy metals proved to be inhibitory of the mannanase. The most effective were cadmium, bismuth, copper and lead (see Table 5.6.). Neither EDTA, EGTA or iodoacetic acid showed any significant inhibitory effect. No results were obtained using mercury or silver salts, because of a blackening of the assay medium, probably caused by precipitation of insoluble salts.
Antimony caused only a slight inhibition whereas arsenic appeared to be even slightly stimulatory. Cadmium is by far the most inhibitory heavy metal. Only 0.5mM cadmium is sufficient to reduce the activity to about a third of the average activity.

	% Activity		
Concentration	10mM	1mM	0.5mM
Positive control	100	100	100
EDTA	85.8	94.8	101.9
EGTA	90.3	96.8	103.9
Antimony	73.5	102.5	102.6
Arsenic	116.7	114.8	106.5
Cadmium	0.1	2.0	40.6
Bismuth	0.6	77.4	96.1
Lead	0.6	77.4	103.2
Copper	1.3	3.2	134.2
Iodoacetic acid	113.5	114.8	107.1

Table 5.6. Effect of common enzyme inhibitors on the activity of the mannanase.

5.1.7. Development of a purification method for the mannanase

5.1.7.1. Disassociation of the enzyme from the cell wall

Several ways to remove the enzyme from the cell walls were tested, including buffers with high and low ionic strength and different detergents.

Cell wall fragments were prepared as usual with sonication of the harvested cells and precipitation of the cell walls by centrifugation. Aliquots of that cell wall fraction were than incubated with different detergents and buffers at 65°C for 30min.

Only washes with 20mM CHAPS resulted in an increase of activity in the supernatant (Table 5.7.). All the other tested detergents or solutions did not remove the activity from the cell wall. Even with 10mM CHAPS as a detergent 34.6% of the total activity remained with the cell pellet during the first wash.

The total activity of the cell fragments plus the supernatant treated with 1M NaCl is less than 50% of the total activity found for the detergent treated cells, probably indicating a partial inhibition of the enzyme. A slight decrease in total activity was also observed when the cell wall was treated with 1M NaCl.

Treated cell fraction	Total increase in reducing sugars (nmoles*/min)	% of total activity
MilliQ SN	0.46	**3.9**
MilliQ CP	11.4	96.1
1M NaCl SN	0.2	**2.7**
NaCl CP	7.33	97.3
Tween20 SN	1.9	**9.4**
Tween 20 CP	18.6	90.6
TritonX100 SN	0.2	**1.6**
TritonX100 CP	16.4	98.4
CHAPS SN	10.6	**65.4**
CHAPS CP	5.6	34.6

Table 5.7. Efficiency of various buffers and detergents in removing the mannanase from the cell wall.
*nmoles/ml of assay.

5.1.7.2. Purification of the mannanase

In order to purify the mannanase four litres of consortium culture were harvested during exponential growth. It was crucial to avoid the pelleting of the poorly soluble konjac glucomannan. The cells, resuspended in 20mM Mops, were sonicated at the highest energy setting for five minutes in an ice bath. The cell fragments were pelleted and incubated with 10ml 20mM CHAPS at 65°C for 30 min, pelleted and the pellet was incubated a second time with 10ml 20mM CHAPS. The supernatants from both washes were pooled and the crude extract was loaded onto a Sepharose Q column under high salt (2M NaCl) conditions. The mannanolytic enzyme was not retained by the column. All activity was found in the void volume. The buffer containing the enzyme was dialysed overnight against 20mM Bis/Tris (pH > 7.5) to remove the salt and loaded onto a MonoQ column attached to a FPLC system and the proteins were eluted with an increasing NaCl gradient. The buffer was 20mM Bis/Tris (pH 8) and the gradient was run from 0-0.5M NaCl. The eluate was collected in 2ml fractions. Protein was detected by measuring the OD_{280}. For the detection of mannanase activity, 10µl of protein containing fractions were added to 490µl of 0.05% konjac glucomannan in 50mM Mops buffer and incubated at 80°C for 20min. The amount of reducing sugars released was determined by PAHBAH assay.
Three fractions showed high mannanase activity. These fractions correlated with a protein peak and eluted at 0.34-0.41M NaCl. Samples from the three fractions were

concentrated by freeze drying and redissolving them in a smaller volume of buffer. Two different concentrations of each fraction were run on a denaturing Phast Gel (see section 2.2.17.) and silverstained.

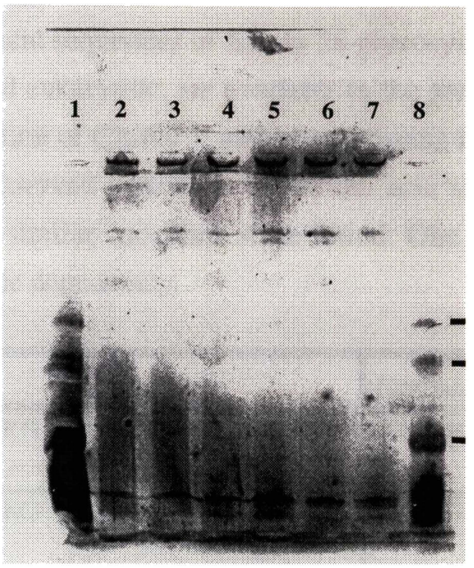


Fig.5.5. Samples from three fractions which exhibited mannanase activity on a 10-15% Phast Gel.
Lane 1: Molecular weight marker: lane 2: fraction 11, 5 fold concentrated: lane 3: fraction 11, 2.5 fold concentrated: lane 4: fraction 12, 5 fold concentrated: lane 5: fraction 12: 2.5 fold concentrated; lane 6: fraction 13, 5 fold concentration; lane 7: fraction 13, 2.5 fold concentrated; lane 8: molecular weight marker (marked from top: 66kDa; 34.7kDa; 24kDa).

All fractions showed the same band pattern. Each lane contained four bands with the estimated molecular masses: 115, 112, 78, 59kDa. The band with the estimated molecular weight of 112kDa might be due to an SDS binding artefact and contain the same protein as the band above. Th dark stain at the bottom of the gel is a printing artefact and not visible in the original gel.

5.1.8. Attempts to clone the mannanase gene

Two different approaches were tried to clone the mannanase gene. First, attempts were made to find an EcoRI digested DNA fragment which hybridizes with a specifically designed oligonucleotide probe in order to clone this fragment into a plasmid vector. An oligonucleotide probe was designed for a conserved amino acid sequence in family 26 mannanases. Second, the cloning of partially Sau3A digested total DNA from the consortium into an expression vector was attempted. Screening of the clones was done either with the Congo Red method or with Remazol Brilliant Blue labelled substrate.

5.1.8.1. Construction of an oligonucleotide probe for mannanase genes

Several gene and amino acid sequences of family 26 glycosyl hydrolases from various species, both bacterial and eukaryotic, are available in the gene and protein databases. Using the alignment function of Clustal V and by comparing the amino acid sequences manually, two highly conserved regions in the amino acid sequences of mannanases and other enzymes with similar functions were found. One of them was suitable to construct a probe with little degeneracy.

Amino acid sequence	Organism	Domain	Enzyme
RPL**HE**ASGG**WFWWG**AKG	*Caldocellulosiruptor saccharolyticus*	Bacteria	Endo-1,4-β mannanase
RPL**HE**AEGR**WFWWG**AKG	*Dictyoglomus thermophilum*	Bacteria	Endo-1,4-β mannanase
RPL**HE**AAGG**WFWWG**AKG	*Rhodothermus marinus*	Bacteria	Endo-1,4-β mannanase
RPL**HE**NDG**DWFWWG**	*Piromyces* sp.	Eukarya, fungi	Mannan endo-1,4 mannosidase
RPF**HE**AAG*__WFWWG__EDG	*Prevotella ruminicola*	Bacteria	β–1,4-endogluconase
RPL**HE**MNGE**WFWWG**AEG	*Bacillus* sp.	Bacteria	Mannan endo-1,4-mannosidase
RPL**HE**MNGE**WFWWG**LTG	*Bacillus subtilis*	Bacteria	Endo-1,4 β mannanase
RLY**HE**NTGS**WFWWG**DKQ	*Pseudomonas fluorescens*	Bacteria	Mannan endo-1,4 mannosidase
RPLHEANGDWY**PW**	*Clostridium thermocellum*	Bacteria	Endoglucanase

Table 5.8. Conserved regions in the amino acid sequence of β-endomannanases or related enzymes from various species.

Using the central G(D/E)WFWWG motif, a 20 nucleotide probe could be designed with only three uncertain nucleotides.

```
5'- GGW GAN TGG TTY TGG TGG GG-3'
     G  D/E  W   F   W   W   G
```

The primer was endlabelled with ^{33}P using T4 polynucleotide kinase as recommended by the supplier.

5.1.8.2. Hybridization of the restricted DNA

DNA from the consortium was digested with 1.5U EcoRI /µg DNA for 90 min at 37°C to ensure complete restriction. The restricted DNA was run on a 1% agarose gel at 20mV overnight and the DNA was transfered onto a Hybond™ membrane in a Southern blot and subsequently hybridized with the ^{33}P labeled probe. Hybridization was done at 50°C degrees considering the nucleotide composition of the probe and the membrane was washed at increasing temperatures up to 65°C with 2x SSC buffer until no radiation could be detected in the washing solution.
Additionally undigested DNA from both the consortium and Tok2 was dot-blotted onto a membrane, hybridized and washed as described above.

No positive signal could be found for the digested DNA or for the dot-blotted DNA despite dropping the hybridization temperature and the washing tempertature down to 40°C and 55°C respectively. Under these conditions the size standard bands hybridised, indicating that the blot and the hybridization principally worked.
Further attempts using three different probes of only 12 nucleotides length (obtained from M. Gibbs, University of Auckland, NZ) which were previously used for the cloning and the sequencing of the mannanase gene from *Caldocellulosiruptor saccharolyticus* (Gibbs et al. 1996) were unsuccessful as well.

5.1.9. Attempts to clone the mannanase gene into an expression vector

It was attempted to clone the mannanase vector into an expression vector. The λ phage based ZAP Express™ Predigested Vector Kit (Stratagene) was used for this and the instructions of the supplier were followed.
Total DNA from a consortium culture was partially digested with Sau3A to obtain random DNA fragments of about 4000bp. These fragments were cloned into the λ vector and plated onto 20 plates. The obtained plaques were washed from the plate and the plaques of five plates each were pooled. The inserts were excised and subcloned into a non-lytic pBK-CMV phagemid vector according to the manufacturer's instructions. *E. coli* host cells were infected with the pBK-CMV phagemid and plated. After growth of colonies, duplicate plates were made onto LB plates containing 0.1% konjac glucomannan and incubated at 37°C overnight. After the development of colonies the plates were incubated at 70°C for another 24h.

Screening for mannanolytic clones was carried out with the Congo Red method (see 2.2.16.1.) or alternatively by the use of dye labelled konjac glucomannan. In the latter case the konjac glucomannan in the duplicate plates was labelled with Remazol Brilliant Blue. Positive clones can be identified by clearing zones around colonies.
No mannanolytic clone could be detected.
The konjac glucomannan did not disperse evenly in the plates. The dye labelled substrate especially formed lumps which could not be dissolved and gave the plates a marbled look. It was not possible to distinguish between clearing zones and areas that had not been stained properly.

5.2. Discussion

5.2.1. The first mannanase from an archaeon

Galactomannans and galactoglucomannans are hemicelluloses which are found in cell walls, especially from softwood, roots, fruits and fungi and are therefore a bountiful carbon source for organisms which are capable of catabolising them. Mannanolytic enzymes have been isolated from a variety of bacteria, fungi and plant tissues but have not previously been reported for archaea. The mannanase from the consortium is therefore the first mannanase found in a member of this domain. In the two euryarchaeotic species *Pyrococcus horikoshii* (Kawarabayasi et al. 1998) and *Methanococcus jannaschii* (Bult et al. 1996) whose genomes have been fully sequenced by now hypothetical proteins have been found which exhibit some homology to amino acid sequences of β-endomannanases. No mannanolytic activity of either organism has been reported as yet.

There is a possibility that the mannanolytic activity is not due to an β-endomannase but to a mannosidase which cleaves β-1,4-glycosidic linked mannose units in an exo-acting fashion. Mannosidases usually act on short chains only, cleaving β-1,4-glycosidic links between two mannose units. Mannosidases would not be able to cleave the konjac glucomannan which is a long, unbranched chain of β-1,4-glycosidic linked mannose and glucose units. The rapid decrease in viscosity which was observed soon after cell growth was perceivable in a consortium culture strongly supports the presence of an endo-acting enzyme. It is very likely that a mannosidase is present in strain Tok1 as well since the degradation of glucomannan is usually performed by several enzymes acting together.

It could be clearly shown that the β-endomannanase activity was not exhibited by strain Tok2 and must therefore be produced from the second member of the consortium, strain Tok1. Strain Tok2 did not show any mannanolytic activity in an enzymatic assay with konjac glucomannan as substrate nor was it able to use glucomannan as a carbon source when it was grown with konjac glucomannan as sole carbon source. The feeble growth which was observed is more likely due to the mono- and disaccharides which are present in the konjac glucomannan as impurities. The rapid decrease of viscosity which is observed for consortium cultures grown on konjac glucomannan is not observed when strain Tok2 is grown on konjac glucomannan. This shows that it does not degrade the long chains of the konjac glucomannan.

