SEQUESTRATION OF METAL AND METALLOID IONS BY THERMOPHILIC BACTERIA

A thesis submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy in Biological Sciences at the University of Waikato by

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2007

Für meine  ertraute,

meine  reundin,

meine  eliebte …

Gewidmet meiner Ehefrau Mauritia, die all dies für mich verkörpert.

Abstract

This Ph. D. thesis presents results and conclusions from studies 1) investigating the interaction between metal and metalloid ions and thermophilic bacteria, and 2) characterizing microbial populations in a geothermally active habitat with relatively high concentrations of metalloid ions and compounds.

In initial cadmium ion toxicity assays, the minimal inhibition concentration for 46 thermophilic bacteria of the genera *Aneurinibacillus*, *Anoxybacillus*, *Bacillus*, *Brevibacillus*, *Geobacillus,* and *Thermus* were determined. The highest tolerances to cadmium ions (Cd^{2+}) in the range of 400 to 3200 µM were observed for species belonging to the genus *Geobacillus*.

The thermophilic Gram-positive bacteria *Geobacillus stearothermophilus* and *G. thermocatenulatus* were selected to describe further biosorption reactions between cadmium ions and chemically reactive functional groups (potential ligands) within and onto the bacterial cell walls. Data obtained from electrophoretic mobility, potentiometric titration and cadmium ion adsorption experiments were used to quantify the number and concentrations of ligands and to determine the thermodynamic stability constants for the ligand-cation complexes. The first reported surface complexation models (SCMs) quantifying metal ion adsorption by thermophilic microorganisms predicted cadmium adsorption and desorption by both studied *Geobacillus* strains over a range of pH values and for different biomasses. The results indicated the functional group, with a deprotonation constant p*K* value of approximately 3.8, to be more dominant in cation biosorption accounting for 66 and 80% of all titrable groups for *G. thermocatenulatus* and *G. stearothermophilus*, respectively. The generated SCMs are different from model parameters obtained from mesophilic species that have been studied to date and might indicate a different biosorption behavior for both studied *Geobacillus* strains.

Another objective of this thesis was to characterize microbial populations in the hot spring Champagne Pool, located in Waiotapu, New Zealand. The thermal

spring is approximately 65 m in diameter and discharges water at 75° C and pH 5.5, which is oversaturated with arsenic and antimony compounds that precipitate and form orange deposits. Recovered nucleic acids and adenosine 5'-triphosphate (ATP) concentrations obtained for Champagne Pool water samples indicated low microbial density and were in good agreement with relatively low cell numbers of $5.6 \pm 0.5 \times 10^6$ cells per ml. Denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone library analyses revealed the abundance of *Sulfurihydrogenibium*, *Sulfolobus* and *Thermofilum*-like populations in Champagne Pool.

Two novel bacteria and one novel archaeon were successfully isolated with a distant phylogenetic relationship to *Sulfurihydrogenibium*, *Thermoanaerobacter*, and *Thermococcus*, respectively. Genotypic and metabolic characteristics differentiated isolate CP.B2^T from described species of the genus *Sulfurihydrogenibium*. CP.B2^T represents a novel genus within the *Aquificales* order, for which the name *Venenivibrio stagnispumantis* gen. nov., sp. nov. is proposed. *V. stagnispumantis* is a thermophilic, chemolithothrophic bacterium, that utilizes molecular hydrogen as electron donor and oxygen as electron acceptor and displayed growth in the presence of up to 8 mM $NaAsO_2$ (As^{3+}) and more than 20 mM $Na_2HAsO_4.7H_2O$ (As^{5+}). However, growth was not observed when $Na_2HAsO_4.7H_2O$ and $NaAsO_2$ were provided as the sole electron acceptor and donor pair. Arsenic resistance was conferred by the genes *arsA* and *arsB*.

Abstract (German)

Die vorliegende Doktorarbeit stellt Ergebnisse and Schlussfolgerungen von Studien vor, welche die Wechselwirkung zwischen Metall- bzw. Metalloidionen und thermophilen Bakterien untersuchen, und die mikrobielle Zusammensetzung in einem geothermalen Habitat mit relativ hohen Konzentrationen an Metalloidionen und -verbindungen beschreiben.

In Kadmiumtoxizitätstests wurde die minimale Wachstumshemmkonzentration für 46 thermophile Bakterien der Gattung *Aneurinibacillus*, *Anoxybacillus*, *Bacillus*, *Brevibacillus*, *Geobacillus* und *Thermus* bestimmt. Die höchsten Toleranzen gegenüber Kadmiumionen (Cd^{2+}), in der Größenordnung von 400 µM bis 3200 µM, wurden für Bakterien der Gattung *Geobacillus* beobachtet.

Die thermophilen, Grampositiven Bakterien *Geobacillus stearothermophilus* und *G. thermocatenulatus* wurden ausgewählt, um deren Biosorptionsverhalten näher zu untersuchen, d.h. die chemischen Reaktionen zwischen Kadmiumionen und reaktionsfähigen funktionellen Gruppen (mögliche Liganden) innerhalb und an den Bakterienzellwände zu beschreiben. Daten aus potentiometrischen Titrations- und Kadmiumionadsorption-Experimenten, die die elektrophoretische Mobilität der Zellen berücksichtigten, wurden verwendet, um die Anzahl und die Konzentrationen der Liganden zu quantifizieren und die thermodynamischen Stabilitätskonstanten für die Ligand-Kation Komplexe festzustellen. Das erste bekannte Oberflächen-Komplexbildungs-Modell, das Biosorption von Metallionen durch thermophile Mikroorganismen quantifiziert, konnte Kadmiumadsorption und -desorption durch die beiden untersuchten *Geobacillus*-Arten in Abhängigkeit von verschiedenen pH-Werten und für unterschiedliche Biomassen vorhersagen. Die Ergebnisse zeigten, dass die funktionelle Gruppe mit einer Deprotonierungskonstanten p*K* von cirka 3.8 dominant in der Biosorption von Kationen zu sein scheint, und für 66% bzw. 80% aller titrierbaren Gruppen von *G. stearothermophilus* und *G. thermocatenulatus* verantwortlich war. Die erstellten Oberflächen-Komplexbildungs-Modelle unterschieden sich von den Modelldaten der bis heute untersuchten mesophilen Arten, und weisen auf ein

anderes Adsorptionsverhalten für die beiden untersuchten *Geobacillus*-Arten hin.

Ein weiteres Ziel dieser Doktorarbeit war, die mikrobielle Population in der heißen Quelle „Champagne Pool“ in Waiotapu, Neuseeland zu charakterisieren. Die Thermalquelle, ungefähr 65 m im Durchmesser, setzt 75° C warmes Wasser mit pH 5.5 frei, welches mit Arsen- und Antimonverbindungen übersättigt ist, die als orangefarbene Niederschlag ausfallen. Nukleinsäure- und Adenosin-5'-triphosphat (ATP)-Konzentrationen, gewonnen aus Wasserproben von „Champagne Pool“, deuteten auf niedrige Zellendichten hin, und bestätigten die ermittelten relativ niedrigen Zellzahlen von 5.6 ± 0.5 $x10^6$ Zellen pro ml. Denaturierende Gradienten-Gelelektrophorese (DGGE) and 16S rRNA Gen-Klonbibliotheken zeigten das Vorkommen von *Sulfurihydrogenibium*-, *Sulfolobus*- und *Thermofilum*-ähnlichen Arten in „Champagne Pool“ an.

Zwei unbekannte Bakterienarten und eine unbekannte Archaeonart konnten erfolgreich isoliert werden, die phylogenetisch entfernt mit *Sulfurihydrogenibium*, *Thermoanaerobacter* und *Thermococcus* verwandt waren. Genotypische und metabolische Eigenschaften unterschieden Isolat CP.B2^T von beschriebenen Arten der Gattung *Sulfurihydrogenibium*. CP.B2^T stellt eine neue Gattung innerhalb der Ordnung *Aquificales* dar, für die der Name *Venenivibrio stagnispumantis* gen. nov., sp. nov. vorgeschlagen wird. *V. stagnispumantis*, ein thermophiles, chemolithothrophes Bakterium, das molekularen Wasserstoff als Elektronendonor und Sauerstoff als Elektronenakzeptor benutzte, konnte in der Anwesenheit von bis zu 8 mM $NaAsO_2$ (As^{3+}) und mehr als 20 mM $Na_2HAsO_4.7H_2O$ (As^{5+}) wachsen. Wachstum wurde jedoch nicht beobachtet, wenn $Na_2HAsO_4.7H_2O$ und $NaAsO_2$ als das alleinige Elektronenakzeptor- und Elektronendonorpaar zur Verfügung gestellt wurde. Arsenresistenz wurde durch die Gene *arsA* und *arsB* vermittelt.

Acknowledgements

The present thesis is a product of interactions not merely with microorganisms but also with other life forms. It is a pleasure to acknowledge those people who contributed to this research work.

Firstly, and most importantly, I love to thank Mauritia Hetzer, who finally became my wife during our stay in New Zealand. Our harmonic relationship was always an endless source of support and encouragement for me. To Mauritia I dedicate this thesis.

I am grateful to Doctor Chris Daughney for giving me the opportunity to be part of the project "Extremophilic microorganisms for metal sequestration from aqueous solutions" and for sponsoring my scholarship. It was very informative and enjoyable to do research work together with you in Wairakei and on the NZAPLUME III cruise.

To Pat and Hugh Morgan I remain indebted for the hearty welcome to New Zealand. I wish to thank my supervisors Professor Hugh Morgan, Professor Ian McDonald, and Doctor Ron Ronimus for their kind help and competent guidance throughout my studies and for giving me so much freedom in my research.

I acknowledge all the colleagues at the University of Waikato in Hamilton and at the Institute of Geological and Nuclear Sciences in Wairakei who contributed to this work.

Last but not least, very special thanks to Stephen Harte, who is the International Student Advisor at the University of Waikato, for his great effort to help my wife and me in dealing with New Zealand Immigration.

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List of abbreviations

° C	Degree Celsius
Å	Ångström(s), 0.1 nm
A	Adenosine, ampere
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
BLAST	Basic local alignment search tool
bp	Base pair(s)
BSA	Bovine serum albumin
C	Cytidine
CMD	Castenholz medium D
CTAB	Cetyltrimethylammoniumbromide
DAPI	4',6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dNTP	2'-deoxynucleotide 5'-triphosphate (dATP, dCTP, dGTP, dTTP)
DSM(Z)	Deutsche Sammlung von Mikroorganismen (und Zellkulturen)
DTT	Dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDS	Energy dispersive X-ray spectroscopy
EDTA	Ethylene diamine tetra-acetic acid
Eh	Oxidation-reduction potential
et al.	Latin: et alii, and others
FISH	Fluorescent *in situ* hybridization
g	Gram(s)
g	Gravitational force

G	Guanosine
h	Hour(s)
H	Adenosine, cytidine or thymidine
HEPES	2-hydroxyethyl-1-piperazinylethanesulfonic acid
http	Hypertext transfer protocol
IEP	Isoelectric point
IPTG	Isopropyl β-D-thiogalactoside
JCM	Japan Collection of Microorganisms
l	Liter(s)
LB	Luria-Bertani broth
µ	Micro, 10^{-6}
m	Milli, 10^{-3} or meter(s)
M	Mega, 10^{6} or mol
min	Minute(s)
MES	2-N-morpholinoethanesulfonic acid
MIC	Minimal inhibition concentration
MOPS	3-N-morpholinopropanesulfonic acid
n	Nano, 10^{-9}
N	Adenosine, cytidine, guanosine or thymidine
N. A.	Not applicable
NCBI	National Centre of Biotechnology Information
N. C.	Does not converge
N. D.	Not determined
NTU	Nephelometric turbidity units
NZ	New Zealand
NZAPLUME	New Zealand and American plume mapping expedition
OD	Optical density
OTU	Operational taxonomic unit
Pa	Pascal (pressure unit)
PCR	Polymerase chain reaction
pH	Negative logarithm of the hydrogen ion concentration
PIPES	Piperazine-1,4-bis-2-ethanesulfonic acid
ppm	Part per million, e.g. 1 mg per 1 kg

ppb	Part per billion, e.g. 1 µg per 1 kg
PVP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
r	Ribosomal
R	Adenosine or guanosine
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RLU	Relative light units
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Second(s)
S	Svedberg, sedimentation coefficient; or cytidine or guanosine
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
T	Thymidine
TAE	Tris-acetic acid EDTA buffer
TBE	Tris-borate EDTA buffer
TE	Tris EDTA buffer
TEM	Transmission electron microscopy
Tris	Tris hydroxylmethyl aminoethane
tRNA	Transfer ribonucleic acid
TSB	Tryptic soy broth
UV	Ultra violet
V	Volt(s); or adenosine, cytidine or guanosine
v/v	Volume per volume, 10% (v/v) is equivalent to 10 ml per 100 ml
w/v	Weight per volume, 10% (w/v) is equivalent to 10 g per 100 ml
W	Watt; or adenosine or thymidine
www	World wide web
x	Time(s)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Y	Cytidine or thymidine

Chapter 1: Introduction

The title “Sequestration of metal and metalloids ions by thermophilic bacteria” has been carefully chosen for the present Ph. D. thesis. Misunderstandings and misrepresentations often result when the same term, in various contexts, has a different meaning. As meanings might vary between scientific and everyday language, even between terminologies of different scientific fields and through time, the basic terms of the title, “thermophilic bacteria”, “metal and metalloid ion” and “sequestration”, will be first introduced in detail.

1.1 Thermophilic bacteria: life on the edge

The philosopher Aristotle (384-322 BC) introduced in his work ‘Categoriae’ a new concept to science by grouping ideas and objects according to their properties. These classification systems help to determine how various ideas or objects are similar and related to each other. The concept is still present in fields of modern-day biology, such as in taxonomy and in phylogeny. Aristotle classified living things as either plants or animals. However, the development of the microscope in the 17th century and new insights in molecular biology at the end of the 20th century (Woese *et al.*, 1990) showed furthermore the presence of bacteria and archaea, respectively. Currently, the most widely applied classification system for organisms in biology consists of three separate domains, the top-level taxa *Eukarya* (containing the kingdoms *Plantae* and *Animalia*), *Bacteria,* and *Archaea*, which is often visualized as the universal phylogenetic tree of life (Figure 1).

Although the term "bacteria" was traditionally used to describe unicellular organisms of microscopic size, it is not accurate as all three domains of life contain members that can fall under this definition: fungi of the domain *Eukarya*, and the entire domains *Bacteria* (except *Thiomargarita* species) and *Archaea*. Nowadays, the traditional meaning of the term “bacteria” is replaced by the terms “microorganism” and “microbes”, whereas the modern term “bacteria” refers exclusively to organisms of the domain *Bacteria*. Although bacteria share

phenotypic properties with fungi and archaea, lateral gene transfer occurred between the groups, and integration of bacterial and archaeal endosymbionts in eukaryotic cells (Martin, 1999); the domains can be genotypically clearly distinguished from each other and be identified by molecular distinctions of biomarkers such as the small-subunit ribosomal nucleic acid molecule (16S rRNA) essential for protein biosynthesis.

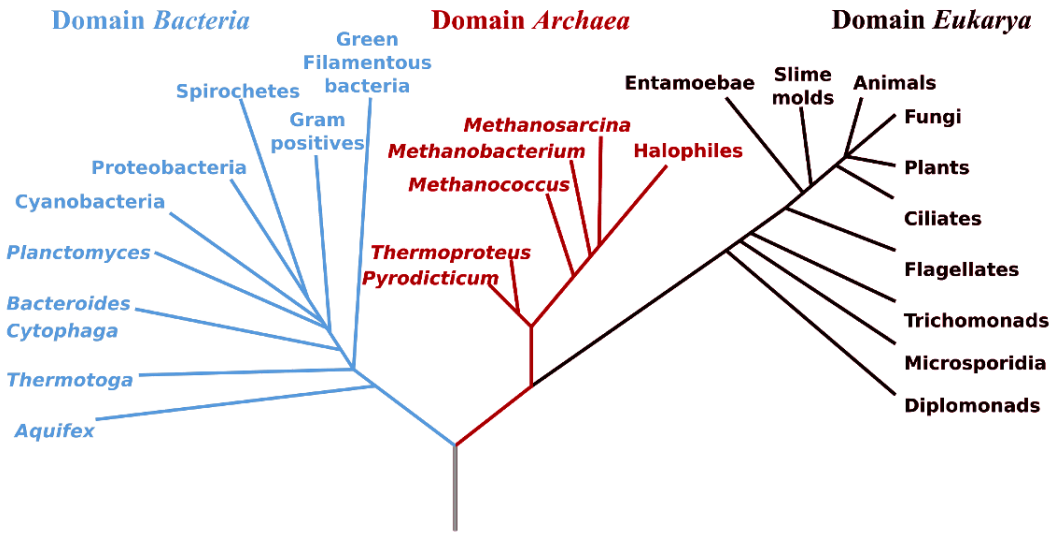

Figure 1. Rooted universal phylogenetic tree displaying the domains *Eukarya*, *Bacteria* and *Archaea* and some groups within the domains based on sequence similarities of 16S rRNA genes (modified from Woese *et al.*, 1990).

Besides phylogenetic groups, there are numerous potential categories by which microorganisms can be classified or grouped. The optimum growth temperature of an organism determines its classification as psychrophilic (Greek: psychros, cold; philos, love), mesophilic (Greek: mesos, middle; philos, love), thermophilic (Greek: thermos, heat; philos, love), or hyperthermophilic (Greek: hyper, beyond; thermos, heat; philos, love):

- under 15° C: psychrophilic (Morita, 1975),
- between 15° C and 45° C: mesophilic,
- between 45° C and 80° C: thermophilic (Madigan *et al.*, 2000),
- above 80° C: hyperthermophilic (Kelly & Adams, 1994).

The current observed maximum growth temperatures of eukaryotic (some fungal species), bacterial (*Aquifex* species), and archaeal monotypic cultures (Strain 121) are 62° C, 95° C, and 121° C, respectively (Deckert *et al.*, 1998; Kashefi & Lovley, 2003; Tansey & Brock, 1972). Thermophilic organisms are able to survive under mild or cold temperatures. Although the first isolated thermophilic bacterium was obtained from the River Seine in France at the end of the 19th century by Miquel (Skinner, 1968), they are generally limited for growth to anthropogenic and natural geologic thermal sources such as compost piles, haystacks, hot water heaters and pipes, terrestrial hot springs and marine hydrothermal vents. As a wide range of geological thermal environments exhibit relatively high concentrations of dissolved metal and metalloid ions (Hirner *et al.*, 1998), organisms within these habitats might have novel adaptive properties to metal and metalloid species. Which leads to the questions: What actually are metals and metalloids?

1.2 Metals and metalloids: a double-edged sword for life

Metals and metalloids, also termed semi-metals, can be distinguished from non-metals by different physical and chemical properties. Usually metals are characterized by good electrical and thermal conduction, malleability (the ability of a material to be deformed under compression without cracking, e.g. when stamped or rolled), ductility (the extent to which a material can be deformed without fracturing when being subject to tensile forces, e.g. when drawn into a thin wire), capacity to form cations (positive ions) and metal bonding. Although metalloids share some of these characteristics with metals, such as formation of cations and metal bonding, metalloids both insulate (like non-metals) and conduct electricity (like metals) as a function of temperature. The metalloids silicon (Si), tellurium (Te), arsenic (As), antimony (Sb), polonium (Po) and astatine (At) occur in negative and positive oxidations states, thus able to form cations and anions (negative ions). Metals, metalloids, and non-metals can be organized by their unique electron configurations and are relatively easily distinguished from each other (Figure 2). So far, 111 chemical elements have been fully characterized: 86 (77%) are metals and eight (7%) are metalloids, which underlies the variety of metal elements.

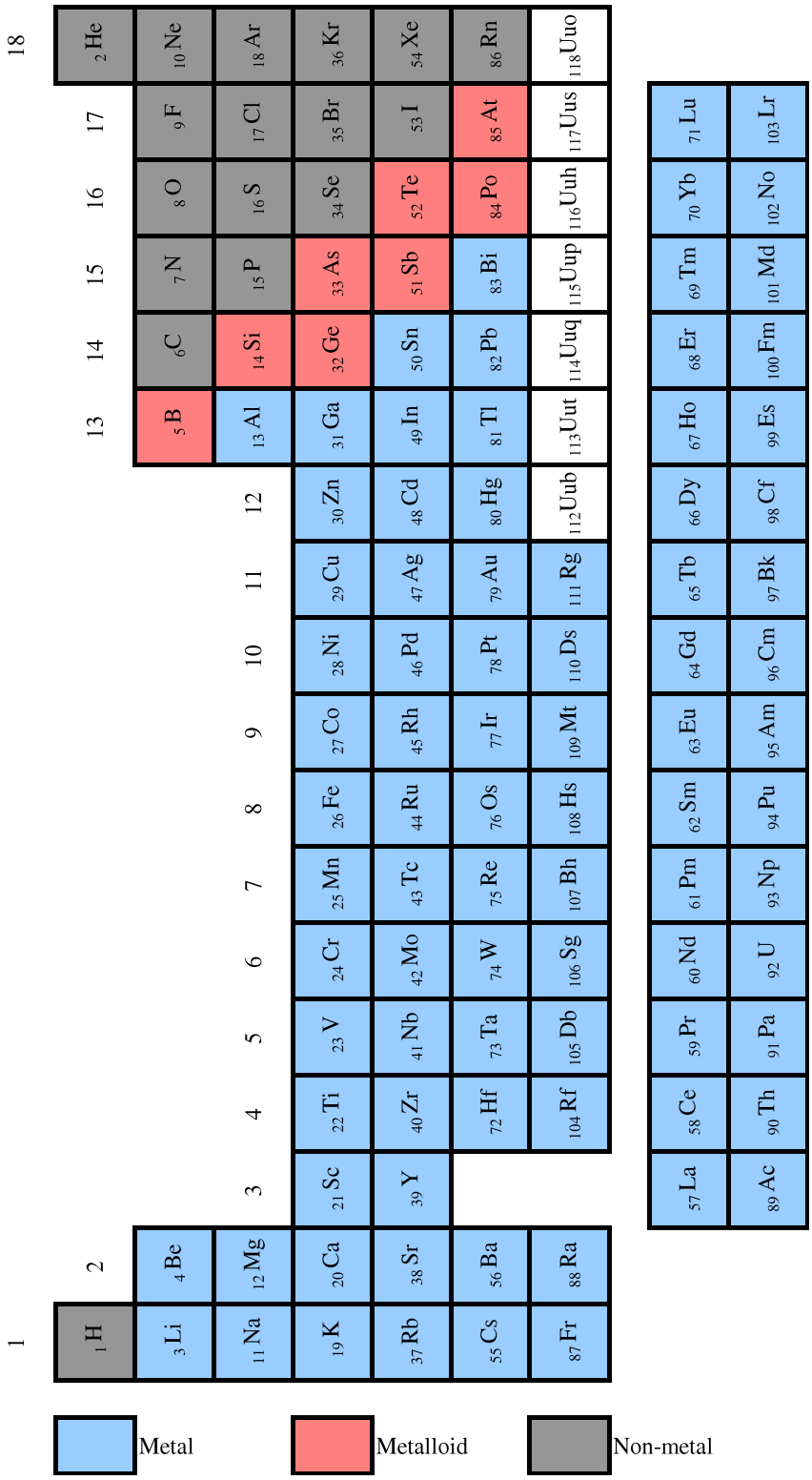

Figure 2. Periodic table displaying elements based on their electron configuration. The white fields refer to discovered elements, but yet not formally named. The subscripted numbers within the fields represent atomic numbers; the one- and two-letter codes the abbreviations used for the elements. Numbers on top of the periodic table indicate group numbers separating metals further in alkali metals (group 1 without hydrogen H), earth alkali metals (group 2), and transition metals (group 3 to 12). The block separated from the main table is the group of rare earth metals.

The terms “metal” and “metalloids” are widely used for elements and the corresponding chemical compounds and species. However, the chemical, physical, and physiological properties of an elemental metal can differ greatly from its chemical species, e.g. the trivalent species of elemental arsenic (As), arsenite (As^{3+}), is several times more toxic for biological systems than its pentavalent form arsenate (As^{5+}) (Bissen & Frimmel, 2003). In this study, the terms “metal” and “metalloid” refer to the corresponding elements, whereas the terms “metal ion” and “metalloid ion” is used for the corresponding chemical species. The common term “heavy metal” has no sound definition. Duffus (2002) summarized a multitude of suggested interpretations for the term used in scientific publications defined by inconsistent values for specific gravity (ranging from above 3.5 g per cm^3 to above 7.0 g per cm^3), atomic number or atomic weight. As the terminology of “heavy metal” is not consistent, the term will be completely avoided in the present thesis.

Why are metal ions biologically important? Cations are essential for a variety of physiological processes fulfilling catalytic and structural functions. Enzymes, a subclass of proteins, make biochemical reactions either possible in the first place or accelerate the rate of reactions significantly by lowering the activation energy for specific chemical reactions. The catalytic centres of many enzymes contain metal ions (Table 1). Cations are important structural elements of biomolecules, e.g. magnesium ions stabilize ribosomes and membranes (Smith & Maguire, 1998). The capacity of cells to maintain physiological conditions (homeostasis) includes the ability to regulate the intracellular concentration of hydrogen ions (H^+). The pH value, defined as the negative logarithm of the hydrogen ion concentration, can be adjusted by influx or efflux of hydrogen ions in or out the cell, thereby lowering or raising the intracellular pH, respectively. The transport process depends on monovalent metal cations (Kroll & Booth, 1983; Matin, 1990). Metalloid ions have been found to be involved for groups of microorganisms in cell-to-cell communication termed quorum sensing (boron ions); incorporated into the 21st described amino acid selenocysteine (selenium ions); and even antimony and cadmium ions, usually toxic for organisms in low concentration, have been found in marine diatoms incorporated into organic molecules such as lipids and one type of enzyme, respectively (Benson & Cooney,

1988; Chen *et al.*, 2002; Lane & Morel, 2000; Zinoni *et al.*, 1986).

Table 1. Some representative metalloproteins and their metabolic functions. Not all of the listed biomolecules are found in a single organism as some are involved in highly specific reactions. There are enzymes containing heterometallic centers, such as Ni-Fe hydrogenase consisting of nickel and iron ions. (Crowley *et al.*, 2000; Kisker *et al.*, 1997; Kobayashi & Shimizu, 1999; Köhler & Völsgen, 1997; Watt & Ludden, 1999)

Ions of	Biomolecule	Biological relevance
Iron (Fe)	Ferredoxin	Electron carrier
	Cytochrome oxidase	Electron carrier
	Hydrogenase	Activates molecular hydrogen (H_2)
Cobalt (Co)	Vitamin B_{12}	Methyl (CH_3^-) group transfer
Nickel (Ni)	Urease	Hydrolyses urea ($(NH_2)_2CO$)
	Hydrogenase	Activates molecular hydrogen (H_2)
	Cis-trans isomerase	Changes structure within a single molecule
Magnesium (Mg)	Phosphatase	Hydrolyses phosphoric acid monoesters (PO_4^{3-})
	Bacteriochlorophylls	Photosynthetic pigments
Molybdenum (Mo)	Nitrogenase	Fixes atmospheric nitrogen gas (N_2)
	Nitrate reductase	Reduces nitrate (NO_3^-) to nitrite (NO_2^-)
Manganese (Mn)	Superoxide dismutase	Disproportionates superoxide (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2)
Zinc (Zn)	RNA and DNA polymerases	Synthesize nucleic acids
	Alcohol dehydrogenase	Oxidizes ethanol (CH_3CH_2OH) to acetaldehyde (CH_3CHO)

Reduction-oxidation (redox) reactions serve as metabolic energy sources. For redox reactions, it is mandatory that reactants take part either in oxidation reactions by accepting electrons (oxidizing agents) or in reduction reactions by providing electrons (reducing agents). Therefore, the involved reactants must be able to occur in different oxidation states. Some metal and metalloid ions fulfill this requirement and are consequently used by many bacterial and archaeal strains as final electron acceptors during dissimilatory metal and metalloid reduction or as electron donor for chemolithothrophic growth (Greek: chemo, chemical; lithos, stone; trophy, nourishment: production of biomass by using oxidation of inorganic compounds as energy source). Redox transformations of various metal and metalloids by organisms have been described including compounds of iron, manganese, chromium, arsenic, mercury, and uranium (Croal *et al.*, 2004; Gihring & Banfield, 2001; Lovley, 1993; Stolz *et al.*, 2006; Stolz & Oremland, 1999; Straub *et al.*, 2001).

Besides the potential beneficial aspects described above, metal and metalloid ions can threaten living cells and even cause their death. Non-essential metal and metalloid species can displace for an organism essential metal and metalloid ions, thereby interfering with cellular functions. Metal and metalloid oxyanions can "mimic" structurally related non-metal species. In cell reactions arsenate (AsO_4^{3-}) and vanadate (VO_4^{3-}) compete with phosphate (PO_4^{2-}), and similarly, chromate (CrO_4^{2-}) and molybdate (MoO_4^{3-}) are analogues of sulfate (SO_4^{2-}) (Ballatori, 2002; Bissen & Frimmel, 2003). Disruptions of phosphate metabolism affect protein biosynthesis, cell integrity, and energy conservation, as phosphates are integral components of nucleic acids (DNA and RNA), lipid membranes, and adenosine 5'-triphosphate (ATP), respectively. Furthermore, the reduction of metal and metalloid oxyanions can result in the formation of radical species possessing an unpaired electron (Nies, 1999). These unpaired electrons are highly reactive and threaten the integrity of nucleic acids (mutations), proteins and lipids and can lead to cell death (Cheeseman & Slater, 1993). Some metal and metalloid ions tend to bind with great affinity to functional groups of proteins, particularly to thiol groups (SH^-), and might modifying the conformation and alter the activity of physiologically-relevant biomolecules (Nies, 1999).

1.3 Sequestration

As discussed earlier, metal and metalloid ions are potentially life-threatening, particularly at elevated concentrations. To guarantee their survival, cells have developed several homeostasis, protection, and detoxification strategies when interacting with metal and metalloids ions. Frequently described responses are:

- Active efflux system

  Efflux systems are energy-dependent processes (consuming ATP) mediated by a specific carrier. The major families of enzymes involved in the export of cations are: 1) P-type ATPase family pumps cadmium (Cd^{2+}), copper (Cu^{2+}), potassium (K^{+}), sodium (Na^{+}), magnesium (Mg^{2+}), and calcium ions (Ca^{2+}) out of the cell; 2) ATP-binding cassette (ABC) family exports nickel (Ni^{2+}), manganese (Mn^{2+}), iron (Fe^{2+}), and molybdenum ions (Mo^{2+}); 3) Resistance-nodulation-cell division family (RND) effluxes Ni^{2+}, cobalt (Co^{2+}), Cd^{2+}, and zinc ions (Zn^{2+}); and 4) Cation diffusion facilitator family (CDF) transports Zn^{2+}, Cd^{2+}, and Co^{2+} (Nies, 2003; Paulsen & Saier, 1997; Saier, 2000; Silver & Phung, 1996; Spada *et al.*, 2002).
- Biotransformation

  The possible enzymatic transformations of metal and metalloid compounds to less toxic forms consist of oxidation, reduction, methylation, and demethylation reactions. Arsenic resistance conferred by the *ars* operon is a model example. Intracellular arsenate is reduced to arsenite by the arsenate reductase (ArsC). This step seems to be irrational at first sight, as arsenite is more toxic than arsenate. However, as mentioned above, arsenate is a structural analogue of the physiologicaly important phosphate, and by reducing it, it is distinguishable from phosphate. Arsenite is then expelled from the cytoplasm through a specific efflux pump encoded by the *arsB* gene, either alone or together with an ATPase (ArsA). Another prominent example is the mercury resistance operon. (Bruins *et al.*, 2000; Nies, 1999; Stolz *et al.*, 2006)
- Intracellular complexation

  Metal and metalloids ion are bound to cellular ligands (complexes one cation) and chelators (complexes more than one cation) such as polyphosphates,

glutathiones (Mendoza-Cozatl *et al.*, 2005), metallothioneins (Palmiter, 1998; Robinson *et al.*, 2001) and in plant cells to phytochelatines (Knauer *et al.*, 1998) to prevent toxic cations interacting with essential biochemical reactions or structures. Intracellular complexation of mercury, cadmium, lead and silver ions have been reported (Singh *et al.*, 2001).

- Compartmentation

  This strategy occurs nearly exclusively in eukaryotic cells as usually bacteria and archaea lack subcellular compartments. Compartmentation in the vacuole has been reported for plants, fungi, algae and yeasts (Hall, 2002; Mendoza-Cozatl *et al.*, 2005).

- Changes in ion permeability

  The permeability of the outer membrane of Gram-negative bacteria might be decreased by altering the conformation of transmembrane transport proteins such as porins and thus reducing the import of the metal and metalloid ions (Li *et al.*, 1997; Zheng *et al.*, 2005).

- Extracellular complexation

  Extracellular complexation includes two separate approaches: 1) The release of biopolymers with anionic character such as exopolysaccharides in the surrounding medium which complex or precipitate metal and metalloid cations (McSwain *et al.*, 2005); and 2) the adsorption of metal and metalloid ions onto and into the cell wall. The latter case is called biosorption and potential ligands, which form complex compounds with cations, are reactive functional groups such as deprotonated hydroxyl (R-OH, R represents the cell to which the functional group is attached), carboxyl (R-COOH), sulfhydryl (R-SH), sulfo ($R\text{-}SO_3H$), phosphono ($R\text{-}PO(OH)_2$), amino ($R\text{–}NH_2$), imino (R=NH), and amido ($R\text{–}CONH_2$) of peptidoglycans, teichoic and teichuronic acids, polysaccharides, and proteins exposed on, or incorporated in, the cell wall (Gadd, 1990; Vecchio *et al.*, 1998).