Strain Tok1 was found in a hot spring which might have a relatively high content of glucomannan due to the dead plant material which is found in and around the spring in

high amounts. Another possible source might be some of the food which is prepared in the hot spring which is frequently used for hangi, a traditional Maori cooking method. Certain vegetables such as carrots contain glucomannan in various amounts, so do some of the containers in which the food is prepared, like wooden boxes and flax baskets. Hemicelluloses are the second most abundant carbon source on earth and terrestrial hot springs often contain dead plant material. Despite their abundance in nature glucomannans and galactoglucomannans are not routinely used in carbon source test for new microbial isolates. It is therefore likely that more thermophilic archaea could be found which are able to degrade mannan or other hemicelluloses.

5.2.2. Cloning attempts

All characterised β-endomannanases (EC 3.2.1.78) which randomly hydrolyse β-1,4 glycosidic links in the backbone of galactoglucomannans and glucomannans fall into two glycosyl hydrolase families which are based on gene and protein sequence similarities as well as structural similarities. Family 26 contains mainly bacterial enzymes and the β-endomannanase domain is either located at the N-terminus of the protein or the mannanase domain takes up the entire protein (Ethier et al. 1998). Family 5 contains both bacterial and eukaryotic enzymes and the location of the β-endomannanase domain within the enzyme is more variable than in family 26. Many of the enzymes which belong to family 5 additionally contain a cellulose binding domain which has not been found in the enzymes belonging to family 26. In order to determine to which family the enzyme of strain Tok1 belongs and to learn something about its structure, attempts were made to clone and sequence the mannanase gene. All enzymes of family 26 contain an amino acid motif which is highly preserved and provided a suitable motif for designing a nucleotide probe due to its high content of tryptophan, an amino acid which is encoded by only one nucleotide codon. A degenerate probe was designed which had only three ambiguities in a total length of 20 nucleotides (section 5.1.8.1.). Attempts to hybridize total DNA from the consortium with this primer failed even when hybridization was carried out under low stringency conditions. Under the least stringent conditions (1xSSC buffer and a washing temperature 10°C lower than the melting temperature of the primer) the molecular weight marker hybridised with the ^{33}P labelled probe which indicates that the Southern blot and the hybridization principally worked. A further attempt to find the β-endomannanase gene using primers which had been successfully used in the cloning and sequencing of the family 26 β-endomannanase of the thermophilic bacterium *Caldocellulosiruptor saccharolyticus* (Gibbs et al. 1996) did not hybridise either with the DNA from the consortium. The β-endomannanase from Tok1 very likely does not belong to the family 26 type enzymes

since none of the probes specifically targeted for this type of enzyme gave any hybridization signals.
It was briefly attempted to find the β-endomannanase gene by cloning it into a phage expression vector. No positive clones could be found by screening for clones with mannanolytic activity. The cloning into an expression vector was made complicated by working with a consortium and therefore with two genomes which doubled the number of clones needed to ensure representation of every possible clone.
The screening for mannanolytic clones was based on the ability of some dyes, such as Congo Red, to bind to carbohydrates. Upon degradation of the glucomannan incorporated in agarose plates, the smaller cleavage fragments migrate and leave zones of clearing around mannanolytic colonies. The low solubility of the dye-labelled substrate made it impossible to obtain plates with an even staining. Clearing zones could not be distinguished from the pattern which was caused by the substrate irregularity in the plates.

5.2.3. Characteristics of the β-endomannanase from Tok1

The β-endomannanase partially purified from the consortium is by far the most thermophilic and the most thermostable β-endomannanase found hitherto. *Thermotoga neapolitana* possesses a β–endomannanase which shows highest enzymatic activity at 90-92°C (Duffaud et al. 1997) and has a half-life of 35 min at 100°C. Optimal temperatures for activity of β-endomannanases isolated from other thermophilic organisms range from 80°C for the β-endomannanase of *Caldocellulosiruptor saccharolyticus* (Bicho et al. 1991) to 65°C for the β-endomannanase of *Bacillus stearothermophilus* (Talbot and Sygusch 1990). The unpurified β-endomannanase from the consortium has an optimum temperature of 110°C and is still highly active at 120°C after 30min incubation. It is possible that some cell compounds contribute to its thermostability and that the thermostability of the purified enzyme would be lower.
The optimum pH for the β-endomannanase activity is around 6 and is therefore slightly lower than the optimum pH for growth of Tok1. Most β-endomannanases described so far are most active at neutral to acidic pHs. Only a β-endomannanase isolated from a human intestinal bacterium belonging to the *Clostridium butyricum- Clostridium beijerinckii* group (Nakajima and Matsuura 1997) has optimal activity at an alkaline pH. Konjac glucomannan seems to lose stability at higher pHs. In the negative controls run in parallel to the enzyme assays at the same pH values the concentration of reducing sugars increased at pH 8 and higher during the incubation periodwhich indicates that glycosidic bonds must have been cleaved independent of enzymatic activity. At pH 9

the increase in reducing sugars in the negative control was as high as in the enzyme assay at this pH. At pH 10 and higher the amount of reducing sugars in the negative control decreases again. PAHBAH assays are not reliable at a pH >10, but the curve of the graph created from the data which was corrected for the non enzymatic degradation (Fig.5.5.) suggest that there is no activity at pH >9.

The β-endomannanase from the consortium exhibited a high substrate preference for konjac glucomannan but was able to hydrolyse as well various xylans such as birchwood, larchwood and oat spelt xylan(Fig. 1.4.). Some activity was also detected against Avicell cellulose. No activity was found against starch, amylose or amylopectin as substrates. The mannanase did not have a significant activity against the other mannan substrates such as locust bean gum and guar gum which are galactoglucomannans (Fig.5.7.). It seems that strain Tok1 does not possess an α-galactosidase to cleave the galactose sidegroups of the galactoglucomannans. Growth of consortium cultures on locust bean gum as sole carbon source is very poor which can be explained by the low activity of the β-endomannanase against galactoglucomannan.

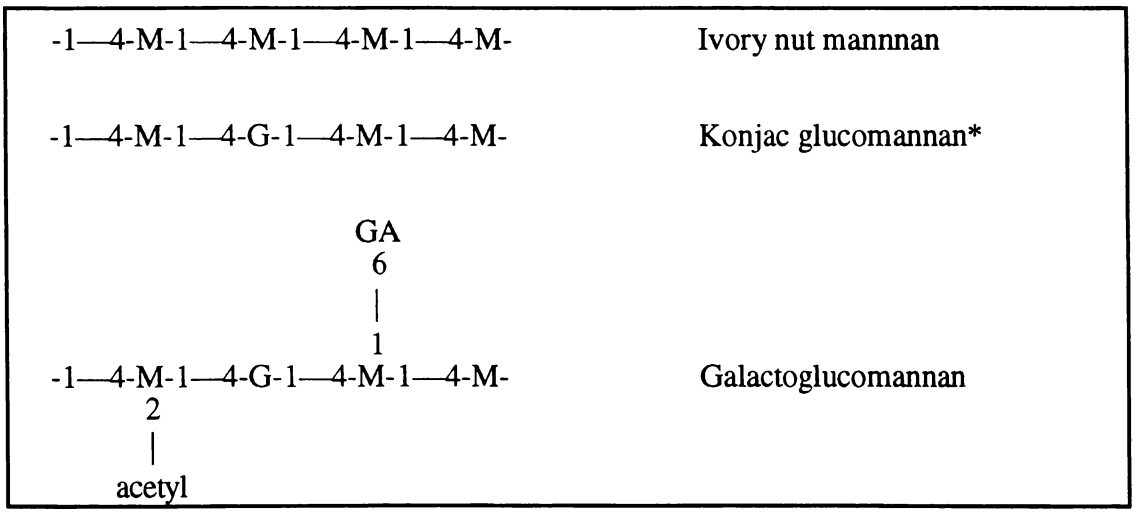


Fig. 5.6. Schematic structure of various mannans.
M: mannose; G: glucose; GA: galactose. *every 60th mannose unit acetylated.

The β-endomannanase did show some activity against ivory nut mannan which is a straight polymer of β-1,4-linked mannose with no sidechains. Ivory nut mannan is poorly soluble even at elevated temperatures which might explain the low activity of the β-endomannanase from strain Tok1 against it.

Most of the β-endomannanases from microorganisms are extracellular with some exceptions such as the β-endomannanases of *Sporocytophaga myxococcoides*, *Aerobobacter mannanolytcus* and *Xanthomonas campestris* (Eriksson et al. 1990). The β-endomannanase from the consortium is evidently bound to the cell wall. No activity

was found in the supernatant or in the cytoplasmic fraction. It was possible to wash the enzyme off the wall after sonication of the cells using the zwitterionic detergent CHAPS.

Some heavy metals such as cadmium, bismuth and lead were inhibitory of enzyme activity. EDTA and EGTA did not inhibit the enzyme activity, indicating that neither Mg^{2+} nor Ca^{2+} is essential for the enzyme activity. Iodoacetic acid, which inhibits enzymes with cysteine, lysine or histidine in the active site, showed no effect. No inhibition was observed with arsenic and some inhibition was found for antimony at concentrations higher than 1mM. Heavy metals and especially arsenic and antimony are frequently found in hot springs in high concentrations. Resistance against those heavy metals might provide an important advantage in this habitat.

Chapter 6 - Conclusion

6.1. Strain Wai21.S1 and the freshwater *Thermococcus* isolates from New Zealand

Strain Wai21.S1 was isolated, characterised and the establishment of strain Wai21.S1 as the second freshwater species of the genus *Thermococcus* under the name "*Thermococcus waimanguensis*" was proposed. Freshwater isolates of the genus *Thermococcus* have been found only in New Zealand hot springs so far. Four terrestrial *Thermococcus* strains -*T. zilligii*, strain Wai21.S1, strain Wt1 and strain RT3- have been isolated altogether and phylogenetic analysis showed a very close relatedness between the four isolates. Physiologically nevertheless *T. zilligii* and strain Wai21.S1 are different and DNA/DNA hybridization studies supported their placement in different species. On the grounds of 16S rRNA gene sequence evidence alone this did not seem likely.

The case of the freshwater *Thermococcus* isolates highlights the need for a thorough investigation of these new strains and the order Thermococcales in general. The genus *Thermococcus* contains by now a high number of species, many of which are on the basis of 16S rRNA gene sequences very close, despite significant physiological differences. It seems necessary to find an additional criterion for the determination of species limits for this genus since the variability of the 16S rRNA gene is obviously not sufficient. The 16S-23S intergenic region which supposedly provides a good tool for categorising species proved to be unsuitable to distinguish between the New Zealand *Thermococcus* strains. It was recently suggested that DNA polymerase genes would be suitable for the phylogenetic analysis of this genus. With the increasing amount of sequence data available this might prove to be a beneficial approach to clarify the taxonomy of the New Zealand fresh water isolates and the remainder species of this genus.

The New Zealand fresh water strains are phylogenetically clearly distant from the marine isolates. Even though phylogenetic analysis does not support the establishment of the freshwater strains in a separate genus, their characteristics and their position as a phylogenetically young branch of the genus *Thermococcus*, evolving most likely from marine ancestors, makes them an extremely interesting object of study. There is evidence from intensive sampling of New Zealand hot springs executed by members of this laboratory and overseas scientists that *Thermococcus*-like organisms can be found in many hot pools of New Zealand.

Thermococcus species are not only interesting from a phylogenetic point of view. Many of them are capable of degrading complex substrates, such as starch, chitin, and amino acids. *T. zilligii* is known to degrade xylan and a xylanase has been isolated and characterised from this organism.
Thermococcus strains are usually very easy to cultivate and grow up to high cell numbers quickly. Therefore they are perfect organisms to screen for interesting and exploitable thermostable enzymes.

6.2. The consortium culture

6.2.1. Strain Tok2

Two strains which were obtained from a consortium growing on the hemicellulose konjac glucomannan were characterised, one of them partially. Strain Tok2 which is a member of the genus *Pyrobaculum* was isolated by plating and 16S rRNA gene sequence data place it, together with two other isolates from New Zealand, "Pyrobaculum neutrophilum" and strain IsoX, close to *Pyrobaculum organotrophum*. Physiological analysis suggest otherwise. DNA/DNA hybridization will have to be carried out to clarify the taxonomic status of the New Zealand isolates. This will be undertaken as soon as all necessary strains for this study could be obtained. In case the DNA/DNA hybridization study supports the establishment of a novel species including all three New Zealand *Pyrobaculum* isolates the name "Pyrobaculum neutrophilum" was proposed. A phylogenetic analysis performed with the Clustal phylogenetic programs including all available 16S rRNA genes sequences of the order Thermoproteales questions the positioning of some organisms of the genus *Thermoproteus*. A thoroughly executed DNA/DNA hybridization study might elucidate not only the position of the New Zealand isolates strain Tok2, "Pyrobaculum neutrophilum" and strain IsoX but might also provide an answer about the right position of *Thermoproteus neutrophilus* which seems to be misplaced in the genus *Thermoproteus*.
In many hot pools in Tokaanu, the geothermal area where the consortium was found, rod-shaped organism were observed which morphologically resembled strain Tok2. It is likely that more Thermoproteales-like isolates could be found.