These strategies have been categorized, whether metal and metalloid species are sequestered or not. The general definition of the term “sequestration” is the act of forming a compound with an ion, atom, or molecule so that the ion, atom, or molecule is no longer available for chemical reactions. Of all the described strategies, only complexation and compartmentation fulfill this definition.

However, in this study, sequestration particularly refers to the interaction between thermophilic bacteria and aqueous metal or metalloid ions, in which the effective concentration of the metal or metalloid ions in a solution is lowered due to complexation. As noted above, compartmentation of metal and metalloid compounds in bacteria might be excluded.

1.4 Objectives

The ability of microorganisms to interact with cationic metal and metalloid species in various ways such as biotransformation, bioaccumulation, and biosorption greatly affects the geochemical cycles of metal and metalloid species. Microbial mediated mobilization and demobilizing of cations influences the distribution and availability of metal and metalloid ions in the environment (Gadd, 2000; Gadd, 2004; Unz & Shuttleworth, 1996). A recent example is the high arsenic concentration of groundwater above the limit recommended by the World Health Organization (10 µg per liter) (WHO, 2004) in parts of Bangladesh, India, Vietnam, Cambodia, Taiwan, Mexico, Argentina, Chile, Hungary, Romania, Canada, and the United States of America (USA) affecting the drinking water supply and the health of many millions of people (Bissen & Frimmel, 2003; Croal *et al.*, 2004). Arsenic compounds are human carcinogens associated with hyperpigmentation and hyperkeratosis of the skin, cardiovascular and cerebrovascular diseases, and reproductive effects including higher rates of spontaneous abortions and stillbirths (Ahmad *et al.*, 2001; Bissen & Frimmel, 2003). Although naturally-occurring arsenic species are immobilized in aquifer sediments, either adsorbed to iron oxyhydroxides (FeOOH; both arsenate and arsenite) or incorporated into arsenopyrite (FeAsS; arsenite), microbial reduction of iron oxyhydroxides or oxidation of arsenopyrite leads to the release of arsenate or arsenite from the sediment to the drinking water (Islam *et al.*, 2005; Zobrist *et al.*, 2000). Microorganisms play a fundamental role in geological processes such as transformation and degradation of minerals containing arsenic (As), antimony (Sb), mercury (Hg), iron (Fe), manganese (Mn), chromium (Cr), molybdenum (Mo), vanadium (V), and uranium (U) (Ehrlich, 1995).

The further understanding of the interactions between microbial cells and metal and metalloid species would support novel, or improve existing, biotechnical applications. Mesophilic microorganisms are commercially employed in bioleaching of copper, uranium, and gold-bearing ores by acidophilic ferrous- and sulfur-oxidizing bacteria involving *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* (formerly *Thiobacillus*) (Rawlings, 2002) and are potential bioremediation agents for recovering metal and metalloid species from waste waters and soils (Gadd, 2000). However, little is known about the interaction between thermophilic microorganisms and metal and metalloid species. This study is part of the biotechnology program "Extremophilic microorganisms for metal sequestration from aqueous solutions". The aim of the program is to describe and predict metal ion biosorption behavior by thermophilic microorganisms to permit future biotechnology applications.

The objectives of the thesis are as follow: 1) to screen the culture collection of the Thermophile Research Unit located at the University of Waikato consisting of approximately 900 archaeal and bacterial strains for metal-tolerant bacteria; 2) based on the initial toxicity experiments to select model organisms and to investigate the interaction of the bacteria and the dissolved metal ion; and 3) try to enrich thermophilic microorganisms from geothermal environments rich in metal or metalloid compounds, and if monotypic organisms could be successfully isolated, to identify and characterize novel isolates.

It has been proposed that thermophilic bacteria, in comparison to psychrophilic and mesophilic microorganisms, have adapted to elevated temperatures by faster metabolic turnover due to replacement of thermal denaturated biomolecules and the ability to stabilize essential molecules and structures such as proteins, nucleic acids and cell membranes (Mulks *et al.*, 1980). Thermophilic bacteria incorporate a higher degree of saturated and iso- and anteiso-branched fatty acids, of elongated fatty acyl chains, and of carbohydrate-enriched lipids into their cell membranes than mesophiles (Edwards, 1990; Konings *et al.*, 2002; Langworthy & Pond, 1986; Sundaram, 1986). Although the current knowledge of metal ion tolerance is almost completely based on mesophilic organisms, thermophiles, which are physiologically and structurally different from mesophiles, might have

elaborated unique strategies to interact with dissolved metal ions. In an initial susceptibility assay of 46 thermophilic bacterial strains with three mesophilic as an out-group, minimal inhibition concentration (MIC) for divalent cadmium cations have been determined (Chapter 2). Bacteria were differentiated into cadmium ion-sensitive and cadmium ion-tolerant organisms with *Geobacillus stearothermophilus* and *G. thermocatenulatus* displaying tolerance to high concentration of cadmium ions and were selected for further investigation into the mode of metal ion-bacteria interaction.

Surface complexation modeling is based upon a set of molecular-scale thermodynamic reactions, which specify electrostatic and chemical interactions occurring at the solution-surface interface. It can be applied in biosorption reactions describing adsorption and desorption of dissolved metal species to potential ligands onto and into bacterial cell walls. Computer analysis of data obtained from acid-base titration and metal ion adsorption experiments on bacterial cell suspensions provide information on number and concentration of ligands and on the stability of the ligand-cation complexes as a function of pH (Figure 3). In Chapter 3 are the first reported surface complexation models (SCMs) quantifying metal ion adsorption by thermophilic microorganisms. SCMs were developed to describe cadmium ion biosorption by *G. stearothermophilus* and *G. thermocatenulatus* cells.

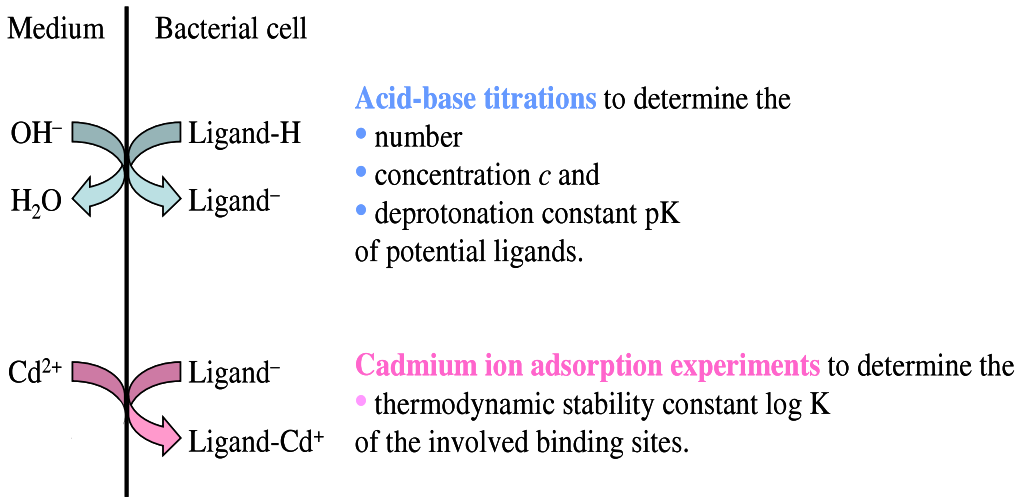

Figure 3. Scheme of surface complexation modeling to predict divalent cadmium cation adsorption by bacteria at the medium-bacterial cell surface interface.

Another aim of this thesis was to study metal ion-tolerant microorganisms obtained from high-temperature environments. New Zealand is famous for its many geothermal features. The North and South Islands of New Zealand lies on the boundary where the Australian and Pacific tectonic plates of the Earth's crust collide. Underneath the North Island, the Pacific plate is subducted below the Indio-Australian plate. When the subducted lithosphere reaches a certain depth, pressure, and heat from the isotopic decay of radioactive elements melts the rock and less dense magma ascends (Price *et al.*, 2005). The thermal energy is transferred to geothermally active areas as magma heats solid surface rock (conduction), or groundwater, which then rises to the surface (convection). Terrestrial thermal activity is noticeable at the surface and associated with volcanoes (magma expelled as lava), fumaroles (local vents that release steam and gases), steaming grounds (areas with diffuse discharge of steam and gases), mud pools (super-heated steam condenses and dissolves surface rock into clay), hot springs (springs that discharge hot water) and geysers (hot springs that erupt periodically).

Figure 4. Water samples collected from Champagne Pool at Waiotapu in New Zealand.

The Waiotapu area located at the center of the North Island is one of the largest geothermal areas in New Zealand. The most prominent feature within the area is the hot spring Champagne Pool (Figure 4) which is around 65 m in diameter and has an estimated volume of 50,000 m^3. The spring water is oversaturated with arsenic and antimony compounds, which precipitate and form the orange deposits at the shelf around Champagne Pool. Although Champagne pool is geochemically well characterized, hardly anything is known about its role as a possible habitat for microbial life. In Chapter 4 microbial density and diversity in Champagne Pool is discussed using first culture-independent and then culture-dependent methods.

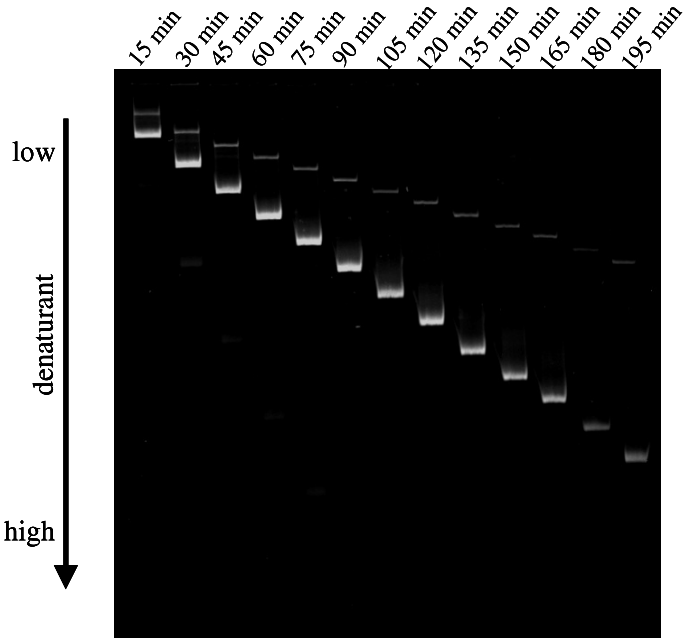

Figure 5. Chronological sequence of a DGGE profile of an environmental DNA sample obtained from a mud pool at Orakei Korako, New Zealand showing migration and separation process of two different archaeal 16S rRNA gene fragments.

Polymerase chain reactions (PCRs) of environmental DNA samples generate fragments of differing sequences (depending on the microbial composition within the samples) but of similar size. In comparison to conventional separation of PCR products by agarose gel electrophoresis, Denaturing Gradient Gel Electrophoresis (DGGE) separates a mix of various DNA species not by nucleotide number but according to the melting behavior based on the nucleotide sequence. PCR

products are electrophoresed through a linear gradient of increasing chemical denaturing concentrations until each DNA species reaches the denaturant concentration equivalent to their individual melting temperature. At that point the two strands of the DNA fragment start separating ("melting") and due the conformation change the molecule slows down its migration and is immobilized at an unique gel position (Figure 5). The DNA fragment can then be excised and analyzed. Based on results obtained from DGGE and 16S rRNA (small-subunit ribosomal nucleic acid) gene clone libraries, a culture medium was designed (Figure 6). This approach has allowed isolation of the novel hydrogen-oxidizing bacterium *Venenivibrio stagnispumantis* (Chapter 5).

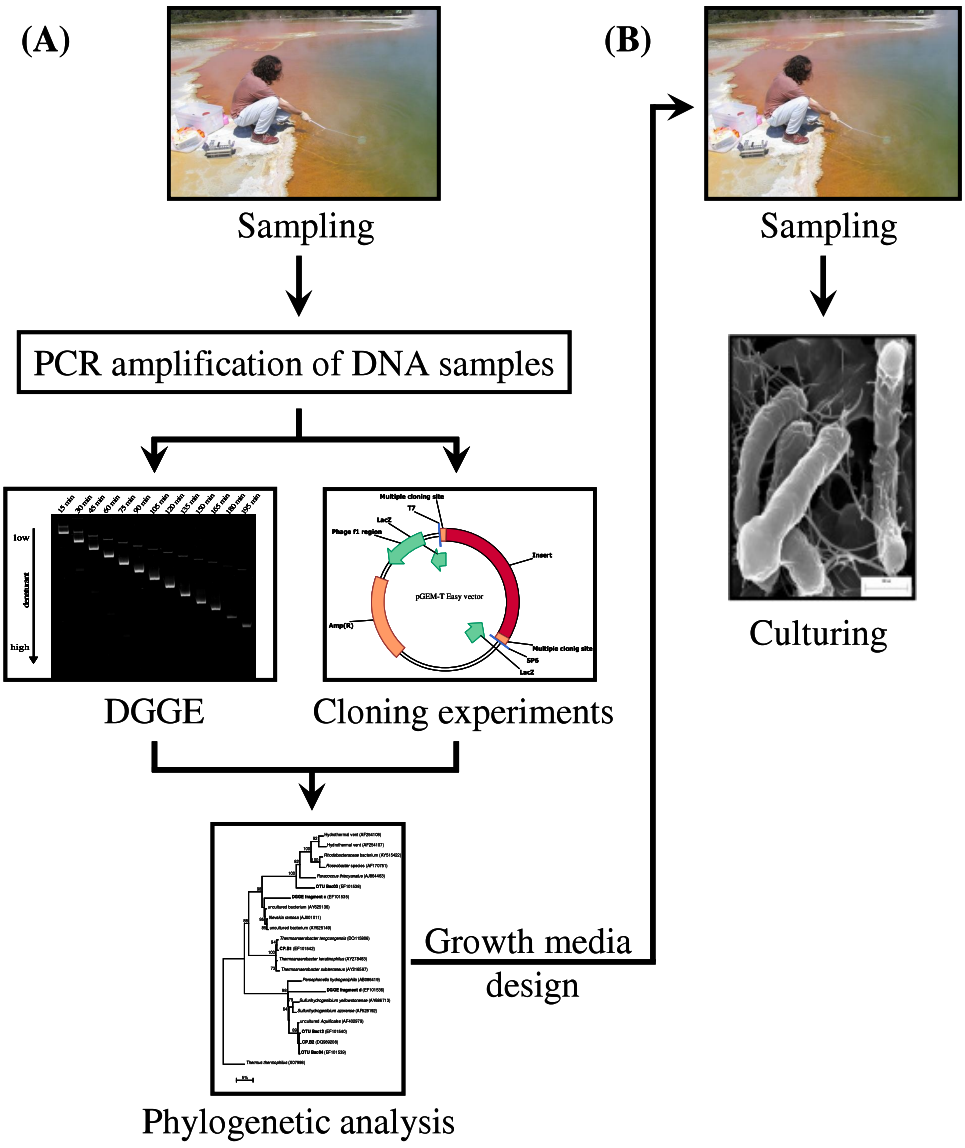

Figure 6. Microbial studies of Champagne Pool consisted of culture-independent (A) and culture-dependent methods (B)

Chapter 2: Cadmium ion toxicity experiments

2.1 Introduction

Cadmium is considered to be a toxic heavy metal, despite the lack of a chemically sound definition for the term heavy metal (Duffus, 2002) and an observed biological benefit of cadmium for zinc-limited marine diatoms (Lane & Morel, 2000). However, cadmium ions and compounds are important environmental pollutants affecting most eukaryotic, bacterial and archaeal cells. Although cadmium is a relatively rare element with an average content of 0.2 mg per kg in the geosphere (Lindsay, 1979), anthropogenic activities have led to local accumulation of cadmium compounds (Vig *et al.*, 2003). Cadmium is a common impurity of zinc ores, and it is most often obtained as a by-product of the smelting of zinc. It is a widely used constituent of nickel-cadmium batteries, of solar cells (cadmium telluride), of cadmium-based solder, of corrosion protective coatings for iron and steel surfaces, of the plastic, polyvinyl chloride (PVC) as stabilizer, and of pigments for coloring plastics, glass, ceramics, and paintings (cadmium sulfide and selenide in cadmium yellow, cadmium orange, and cadmium red). Besides industrial applications, cadmium-contaminated zinc sulfate in fertilizers is another important source of environmental pollution of cadmium compounds.

In this study, 46 thermophilic and 3 mesophilic strains of the genera *Thermus* and *Bacillus* (Table 2 and Table 3) available in the culture collection of the Thermophile Research Unit (University of Waikato, Hamilton, NZ) were tested in susceptibility assays to determine their tolerance to cadmium ions (Cd^{2+}). Thermophilic bacteria of both these genera are aerobic and have overlapping ranges of growth temperatures and pH values, but differ significantly in cell wall composition. Aerobic bacteria were selected as the relatively simple cultivation technique allows a quick determination of cadmium ion-tolerant model organisms for further investigations (Chapter 3).