6.2.2. Strain Tok1

The second member of the consortium, the coccoid organism Tok1 could not be purified, despite the application of every microbiological isolation method possible with the equipment available in this lab. Strain Tok1 was therefore characterised as a member of the consortium culture as far as this was possible.
Strain Tok1 is a highly interesting organism because of its ability to utilise konjac glucomannan, and because of its phylogenetic position close to the base of the "Igneococcales" branch, marking it as one of the oldest organisms of this branch.
No archaea were previously known to degrade the hemicellulose konjac glucomannan or any other mannan. Strain Tok1 is not only capable of utilising konjac glucomannan but it is the only carbon source of the many tested, which it can metabolize at all. Other features of strain Tok1 which are rather unusual among the order "Igneococcales" and the domain Archaea in general are its sensitivity to elemental sulfur and to yeast extract. Many physiological aspects of strain Tok1 can not be investigated while it remains in mixed culture which is a problem many microbiologists have encountered (W. Zillig, Max-Planck Institut, Martinsried, pers.comm, J. Wiegel, University of Atlanta, Athens, pers.comm.). The establishment of techniques for investigation of organisms in a consortium, including molecular and biochemical as well as microbiological methods, is necessary in order to study these organisms (for an example of such a combined approach see Schleper et al. 1997a). Our knowledge about microorganisms will otherwise be limited to the few which can be isolated and to short gene sequences obtained from environmental samples. The recent approach of studying microbial communities with molecular methods provided an insight into the magnitude and complexity of microbial life but an organism like strain Tok1 would have been reduced to a 16S rRNA gene fragment among thousands of other cloned sequences in the databases. This would of course reveal the phylogenetic position of strain Tok1 but nothing of its unique physiology would have been known. Classic microbiological investigations are still necessary to answer some of the many questions molecular environmental studies have raised.

6.3. A β-endomannanase from the consortium

A β-endomannanase was isolated from the consortium culture and partially purified. The characterisation of the enzyme in the crude extract revealed that it is produced by strain Tok1. It is the first mannanolytic enzyme found in an archaeon. It is the β-endomannanase with the highest optimum temperature for activity known so far.

The characterised β-endomannanase from bacteria and eukarya fall into two different groups which have independent evolutionary origins. In this light the characterisation of the enzyme and the sequencing of its gene would be of fundamental interest for evolutionary studies beyond their possible application in industry.

Appendix 1 - GenBank accession numbers for nucleotide sequences

16S rRNA gene sequences

Euryarchaeota:

Archaeoglobus fulgidus	Y00275
Haloarcula vallismortis	D50851
Halobacterium halobium	AJ002949
Halococcus turkmenicus	AB004878
Haloferax volcanii	U68540
Halogeometricum borinquense	AF002984
Methanobacterium bryantii	AF028688
Methanobrevibacter smithii	AF054208
Methanocalculus halotolerans	AF033672
Methanococcus jannaschii	M59126
Methanococcus maripuladis	AF005049
Methanocorpusculum parvum	M59147
Methanoculleus marisnigri	AF028693
Methanohalobium evestigatum	U20149
Methanoplanus limicola	M59143
Methanopyrus kandleri	M59932
Methanospirillum stadtmanae	M59139
Natronobacterium innermongoliae	AF009601
Natronococcus occultus	Z28378
Picrophilus oshimae	X84901
Pyrococcus abyssii	Z70246
Pyrococcus furiosus	U20163
Thermococcus acidaminovorans	Y15935
Thermococcus barossii	U76535
Thermococcus celer	M21529
Thermococcus chitonophagus	X99570

Thermococcus fumiculans	Z70250
Thermococcus gorgonarius	Y16226
Thermococcus hydrothermalis	Z70244
Thermococcus litoralis	Z70252
Thermococcus mexicalis	Z75218
Thermococcus pacificus	Y16227
Thermococcus peptinophilus (SM-2)	D37983
Thermococcus profundus	Z75233
Thermococcus siculi	AB010893
Thermococcus stetteri	Z75240
Thermococcus strain Rt3	AF017455
Thermococcus zilligii	U76534
Thermoplasma acidophilum	M38637

Crenarchaeota:

Acidianus brierleyi	D26489
Acidianus infernus	D85505
Caldococcus noboribetsu	D85038
Desulfurococcus mobilis	M36474
Hyperthermus butylicus	X99553
Igneococcus islandicus	X99562
Metallosphaera sedula	X90481
Pyrobaculum aerophilum	L07510
Pyrobaculum islandicum	L07511
Pyrodictium occultum	M21087
Pyrolobus fumarius	X99555
Staphylothermus marinus	X99560
Stetteria hydrogenophila	Y07784
Stygiolobus azoricus	D85520
Sulfolobus acidocaldarius	D14053
Sulfolobus solfataricus	D26490
Sulfophobococcus zilligii	X98064
Sulfurisphaera ohwakuensis	D85507
Thermocladium modestius	AB005296
Thermodiscus maritimus	X99554
Thermofilum pendens	X14835

Thermoproteus neutrophilus	AB009618
Thermoproteus tenax	M35966
Thermosphaera aggregans	X99556

β-endo-mannanase gene sequences

Bacillus sp.	AB016163
Bacillus subtilis	D37964
Caldicellulosiruptor saccharolyticus	U39812
Clostridium thermocellum	M31903
Dictyoglomus thermophilum	AF013989
Piromyces sp.	X97520
Prevotella ruminicola	U96771
Pseudomonas fluorescens	X82179
Rhodothermus marinus	X90947

Appendix 2 - Clustal V alignment of 16S rDNA sequences of selected *Thermococcus* strains

```
Wai21S          GGGGTCCGACTAAGCCATGCGA---GTCATGGGG-CGCCTCGC----GCG
T. zilligii     GGGGTCCGACTAAGCCATGCGA---GTCATGGGG-CGCCTCGC----GCG
Rt3             GGGGTCCGACTAAGCCATGCGA---GTCATGGGG-CGCCTTGC----GCG
T. fumicolans   GGGGTCCGACTAAGCCATGCGACGAGTCAAGGGGGTGTCCCTCTGGGACN
T. celer        GGGGTCCGACTAAGCCATGCGA---GTCATGGGG-CGCCTTGC----GCG
                ***********************   **** ****  * *   *     *

Wai21S          CACCGGCGGACGGCTCAGTAACACGTCGGTAACCTACCCTCGGGAGGGGG
T. zilligii     CACCGGCGGACGGCTCAGTAACACGTCGGTAACCTACCCTCGGGAGGGGG
Rt3             CACCGGCGGACGGCTCAGTAACACGTCGGTAACCTACCCTCGGGAGGGGG
T. fumicolans   NACCGGCGGACGGCTCAGTAACACGTCGGTAACCTACCCTCGGGAGGGGG
T. celer        CACCGGCGGACGGCTCAGTAACACGTCGGTAACCTACCCTCGGGAGGGGG
                 ************************************************

Wai21S          ATAACCCCGGGAAACTGGGGCTAATCCCCCATAGGTCTGAGGTACTGGAA
T. zilligii     ATAACCCCGGGAAACTGGGGCTAATCCCCCATAGGTCTGAGGTACTGGAA
Rt3             ATAACCCCGGGAAACTGGGGCTAATCCCCCATAGGTCTGAGGTACTGGAA
T. fumicolans   ATAACCCCGGGAAACTGGGGCTAATCCCCCATAGGCCTGAGGTACTGGAA
T. celer        ATAACCCCGGGAAACTGGGGCTAATCCCCCATAGGCCTGAGGTACTGGAA
                ********************************** **************

Wai21S          GGTCCTCAGGCCGAAAGGGACTCTGTCCGCCCGAGGATGGGCCGGCGGCC
T. zilligii     GGTCCTCAGGCCGAAAGGGACTCTGTCCGCCCGAGGATGGGCCGGCGGCC
Rt3             GGTCCTCAGGCCGAAAGGGACTCTGTCCGCCCGAGGATGGGNCGGCGGCC
T. fumicolans   GGTCCTCAGGCCGAAAGGGGCTCTGCCCGCCCGAGGATGGGCCGGCGGCC
T. celer        GGTCCTCAGGCCGAAAGGGGCTCTGCCCGCCCGAGGATGGGCCGGCGGCC
                ******************* ***** *************** ********

Wai21S          GATTAGGTAGTTGGTGGGGTAACGGCCCACCAAGCCTAAGATCGGTACGG
T. zilligii     GATTAGGTAGTTGGTGGGGTAACGGCCCACCAAGCCTAAGATCGGTACGG
Rt3             GATTAGGTAGTTGGTGGGGTAACGGCCCACCAAGCCTAAGATCGGTACGG
T. fumicolans   GATTAGGTAGTTGGTGGGGTAACGGCNCACCANGCCGAANACCGGTACGG
T. celer        GATTAGGTAGTTGGTGGGGTAACGGCCCACCAAGCCGAAGATCGGTACGG
                ************************** ***** *** ** * ********

Wai21S          GCCATGAGAGTGGGAGCCCGGAGATGGACACTGAGACAAGGGTCCAGGCC
T. zilligii     GCCATGAGAGTTGGAGCCCGGAGATGGACACTGAGACAAGGGTCCAGGCC
Rt3             GCCATGAGAGTGGGAGCCCGGAGATGGACACTGAGACAAGGGTCCAGGCC
T. fumicolans   GCTGTGAGAGCAGGAGCCCGGAGATGGACACTGAGACACGGGTCCAGGCC
T. celer        GCCATGAGAGTGGGAGCCCGGAGATGGACACTGAGACACGGGTCCAGGCC
                **  ******  ************************** ***********

Wai21S          CTACGGGGCGCAGCAGGCGCGAAACCTCCGCAATGCGGGAAACCGCGACG
T. zilligii     CTACGGGGCGCAGCAGGCGCGAAACCTCCGCAATGCGGGAAACCGCGACG
Rt3             CTACGGGGCGCAGCAGGCGCGAAACCTCCGCAATGCGGGAAACCGCGACG
T. fumicolans   CTACGGGGCGCANNAGGCGCGAAACCTCCGCAATGCGGGAAACCGCGACG
T. celer        CTACGGGGCGCAGCAGGCGCGAAACCTCCGCAATGCGGGCAACCGCGACG
                ************  ************************* **********

Wai21S          GGGGGACCCCCAGTGCC-GTGGCATCGCCACGGCTTTTCCGGAGTGTAAG
T. zilligii     GGGGGACCCCCAGTGCC-GTGGCAATGCCACGGCTTTTCCGGAGTGTAAG
Rt3             GGGGGACCCCCAGTGCC-GTGGCATCACCACGGCTTTTCCGGAGTGTAAG
T. fumicolans   GGGGGACCCCGAGTGCCCGTGGCATCGCCGCANNTTTTCCGGAGTGTAAA
T. celer        GGGGGACCCCCAGTGCC-GTGGCAACGCCACGGCTTTTCCGGAGTGTAAA
                ********** ****** ******   ** *   ***************
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```
Wai21S          GAGCTCCGGGAATAAGGGCTGGGCAAGGCCGGTGGCAGCCGCCGCGGTAA
T. zilligii     GAGCTCCGGGAATAAGGGCTGGGCAAGGCCGGTGGCAGCCGCCGCGGTAA
Rt3             GAGCTCCGGGAATAAGGGCTGGNCAAGGCCGGTGGCANCCNCCGCGGTAA
T. fumicolans   AAGCTCCGGGAATAAGGGCTGGGCAAGGCCGGTGGCAGCCGCCGCGGTAA
T. celer        AAGCTCCGGGAATAAGGGCTGGGCAAGGCCGGTGGCAGCCGCCGCGGTAA
                ********************* ************** ** *********

Wai21S          TACCGGCGGCCCGAGTGGTGGCCACTATTATTGGGCCTAAAGCGTCCGTA
T. zilligii     TACCGGCGGCCCGAGTGGTGGCCACTATTATTGGGCCTAAAGCGTCCGGA
Rt3             TACCGNCGGCCCGAGTGGTGNCCACTATTATTGGGCCTAAAGCGTCCGTA
T. fumicolans   TACCGGCGGCCCGAGTGGTGGCCGCTATTATTGGGCCTAAAGCGTCCGTA
T. celer        TACCGGCGGCCCGAGTGGTGGCCGCTATTATTGGGCCTAAAGCGTCCGTA
                ***** ************** ** ************************ *

Wai21S          GCCGGGCCCGTAAGTCCCTGGCGAAATCTCACGGCTCAACCGTGGGGCTC
T. zilligii     GCCGGGCCCGTAAGTCCCTGGCGAAATCTCACGGCTCAACCGTGGGGCTC
Rt3             GCCGGGCCCGTAAGTCCCTGGCGAAATCTCACGGCTCAACCGTGGGGCTC
T. fumicolans   GCCGGGCCCGTAAGTCCCTGGCGAAATCCCACGGCTCAACCGTGGG-CTT
T. celer        GCCGGGCCCGTAAGTCCCTGGCGAAATCCCACGGCTCAACCGTGGGGCTT
                **************************** ***************** **

Wai21S          GCTGGGGATACTGCGGGCCTTGGGACCGGGAGAGGCGGAGGGTACCCCTG
T. zilligii     GCTGGGGATACTGCGGGCCTTGGGACCGGGAGAGGCGGAGGGTACCCCTG
Rt3             GCTGGGGATACTGCGGNCCTTGGGACCGGGAGAGGCGGAGGGTACCCCTG
T. fumicolans   GCTGGGGATACTGCGGGCCTTGGGACCGGGAGAGGCGGGGGGTACCCCTG
T. celer        GCTGGGGATACTGCGGGCCTTGGGACCGGGAGAGGCCGGGGGTACCCCTG
                ***************** ******************* * ***********

Wai21S          GGGTAGGGGTGAAATCCTATAATCCCAGGGGGACCGCCAGTGGCGAAGGC
T. zilligii     GGGTAGGGGTGAAATCCTATAATCCCAGGGGGACCGCCAGTGGCGAAGGC
Rt3             GGGTAGGGGTGAAATCCTATAATCCCAGGGGGACCGCCAGTGGCGAAGGC
T. fumicolans   GGGTAGGGGTGAAATCCTATAATCCCAGGGGGACCGCCAGTGGCGAAGGC
T. celer        GGGTAGGGGTGAAATCCTATAATCCCAGGGGGACCGCCAGTGGCGAAGGC
                **************************************************

Wai21S          GCTCCGCTGGAACGGGTCCGACGGTGAGGGACGAAGGCCAGGGGAGCAAA
T. zilligii     GCTCCGCTGGAACGGGTCCGACGGTGAGGGACGAAGGCCAGGGGAGCAAA
Rt3             GCTCCGCTGGAACGGGTCCGACGGTGAGGGACGAAGGCCAGGGGAGCAAA
T. fumicolans   GCCCCGCTGGAACGGGTCC-ACGGTGAGGGACGAAGGCCAGGGGAGCAAA
T. celer        GCCCGGCTGGAACGGGTCCGACGGTGAGGGACGAAGGCCAGGGGAGCGAA
                ** * ************** **************************** **

Wai21S          CCGGATTAGATACCCGGGTAGTCCTGGCTGTAAAGGATGCGGGCTAGGTG
T. zilligii     CCGGATTAGATACCCGGGTAGTCCTGGCTGTAAAGGATGCGGGCTAGGTG
Rt3             CCGGATTAGATACCCGGGTAGTCCTGGCTGTAAAGGATGCGGGCTAGGTG
T. fumicolans   CCGGATTAGATACCCGGGTAGTCCTGGCTGTAAAGGATGCGGGCTAGGTG
T. celer        CCGGATTAGATACCCGGGTAGTCCTGGCTGTAAAGGATGCGGGCTAGGTG
                **************************************************

Wai21S          TCGGGTGAGCTTCGGGCTCGCCCGGTGCCGGAGGGAAGCCGTTAAGCCCG
T. zilligii     TCGGGTGAGGTTCGGGCTCGCCCGGTGCCGGAGGGAAGCCGTTAAGCCCG
Rt3             TCGGGTGAGCTTCGGGCTCGCCCGGTGCCGGAGGGAAGCCGTTAAGCCCG
T. fumicolans   TCGGGTGAGCTTCGGGCTCGCCCGGTGCCGTAGGGAAGCCGTTAAGCCCG
T. celer        TCGGGCGAGCTTCGAGCTCGCCCGGTGCCGAAGGGAAGCCGTTAAGCCCG
                ***** *** **** *************** ******************

Wai21S          CCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCGGGG
T. zilligii     CCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCGGGG
Rt3             CCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCGGGG
T. fumicolans   CCGCCTGGGGAGTACGGCCGCAAGCGTGAAACTTAAAGGAATTGGCGGGG
T. celer        CCGCCTGGGGAGTACGGCCGCATGGCTGAAACTTAAAGGAATTGGCGGGG
                ********************** *  ************************
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```
Wai21S          GAGCACCACAAGGGGTGGAGCGTGCGGTTTAATTGGATTCAACGCCGGGA
T. zilligii     GAGCACCACAAGGGGTGGAGCGTGCGGTTTAATTGGATTCAACGCCGGGA
Rt3             GAGCACCACAAGGGGTGGAGCGTGCGGTTTAATTGGATCCAACGCCGGGA
T. fumicolans   GAGCACTACAAGGGGTGGAGCGTGCGGTTTAATTGGATTCAACGCCGGGA
T. celer        GAGCACTACAAGGGGTGGAGCGTGCGGTTTAATTGGATTCAACGCCGGGA
                ****** ******************************** ***********

Wai21S          ACCTCACCGGGGGCGACGGCAGGATGAAGGCCAGGCTGAAGGTCTTGCCG
T. zilligii     ACCTCACCGGGGGCGACGGCAGGATGAAGGCCAGGCTGAAGGTCTTGCCG
Rt3             ACCTCACCGGGGGCGACGGCAGGATGAAGGCCAGGCTGAAGGTCTTGCCG
T. fumicolans   ACCTCACCGGGGGCGACGGCAGGATGAAGGCCAGGCTGAAGGTCTTGCCG
T. celer        ACCTCACCGGGGGCGACGGCAGGATGAAGGCCAGGCTGAAGGTCTTGCCG
                *************************************************

Wai21S          GACACGCCGAGAGGAGGTGCATGGCCGCCGTCAGCTCGTACCGTGAGGCG
T. zilligii     GACACGCCGAGAGGAGGTGCATGGCCGCCGTCAGCTCGGACCGTGAGGCG
Rt3             GACACGCCGAGAGGAGGTGCATGGTCGCCGTCAGCTCGTACCGTGAGGCG
T. fumicolans   GACACGCCGAGAGGAGGTGCATGGCCGCCGTCAGCTCGTACCGTGAGGCG
T. celer        GACACGCCGAGAGGAGGTGCATGGCCGCCGTCAGCTCGTACCGTGAGGCG
                ************************ ************* ***********

Wai21S          TCCACTTAAGTGTGGTAACGAGCGAGACCCGCGCCCCCAGTTGCCAGTCC
T. zilligii     TCCACTTAAGTGTGGTAACGAGCGAGACCCGCGCCCCCAGTTGCCAGTCC
Rt3             TCCACTTAAGTGTGGTAACGAGCGAGACCCGCGCCCCCAGTTGTCAGTCC
T. fumicolans   TCCACTTAAGTGTGGTAACGAGCGAGACCCGCGCCCCCAGTTGCCAGCCC
T. celer        TCCACTTAAGTGTGGTAACGAGCGAGACCCGCGCCCCCAGTTGCCAGTCC
                ****************************************** *** **

Wai21S          TCCCCGCTGGGGAGGAGGCACTCTGGGGGGACCGCCGGCGATAAGCCGGA
T. zilligii     TCCCCGCTGGGGAGGAGGCACTCTGGGGGGACCGCCGGCGATAAGCCGGA
Rt3             TCCCCGTTGGGGAGGAGGCACTCTGGGGGGACCGCCGGCGATAAGCCGGA
T. fumicolans   TTCCCGCTGGGAAGGGGGCACTCTGGGGGGACTGCCGGCGATAAGCCG-A
T. celer        TTCCCGCTGGGGAGGAGGCACTCTGGGGGGACCGCCGGCGATAAGCCGGA
                * **** **** *** ***************** *************** *

Wai21S          GGAAGGAGCGGGCGACGGTAGGTCAGTATGCCCCGAAACCCCCGGGCTAC
T. zilligii     GGAAGGAGCGGGCGACGGTAGGTCAGTATGCCCCGAAACCCCCGGGCTAC
Rt3             GGAAGGAGCGGGCGACGGTAGGTCAGTATGCCCCGAAACCCCCGGGCTAC
T. fumicolans   GGAAGGAGCGGGCGACGGTAGGTCAGTATGCCCCGAAACCCCCGGGCTAC
T. celer        GGAAGGAGCGGGCGACGGTAGGTCAGTATGCCCCGAAACCCCCGGGCTAC
                **************************************************

Wai21S          ACGCGCGCTACAATGGGCGGGACAATGGGATCCGACCCCGAAAGGGGAAG
T. zilligii     ACGCGCGCTACAATGGGCGGGACAATGGGATCCGACCCCGAAAGGGGAAG
Rt3             ACGCGCGCTACAATGGGCGGGACAATGGGATCCGACCCCGAAAGGGGAAG
T. fumicolans   ACGCGCGCTACAATGGGCGGGACAATGGGATCCGACCCCGAAAGG--AAG
T. celer        ACGCGCGCTACAATGGGCGGGACAATGGGATCCGACCCCGAAAGGGGAAG
                ********************************************  ***

Wai21S          GGAATCCCCTAAACCCGCCCCCAGTTCGGATTGCGGGCTGCAACTCGCCC
T. zilligii     GGAATCCCCTAAACCCGCCCCCAGTTCGGATTGCGGGCTGCAACTCGCCC
Rt3             GAAATCCCCTAAACCCGCCCTCAGTTCGGATTGCGGGCTGCAACTCGCCC
T. fumicolans   GGAATCCCCTAAACCCGCCCCCAGTTCGGATCGCGGGCTGCAACTCGCCC
T. celer        GGAATCCCCTAAACCCGCCCCCAGTTCGGATCGCGGGCTGCAACTCGCCC
                * ****************** ********** ******************

Wai21S          GCATGAAGCTGGAAT
T. zilligii     GCATGAAGCTGGAAT
Rt3             GCATGAAGCTGGAAT
T. fumicolans   GCGTGAAGCTGGAAT
T. celer        GCGTGAAGCTGGAAT
                ** ************
```

Appendix 3 - Clustal V alignment of 16S-23S intergenic region of terrestrial *Thermococcus* strains

```
Wai21S          GGCNAATTTGCA-CCGGCCGGGACGTTTCCTGGACCAGCTCGCCTGTGCA
AN1             GTCCANTTTGCNATCGGCCGGGACGTTTCCTGGACCAGCTCGCCTGTGCA
Wt1             GGCTTATT-GCA-CCGGGCGGGACGTTTCCTGGACCAGCTCGCCTATGCA
                * *   ** **   *** ************************** ****

Wai21S          CGGCCCTCACCGTGACCCCTGTTCGGGGTCTCAGGCCCTTCCACCCCGAG
AN1             CGGCCCTCACCGTGACCCCTGTTCGGGGTCTCAGGCCCTTCCACCCCGAG
Wt1             CGGCCCTCACCGTGACCCCTGTTCGGGGTCTCAGGCCCTTCCACCCCGAG
                **************************************************

Wai21S          CCTGGCTCGGGATGTGCACCTCTTCGTGGTGGACCGGCCGGGATTCGAAC
AN1             CCTGGCTCGGGATGTGCACCTCTTCGTGGTGGACCGGCCGGGATTCGAAC
Wt1             CTTGGCTCGGGATGTGCACCTCTTCGTGGTGGACCGGCCGGGATTCGAAC
                * **************************************************

Wai21S          CCGGGGCCTCCGCCTTGCAAGGGCGGCGCTCATACCAGGCTGAGCTACCG
AN1             CCGGGGCCTCCGCCTTGCAAGGGCGGCGCTCATACCAGGCTGAGCTACCG
Wt1             CCGGGGCCTCCGCCTTGCAAGGGCGGCGCTCATACCAGGCTGAGCTACCG
                **************************************************

Wai21S          GCCCACCCGTGGCAGGCCCGACATCCCTTAAACCCCCCGGACGGGATTTC
AN1             GCCCACCCGTGGCAGGCCCGACATCCCTTAAACCCCCCGGACGGGATTTC
Wt1             GCCCACCCGTGGCAGGCCCGACATCCCTTAAACCCCCCGGACGGGATTTC
                **************************************************

Wai21S          CGGCGATAGGAGGTGATCGAGCCGCAGGTTCCCCTACGGCTACA
AN1             CGGCGATAGGAGGTGATCGAGCCGCAGGTTCCCCTACGGCTACA
Wt1             CGGCGATAGGAGGTGATCGAGCCGCAGGTTCCCCTACGGNTACA
                ************************************* ****
```