Gram-staining is a standard method to differentiate bacteria based on differences in the structure of their cell walls into two large groups, either Gram-negative or

Gram-positive (Hucker & Conn, 1927), and into a small group of Gram-variable (Beveridge, 1990). Gram-positive cells contain multilayers of peptidoglycan, whereas Gram-negative cells have a single peptidoglycan layer surrounded by a lipopolysaccharide bilayer (outer membrane). *Thermus* cells stain Gram-negative and *Bacillus* cells react Gram-positive. However, in the peptidoglycan of *Thermus*-like organisms the dibasic amino acid ornithine is present in place of the more typical Gram-negative constituent, diaminopimelic acid (Pask-Hughes & Williams, 1978). Potential differences of the cadmium ion toxicity between bacteria of both genera may reflect the different types of cell wall structures.

The first *Thermus* isolate, *Thermus aquaticus*, was described in 1969. The isolation of its thermostable DNA polymerase (*Taq* polymerase) and use in polymerase chain reactions (PCRs) to amplify *in vitro* nucleic acids has greatly influenced molecular biology (Chien *et al.*, 1976). Since then several species have been obtained from thermal environments. Hudson (1986) grouped *Thermus* isolates obtained from New Zealand, Iceland and USA in 14 clusters (A to N) by numerical classification. In the cadmium ion toxicity assays, at least one representative from each cluster was selected except for the smallest clusters C and N (Table 3). All strains listed in Table 2 were assigned to the genus *Bacillus* until the last decade. However, as more and more information about DNA base compositions became available, a major reorganization for the genus *Bacillus* began (Zeigler, 2001). Due to the changes in nomenclature, the bacteria listed in Table 2 now belong to the genera *Aneurinibacillus*, *Anoxybacillus*, *Bacillus*, *Brevibacillus*, and *Geobacillus*.

2.2 Materials and Methods

2.2.1 Bacteria and culture conditions

The following thermophilic bacteria were tested in toxicity experiments for cadmium ion tolerance (Table 2 and Table 3): *Aneurinibacillus thermoaerophilus* (DSM 10154); *Anoxybacillus flavithermus* (milk powder isolate); *Bacillus sp.* (DSM 2349), *B. caldolyticus* (DSM 405), *B. caldotenax* (DSM 406), *B. caldovelox* (DSM 411), *B. coagulans* (DSM 1, as mesophilic out-group for

comparison), *B. licheniformis* (milk powder isolate), *B. mycoides* (DSM strain 299, as mesophilic out-group), *B. smithii* (DSM 460, 4216), *B. sphaericus* (DSM 462, 463); *Brevibacillus brevis* (DSM 5507), *B. borstelensis* (DSM 6453, as mesophilic out-group); *Geobacillus thermocatenulatus* (DSM 730), *G. thermodenitrificans* (DSM 13147, 13148, 13149), *G. thermoglucosidasius* (DSM 2543), *G. thermoleovorans* (DSM 5366), *G. stearothermophilus* (DSM 458, 6790, 13240); *Thermus aquaticus* (DSM 625, ATCC 27737), *Thermus sp.* T351 (ATCC 31674), *T. thermophilus* (DSM 579), *T. rubber* (DSM 1279); fifteen environmental strains isolated from various hot springs in New Zealand: HS.8A1 (Hanmer Springs); KP.A3, RT.4A1, RT.8A1, RT26.A1, RT.41A, RT.355A1 (all from Rotorua); OH.A2 (Ohaki); TOK10.A3, TOK13.A4, TOK.20A1 (all from Tokaanu); TP.10A2 (Taupo); WAI21.A2, WAI.33A2, WAI.33A3 (all from Waimangu); and five isolates obtained from overseas: NMX.2A1 and YSP.2AA1 (both from the USA); and ZHGI.A1, ZHGIC.A1 and ZVIE.A3 (all from Iceland).

Prior to the experiments, all bacterial strains were successively sub-cultured at least twice in fresh medium. Castenholz medium D (CMD) was used to cultivate the *Thermus* species (Table 3) and contained (per liter): 0.10 g of nitrilotriacetic acid, 0.10 g of $MgSO_4.7H_2O$, 0.06 g of $CaSO_4.2H_2O$, 8.00 mg of NaCl, 0.10 g of KNO_3, 0.69 g of $NaNO_3$, 0.11 g of Na_2HPO_4, 0.47 mg of $FeCl_3.6H_2O$, 1.00 g of trypticase peptone, 1.00 g of yeast extract, and Nitsch's trace elements (per liter medium: 2.20 mg of $MnSO_4.4H_2O$, 0.50 mg of $ZnSO_4.7H_2O$, 0.50 mg of H_3BO_4, 0.016 mg of $CuSO_4$, 0.025 mg of $Na_2MoO_4.2H_2O$, and 0.046 mg $CoCl_2.6H_2O$. The pH value of the medium was 7. The basal medium to grow RT26.A1 and WAI21.A2 contained (per liter): 0.25 g of $CaCl_2.2H_2O$, 0.50 g of $MgSO_4.7H_2O$, 0.20 g of $(NH_4)_2SO_4$, 2.00 g of yeast extract, 5.00 g of glucose, 3.00 g of KH_2PO_4, and trace element solution SL-6 (per liter medium: 0.10 mg of $ZnSO_4.7H_2O$, 0.03 mg of $MnCl_2.4H_2O$, 0.30 mg of H_3BO_3,, 0.20 mg of $CoCl_2.6H_2O$, 0.01 mg of $CuCl_2.2H_2O$, 0.02 mg of $NiCl_2.6H_2O$, and 0.03 mg of $Na_2MoO_4.2H_2O$). The final pH value of the medium was adjusted to 4. The culture medium for the other bacterial strains (Table 2) except RT26.A1 and WAI21.A2 was 30 g per liter tryptic soy broth (TSB, Difco) adjusted to a pH value of 7.

2.2.2 Toxicity experiments

All bacterial strains mentioned above were tested for tolerance to elevated cadmium ion levels ($CdCl_2.2.5H_2O$). Growth curves for strains displaying tolerance to cadmium ion concentration above 500 µM were also obtained in the presence of different arsenate concentrations ($Na_2HAsO_4.7H_2O$). 160 µl medium per well in microtiter plates and 5 ml medium in test tubes supplemented with different defined concentrations of $CdCl_2.2.5H_2O$ were inoculated using 5% inocula of freshly grown cultures. The growth was monitored in microtiter plates at a wavelength of 405 nm (FLUOstar Optima, BMG Labtechnologies) and in test tubes at 600 nm (Ultrospec 3000, Pharmacia Biotech). The minimum inhibitory concentration (MIC) was defined as absence of growth of the species after 16 h (microtiter plates) and 2 d (test tubes) at the lowest tested cadmium ion concentration. Additionally, samples were subsequently observed under a phase-contrast microscope (Olympus BH-2).

2.2.3 Small-scale plasmid isolation

Small-scale plasmid isolation according to Ronimus (1993), a derivation of the alkaline lysis method of Birnboim and Doly (1979), was performed to detect the presence of extrachromosomal DNA. Plasmid-encoded genes for cadmium resistance are commonly found in bacteria. *G. stearothermophilus* (DSM 6790) and *G. thermocatenulatus* (DSM 730) cells were grown in the presence of 200 µM Cd^{2+} and without Cd^{2+} prior to the plasmid isolation. *Escherichia coli* cells (strain JM109) were used as a control. A 3 ml aliquot of cell suspension was centrifuged at 12,000×*g* (Eppendorf Centrifuge 5415D) for 30 s and the resulting pellet was resuspended in the "Solution A" (50 mM glucose, 23 mM Tris, 10 mM EDTA; pH 8.0). The suspension was centrifuged at 12,000×*g* for 15 s and cells were resuspended in 20 µl lysozyme (10 mg per ml) and incubated for 15 min at 37° C. Then 200 µl "Solution A" and 400 µl "Solution B" (1% SDS, 0.2 M NaOH) were added and the cells were incubated in the lysis buffer on ice for 3 min. 300 µl of "Solution C" (3.0 M potassium acetate, 5.0 M glacial acetic acid; pH 4.8) was added, mixed for 10 min and then incubated on ice for 10 min. The suspension

was centrifuged at 12,000×*g* for 10 min to pellet chromosomal DNA and high molecular weight RNA. A 750 µl aliquot of the supernatant was transferred into 750 µl isopropanol. After 10 min incubation at room temperature, the sample was centrifuged at 12,000 *g* for 7 min. The pellet (extrachromosomal DNA and low molecular weight RNA) was then washed twice in 70% ethanol, air-dried and resuspended in 100 µl TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA) containing 1 µl of 10 mg per ml RNase. The sample was incubated at 37° C for 20 min to digest any co-isolated RNA. The presence of plasmids was determined by electrophoresis in 0.7% agarose gel and TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) at 86 V for 1 h.

2.3 Results

2.3.1 Cadmium ion susceptibility tests

The values of the minimum inhibitory concentration (MIC) of Cd^{2+} for the bacterial species tested are displayed in Table 2 and Table 3. It is noteworthy that the thermophilic species displaying the highest cadmium ion tolerance in the experiments belong to a phylogenetic group proposed in 1991 as "group 5" (Ash *et al.*, 1991) that were later defined as the genus *Geobacillus* (Nazina *et al.*, 2001). In comparison to the mesophilic out-group *B. mycoides*, the thermophilic species were more sensitive to Cd^{2+}, while *G. thermoleovorans* tolerated similar Cd^{2+} concentration as *B. mycoides* as high as 3.2 mM. The highest MIC value for *Thermus* strains was 400 µM of Cd^{2+} and was determined for *T. aquaticus* and *T. thermophilus*. The lower values obtained for *Thermus* isolates indicate the cells to be more sensitive to cadmium ions. However, the use of different growth media, CMD for *Thermus* species and TSB for *Bacillus*-like organisms, might have influenced the tolerance to Cd^{2+}. Therefore, the cadmium ion toxicity experiments were repeated for those bacteria displaying a high tolerance to Cd^{2+}. Selected *Bacillus* species were used in the minimal medium CMD, and selected *Thermus* strains were grown in the complex medium TSB, respectively. This approach enables the comparison of the cadmium toxicity between bacteria of both genera in the same medium and the effects of the alternated media composition on the cadmium ion toxicity within a genus. The results clearly demonstrated that the

medium composition affected the ability of the tested bacteria to tolerate Cd^{2+}. All selected mesophilic and thermophilic bacteria exhibited a lower tolerance to Cd^{2+} in the other medium (Table 4).

Table 2. Minimum inhibitory concentration (MIC) of Cd^{2+} for the microorganisms studied and the temperature at which the toxicity experiments were conducted. Strains marked in bold displayed the highest tolerance and growth curves in the presence of different Cd^{2+} concentrations were obtained for these bacteria.

Microorganism	Strain[a]	T [° C]	MIC [Cd^{2+} µM]
Bacillus mycoides	299	25	**>3200**
B. coagulans	1	40	80
Brevibacillus borstelensis	6453	40	**600**
Aneurinibacillus	10154	55	80
Bacillus sp.	2349	55	80
B. licheniformis	Milk powder	55	80
B. smithii	460	55	80
B. smithii	4216	55	80
B. sphaericus	462	55	200
Brevibacillus brevis	5507	55	80
Geobacillus stearothermophilus	458	55	400
G. stearothermophilus	6790	55	**600**
G. thermocatenulatus	730	55	**600**
G. thermodenitrificans	13147	55	400
G. thermodenitrificans	13148	55	80
G. thermodenitrificans	13149	55	80
G. thermoglucosidasius	2543	55	**600**
G. thermoleovorans	5366	55	**>3200**
KP.A3	Rotorua	55	80
Anoxybacillus flavithermus	Milk powder	60	200
Bacillus caldolyticus	405	60	400
B. caldotenax	406	60	400
B. caldovelox	411	60	200
B. sphaericus	463	60	200
Geobacillus stearothermophilus	13240	60	200
Tok10.A3	Tokaanu	60	80
Tok13.A4	Tokaanu	60	**600**
Rt26.A1	Rotorua	65	80
Wai21.A2	Waimangu	65	80

[a] Numbers refer to the corresponding DSM strain (Deutsche Sammlung von Mikroorganismen, the German Collection of Microorganisms).

Table 3. Minimum inhibitory concentration (MIC) of Cd^{2+} for the investigated *Thermus* isolates and the temperature at which the experiments were conducted. Hudson (1986) grouped the studied strains in different clusters. The corresponding cluster names are displayed.

Thermus species	Cluster	Strain[a]	T [° C]	MIC [Cd^{2+} µM]
Thermus ruber	-	DSM 1279	55	80
Thermus sp. T351	H	ATCC 31674	70	200
T. aquaticus	I	DSM 625	70	200
T. aquaticus	I	ATCC 27737	70	400
T. thermophilus	J	DSM 579	70	400
HS.8A1	A	Maruia Springs	70	80
OH.A2	L	Ohaki	70	200
RT.4A1	F	Rotorua	70	80
RT.8A1	M	Rotorua	70	200
RT.41A	H	Rotorua	70	200
RT.355A1	N	Rotorua	70	200
TOK.20A1	G	Tokaanu	70	200
TP.10A2	E	Taupo	70	80
WAI.33A2	E	Waimangu	70	80
WAI.33A3	K	Waimangu	70	80
NMX.2A1	A	Jemez	70	200
YSP.2AA1	I	Yellowstone NP	70	80
ZHGI.A1	L	Iceland	70	80
ZHGIC.A1	D	Iceland	70	200
ZVIE.A3	B	Iceland	70	200

[a] Numbers refer to the corresponding DSM (Deutsche Sammlung von Mikroorganismen, the German Collection of Microorganisms) or ATCC strain (American Type Culture Collection).

Subsequent studies of the species showing MIC values of more than 500 µM Cd^{2+} revealed two different patterns of growth response. An increasing concentration of Cd^{2+} resulted either in a decrease of the maximum optical density with no change in the onset of growth and was observed for *B. mycoides*, *G. thermocatenulatus*, and *G. thermoleovorans* (Figure 7 A, Appendix A: Figure 34) or, alternatively a delay of the onset of growth but no significant effect on maximum optical density and was observed for *G. stearothermophilus*, *G. thermoglucosidasius*, and Tok13.A4 (Figure 7 B, Appendix A, Figure 35), respectively. These results may indicate two different strategies to interact with elevated concentrations of Cd^{2+}.

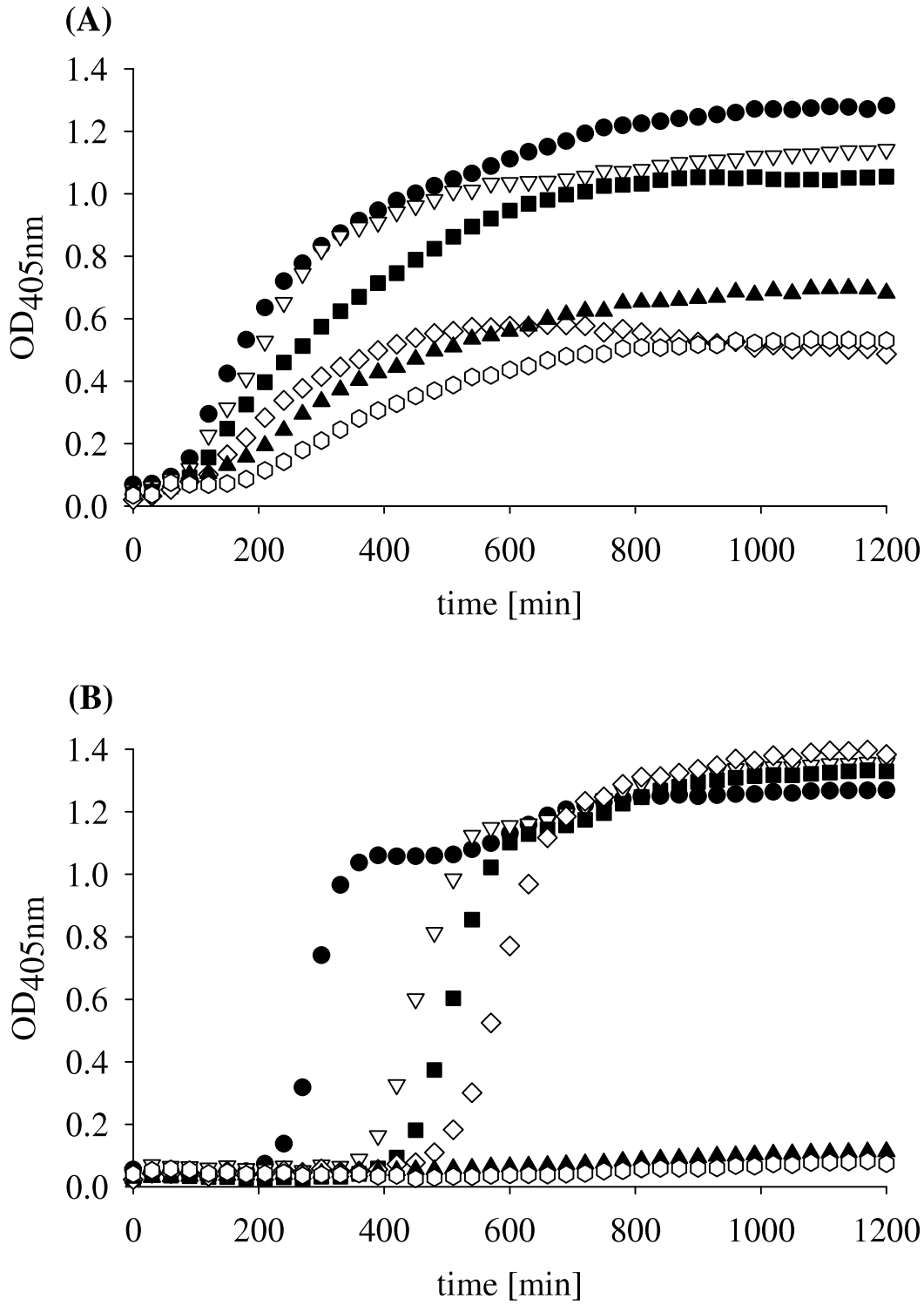

Figure 7. Microbial growth monitored spectrophotometrically at 405 nm as a function of concentration of Cd^{2+} (black circles: 0 µM, white triangles: 100 µM, black squares: 200 µM, white diamonds: 400 µM, black triangles: 600 µM, white hexagons: 800 µM). (A) Growth curves for *G. thermoleovorans* (DSM 5366) and (B) growth curves for *G. stearothermophilus* (DSM 6790). For clarity, only every second data point is shown.