Appendix 4 - Clustal V alignment of 16S rDNA sequences of strains of the Order Thermoproteales

```
Tok2                CCATGCGAGNCGCGCGCCCGGNGG---CGCCCGGGAGCGGCGCACGGCTC
P. neutroph *       CCATGCGAGTCGCGCGCCCGGGGG---CGCCCGGGAGCGGCGCACGGCTC
T.neutroph †        CCATGCGAGTCGCNCGCCCGGGGG---CGCCCGGGAGCGGCGCACGGCTC
T. modestius        CCATGCGAGTCGGATGTCTTGGGGATCCGCCAGGGCATGGCGGACGGCTC
T. pendens          CCATGGAAGTC-TAGGAGCCGGGGCTACGGCCGGCTCCGGCGGACGGCTC
T. tenax            CCATGCGAGTCGCGCGCCC-GGGG---CGCC-GGGCGCGGCGCACGGCTC
P. islandicum       CCATGCGAGTCNCGCGCCCGGGCG---CGC--GGGAGCGGCGCACGGCTC
P. aerophilum       CCATGCGAGTGGT-CGCGCCGGGG---GGCCCGGGAGCGGCGCACGGCTC
S. solfatar ‡       CCATGGGAGTCTTACACTCCCGGGTAAGG---GAGTGTGGCGGACGGCTG
                    *****  **             * *    *   *     **** ******

Tok2                AGTAACACGTGCCTAACCTAACCTCGGGAGGGGGACACCCCCGGGAAACT
P. neutroph         AGTAACACGTGCCTAACCTAACCTCGGGAGGGGGACACCCCCGGGAAACT
T. neutroph         AGTAACACGTGCCCAACCTACCCTCGGGAGGGGGACACCCCCGGGAAACT
T. modestius        AGTAACACGTGGCCAACCTACCCTAGGGAGGGGAATAACCCCGGGAAACT
T. pendens          AGTAGCACGTGGCTAACCTACCCTCGGGAGGGGGATAACCCCGGGAAACT
T. tenax            AGTAACACGTACCCAACCTAACCTCGGGAGGGGGACAACCCCGGGAAACT
P. islandicum       AGTAACACGTGCCTAACCTACCCTCGGGAGGGGGATACCCCCGGGAAACT
P. aerophilum       AGTAACACGTGCCTAACCTAACCTCGGGAGGGGGACACCCCCGGGAAACT
S. solfatar         AGTAACACGTGGCTAACCTACCCTCGGGACGGGGATAACCCCGGGAAACT
                    **** *****  * ****** *** **** *** * * ************

Tok2                GGGGTCAATCCCCCATAGGGGAAGGGCGCTGGAAGGCCCCTTCCCCGAAA
P. neutroph         GGGGTCAATCCCCCATAGGGGAAGGGCGCTGGAAGGCCCCTTCCCCGAAA
T. neutroph         GGGGCCAATCCCCCATAGGGGAGGGGCGCTGGAAGGCCCCCTCCCCCAAA
T. modestius        GGGGCTAATTCCCCATAGGAGAGGGAAGCTGGAAGGCTCCCTCCCCGAAA
T. pendens          GGGGATAAACCCCCATAGGCGCGGACACCTGGAATGGGTCCGCGCTGAAA
T. tenax            GGGGCTGATCCCCCATAGGGGAAGGGCGCTGGAAGGCCCCTTCCTCCAAA
P. islandicum       GGGGTCAATCCCCCATAGGGGAGGGGTGCTGGAAGGCCCCCTCCCCNAAA
P. aerophilum       GGGGTCAATCCCCCATAGGGGAAGGGCGCTGGAAGGCCCCTTCCCCGAAA
S. solfatar         GGGGATAATCCCCGATAGGGAAGGAGTCCTGGAATGGTTCCTTCCCTAAA
                    ****   *  *** *****    *    ****** *   *       ***

Tok2                GGGCTGGCGGCCG--ATCCGCCGCCAGTCGCCCGAGGGTGGGGGCACGGC
P. neutroph         GGGCCGGCGGCCG--ATCCGCCGCCAGCCGCCCGAGGGTGGGGGCACGGC
T. neutroph         GGGATGGCGGCCG--ATCCGCCGCCATCCGCCCGAGGATGGGGGCACGGC
T. modestius        GGAACCGCGTCCGGCACCCGGCGCGATTCGCCCTAGGATGGGGCTGCGGT
T. pendens          GGGCCACGGTACCATGCTTACCGTGG-CCGCCCGAGGATGGGGCTGCGCC
T. tenax            GGGATCGCGGGCG--ATCTCCCGCGGTCCGCCCGAGGGTGGGGGTACGGC
P. islandicum       GGGATG----CCG--ATCCGCCGCCATCCGCCCGAGGATGGGGNCACGGC
P. aerophilum       GGGAT--CGGCCG--ATCCGCCGCCGTCCGCCCGAGGGTGGGGGCACGGC
S. solfatar         GGGCTATAGGCTATTTCCCGTTTGTAGCCGCCCGAGGATGGGGCTACGGC
                    **                          ***** *** *****   **

Tok2                CCATCA-GGTAGTTGGCGGGTTAAAGGCCCGCCAAGCCGAAGACGGGTAG
P. neutroph         CCATCA-GGTAGTTGGCGGGTTAAAGGCCCGCCAAGCCGAAGACGGGTAG
T. neutroph         CCATCA-GGTAGTTGGCGGGTTAAAGGCCCGCCAAGCCGAAGACGGGTAG
T. modestius        CCATCATGGTTGTTGGTGGGGTAATGGCCCACCAAGCCGAAGACGGATGG
T. pendens          CTATCA-GGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGATAACGGGTGG
T. tenax            CCATCA-GGTTGTTGGCGGGGTAACGGCCCGCCAAGCCGAAGACGGGTAG
P. islandicum       CCATCA-GGTAGTTGGCGGGTTAAAGGCCCGCCAAGCCGAAGACGGGTAG
P. aerophilum       CCATCA-GGTAGTTGGCGGGTTAAAGGCCCGCCAAGCCGAAGACGGGTAG
S. solfatar         CCATCA-GGCTGTCGGTGGGGTAAAGGCCCACCGAACCTATAACGGGTAG
                    * **** **  ** ** *** *** ***** ** * ** *  **** * *
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```
Tok2            GGGCGGTGAGAGCCGTGAGCCCCGAGATGGGCACTGAGACAAGGGCCCAG
P. neutroph     GGGCGGTGAGAGCCGCGAGCCCCGAGATGGGCACTGAGACAAGGGCCCAG
T. neutroph     GGGCGGTGAGAGCCGCGAGCCCCGAGATCGGCACTGAGACAAGGGCCGAG
T. modestius    GGGCCGTGAGAGCGG-GAGCCCCGAGATGGGCACTGAGACAAGGGCCCAG
T. pendens      GGGCCGTGAGAGCGG-GAGCCCCGAGATGGGCACTGAGACAAGGGCCCAG
T. tenax        GGGCGGTGAGAGCCGTGAGCCCCGAGATGGGCACTGAGACAAGGGCCCAG
P. islandicum   GGGCGGTGAGAGCCGTGAGCCCCGAGATCGGCACTGAGACAAGGGCCGAG
P. aerophilum   GGGCGGTGAGAGCCGCGAGCCCCGAGATCGGCACTGAGACAAGGGCCGAG
S. solfatar     GGGCCGTGGAAGCGG-GAGCCTCCAGTTGGGCACTGAGACAAGGGCCCAG
                **** ***  *** * ***** * ** * ****************** **

Tok2            GCCCTACGGGGTGCAGCAGGCGCGAATACTCCGCAATGCGGGAAACCGCG
P. neutroph     GCCCTACGGGGTGCAGCAGGCGCGAATACTCCGCAATGCGGGCAACCGCG
T. neutroph     GCC-TACGGGGTGCAGCAGGCGCGAATACTCCGCAATGCGGGCAACCGCG
T. modestius    GCCCTATGGGGTGCAGCAGGCGCGAAACCTCCGCAATGCAGGAAACTGCG
T. pendens      GCCCTACGGGGTGCACCAGGGGCGAAACTTCCGCAATGCGGGAAACCGTG
T. tenax        GCCCTACGGGGTGCAGCAGGCGCGAATACTCCGCAATGCGGGCAACCGCG
P. islandicum   GCCCTACGGGGCGCAGCAGGCGCGAATACTCCGCAATGCGGGCAACCGCG
P. aerophilum   GCCCTACGGGGTGCAGCAGGCGCGAATACTCCGCAATGCGGGCAACCGCG
S. solfatar     GCCCTACGGGGCGCACCAGGCGCGAAACGTCCCCAATGCGCGAAAGCGTG
                *** ** **** *** **** *****   *** ******  * **  * *

Tok2            ACGGGGCTACCCCGAGTGCCGGGCGAAGAGCCC-GGCTTTTGCCCGGTCT
P. neutroph     ACGGGGCTACCCCGAGTGCCGGGCGAAGAGCCC-GGCTTTTGCCCGGTCT
T. neutroph     ACGGGGCTACCCCGAGTGCCGGGCGAAGAGCCC-GGCTTTTGCCCGGTCT
T. modestius    ACGGGGTTACCCCGAGTGCTACCCGATGAGGGT-AGCTTTTCCCCGGTGT
T. pendens      ACGGAGTCACCCCGAGTGCCACCCGAAGAGGGT-GGCTTTTGCCCGGTCT
T. tenax        ACGGGGCCACCCCGAGTGCCGGGCGAAGAGCCC-GGCTTTTGCCCGGTGT
P. islandicum   ACGGGGCTACCCCGAGTGCCGGGCGAAGAGCCC-GGCTTTTGCCCGGTCT
P. aerophilum   ACGGGGCTACCCCGANNNNNGGGCGAAGAGCCCCGGCTTTTNCCCGGTCT
S. solfatar     AGGGCGCTACCCCGAGTGCC-TCCGCAAGGA---GGCTTTTCCCCGCTCT
                * ** *  *******         **    *     ****** **** * *

Tok2            AAAAAGCCGGGAGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGG
P. neutroph     AAAAAGCCGGGAGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGG
T. neutroph     AAAAAGCCGGGAGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGG
T. modestius    AGAAAGCCGGGGGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGG
T. pendens      AAAAAGCCGGGCGAATAAGCGGGGGGCAAGCTTGGTGTCAGCCGCCGCGG
T. tenax        AAGGAGCCGGGCGAATAAGCGGGGGGTAAGTCTGGTGTCAGCCGCCGCGG
P. islandicum   AAAACGCCGGGCGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGG
P. aerophilum   AAAACGCCGGGCGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGG
S. solfatar     AAAAAGGCGGGGGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGG
                *    * **** ************** ***  ******************

Tok2            TAATACCAGCTCCGCGAGTGGTCGGGGTGTTTACTGGGCTTAAAGCGCCC
P. neutroph     TAATACCAGCTCCGCGAGTGGTCGGGGTGTTTACTGGGCTTAAAGCGCCC
T. neutroph     TAATACCAGCTCCGCGAGTGGTCGGGGTGTTTACTGGGCTTAAAGCGCCC
T. modestius    TAATACCAGCTCCGCGAGTGGTCGGGGTGATTACTGGGCCTAAAGCGCCC
T. pendens      TAATACCAACCCCGCGAGTGGTCGGGACGTTTATTGGGCCTAAAGCGTCC
T. tenax        TAATACCAGCCCCGCGAGTGGTCAGGGTGATTACTGGGCTTAAAGCGCCC
P. islandicum   TAATACCAGCACCGCGAGTGGTCGGGGTGTTTATTGGGCTTAAAGCGCCC
P. aerophilum   TAATACCAGCTCCGCGAGTGGTCGGGGTGTTTACTGGGCTTAAAGCGCCC
S. solfatar     TAATACCAGCTCCGCGAGTGGTCGGGGTGATTACTGGGCCTAAAGCGCCT
                ******** * ************ **  * *** ***** ******* *
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Tok2            GTAGCCGGCCCGGCAAGTCGC-TCCTGAAATCCCCGGGCTCAACCCGGGG
P. neutroph     GTAGCCGGCCCGGCAAGTCGC-TCCTGAAATCCCCGGGCTCAACCCGGGG
T. neutroph     GTAGCCGGCCCGGCAAGTCGC-TCCTGAAATCCCAGGGCCCAACCCTGGG
T. modestius    GTAGCCGGCCCGGCAAGCCTTGCTCTGAAATCCCCGGGCTCAACCCGGGG
T. pendens      GTAGCCGGCCCGGTAAGTCCC-TCCTTAAAGCCCACGGCTCAACCGTGGG
T. tenax        GTAGCCGGCCCGGCAAGTCGC-TCCTGAAATCCCCAGGCTCAACCTGGGG
P. islandicum   GTAGCCGGCCCGGCAAGTCGC-TCCTGAAATCCCCGGGCTCAACCCGGGG
P. aerophilum   GTAGCCGGCCCGNNAAGTCGC-TCCTGAAATCCCCGGGCTCAACCCGGGG
S. solfatar     GTAGCCGGCCCACCAAGTCGCCCCTTAAAGTCCCC-GGCTCAACCGGGGA
                ***********   *** *      * **  ***  *** ***** **

Tok2            GCCGGGGGCGATACTGCCGGGCTAGGGGGCGGGAGAGGCCGCCGGTACTC
P. neutroph     GCCGGGGGCGATACTGCCGGGCTAGGGGGCGGGAGAGGCCGCCGGTACTC
T. neutroph     GCGGGGGGCGATACTGCCGGGCTAGGGGGCGGGAGAGGCCGCCGGTACTC
T. modestius    GCTGGGGAAGGTACTGCCGGGCTTGGGGGCGGGAGAGGCCGAGGGTACTC
T. pendens      AGCGGAGG-GATACTGCCGGGCTAGGGGGCGGGAGAGGCCGGGGGTACTC
T. tenax        GCAGGGGGCGATACTGCCGGGCTAGGGGGCGGGAGAGGCCGCCGGTACTC
P. islandicum   GCGGGGGGCGATACTGCCGGGCTAGGGGGCGGGAGAGGCCGCCGGTACTC
P. aerophilum   GCGGGGGGCGATACTGCCGGGCTAGGGGGCGGGAGAGGCCGCCGGTACTC
S. solfatar     ACTGGGGGCGATACTGGTGGGCTAGGGGGCGGGAGAGGCGGGGGGTACTC
                   ** *  * *****  ***** *************** *  *******

Tok2            CGGGGGTAGGGGCGAAATCCGTTAATCCCCGGAGGACCACCAGTGGCGAA
P. neutroph     CGGGGGTAGGGGCGAAATCCGTTAATCCCCGGAGGACCACCAGTGGCGAA
T. neutroph     CGGGGGTAGGGGCGAAATCCGTTAATCCCCGGAGGACCACCAGTGGCGAA
T. modestius    CTGGGGTAGGGGCGAAATCCGATAATCCCAGGAGGACCACCAGTGGCGGA
T. pendens      CTGGGGTAGGGGCGAAATCCTATAATCCCAGGAGGACCACCAGTGGCGAA
T. tenax        CGGGGGTAGGGGCGAAATCCTATAATCCCCGGAGGACCACCAGTGGCGAA
P. islandicum   CGGGGGTAGGGGCGAAATCCGTTAATCCCCGGAGGACCACCAGTGGCGAA
P. aerophilum   CGGGGGTAGGGGCGAAATCCGTTAATCCCCGGAGGACCACCAGTGGCGAA
S. solfatar     CCGGAGTAGGGGCGAAATCCTTAGATACCGGGAGGACCACCAGTGGCGGA
                * ** ***************    ** ** ******************* *

Tok2            AGCGGGCGGCCAGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGC
P. neutroph     AGCGGGCGGCCAGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGC
T. neutroph     AGCGGGCGGCCAGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGC
T. modestius    GGCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCTGGGGGAGC
T. pendens      GGCGCCCGGCTAGCACGCGCCCGACGGTGAGGGACGAAAGCTGGGGGAGC
T. tenax        AGCGGGCGGCCAGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGC
P. islandicum   AGCGGGCGGCCAGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGC
P. aerophilum   AGCGGGCGGCCAGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGC
S. solfatar     AGCGCCCCGCTAGAACGCGCCCGACGGTGAGAGGCGAAAGCCGGGGCAGC
                 ***  * **  * ***************** * ******* **** ***

Tok2            AAAGGGGATTAGATACCCCTGTAGTCCCGGCCGTAAACGATGCGGGCTAG
P. neutroph     AAAGGGGATTAGATACCCCTGTAGTCCCGGCCGTAAACGATGCGGGCTAG
T. neutroph     AAAGGGGATTAGATACCCCTGTAGTCCCGGCCGTAAACGATGCGGGCTAG
T. modestius    AAAGGGGATTAGATACCCCTGTAGTCCCAGCTGTAAACTATGCGGGCTAG
T. pendens      AAAGGGGATTAGATACCCCCGTAGTCCCAGCTGTAAACGATGCGGGCTAG
T. tenax        AAAGGGGATTAGATACCCCTGTAGTCCCGGCCGTAAACGATGCGGGCTAG
P. islandicum   AAAGGGGATTAGATACCCCTGTAGTCCCGGCCGTAAACGATGCGGGCTAG
P. aerophilum   AAAGGGGATTAGATACCCCTGTAGTCCCGGCCGTAAACGATGCGGGCTAG
S. solfatar     AAACGGGATTAGATACCCCGGTAGTCCCGGCTGTAAACGATGCGGGCTAG
                *** *************** ******** ** ****** ***********
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Tok2              CTGTCGGTCGGGCTTAGGGCCCGGCCGGTGGCGAAGGGAAACCGTTAAGC
P. neutroph       CTGTCGGTCGGGCTTAGGGCCCGGCCGGTGGCGAAGGGAAACCGTTAAGC
T. neutroph       CTGTCGGCCGGGCTTAGGGCCCGGCCGGTGGCGAAGGGAAACCGTTAAGC
T. modestius      CTGTTGGGCGGGCTTAGAGCCCGCCCAGTGGCGTAGGGAAGCCGTTAAGC
T. pendens        GTGTTGGACGGGCTTCGAGCCCGTCCAGTGCCGTAGGGAAGCCGTTAAGC
T. tenax          CTGTCGGCCGGGCTTAGGGCCCGGCCGGTGGCGTAGGGAAACCGTTAAGC
P. islandicum     CTGTCGGCCGGGCTTAGGGCCCGGCCGGTGGCGAAGGGAAACCGTTAAGC
P. aerophilum     CTGTCGGTCGGGCTTAGGGCCCGGCCGGTGGCGAAGGGAAACCGTTAAGC
S. solfatar       GTGTCGAGTAGGCTTAGAGCCTACTCGGTGCCGCAGGGAAGCCGTTAAGC
                   *** *    ***** * ***    * *** ** ****** *********

Tok2              CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
P. neutroph       CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
T. neutroph       CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
T. modestius      CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
T. pendens        CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
T. tenax          CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
P. islandicum     CCGCCGCCTGGGGAGTACGGCCGCAAGGCTNAAACTTAAAGGAATTGGCG
P. aerophilum     CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
S. solfatar       CCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTTAAAGGAATTGGCG
                  ******************** ****** ** ********************

Tok2              GGGGGGCACCACAAGGGGTGAAGC
P. neutroph       GGGGGGCACCACAAGGGGTGAAGC
T. neutroph       GGGGGGCACCACAAGGGGTGAAGC
T. modestius      GGGGGGCACCACAAGG--------
T. pendens        GGGGAGCACCACAAGGGGTGAAGC
T. tenax          GGGGGGCACCACAAGGGGTGAAGC
P. islandicum     GGGGGGCACCACAAGGGGTGAAGC
P. aerophilum     GGGGGGCACCACAAGGGGTGAAGC
S. solfatar       GGGGAGCACCACAAGGGGTGGAAC
                  **** *************** * *
```