Similar types of growth responses due to Cd^{2+} exposure could be monitored for *G. thermoleovorans* and *G. stearothermophilus* (DSM 6790) when the cells were cultivated in the presence of As^{5+} (Appendix A: Figure 36). However, the growth curves of the other bacteria studied could not be assigned to one of the described types, as the As^{5+} concentrations were too low to inhibit effectively their growth (Appendix A: Figure 37, Figure 38) and no general conclusion can be drawn.

Table 4. Minimum inhibitory concentration (MIC) of Cd^{2+} [µM] for nine selected bacteria when cultivated in the complex media TSB and in the minimal medium CMD.

Species	Strain[a]	T [° C]	MIC (TSB)	MIC (CMD)
B. mycoides	DSM 299	25	>3200	80
G. stearothermophilus	DSM 6790	55	600	80
G. thermocatenulatus	DSM 730	55	600	80
G. thermoglucosidasius	DSM 2543	55	600	80
G. thermoleovorans	DSM 5366	55	>3200	80
TOK.13A4	Tokaanu	65	600	80
Thermus sp.T351	ATCC 31674	70	no growth	200
T. aquaticus	ATCC 27737	70	80	400
TOK.20A1	Tokaanu	70	no growth	200

[a] Numbers refer to the corresponding DSM (Deutsche Sammlung von Mikroorganismen, the German Collection of Microorganisms) or ATCC strain (American Type Culture Collection).

Although *G. stearothermophilus* (DSM 6790) cells showed the same growth pattern when pre-cultured in the presence of 200 µm Cd^{2+} prior to the cadmium ion susceptibility tests the cells displayed higher MIC values and the delay of the onset of growth was reduced in comparison to untreated *G. stearothermophilus* cells (Figure 8). It was assumed that the last described pattern might be the effect of inducible gene expression of a cadmium-resistance factor-carrying plasmid. Therefore, experiments were conducted to isolate plasmids that might be present.

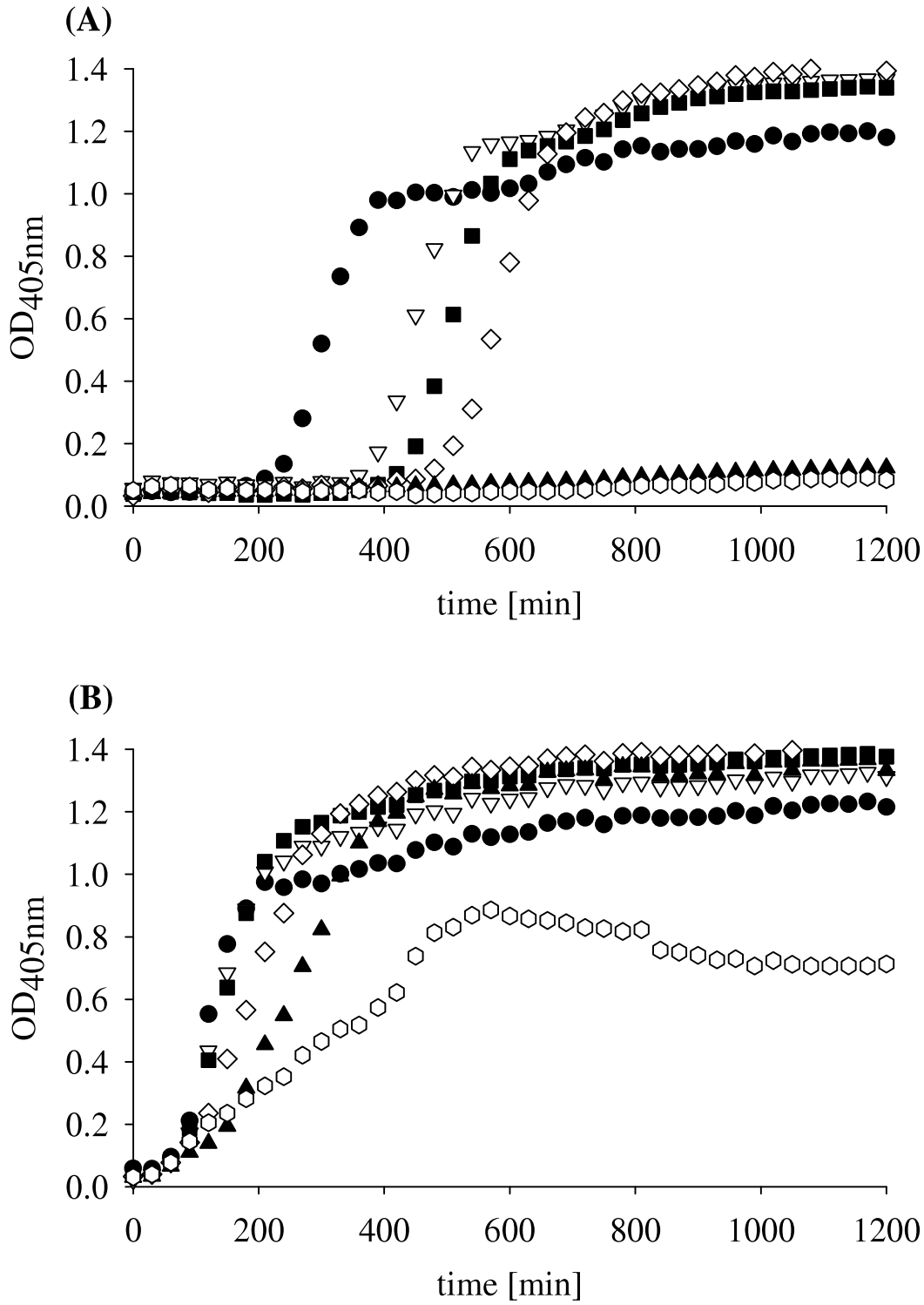

Figure 8. Microbial growth monitored spectrophotometrically at 405 nm as a function of concentration of Cd^{2+} (black circles: 0 µM, white triangles: 100 µM, black squares: 200 µM, white diamonds: 400 µM, black triangles: 600 µM, white hexagons: 800 µM). Growth curves for *G. stearothermophilus* (DSM 6790) previously cultured (A) without Cd^{+2} and (B) in the presence of 200 µM Cd^{+2}. For clarity, only every second data point is shown**.**

2.3.2 Plasmid isolation

Although plasmid-encoded genes for cadmium ion resistance are widely distributed within the bacterial domain, plasmid isolations from *G. stearothermophilus* (DSM 6790) or *G. thermoleovorans* cells were negative. No extrachromosomal DNA was obtained (Figure 9).

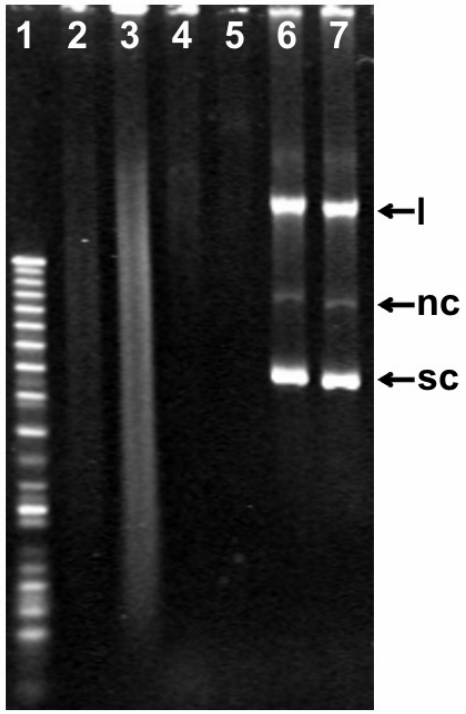

Figure 9. Image of an ethidium bromide-stained 0.7% agarose gel showing samples after plasmid extraction. Lanes: 1, 1 Kb Plus DNA ladder (Invitrogen); 2, *G. stearothermophilus* (DSM 6790); 3, *G. stearothermophilus* (DSM 6790) cultured in the presence of 200 µm Cd^{2+}; 4, *G. thermoleovorans* (DSM 5366); 5, *G. thermoleovorans* (DSM 5366) cultured in the presence of 200 µm Cd^{2+}; 6 and 7, *E. coli* JM109 as control. Abbreviations: l, linear plasmid DNA; nc, nicked circular plasmid DNA; sc, supercoiled circular plasmid DNA.

2.4. Discussion

The physiochemical composition of microbial cell walls can vary considerably within a species with organic and inorganic composition of the growth medium (Malouin *et al.*, 1991; Schär-Zammaretti *et al.*, 2005). The cadmium ion susceptibility assays revealed that the determined MIC values for Cd^{2+} for the studied bacteria depend on the growth medium used (Table 4). The results suggest

either that the *Thermus* cells in the complex media TSB and the *Bacillus*-like cells in the minimal medium CMD were already in a stressed situation and unable to tolerate additionally toxic cations or, that the alternate growth medium might have lead to changes of the bacterial cell surface properties and modified the interaction with cations.

Members of the genus *Geobacillus* displayed the highest Cd^{2+} tolerance with MIC values ranging from 80 to more than 3200 µM (Table 2). Although the diversity of growth media and conditions complicate a meaningful comparison with published studies, some of the results were higher than previously reported MIC values ranging from 10 to 500 µM of Cd^{2+} for thermophilic *Bacilli* (Llanos *et al.*, 2000). No correlation could be established in general for growth temperature and MIC values, and particularly for the studied *Thermus* strains between their cluster affiliation and their tolerance to Cd^{2+}.

Further studies on the strains displaying MIC values of more than 500 µM Cd^{2+} showed two different types of growth curves when exposed to elevated Cd^{2+} concentrations (Figure 7). An elongation of lag growth phase has been previously described for *Bacillus circulans* (Cd^{2+}), *Desulfovibrio vulgaris* (Cu^{2+}, Hg^{2+}) and *Flavobacterium sp.* (Sb^{3+}) when cells were exposed to increasing concentration of metal ions (Chang *et al.*, 2004; Jenkins *et al.*, 2002; Yilmaz, 2003). It is postulated that the response might be due to induced DNA repair mechanism (Bruins *et al.*, 2003). *G. stearothermophilus* (DSM 6790) and *G. thermoleovorans* cells were selected as representatives for each observed pattern. Although plasmid-encoded genes for metal ion tolerance are widely distributed through the domain *Bacteria* (Silver & Phung, 1996) and plasmid-mediated cadmium ion tolerance has been described for *Pseudomonas* (Bruins *et al.*, 2003; Malik & Jaiswal, 2000), *Staphylococcus* (Oger *et al.*, 2003; Udo *et al.*, 2000), plasmids could not be detected for *G. stearothermophilus* (DSM 6790) and *G. thermoleovorans* cells. The successful isolation of plasmids and following sequencing identification of the genetic carrier, may have given deeper insights in the tolerance mechanism and the transfer of plasmids into other bacteria could have been used as biotechnological application to generate multi-metal ion tolerances.

Regarding the known detoxification strategies summarized in Chapter 1, biotransformation and compartmentation can be excluded: Cadmium only occurs in one oxidation state and compartments could not be observed for *Geobacillus* strains. Biosorption reactions might play an important role in microbial tolerance to cadmium ions either solely or in combination with the remaining possibilities, efflux system, and regulation of the ion permeability, which are mediated by chromosomal genes. *G. stearothermophilus* (DSM 6790) and *G. thermocatenulatus* (DSM 730) displayed the same MIC value and had identical optimal growth temperature, but different growth responses in the presence of Cd^{2+} were observed. These two species were selected as model organisms for further experiments (Chapter 3).

2.5 Acknowledgements

The help of Ryan Todorovich in initial cadmium susceptibility tests on *Thermus* isolates is appreciated.

Chapter 3: Cadmium ion biosorption

Cadmium ion biosorption onto the thermophilic bacteria *Geobacillus stearothermophilus* and *G. thermocatenulatus*

(A condensed version of this chapter has been published in
Applied and Environmental Microbiology, June 2006)

3.1 Abstract

This study reports surface complexation models (SCMs) quantifying metal ion adsorption by thermophilic microorganisms. In initial cadmium ion toxicity tests, members of the genus *Geobacillus* displayed the highest tolerance to $CdCl_2.2.5H_2O$ (as high as 400 to 3200 μM). The thermophilic, Gram-positive bacteria *Geobacillus stearothermophilus* and *G. thermocatenulatus* were selected for further electrophoretic mobility, potentiometric titration, and Cd^{2+} adsorption experiments to characterize Cd^{2+} complexation by functional groups within and on the cell wall. Distinct one-site SCMs described the extent of cadmium ion adsorption by both studied *Geobacillus* strains over a range of pH values and metal ion-to-bacteria concentration ratios. The results indicate that a functional group with a deprotonation constant p*K* value of approximately 3.8 accounts for 66% and 80% of all titratable sites for *G. thermocatenulatus* and *G. stearothermophilus*, respectively, and is dominant in Cd^{2+} adsorption reactions. The results suggest a different type of functional group may be involved in cadmium biosorption for both thermophilic strains investigated here, compared to previous reports for mesophilic bacteria.

3.2 Introduction

As described in Chapter 1, various metal and metalloid compounds can be either essential or toxic for living organisms, depending on form and concentration. Therefore, organisms have developed several detoxification strategies including active efflux system, biotransformation, changes in ion permeability,

compartmentation and intra- and extracellular complexation (Bruins *et al.*, 2000; Gadd & Griffiths, 1978; Mendoza-Cozatl *et al.*, 2005; Rosen, 1996; Silver & Phung, 1996). Extracellular complexation of metal species by cell wall components is one of the more important interaction mechanisms, and it has been the subject of many previous studies (for reviews see Beveridge, 1989; Daughney & Fortin, 2001; Gadd, 1988; Schultze-Lam *et al.*, 1993; Shumate & Strandberg, 1985; Volesky & Holan, 1995). Previous investigations indicate that adsorption of metal ions by bacterial cells is affected by 1) the characteristics of the sorbate; 2) the characteristics of the bacteria such as the cell wall composition, growth phase and metabolic state; 3) the system composition (e.g. pH, ionic strength, metal ion-to-bacteria concentration ratio, presence of competing sorbates, sorbents or ligands); 4) the temperature; and 5) the reaction time. Many of these variables are known to affect metal ion adsorption by a variety of electrically charged colloid-sized particles, but some are specifically relevant to biological sorbents, for example the type and concentration of reactive binding sites found within the cell wall.

Several recent investigations have used surface complexation models (SCMs) to describe the extent of metal ion adsorption by bacteria as important physical, chemical, and biological parameters are independently varied (Borrok *et al.*, 2004a; Borrok & Fein, 2005; Daughney & Fein, 1998; Daughney *et al.*, 2001; Fein *et al.*, 1997; Haas, 2004; Yee & Fein, 2001). These SCMs are based upon a set of molecular-scale thermodynamic reactions, each describing adsorption of a particular dissolved chemical species to a particular type of cell wall functional group using a single stability constant (K). SCMs specifically account for the speciation of the sorbate and the bacterial surface. Some previous investigations indicate that different bacterial species display similar types of reactive surface functional groups (e.g. carboxyl, phosphono, hydroxyl, amino) and exhibit broadly similar reactivity toward certain metal ions (Borrok *et al.*, 2004a; Yee & Fein, 2001). However, in other cases, the absolute and relative concentrations of cell wall functional groups and their reactivities towards metal ions have been observed to vary substantially between species, and even for a single species as a function of population growth phase, growth conditions, or cell metabolic state (Claessens *et al.*, 2004; Daughney *et al.*, 2001; Haas, 2004).