* *P. neutroph - P. neutrophilum*

† *T. neutrop - T. neutrophilus*

‡ *S. solfatar - S. solfataricus*

Appendix 5 - Clustal V alignment of 16S rDNA sequences of strains of the Order "Igneococcales"

```
Tok1            CCATGGAAGTCGTACGC-CCACCAAGTG-GTGGGCGTGGCGGACGGCTGA
T. aggregans    CCATGGGAGTCGCACGCTCCGCCGC-TGCGGGG-CGTGGCGCACGGCTGA
S. marinus      CCATGGGAGTCGTACGCTCCGCCGC-TGCGGAG-CGTGGCGGACGGCTGA
P. occultum     CCATGGGAGTCGTGCGCCCCCGG--ACGCGGGGGCGCGGCGGACGGCTGA
P. fumarius     CCATGGGAGTCGTGGGCCCCCGGGTACGCGGGGGCCCGGCGGACGGCTGA
D. mobilis      CCATGGGAGTCGCACGCTCCGCCGC-TGCGGGG-CGTGGCGGACGGCTGA
H. butylicus    CCATGGGAGTCGTNCNCCCCCGG--ATGCGGGGGCNCGGCGGACGGCTGA
S. h-phila*     CCATGGGAGTCGAACGCCCCGCCGC-TGCGGGG-AGTGGCGCACGGCTGA
I. islandicus   CCATGGGAGTCGAACGC-CCGCCGC-CGCGG-G-CGTGGCGGACGGCTGA
T. maritimus    CCATGGGAGTCGAACGCCCCGCCGC-TGCGGGG-CGTNGCGGACGGCTGA
S. zilligii     CCATGGGAGTCGTACGCTCCGCCGC-TGCGGGG-CGTGGCGGACGGCTGA
C.noboribetsu   CCATGGGAGTCGCGCGCCCAGCCGC-CGCTGGG---CGGCGCACGGCTGA
                ****** *****   ** *        *    *     *** ********

Tok1            GTAACACGTGGCTAACCTACCCTCGGGACGGGGATAGCCCCGGGAAACTG
T. aggregans    GTAGCACGTGGCTAACCTGCCCTCGGGAGGGGGATAACACCGGGAAACTG
S. marinus      GTAACACGTGGCTAACCTACCCTCGGGAGGGGGATAACACCGGGAAACTG
P. occultum     GTAACACGTGGCCAACCTACCCTCGGGACGGGGATAACCCCGGGAAACTG
P. fumarius     GTAACACGTGGCCAACCTACCCTCGGGACGGGGATAACCCCGGGAAACTG
D. mobilis      GTAACACGTGGCTAACCTACCCTCGGGAGGGGGATAACACCGGGAAACTG
H. butylicus    GTAACACGTGGCTAACCTACCCTCGGGACGGGGATAACCCCGGGAAACTG
S. h-phila      GTAACACGTGGCTAACCTACCCTCGGGAGGGGGATAACCCCGGGAAACTG
I. islandicus   GTAACACGTGGCTAACCTACCCTCGGGAGGGGGATAACACCGGGAAACTG
T. maritimus    GTAACGCGTGGCTAACCTACCCTCGGGACGGGGATAACTCCGGGAAACTG
S. zilligii     GTAACACGTGGCCAACCTACCCTCGGGAGGGGGATAACACCGGGAAACTG
C.noboribetsu   GTAACACGTAGCTAACCTACCCTCGGGACGGGGATAACCCCGGGAAACTG
                *** * *** ** ***** ********* ******* * ***********

Tok1            GGGCTAATCCCCGATAGGTGGAGGGGCCTGGAAC-GGTCCTCCACCGAAA
T. aggregans    GTGCTAATCCCCCATAGGGGAAGGCGCCTGGAA-GGGTCCTTCCTCGAAA
S. marinus      GTGCTAATCCCCCATAGGGGAGGATGCCTGGAA-GGGTTCCTCCCCGAAA
P. occultum     GGGCTAATCCCCGATAGGCGAGGGGGCCTGGAACGGGTCCCTCGCCGAAA
P. fumarius     GGGCTAATCCCCGATAGGCGAGGGGGCCTGGAACGGGTCCCTCGCCGAAA
D. mobilis      GTGCTAATCCCCCATAGGGGAGGAGGCCTGGAA-GGGTTCCTCCCCGAAA
H. butylicus    GGGCTAATCCCCGATAGGCGAGGGGGCCTGGAACGGGTCCCTCGCCGAAA
S. h-phila      GGGCTAATCCCCCATAGGCGGGGAGGCCTGGAA-GGGTTCCCCGCCGAAA
I. islandicus   GTGCTAATCCCCCATAGGGGCGGTGGCCTGGAA-GGGTACCGCCCCGAAA
T. maritimus    GAGCTAATCCCCGATAGGCGGGGAGGCCTGGAA-TGGTTCCCCGCCGAAA
S. zilligii     GTGCTAATCCCCCATAGGGGAGGAGGCCTGGAA-GGGTTCCTCCCCGAAA
C.noboribetsu   GGGCTAATCCCCGATAGGCGAGGGGGCCTGGAAC-GGTCCCTCGCCGAAA
                * ********** ***** *  *  ********  *** *  *  *****

Tok1            GGGTCAGACCGCGATGAAGGTCTG---ACCGCCCGAGGATGGGGCTGCGG
T. aggregans    AGGCCCGGCAGGGGTTAGCGCTGCCGAGCCGCCCGAGGATGGGGCTACGG
S. marinus      GGGTGCGGTGGGGGTTATCGCCGCCGCGCCGCCCGAGGATGGGGCTGCGG
P. occultum     GGGCGCGC--GGAGCCTCC--CCGCGCGCCGCCCGAGGATGGGGCTGCGG
P. fumarius     GGGTGCGG--GGGGCATCCTCCCCCGCGCCGCCCGAGGATGGGGCTGCGG
D. mobilis      GGGTGTGGCAGGGGTTAACGCTGCTACACCGCCCGAGGATGGGGCTACGG
H. butylicus    GGNNGCGC--GGAGCTTCC--CCGCGCGCCGCCCGAGGATGGGGCTGCGG
S. h-phila      GGGGCCGCGGGGGGTTATCGCCCGCGGTCCGCCCGAGGATGGGGCTGCGG
I. islandicus   GGGGGAGGCGGGGGTTATCGCCGCCTCTCCGCCCGAGGATGCGGCTGCGC
T. maritimus    GGCGCCGCCGGGGGTTATCGCCGGCGGTGCGCCCGAGGATGGGGCTGCGT
S. zilligii     GGGCGTAGCGGGGGTTATCGCCGCTACGCCGCCCGAGGATGGGGCTGCGG
C.noboribetsu   GGGACCCTGGGGGGTTATCGCCTGGGGTCCGCCCGAGGATGGGGCTGCGG
                 *        *                  ************ **** **
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Tok1              CCCATCATGGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGATAACGGGTA
T. aggregans      CCCATCA-GGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGATAACGGGTG
S. marinus        CCCATCA-GGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGATAACGGGTA
P. occultum       CCCATCA-GGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGATAACGGGTA
P. fumarius       CCCATCA-GGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGACGACGGGTA
D. mobilis        CCCATTA-GGTTGTTGGCGGGGTAACGGCCCGCCAAGCCGATAATGGGTA
H. butylicus      CCCATCA-GGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGATAACGGGTA
S. h-phila        CCCATCA-GGTAGTTGGCGGGGTAATGGCCCGCCAAGCCGACGACGGGTA
I. islandicus     CCTATCA-GGTAGTTGGCGGGGTAACGGCCCGCCAAGCCTAAGACGGGTA
T. maritimus      CCCATCACGGTAGTTGGCGGGGTAATGGCCCGCCAAGCCTACGACGGGTA
S. zilligii       CCCATCA-GGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGAAGACGGGTA
C.noboribetsu     CCCATCATGGTTGTTGGCGGGGTAATGGCCCGCCAAGCCGACGACGGGTA
                  ** ** * *** ************* ************* *  * ****

Tok1              GGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAG
T. aggregans      GGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAG
S. marinus        GGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAG
P. occultum       GGGGCCGTGAGAGCGGGAGNCCCCAGATGGGCACTGAGACAAGGGCCCAG
P. fumarius       GGGGCCGTGGGAGCGGGAGCCCCCAGATGGGCACTGAGACAANGGCCCAG
D. mobilis        GGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAG
H. butylicus      GGGGCCGTGGGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAG
S. h-phila        GGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAG
I. islandicus     GGGGCCGTGGGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAG
T. maritimus      GGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGNCCAG
S. zilligii       GGGGCCGTGAGAGCGGGAGCCCCCAGTTGGGCACTGAGACAAGGGCCCAG
C.noboribetsu     GGGGCCGTGGGAGCGGGAGCCCC-AGATGGGCCCTGAGACAAGGGCCCAG
                  ********* ************* ** ***** ******************

Tok1              GCCCTACGGGGTGCACCAGGCGCGAAACCTCCGCAATGCGGGAAACCGTG
T. aggregans      GCCCTAAGGGGCGCACCAGGCGCGAAACCTCCGCAATGCNGGAAACCGTG
S. marinus        GCC-TACGGGGTGCACCAGGCGCGAAACCTCCGCAATGCGGGCAACCGTG
P. occultum       GCCCTACGGGGCGCACCAGGCGCGAAACCTCCGCAATGCGGGCAACCGTG
P. fumarius       NNCCTACGGGNCGCACCAGGCGCGAAACCTCCGCAATGCGGGCAACCGTG
D. mobilis        GCCCTACGGGGCGCACCAGGCGCGAAACCTCCGCAATGCGGGAAACCGTG
H. butylicus      GNCCTACGGGGCGCACCAGGCGCGAAACCTCCGCAATGCGGGCAACCGTG
S. h-phila        GCCCTACGGGGCGCACCAGGCGCGAAAACTCCGCAATGCGGGCAACCGTG
I. islandicus     GCCCTACGGGGCGCACCAGGCGCGAAAACTCGCCAATGCGGGCAACCGTG
T. maritimus      GCN-TACGGGGNGCACCAGGCGCGAAAACTCCGCAATGCAGGCAACCGTG
S. zilligii       GCCCTACGGGGCGCACCAGGCGCGAAACCTCCGCAATGCGGGAAACCGTG
C.noboribetsu     GCCCTACGGGGCGCACCAGGCGCGAAACCTCCGCAATGCGGGAAACCGTG
                  *** ** ***  *************** ***  ****** ** *******

Tok1              ACGGGGTCACCCCGAGTGCCCCGA--AGAGGGGCTTTTCCCCGCTGTAAG
T. aggregans      ACGGGGCCACCCCGAGTGCCCCCTTTCCGGGGGCTTTTCCCCGCTGTAGG
S. marinus        ACGGGGTCACCCCGAGTGCCCCCTATATGGGGGCTTTTCCCCGCTGTAGG
P. occultum       ACGGGGTCACCCCGAGTGCCGCCGATAAGGCGGCTGTTCCCCGCTGTAGG
P. fumarius       ACGGGGTCACCCCGAGTGCC-CCCTTGCGGGGGCTGTTCCCCGCTGTAGA
D. mobilis        ACGGGGCCACCCCGAGTGCCCCCT-TACGGGGGCTTTTCCCCGCTGTAGG
H. butylicus      ACGGGGTCACCCCGAGTGCCGCCGATAAGGCGGCTGTTCCCCGCTGTAAG
S. h-phila        ACGGGGCTACCCCGAGTGCCCCCTCGTGGGGGGCTTTTCCCCGCTGTAGG
I. islandicus     ACGGGGTTACCCCGAGTGCCCCCATTACGGGGGCTTTTCCCCGCTGTAAA
T. maritimus      ACGGGGCTACCCCGAGTGCCCCCCTCGTGGGGGCTTTTCCCCGCTGCAGG
S. zilligii       ACGGGGTCACCCCGAGTGCCCCCGATAAGGGGGCTGTTCCCCGCCGTAGG
C.noboribetsu     ACGGGGTCACCCCGAGTGCTCCCG-TAAGGGAGCTTTTCCCCGCTGCAAG
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Tok1            TAGGCGGGGG-AATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTA
T. aggregans    AAGGCGGGGG-AATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCCGTA
S. marinus      AAGGCGGGGG-AATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTA
P. occultum     AAGGCGGGGG-AGTAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTA
P. fumarius     AAGGCGGGGG-AGTAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTA
D. mobilis      AAGGCGGGGG-AATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTA
H. butylicus    AAGGCGGGGG-AGTAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTA
S. h-phila      AAGGCGGGGG-AATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTA
I. islandicus   CAGGCGGGGGTAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTA
T. maritimus    TAGGCGGGGG-AATAAGCGGGGGGCAAGACTGGTGTCAGCCGCCGCGGTA
S. zilligii     ATGGCGGGGGTAGTAAGCGGGGGGCAAGTCCGGTGTCAGCCGCCGCGGTA
C.noboribetsu   GAGGCGGGGG-AATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTA
                  ******** * *************** * *************** ***

Tok1            ATACCAGCCCCGCGAGTGGTCGGGACGTTTACTGGGCCTAAAGCGCCCGT
T. aggregans    ATACCAGCCCCGCGAGTGGTCGGGACGGTTATTGGGCCTAAAGCGCCCGT
S. marinus      ATACCAGCCCCGCGAGTGGTCGGGACGATTATTGGGCCTAAAGCGCCCGT
P. occultum     ATACCAGCCCCGCGAGCGGTCGGGATGATTACTGGGCCTAAAGCGCCCGT
P. fumarius     ATACCAGCCCCGCGAGCGGTCGGGACGATTATTGGGCCTAAAGCGCCCGT
D. mobilis      ATACCAGCCCCGCGAGTGGTCGGGACGATTATTGGGCCTAAAGCGCCCGT
H. butylicus    ATACCAGCCCCGCGAGCG-TCGGGATGCTTACTGGGCCTAAAGCGCCCGT
S. h-phila      ATACCAGCCCCGCGAGTGGTCGGGATGATTACTGGGCCTAAAGCGCCCGT
I. islandicus   ATACCAGCCCCGCGAGTGGTCGGGACGTTTATTGGGCCTAAAGCGCCCGT
T. maritimus    ATACCAGCCCCGCGAGTGGTCGGGGTGCTTACTGGGCCTAAAGCGCCCGT
S. zilligii     ATACCGGCCCCGCGAGTGGTCGGGGTGATTATTGGGCCTAAAGCGCCCGT
C.noboribetsu   ATACCAGCCCCGCGAGTGGTCGGGACGTTTACTGGGCCTAAAGCGCCCGT
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Tok1            AGCCCGGCCCAGTAAGTCCCCACAGAAATCCTCGGGCTCAACCCGAGGGC
T. aggregans    AGCC-GGCCCGGCAAGTCCCCTCCTAAATCCCCGGGCTCAACCTGGGGAC
S. marinus      AGCC-GGCCTGGCAAGTCCCCTCCTAAATCCCCGGGCTCAACCCGGGGAG
P. occultum     AGCC-GGCCCGGTAAGTCCCCCCCTAAAGCCCCGGGCTCAACCCGGGGAG
P. fumarius     AGCC-GGCCCGGCAAGTCCCCCCCTAAAGCCCCGGGCTCAACCCGGGGAG
D. mobilis      AGCC-GGCCCGGCAAGTCCCCTCCTAAATTCCCGGGCTCAACCCGGGGAC
H. butylicus    AGCC-GGCCCGGTAAGTCCCCCCCTAAAGCCCCGGGCTCAACCCGG--AG
S. h-phila      AGCC-GGCTCGGCAAGTCCCCTCCTAAAGCCCCGGGCTCAACCCGGGGAG
I. islandicus   AGCC-GGCCTGGTAGGTCCCCTCCTAAAACCCGGGGCTCAACCCCGGGGG
T. maritimus    AGCC-GGCTCGGCAAGTCCCCTCCTAAAGCCCCGGGCTCAACCCGGGGAG
S. zilligii     AGCC-GGCCCGGTAAGTCTCCCCTAAAATCCCCGGGCTCAACCCGGGGAT
C.noboribetsu   AGCC-GGCCCCGTAAGTCCCTCCTGAAAGCCCTGGGCTCAACCCAGGGAG
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Tok1            CTGTGGGG-ATACTGCTGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCC
T. aggregans    TGGGGGGG-ATACTGCCGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCC
S. marinus      TGGAGGGG-AAACTGCCAGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCC
P. occultum     TGGGGGGG-ATACTGCCGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCC
P. fumarius     TGGGGGGGGATACTGCCGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCC
D. mobilis      TGGAGGGG-ATACTGCCGGGCTAGGGGGTGGGAGAGGCCGAGGGTACTCC
H. butylicus    TGGGGGGG-ATACTACCGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCC
S. h-phila      TGGAGGGG-ATACTGCCGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCC
I. islandicus   TGGAGGGG-AAACCACCAGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCC
T. maritimus    TGGAGGGG-ATACTGCCGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCC
S. zilligii     TGGGGGAG-ATACTGCCGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCC
C.noboribetsu   TGG-GGGGGATACTGCGGGGCTAGGGGGCGGGAGAGGCCGGGGGTACCCC
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Tok1            CGGGGTAGGGGCGAAATCCGATAATCCCGGGAGGACCACCAGTGGCGAAG
T. aggregans    CGGGGTAGGGGCGAAATCCTATAATCCCGGGAGGACCACCAGTGGCGAAG
S. marinus      CGGGGTAGGGGCGAAATCCTATAATCCCGGGAGGACCACCAGTGGCGAAG
P. occultum     CGGGGTAGGGGCGAAATCCGATAATCCCGGGAGGACCACCAGTGGCGAAG
P. fumarius     CGGGGTAGGGGCGAAATCCTATAATCCCGGGAGGACCACCAGTGGCGAAG
D. mobilis      CGGGGTAGGGGCGAAATCCTATAATCCCGGGAGGACCACCAGTGGCGAAG
H. butylicus    CGGGGTAGGGGCGAAATCCGATAATCCCGGGAGGACCACCAGTGGCGAAG
S. h-phila      CGGGGTAGGGGCGAAATCCATTAATCCCGGGAGGACCACCAGTGGCGAAG
I. islandicus   CGGGGTAGGGGCGAAATCCGATAATCCCGGGAGGACCGCCAGTGGCGAAG
T. maritimus    CGGGGTAGGGGCGAAATCCATTAATCCCGGGAGGACCACCAGTGGCGAAG
S. zilligii     CGGGGTAGGGGCGAAATCCGTTAATCCCGGGAGGACCACCCGTGGCGAAG
C.noboribetsu   AGGGGTAGGGGCGAAATCCGATAATCCCTGGGGGACCACCAGTGGCGAAA
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Tok1            GCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCG
T. aggregans    GCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCA
S. marinus      GCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCG
P. occultum     GCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCA
P. fumarius     GCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGCAAGCCGGGGGAGCA
D. mobilis      GCGCTCGGCTGGAACACGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCG
H. butylicus    GCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCA
S. h-phila      GCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCG
I. islandicus   GCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCG
T. maritimus    GCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCG
S. zilligii     GCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCG
C.noboribetsu   GCGCCCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCG
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Tok1            AACCGGATTAGATACCCGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGG
T. aggregans    AACCGGATTAGATACC-GGGTAGTCCCGGCTGTAAACGATGCGGGCTAGC
S. marinus      AACCGGATTAGATACCCGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGG
P. occultum     AACCGGATTAGATACCCGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGG
P. fumarius     AACCGGATTAGATACCCGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGG
D. mobilis      AACCGGATTAGATACCCGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGG
H. butylicus    AACCGGATTAGATACC-GGGTAGTCCCGGCTGTAAACGATGCGGGCTAGG
S. h-phila      AACCGGATTAGATACCCGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGG
I. islandicus   AACCGGATTAGATACC-GGGTAGTCCCGGCTGTAAACGATGCGGGCTAGG
T. maritimus    AACCGGATTAGATACCNGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGG
S. zilligii     AACCGGATTAGATACCCGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGC
C.noboribetsu   AACCGGATTAGATACCCGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGG
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Tok1            TGTTGGACGGGCTTT-GAGCCCGTCCAGTGCCGCAGGGAAGCCGTTAAGC
T. aggregans    TGTTGGGTGGGCTT-AGAGCCCACCCAGTGGCGCAGGGAAGCCGTTAAGC
S. marinus      TGTTGGGCGGGCTT-AGAGCCCGCCCAGTGCCGCAGGGAAGCCGTTAAGC
P. occultum     TGTTGGGCGGGCTT-GGAGCCCGCCCAGTGCCGCAGGGAAGCCGTTAAGC
P. fumarius     TGTNNNNNNNNGCTT-TGAGCCCGCCCAGTGCCGCAGGGAAGCCGTTAAGC
D. mobilis      TGTTGGGTGGGCTT-AGAGCCCACCCAGTGCCGCAGGGAAGCCGTTAAGC
H. butylicus    TGNNNNNNNNNNCTT-GGAGCCCGCCCAGTGCCGCAGGGAAGCCGTTAAGC
S. h-phila      TGTNNNNNNNNNNNNNNNNNNNNCC-AGTGCCGCAGGGAAACCGTTAAGC
I. islandicus   TGTTGGGTGGGCTT-CGAGCCCGCCCAGTGCCGCAGGGAAGCCGTTAAGC
T. maritimus    TGTTGGNCGGGCGAGAGGGCCCGCCCAGTGCCGCAGGGAAGCCGTTAAGC
S. zilligii     TGTTGGGTGGGCTT-AGAGCCCACCCAGTGGCGCAGGGAAGCCGTTAAGC
C.noboribetsu   TGTCGGGCGGGCGTTAGAGCCCGCC-GGTGCCGCAGGGAAGCCGTTAAGC
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Tok1            CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
T. aggregans    CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
S. marinus      CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
P. occultum     CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