The implication is that cell wall structure, while generally similar for the bacteria that have been studied to date, can still influence the nature and extent of metal ion interaction with the cell wall. Note that all previous SCMs have been developed for mesophilic bacteria; no single investigation has been ever published addressing metal ion adsorption onto thermophilic microorganisms. Metal ion adsorption reactions onto thermophilic microorganisms may therefore differ quantitatively and qualitatively from the mesophilic species that have been studied to date. A wide range of geological and anthropogenic thermal environments exhibit high concentrations of dissolved metals. In response to these conditions, microorganisms isolated from theses habits may have unique cell wall structures. Thus, studies of thermophilic microorganisms can supplement our present knowledge of metal biosorption that is completely based on mesophilic organisms.

In initial cadmium ion toxicity experiments (Chapter 2), 46 thermophilic bacteria of the genera *Aneurinibacillus*, *Anoxybacillus*, *Bacillus*, *Brevibacillus*, *Geobacillus,* and *Thermus* were tested. The highest tolerance to cadmium ($CdCl_2.2.5H_2O$), 400 to 3200 µM, was observed for species belonging to the genus *Geobacillus*. *G. thermocatenulatus* (Golovacheva *et al.*, 1975) and *G. stearothermophilus* (Donk, 1920) were selected for further investigation based on their tolerance to Cd^{2+}. The aim of this investigation was to develop a SCM for cadmium ion adsorption onto the two thermophilic bacteria. Cadmium was also selected in the present investigation because SCMs describing the adsorption of cadmium ions by various mesophilic bacteria are available for comparison (Borrok *et al.*, 2004a; Borrok & Fein, 2005; Daughney & Fein, 1998; Daughney *et al.*, 2001; Fein *et al.*, 1997; Yee & Fein, 2001).

3.3 Materials and Methods

3.3.1 Culture conditions

Prior to the titration and cadmium ion adsorption experiments, *G. stearothermophilus* (DSM 6790) and *G. thermocatenulatus* (DSM 730) cells were

successively sub-cultured at least twice in new TSB medium adjusted to a final pH value of 7. For acid base titration and cadmium ion adsorption experiments cells were grown in 1 liter of TSB at 60° C in an orbital mixer incubator at 100 rpm. After 24 h, bacteria were harvested in the exponential growth phase by centrifugation at 8230×*g* (Beckmann Coulter Avanti J-E Centrifuge with fixed-angle rotor JLA-9.100) and washed five times in 250 ml 10 mM $NaNO_3$ to ensure complete removal of the growth medium and adsorbed cations from the bacterial surface. Between each wash step, the bacterial suspension was centrifuged for 10 min at 8230×*g* to form a pellet and the supernatant was discarded. After the final wash, the bacterial pellet was resuspended in a known weight of 10 mM $NaNO_3$ solution and the optical density at 600 nm (Hach DR2010) relative to the electrolyte was determined.

Prior to the experiments, to determine electrophoretic mobility, *G. stearothermophilus* (DSM 6790) and *G. thermocatenulatus* (DSM 730) were incubated in 200 ml TSB at 57° C at 100 rpm for 24 h. The optical density at 600 nm was measured and cells were harvested by centrifugation (8 min, 9820×*g*, 4° C). The pellet was resuspended in 1 mM $NaNO_3$ solution, divided into 3 aliquots and washed three times in 150 ml 1 mM, 10 mM, or 100 mM $NaNO_3$, respectively. The final pellets were resuspended in 1 ml of the respective electrolyte concentration and kept on ice.

The ratio of bacterial biomass to optical density at 600 nm was determined using two different methods. First, a bacterial suspension of known optical density was filtered through a pre-weighed filter membrane (pore size 0.45 µm, Millipore) and flushed with distilled water several times. The filter was dried at 80° C to constant weight. Second, aliquots of a bacterial suspension with known optical density and weight were centrifuged at 1850×*g* (Jouan CR 4.11) for 1 h, stopping three times to decant the supernatant. After 1 h, the wet weight of the bacterial pellet was determined. The dry mass of the pellet was recorded after drying at 80° C to constant weight. The cell number in relation to optical density was determined with a Thoma counting chamber (depth 0.02 mm).

Cell dimensions were recorded by evaluating images obtained from electron microscope. For transmission electron microscopy (TEM), cells were harvested by centrifugation (8 min, 9820×*g*) and three times washed in 10 mM $NaNO_3$. Specimens were fixed first in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.3% ruthenium red and then postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer with 0.3% ruthenium red according to Handley *et al.* (1988). The cells were embedded in agarose, following stepwise dehydration with ethanol and infiltration with Quetol 651 epoxy resin (ProSciTech) using a Lynx el Tissue Processor (Australian Biomedical Corporation). Thin sections of 80 to 90 nm were cut on a Reichert-Jung Ultracut E ultramicrotome and placed onto formvar coated slot grids. The grids were stained with uranyl acetate and lead citrate using an LKB Ultrostainer and examined using a Philips CM100 transmission electron microscope.

3.3.2 Acid-base titrations

Acid-base titrations were conducted using an automated potentiometric titrator (Metrohm titrator 736 GP Titrino) with a glass electrode (Metrohm 6.0130.100) and a separate Ag/AgCl reference electrode (Metrohm 6.07.26.110). The CO_2-free NaOH and the HNO_3 titrants were calibrated against potassium hydrogen phthalate and standardized NaOH, respectively. A known weight (c. 50 g) of homogenized bacterial suspension was placed in an air-tight polystyrene vessel, and the pH was adjusted to approximately 4 by addition of an aliquot of standardized HNO_3. The suspension was purged with humid N_2 gas for 30 min in order to remove dissolved CO_2. Then the suspension of bacteria cells was titrated in an up-pH direction while continuously stirred and under a positive-pressure N_2-atmosphere. Following the first titration, the suspension was again adjusted to pH 4, and a second up-pH titration was performed to evaluate reproducibility and the reversibility of the bacterial surface protonation reactions. For each titration, a stability of 5 mV per min was obtained prior to the addition of the next aliquot of titrant. All titrations were performed in a temperature-controlled room at 20 ± 2° C. At the end of each titration, the dry mass of the titrated bacteria was determined by drying the cells at 80° C, after accounting the dry weight of the electrolyte solution ($NaNO_3$), the added acid and base.

3.3.3 Metal ion adsorption

Batch cadmium ion adsorption experiments were conducted for each bacterial species as a function of pH and bacteria-to-metal ion concentration ratio. A weighed aliquot from a bacterial parent solution was dried to constant weight at 80° C, as described above, to determine the dry weight of bacteria. The remainder was used to prepare three suspensions (c. 150 g each), having 100%, 50% and 25% of the bacterial concentration as the parent suspension. These three suspensions were supplemented with 1000 ppm cadmium atomic absorption standard solution (Merck, $Cd(NO_3)_2$ in 0.5% HNO_3) to a final concentration of 5 ppm. After the addition of the metal, the optical density at 600 nm for each sample was recorded and each suspension was divided into 12 individual reaction vessels. The pH value in each reaction vessel was adjusted by adding small volumes of 100 mM NaOH solution to cover a pH range of 3.5 to 10.0 for each suspension. After a sufficient equilibration time of 2 h (Borrok *et al.*, 2004b) in an orbital mixer incubator (Ratek) set at 100 rpm and 20° C, the samples were centrifuged at 6870×*g* (Beckmann Coulter Avanti J-E Centrifuge with swinging-bucket rotor JLA-5.3) for 15 min. A portion of the supernatant was decanted and acidified with concentrated HNO_3 solution (ARISTAR grade) for subsequent metal analysis by flame atomic adsorption spectroscopy (Perkin Elmer Atomic Adsorption Spectrometer Analyst 800). The remaining supernatant was used for measurement of final equilibrium pH. The concentration of metal ions adsorbed to bacteria in each sample was calculated by subtracting the cadmium ion concentration remaining in the supernatant from the original concentration of 5 ppm.

3.3.4 Electrophoretic mobility measurements

Electrophoretic mobility measurements were carried out using a Malvern Zetasizer Nano ZS at 25° C. 10 ml volumes of 1 mM, 10 mM, and 100 mM $NaNO_3$ were prepared and adjusted to pH values between 1.8 and 9.5 by addition of HNO_3 and NaOH respectively. Additionally, 10 ml volumes of 30 µM, 300 µM, and 3 mM $Cd(NO_3)_2$ solution with final pH values of 4.5 to 5.5 were prepared. The ionic strength of all cadmium solutions was maintained at 10 mM

$NaNO_3$. 3 to 100 µl of the bacterial suspension was added to the 10 ml electrolyte solutions and the pH was measured. The samples were incubated for 2.5 to 5.0 h prior to measurement. The "auto" measurement duration was selected, which reports the electrophoretic mobility as an average of 10 to 30 runs. The isoelectric point values were located by determining the pH value where the electrophoretic mobility of the bacteria was zero.

3.3.5 Surface complexation model (SCM)

Surface complexation models (SCMs) were developed using the computer program FITMOD, a modified version of FITEQL (Westall, 1982). From the experimental acid-base titration data, FITMOD determines the concentrations and deprotonation constants of binding sites on the bacteria. As an example, for the following deprotonation reaction of carboxylic groups

$$R\text{-}COOH \Leftrightarrow R\text{-}COO^- + H^+ \quad (1)$$

where R represents the cell to which the functional group is attached, the stability constant *K* can be determined using the corresponding mass action equation

$$K_{\text{carboxyl}} = \frac{[R\text{-}COO^-] \times a_{H^+}}{[R-COOH]} \quad (2)$$

where *a* refers to the activity of the proton and square brackets represent surface site concentrations in moles per kg solution. The adsorption of cadmium cations onto carboxylic surface binding sites can be expressed by

$$Cd^{2+} + R\text{-}COO^- \Leftrightarrow R\text{-}COOCd^+ \quad (3)$$

FITMOD calculates the thermodynamic stability constants *K* for the metal complexes using the corresponding mass action equation (4).

$$K_{Cd^{2+}\text{-carboxyl}} = \frac{[R\text{-}COOCd^{+}]}{[R-COO^{-}] \times a_{Cd^{2+}}} \quad (4)$$

All SCMs incorporated the Donnan electrostatic model (Yee *et al.*, 2004).

SCM development required information about biophysical parameters such as the cell wall thickness, the dry-to-wet mass ratio, and specific surface area (Table 5). Methods to determine the first two parameters have been described above. The bacterial surface area *A*, was calculated assuming that the cells were cylindrical shaped with a radius *r*, a length *l*, a density *p* equal to 1 kg per liter by following equation

$$A = \frac{2}{p} \times \frac{r + l}{r \times l} \quad (5)$$

where *r* and *l* are expressed in µm, and *A* in m^2 per g wet bacteria (Chatellier & Fortin, 2004). Cell radius *r* and cell length *l* were determined by examining TEM micrographs (Figure 10).

Table 5. Selected biophysical parameters for *G. thermocatenulatus* (DSM 730) and *G. stearothermophilus* (DSM 6790).

Parameter	*G. thermocatenulatus*	*G. stearothermophilus*
Dry-to-wet mass ratio	1 to 15	1 to 8
Isoelectric point [pH]	3.25	3.95
Cell wall thickness [nm]	49.5 ± 3.5	35.0 ± 1.6
Cell length [µm]	4.3 ± 1.9	3.1 ± 0.3
Cell width [µm]	0.4 ± 0.04	0.7 ± 0.02
Surface area [m^2/g wet cells]	8.96	6.38

3.4 Results

3.4.1 Potentiometric titration experiments

Acid-base titration curves for *G. stearothermophilus* and *G. thermocatenulatus* cells in 10 mM $NaNO_3$ are displayed in Figure 11. These figures depict the concentration of protons adsorbed onto the bacterial surface as a function of pH. Titration experiments were conducted in triplicate for *G. thermocatenulatus* and in duplicate for *G. stearothermophilus* (Appendix C: Table 19, Table 20, Table 21, Table 22, and Table 23). Individual titration curves consisting of two titrations from low to high pH values are in excellent agreement with each other after taking dilution into account, indicating reproducibility of the data and complete reversibility of the adsorption reactions during the experiments. The curves are normalized to the dry (Figure 11 A) and wet cell mass of bacteria (Figure 11 B) to allow direct visual comparison between the two species. In comparison to titrated electrolyte solution without bacteria, *G. stearothermophilus* and *G. thermocatenulatus* cells significantly buffer pH over the entire range studied in this investigation, which is in agreement with published data for mesophilic microorganisms (Claessens *et al.*, 2004; Daughney & Fein, 1998; Fein *et al.*, 1997) and one thermophilic bacterium (Wightman *et al.*, 2001).

G. thermocatenulatus compared to *G. stearothermophilus* shows a slightly higher buffering capacity against addition of the base when titration data are normalized to dry cell mass (Figure 11 A). In contrast, when data are normalized to wet cell mass, *G. stearothermophilus* displays a stronger buffering capacity than *G. thermocatenulatus* (Figure 11 B). The difference is due to the different bacterial dry-to-wet mass ratio, 1 to 15 and 1 to 8, for *G. thermocatenulatus* and *G. stearothermophilus*, respectively (Figure 11). This result is unexpected as the data show a smaller surface area and thinner cell wall for *G. stearothermophilus*, hence indicating a smaller volume that may adsorb cations. Bacterial concentrations during titration were approximately $3.8 \pm 0.06 \times 10^8$ cells per ml for *G. thermocatenulatus*, and ranged from $4.8 \pm 0.06 \times 10^8$ to $5.3 \pm 0.07 \times 10^8$ cells per ml for *G. stearothermophilus*, representing a total bacterial dry mass of 1.59 ± 0.07 g. The bacterial surface areas calculated from biophysical parameters were 8.96 and 6.38 m^2 per g wet cells for *G. thermocatenulatus* and *G. stearothermophilus* cells, respectively. The values are much lower than values reported in other SCM studies (Yee *et al.*, 2004) where surface areas as high as 290 m^2 per g wet mass

for *B. subtilis* have been reported (Wightman *et al.*, 2001).

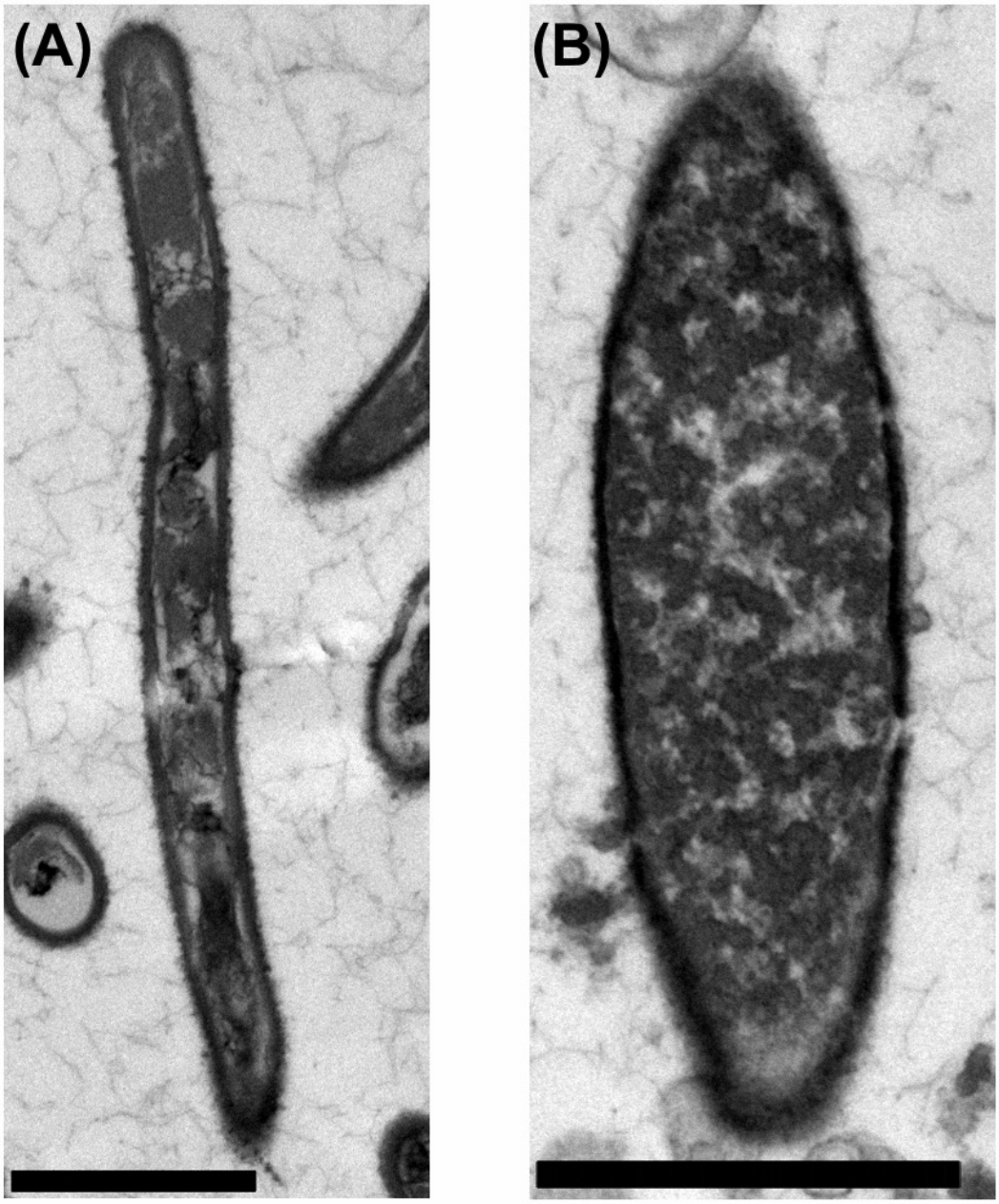

Figure 10. Transmission electron micrographs of *G. thermocatenulatus* (A) and *G. stearothermophilus* (B). Bars 1 µm.