P. fumarius     CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
D. mobilis      CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGGCG
H. butylicus    CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
S. h-phila      CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
I. islandicus   CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
T. maritimus    CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCG
S. zilligii     CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGGCG
C.noboribetsu   CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTTAAGGAATTGGCG
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Tok1            GGGGAGCACCACAAGGGGTGGAGCCTGCGGCTTAATTGGAGTCAACGCCG
T. aggregans    GGGGAGCACCACAAGGGGTGGAGCCTGCGGTTCAATTGGAGTCAACGCCG
S. marinus      GGGGAGCACCACAAGGGGTGGAGCCTGCGGTTTAATTGGAGTCAACGCCG
P. occultum     GGGNAGCACCACAAGGGGTGGAGCCTGCGGCTTAATTGGAGTCAACGCCG
P. fumarius     GGGGAGCACCACAAGGGGTGGAGCCTGCGGCTTAATTGGAGTCAACGCCG
D. mobilis      GGGGAGCACCACAAGGGGTGGAGCCTGCGGTTCAATTGGAGTCAACGCCG
H. butylicus    GGGGAGCACCACAAGGGGTGGNNNNTGCGGCTTAATTGGAGTCAACGCCG
S. h-phila      GGGGAGCACCACAAGGGGTGGAGCCTGCGGCTTAATTGGAGTCAACGCCG
I. islandicus   GGGGAGCACCACAAGGGGTGGCAGGTGCGGCTTAATTGGAGTCAACGCCG
T. maritimus    GGGGAGCACCACAAGGGGTGGAGCCTGCGGCTTAATTGGAGTCAACGCCG
S. zilligii     GGGGAGCACCACAAGGGGTGGAGCCTGCGGTTCAATTGGAGTCAACGCCG
C.noboribetsu   GGGGGGCAC-ACAAGGGGTGGAGCCTGCGGCTCAATTGGAGTCAACGCCG
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Tok1            GGAATCTT-ACCGGGGGCGACAGCAGGATGATGGCCAGGCTAACGACCTT
T. aggregans    GGAATCTC-ACCGGGGGCGACAGCAGGATGACGGCCAGGCTAACGACCTT
S. marinus      GGAACCTC-ACCGGGGGCGACAGCAGGATGACGGCCAGGCTAACGGCCTT
P. occultum     GGAATCTT-ACCGGGGGCGACAGCAGGATGACGGCCAGGCTGACGACCTT
P. fumarius     GGAACCTT-ACCGGGGGCGACAGCAGGATGACGGCCAGGCTGACGACCTT
D. mobilis      GGAATCTC-ACCGGGGGAGACAGCAGGATGACGGCCAGGTTAAAGGCCTT
H. butylicus    GGAATCTT-ACCGGGGGCGACAGCAGGATGACGGCCAGGCTGACGACCTT
S. h-phila      GGAACCTCCACCGGGGGCGACAGCAGGATGACGGCCAGCCTGAAGGGCTT
I. islandicus   GGAACCTC-ACCGGGGGCGACAGCAGGATGAAGGTCAGGCTGAAGACCTT
T. maritimus    GGAACCTCCACCGGGGGCGACAGCAGGATGATGGCCAGGCTGAAGGCCTT
S. zilligii     GGAATCTT-ACCGGGGGAGACAGCAGGATGACGGCCAGGTTAAAGGCCTT
C.noboribetsu   GGAACCT-CACCGGGGGCGACA-CAGGATGACGGCCAGGCTAACGACCTT
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Tok1            GCCCGACACGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGA
T. aggregans    GCCTGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGA
S. marinus      GCCCGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGA
P. occultum     GCCCGACACGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGA
P. fumarius     GCCCGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGA
D. mobilis      GCCTGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCTGTGA
H. butylicus    GCCCGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTNNTGCCGTGA
S. h-phila      GCCCGACGCGCTGAGGGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGA
I. islandicus   ACCTGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGA
T. maritimus    GCCTGACGCGCTGAGGGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGA
S. zilligii     GCCCGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGTCGTGA
C.noboribetsu   GCCCGACGCGCTGAGGGGAGGTGCATGGCCGTCGCCAGCTCGTGCTGTGA
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Tok1            GGCGTCCGGTTAAGTCCGGCAACGAGCGAGATCCCTGCCCCCAGTTGCTA
T. aggregans    GGTGTCCGGTTAAGTCCGGAAACGAGCGAGACCCCTGNCCCCAGTTGCGA
S. marinus      GGTGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCGCCCCTAGTTGCGA
P. occultum     GGTGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCACCCCTAGTTGCTA
P. fumarius     GGTGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCACCCCTAGTTGCTA
D. mobilis      AGTGTCCGGTTAAGTCCGGAAACGAGCGAGACCCCCACCCCTAGTTGCTA
H. butylicus    GGTGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCACCCCTAGTTGCTA
S. h-phila      GGTGTCCGGTTAAGTCCGGAAACGAGCGAGACCCCCGCCCCTAGTTGCTA
I. islandicus   GGTGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCACCCCTACTTGCTA
T. maritimus    GGTGTCCTGTTAAGTCAGGCAACGAGCGAGACCCCCGCCCCCATTTGCCA
S. zilligii     GATGTCTGGTTAAGTCCAGCAACGAGCGAGACCCCCACCCCCAGTTGCTA
C.noboribetsu   AGTGTCCTGTTAAGTCAGGCAACGAGCGAGACCCCCGCCCCTAGTTGCGA
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Tok1            CCCCGACCTTCGGGTCGGGGGCACACTGGGGGGACTGCCCGCCG-ATAAG
T. aggregans    NNNNNNNCTACGGCCCTGGGGCACACTGGGGGGACTGCC-GCCGTTCAAG
S. marinus      CCCGGAGCTCTGGCTCCGGGGCACACTAGGGGGACTGCC-GCCGTATAAG
P. occultum     CCCCGGGG-CCACCCCGGGGGCACACTAGGGGGACTGCC-GNCGTCCAAG
P. fumarius     CCCCGGGGGCCACCCCGGGGGCACACTAGGGGGACTGCC-GCCGTCCAAG
D. mobilis      CCCGGGGCTACGGCTCCGGGGCACACTAGGGGGACTGCC-GCCGTTTAAG
H. butylicus    CNNNGGGG-CCACCCCGGGGGCACACTAGGGGGACTGCC-GCCGTCCAAG
S. h-phila      CCCCGGGGGAGACCCCGGGGGCACACTAGGGGGACTGCC-GCCGT-TAAG
I. islandicus   CCCGGGGCTCCGGCCCCGGGGCACAGTAGGGGGACTGCC-GCCGTATAAG
T. maritimus    TCCCAGGGGAGACCCTGGGGGCACTATGGGGGGACTGCC-GCCGC-TAAG
S. zilligii     CCCCGGCCTCAGGGCCGGGGGCACACTGGGGGGACTGCC-GCCGTTTAAG
C.noboribetsu   CCCAGCGGGCGACCGCTGGGGCACACTAGGGGGACTGCC-GCCGC-TAAG
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Tok1            GCGGANGAAGGAGGGGGCCACGGCANGTCAGCATGCCCCGAATCCCCCGG
T. aggregans    GCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAATCCCCCGG
S. marinus      GCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAATCCCCCGG
P. occultum     GCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAATCCCCCGG
P. fumarius     GCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAATCCCCCGG
D. mobilis      GCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAACCCCCCGG
H. butylicus    GCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAATCCCCCGG
S. h-phila      GCGGAGGAAGGAGGGGGCAACGGCAGGTCAGCATGCCCCTAAACCCCCGG
I. islandicus   GCGGAGGAAGGAGGGGGCTATGGCAGGTCAGCATGCCCCGAAACCCCCGG
T. maritimus    GCGGAGGAAGGAGGGGGCAACGGCAGGTCAGCATGCCCCTAAACCCCCGG
S. zilligii     GCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAATCCCCCGG
C.noboribetsu   GCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCTAAACCCCCGG
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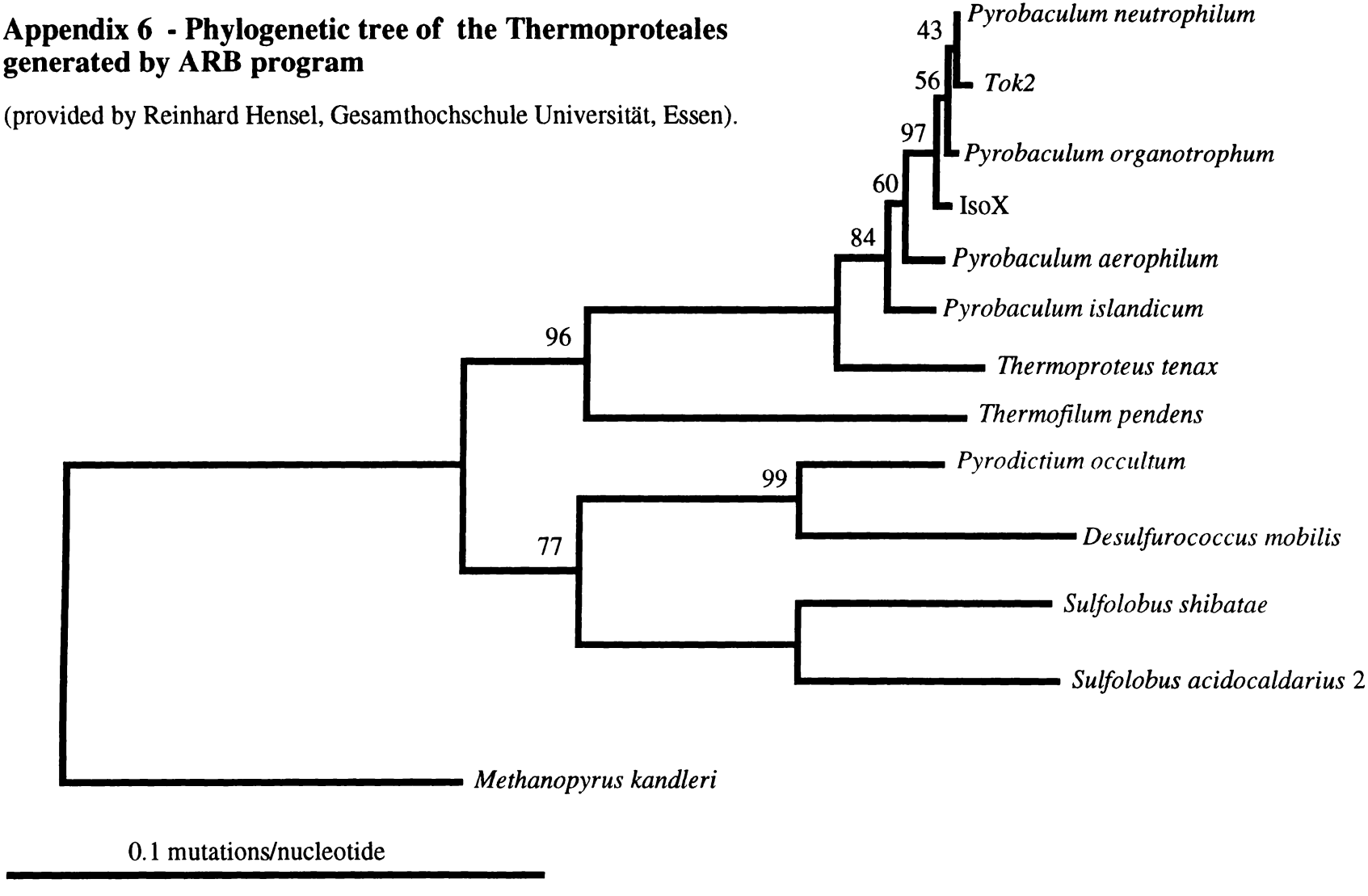
Tok1            GGCTTGCACGCGGGCTTCAATNGCGGGGACAAGCGGNTTCCGACCCCCGA
T. aggregans    G--CTACACGCGGGCTACAATGGCGGGGACA-GCGGGTTCCGACCCCGAA
S. marinus      G--CTACACGCGGGCTACAATGGCGGGGACA-GCGGGATCCGACCCCGTA
P. occultum     G--CTGCACGCGGGCTACAATGGCGGGGACA-GCGGGATCCGACCCCGAA
P. fumarius     G--CTGCACGCGGGCTACAATGGCGGGGACA-GCGGGACCCGACCCCGAA
D. mobilis      G--CTACACGCGGGCTACAATGGCGGGGACA-GCGGGATCCGACCCCGAA
H. butylicus    G--CTGCACGCGGGCTACAATGGCGGGGACA-GCGGGATCCGACCCCGAA
S. h-phila      G--CTGCACGCGGGCTACAATGGCGGGGACA-GCGGGTGCCGACCCCGAA
I. islandicus   G--CTGCACGCGGGCTACAATGGCGGGGACA-GCGGGTTGCGACCCCGAA
T. maritimus    G--CTGCACGCGGGCTACAATGGCGGGGACA-GCGGGATCCGACCCCGAA
S. zilligii     G--CTACACGCGGGCTACAATGGCGGGGACA-GCGGGTGCCGACCCCGAA
C.noboribetsu   G--CTGCACGCGGGCTACAATGGCGGGGACA-GCGGGATCCGACCCCGAA
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Tok1              AAAGGGGGGGAG
T. aggregans      AGGGGGAGGCAA
S. marinus        AGGGGGAG----
P. occultum       AGGGGGAGGCAA
P. fumarius       AGGGGGAGGCA-
D. mobilis        AGGGGGAGGCAA
H. butylicus      AGGGGGAGGCAA
S. h-phila        AGGGGGAGGCAA
I. islandicus     AGGGGGAG----
T. maritimus      AGGGGGAGGCA-
S. zilligii       AGGGGGAGGCAA
C.noboribetsu     AGGGGGAGGCAA
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S. h-phila - *S. hydrogenophila*

Appendix 6 - Phylogenetic tree of the Thermoproteales generated by ARB program

(provided by Reinhard Hensel, Gesamthochschule Universität, Essen).



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