To develop SCMs for the titration data, the electrophoretic mobility of the bacterial cells were taken into consideration. In theory, the net concentration of supplied protons can be quantified by

$$[H^+]_{net\ added} = [H^+]_{added} - [OH^-]_{added} + [H^+]_{adsorbed} - [H^+]_{desorbed} \quad (6)$$

where $[H^+]_{added}$ and $[OH^-]_{added}$ refer to the known concentrations of the added HNO_3 and the NaOH titrants, and where $[H^+]_{adsorbed}$ and $[H^+]_{desorbed}$ are the concentrations of protons consumed and released by the bacteria, respectively. The isoelectric point (IEP), when positive and negative charges of the chemical functional groups onto and within the microbial surface are in balance, can be described with the following relationship:

$$[H^+]_{adsorbed} = [H^+]_{desorbed} \quad (7)$$

The net concentration of added protons at the IEP results out of equations (6) and (7) and can be determined by

$$[H^+]_{net\ added} = [H^+]_{added} - [OH^-]_{added} \quad (8)$$

which as well represent the titration of a pure electrolyte solution. It is assumed that titration data obtained without and with cells at the corresponding IEP to be identical. Therefore, titration curves for both studied strains were normalized to intersect the titration curve of a pure electrolyte solution at the measured IEP at pH values of 3.25 for *G. thermocatenulatus* and 3.95 for *G. stearothermophilus* cells (Figure 12). IEP values commonly found for bacterial cells range from 2 to 4 depending on the species (Harden & Harris, 1953; van der Wal *et al.*, 1997). Although titrations at pH values below 4 or above 10 were avoided to minimize cell damage (Claessens *et al.*, 2004; Haas, 2004), the titration data were constrained by the electrophoretic mobility of the cells by iteration (Burnett *et al.*, 2006).

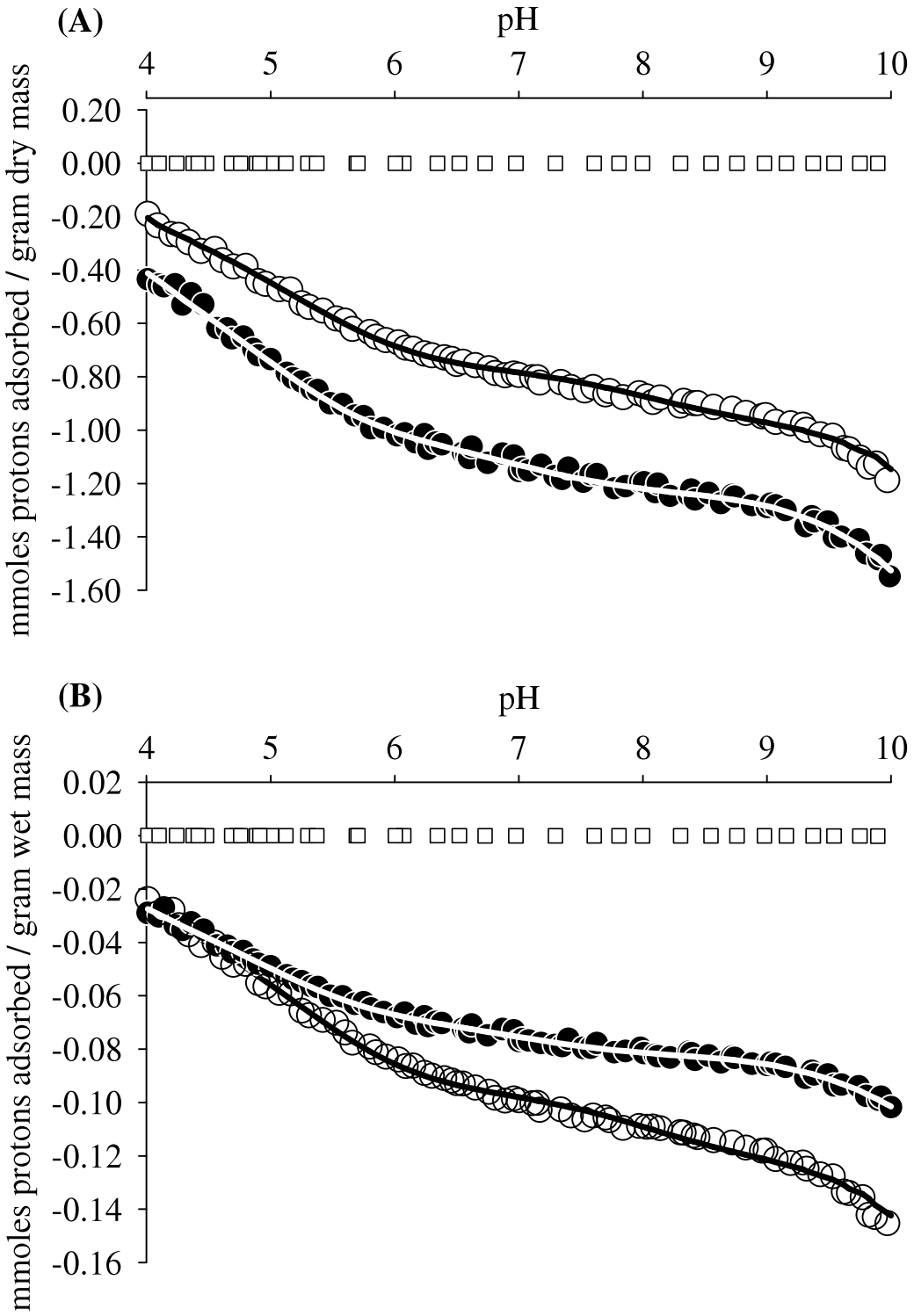

Figure 11. Acid-base titration data normalized to dry mass (A) and wet mass (B) obtained for *G. stearothermophilus* DSM 6790 (white circles) and *G. thermocatenulatus* DSM 730 (black circles) cells. Solid lines represent the best-fitting discrete multi-site model calculated by FITMOD. White squares represent titrated electrolyte solution without bacteria. For clarity, only every third data point is displayed.

A discrete multiple-site SCM can account for the buffering capacity of the bacterial species observed over the entire range studied between pH 4.0 and 10.0 (Figure 11). Best-fit approximations of the titration data resulted in a three-site model for *G. thermocatenulatus*, whereas only two individual binding sites were necessary to fully interpret the titration data of *G. stearothermophilus*. Besides the excellent visual agreement of measured experimental data and calculated SCM, the quality of fit was quantified using the overall variance $V(Y)$, which is the mean of weighted sum of squares of errors Y in the stoichiometric equation. The program FITMOD computes the variance as an output parameter. Low $V(Y)$ numbers indicate a better model fit to the data. For adsorption data, values of $V(Y)$ between 1 and 20 indicate a reasonable fit (Westall, 1982). The $V(Y)$ values for each SCM and corresponding pK values (negative log equilibrium constants for deprotonation reactions) and concentration c of proton-reactive surface sites are compiled in Table 6. The low variance values indicate a good fit to the experimental data. Values of pK are similar for both bacterial strains. The most abundant proton binding site has a pK value of approximately 3.8 and accounts for 66% and 80% of the total concentration of binding sites for *G. thermocatenulatus* and *G. stearothermophilus*, respectively.

Despite the present knowledge of the cell wall composition and structure of Gram-positive bacteria (Scheffers & Pinho, 2005; Schleifer & Kandler, 1972), such as *Geobacillus* species, the clear identification of a proton-reactive binding site solely based on its pK value remains complicated. Stability constants of individual functional groups can vary significantly depending on various parameters such as temperature, ionic strength (Borrok & Fein, 2005) and relative position of the functional group in the biomolecule. Due to this interpretation problem, the pK values will not be assigned to possible functional groups and the detected reactive binding sites will be referred as sites 1, 2, and 3. The best-fitting SCMs included deprotonation reactions onto the bacterial surface may be characterized for site 1 and 2 by

$$\text{R-LH} \Leftrightarrow \text{R-L}^- + \text{H}^+ \qquad (9)$$

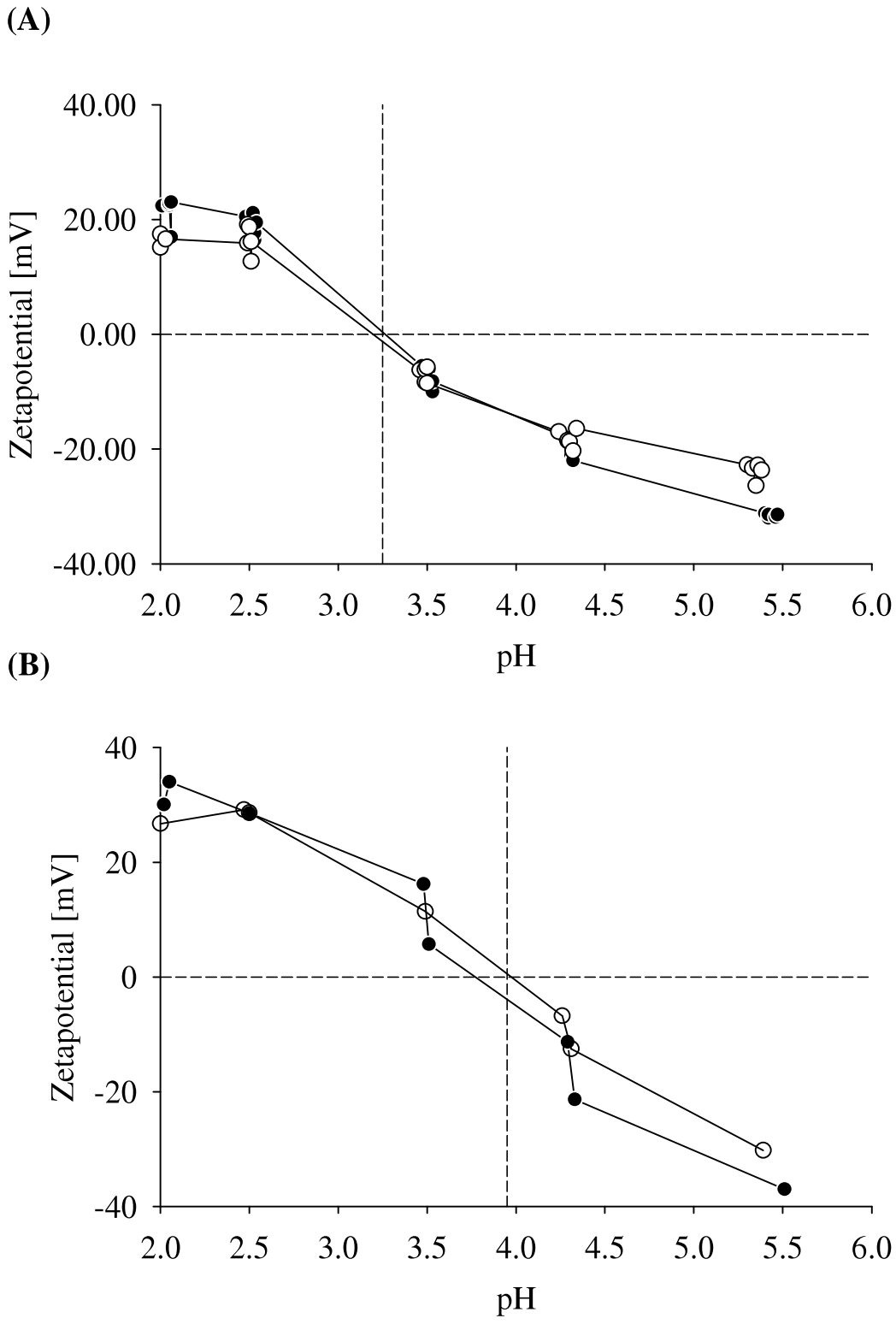

Figure 12. Zetapotentials obtained for *G. thermocatenulatus* (A) and *G. stearothermophilus* cells (B) at ionic strengths of 1 mM (black circles) and 10 mM $NaNO_3$ (white circles).

and for site 3

$$R\text{-}LH^{+} \Leftrightarrow R\text{-}L + H^{+} \quad (10)$$

where L represents the functional group acting as a ligand. These formulations are consistent with the electrophoretic mobility data, which indicate that both bacteria maintain a positive cell surface charge at pH values below their IEP and negative surface charge at pH above their IEP. Although site 3 is required for both *Geobacillus* strains to explain the positive charge under acidic conditions, it does not play an active role in proton adsorption for *G. stearothermophilus* under the conditions covered in the experiments.

The cell wall thickness, the bacterial surface area and the concentration of the dominant site 1 indicate a density of 232.6 and 322.0 sites per nm^3 for *G. thermocatenulatus* and *G. stearothermophilus*, respectively. Theoretically, an ionic radius for cadmium of 0.1 nm results in a maximum density of 238.7 Cd^{2+} per nm^3 that may be complexed by functional groups in or on the bacterial cell wall. Therefore, it is assumed that all reactive groups of site 1 that are accessible by protons are also accessible by the larger cadmium ion.

3.4.2 Cadmium ion biosorption

Cadmium ion adsorption experiments performed as a function of pH and biomass revealed that both parameters strongly influenced the adsorption onto the microbial surfaces (Figure 14). Experiments were performed in 10 mM $NaNO_3$ in the presence of 5 ppm $CdCl_2.2.5H_2O$ (44.5 µM) with bacteria-to-metal ion concentration ratios of 1:1, 1:2, and 1:4 resulting in total bacterial dry mass of 1.15 ± 0.20 g, 0.57 ± 0.09 g, and 0.30 ± 0.05 g, respectively. At a given pH, an increasing concentration of organic mass resulted in an increase in Cd^{2+} adsorption. *G. thermocatenulatus* cells (1.31 g dry mass) were able to adsorb up to 95% Cd^{2+} (Figure 14 A), whereas *G. stearothermophilus* cells (1.32 g dry mass) absorbed up to 85% (Figure 14 B). At a given biomass concentration, increasing pH results in increasing Cd^{2+} adsorption. Depending on the biomass concentration, most adsorption occurred in the pH range of 3.5 to 5.5,

demonstrating the importance of site 1 (pK = 3.8). This pH dependence is due to the deprotonation of cell wall functional groups that occurs with increasing pH, progressively resulting in increasing Cd^{2+} biosorption until saturation of binding sites occurs. Negative control experiments (no bacterial cells) indicated that there was a significant loss of Cd^{2+} at pH values above 8 within 2 hours (Figure 13). Thermodynamic calculations using the computer program MINTEQA2 (Allison *et al.*, 1991) suggest that for a solution containing 5 ppm Cd^{2+} in equilibrium with the atmosphere, otavite ($CdCO_3$) should precipitate at pH values above 7.

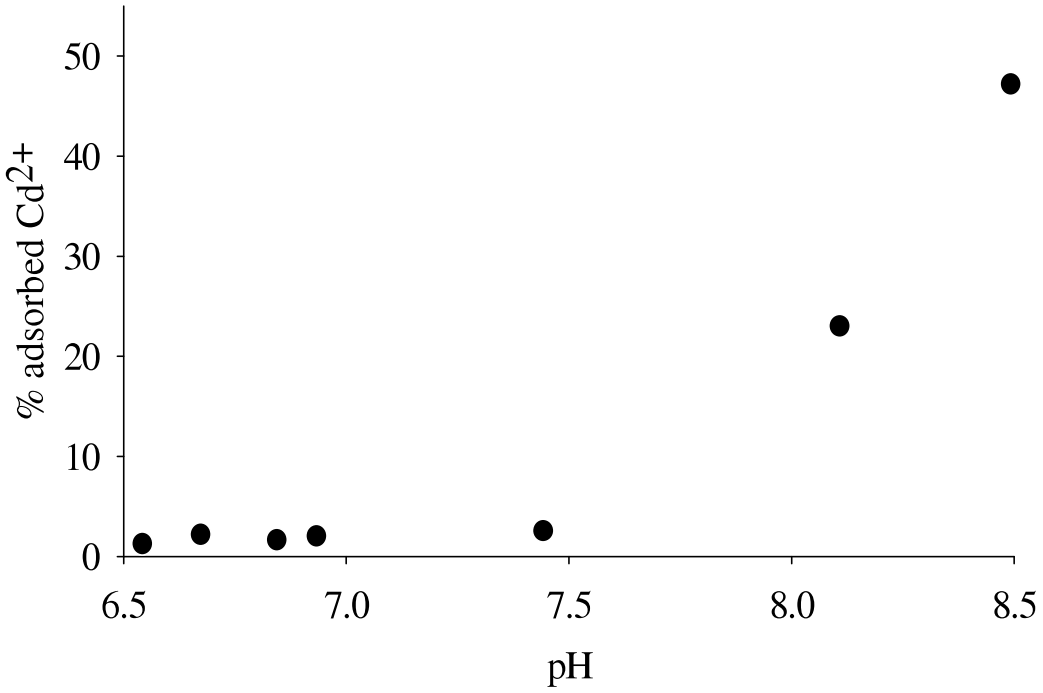

Figure 13. Cadmium ion adsorption experiment using 10 mM $NaNO_3$ solution without bacterial cells. Thermodynamic calculations indicate that Cd^{2+} precipitates at higher pH values.

The computer code FITMOD, incorporating the Donnan electrostatic model, was used to develop SCMs to describe the Cd^{2+} adsorption data. The SCMs used the concentrations and deprotonation constants of cell wall surface functional groups obtained from the potentiometric titration experiments (Table 6). SCMs did not consider data obtained at pH values above 8, due to the possibility of biasing caused by loss of Cd^{2+} by precipitation. For both bacteria, three individual

cultures each with three different biomass-metal ion ratios were investigated resulting in a total of nine data subsets per bacterium.

Initially, SCMs with unique thermodynamic stability constants *K* for the metal complexes were calculated for each individual subset (Appendix B: Table 15, Table 16, Table 17, and Table 18) A discrete one-site SCM fitted all data subsets. Additionally, for the two datasets displaying the lowest biomass-metal ion ratio for *G. stearothermophilus* (0.26 and 0.34 g dry mass), a two-site SCM could be applied (Appendix B: Table 18). The multi-site model for both datasets just slightly improves the fit in terms of lower *V*(*Y*) value. However, it indicates that at lower cell concentrations site 1 may be saturated, hence Cd^{2+} complexation reaction significantly occurs at site 2 (Daughney & Fein, 1998).

Subsequently, a single one-site SCM was developed to fit all of the data for each bacterium. These one-site SCMs account for variation in pH and metal ion-to-bacteria concentration ratio. Good agreement with the analytical data was obtained, yielding an overall *V*(*Y*) value of 1.8 and 5.9 for *G. thermocatenulatus* and *G. stearothermophilus*, respectively (Table 6). The higher log *K* value of 2.20 ± 0.16 obtained for *G. stearothermophilus* indicates a stronger interaction between Cd^{2+} and site 1, compared to *G. thermocatenulatus* (log *K* = 1.30 ± 0.22). The fit of the single best-fitting SCM to the experimental data is displayed in Figure 14.

3.5 Discussion

This study uses a combined approach of metal ion susceptibility tests and complexation modeling analysis. Initial cadmium ion toxicity experiments revealed a high MIC value of Cd^{2+} for *G. stearothermophilus* (DSM 6790) and *G. thermocatenulatus* (DSM 730) in comparison to 44 other studied thermophilic bacteria.

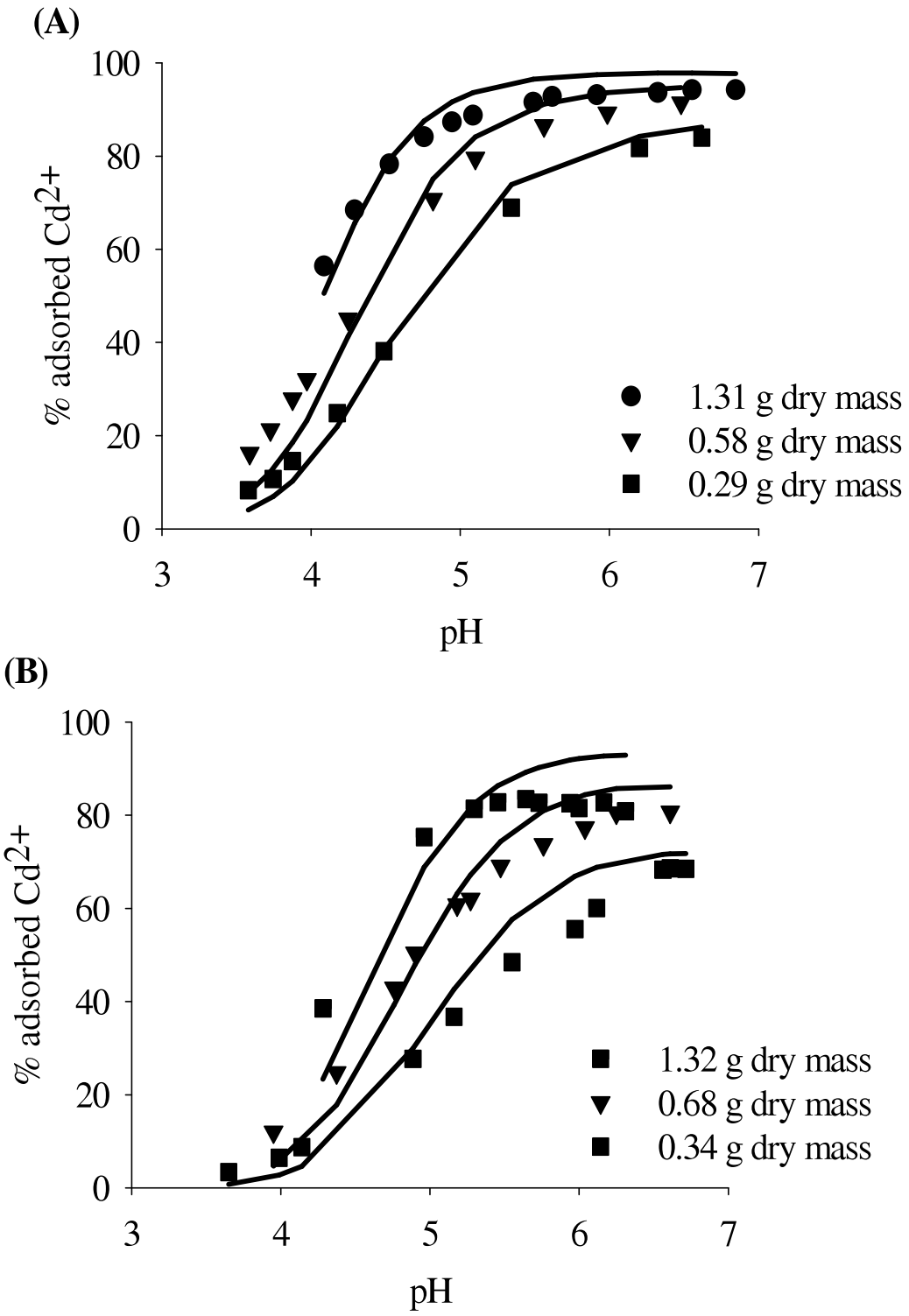

Figure 14. Percentage of cadmium ions adsorbed onto *G. thermocatenulatus* (A) and *G. stearothermophilus* (B) as a function of pH and dry biomass (black circles: 1.3 g, white triangles: 0.6 g, black squares: 0.3 g). Solid lines represent the best-fitting discrete one-site model developed by FITMOD. For clarity, for each species only data from one of three individual cultures are shown.

Calculations of SCMs for both microorganisms were performed using the Donnan model. In theory, the advantage of the Donnan model over other electrostatic models is that it considers the cell wall as a volume that is ion-penetrable, whereas other electrostatic models (e.g. Constant capacitance and Stern models) only account for ion-impenetrable planar surfaces (Yee *et al.*, 2004). Although the Donnan model assumes the surface binding sites to be uniformly distributed over the cell wall volume, some previous research on *Bacilli* suggests that binding sites may be concentrated on the cell poles (Sonnenfeld *et al.*, 1985a; Sonnenfeld *et al.*, 1985b). It is acknowledged that additional research is warranted to better define the most appropriate format for SCMs for bacterial surfaces, in particular with regard to the modeling of the effects of cell wall electric charge. However, in the absence of contradictory data, the Donnan model is employed under the assumption of uniform site distribution to quantify electrostatic interactions between aqueous metal ions and chemically functional groups within and on the cell walls.

Some of the model parameters are similar to previously published results (Table 6), although the diversity of biosorbants and modeling approaches described in the literature somewhat limit the comparisons that can be made. The deprotonation constant p*K* values determined for site 2 and site 3 for both *Geobacillus* strains are in good agreement with recent results reported for the thermophilic species TOR-39 (Wightman *et al.*, 2001) and *Anoxybacillus flavithermus* (Burnett *et al.*, 2006), the mesophilic bacterium *Bacillus subtilis* (Daughney *et al.*, 2001) and five natural bacterial consortia (Borrok *et al.*, 2004a). The p*K* values of site 1 for *G. stearothermophilus* and *G. thermocatenulatus* are considerably lower than those reported for the other bacteria, and may indicate that a different type of functional group is involved in complexation reactions for species of the genus *Geobacillus*.

Interestingly, the average of the p*K* values of site 1 and site 2 of the four-site model for the bacterial consortia (Borrok *et al.*, 2004a) is almost equivalent to the deprotonation constant for site 1 for the *Geobacillus* strains. Yee and Fein (Yee & Fein, 2001) proposed that metal-bacteria adsorption is independent of the bacterial species involved and therefore a generalized adsorption model may be applied. It is acknowledged that the differences between the SCMs developed in this

investigation and the generalized model of Yee and Fein (Yee & Fein, 2001) may relate to the different experimental methods or modeling approaches employed, but it is also possible that the generalized model is simply inappropriate for the two studied *Geobacillus* strains and therefore not universally applicable.

Although in all of the above-mentioned previous investigations site 1 is proportionally the most abundant binding site, only for the thermophiles *A. flavithermus*, *G. thermocatenulatus*, and *G. stearothermophilus* does it account for more than 62% of the total concentration of binding sites. The significantly lower proportions of site 1 observed for the other bacteria (below 44%) may indicate that *A. flavithermus*, *G. thermocatenulatus*, and *G. stearothermophilus* as thermophiles differ in cell wall composition from the other mesophilic bacteria and therefore expose different chemically reactive functional groups on and within their cell walls.

As mentioned above, site 1 (pK = 3.8) was not assigned to a particular identity or functional group structure. Many studies assume binding sites with low pK values to be carboxylic because short carboxylic acids have pK values below 4.5 and carboxylic acids are present in the cell wall. However, it must be borne in mind that functional groups can only be reliably identified by other methods such as X-ray spectroscopy analysis. X-ray adsorption spectroscopy experiments conducted by Boyanov and colleagues (2003) suggest that Cd^{2+} adsorption onto *Bacillus subtilis* at a pH value of 3.4 is predominated by phosphono ligands, whereas carboxylic ligands are the dominant binding sites in the range of pH 5.0 to 7.8.

The thermodynamic stability constants K for the cadmium ion-binding are not as high for the *Geobacillus* strains as for the bacteria described in the literature (Borrok *et al.*, 2004a; Burnett *et al.*, 2006; Daughney *et al.*, 2001; Wightman *et al.*, 2001; Yee & Fein, 2001). It is acknowledged that differences may be due to different modeling approaches employed. Alternatively, this may indicate a weaker interaction between the cadmium ions and the reactive surface sites of the *Geobacillus* strains compared to other species investigated to date.

Table 6. Average model parameters for *G. thermocatenulatus* and *G. stearothermophilus* in comparison to other bacterial species

Species	Model	Parameter	Site 1	Site 2	Site 3	Site 4	$V(Y)$[d]
G. thermocatenulatus (this study)	Donnan	pK[a]	3.84 ± 0.03	5.97 ± 0.08	8.70 ± 0.30		3.4
G. thermocatenulatus (this study)	Donnan	c[b]	7.03 ± 0.14	1.20 ± 0.10	2.50 ± 0.52		
G. thermocatenulatus (this study)	Donnan	log K[c]	1.30 ± 0.22				1.8
G. stearothermophilus (this study)	Donnan	pK[a]	3.73 ± 0.04	6.46 ± 0.07			4.9
G. stearothermophilus (this study)	Donnan	c[b]	9.74 ± 0.03	2.40 ± 0.04			
G. stearothermophilus (this study)	Donnan	log K[c]	2.20 ± 0.16				5.9
TOR-39 (Wightman *et al.*, 2001)	Constant capacitance	pK[a, f]	4.5 ± 0.2	5.8 ± 0.1	8.2 ± 0.1		0.9
TOR-39 (Wightman *et al.*, 2001)	Constant capacitance	c[b]	6 ± 2	5 ± 1	3 ± 1		
A. flavithermus (Burnett *et al.*, 2006)	Donnan	pK[a]	4.94 ± 0.35	6.85 ± 0.57	7.85 ± 0.27		15.5
A. flavithermus (Burnett *et al.*, 2006)	Donnan	c[b, e]	3.57 ± 0.58	1.20 ± 0.56	0.95 ± 0.20		
B. subtilis (Daughney *et al.*, 2001)	Constant capacitance	pK[a]	4.80 ± 0.1	6.49 ± 0.3	8.52 ± 0.6		8.5-13.5
B. subtilis (Daughney *et al.*, 2001)	Constant capacitance	c[b]	6.78 ± 3.9	4.35 ± 2.6	6.17 ± 2.2		
B. subtilis (Daughney *et al.*, 2001)	Constant capacitance	log K[c]	3.62 ± 0.5	4.11			11

Table 6. Continued.

Species	Model	Parameter	Site 1	Site 2	Site 3	Site 4	$V(Y)$[d]
Average model on nine individual bacterial species (Yee & Fein, 2001)	Constant capacitance	pK[a]	5.0 ± 0.2	7.2	9.7		N. A.[g]
Average model on nine individual bacterial species (Yee & Fein, 2001)	Constant capacitance	c[b, e]	20 ± 8	8	17		
Average model on nine individual bacterial species (Yee & Fein, 2001)	Constant capacitance	log K[c]	4.0 ± 0.3	N. A.	N. A.		3.3-34.6
Average model on five natural bacterial consortia (Borrok *et al.*, 2004a)	Non-electrostatic	pK[a]	3.12 ± 0.13	4.70 ± 0.11	6.57 ± 0.17	8.99 ± 0.21	N. A.
Average model on five natural bacterial consortia (Borrok *et al.*, 2004a)	Non-electrostatic	c[b]	6.65 ± 1.96	6.78 ± 2.65	3.68 ± 1.71	4.47 ± 2.19	
Average model on five natural bacterial consortia (Borrok *et al.*, 2004a)	Non-electrostatic	log K[c]	2.83 ± 0.30	2.70 ± 0.47	3.95 ± 0.22	5.22 ± 0.40	N. A.

[a] Negative logarithm of deprotonation constant for subscripted surface functional groups with 1σ errors.

[b] Concentration of subscripted surface sites with 1σ errors in $\times 10^{-5}$ mol/g of wet cells.

[c] Logarithm of stability constant for Cd^{2+} complexation reaction with 1σ errors.

[d] Variance computed by FITMOD. A lower value indicates a better model fit.

[e] Concentration of surface sites were published in mol/g of dry bacteria. The dry-to-wet mass ratio for *A. flavithermus* is 1 to 6.7 and for the nine bacterial species 1 to 9.9.

[f] Wightman *et al.* determinate deprotonation constant at 50° C.

[g] N. A., not applicable.

The variation between the SCM parameters of the two *Geobacillus* strains investigated here are too small to permit identification of any relationship between biosorption and growth responses to elevated concentrations of Cd^{2+}. *G. thermocatenulatus* and *G. stearothermophilus* may have evolved several metal ion detoxification strategies that account for the observed types of growth curves. The genome of *G. stearothermophilus* (DSM 13240) is being sequenced at the present time. A search of the genome data currently available shows evidence that *G. stearothermophilus* (DSM 13240) contains sequence information required for cadmium ion efflux, such as cadmium-transporting ATPases (http://www.genome.ou.edu/bstearo.html). When completely sequenced the genome may reveal even more cadmium ion detoxification systems.

This study demonstrates that the extent of cadmium ion adsorption by *G. thermocatenulatus* and *G. stearothermophilus* can be predicted for a range of pH values and metal ion-to-bacteria concentration ratios. SCMs can be applied to describe cation-bacteria interaction under a given set of conditions and may be valuable for development and optimization of bioremediation schemes. As for both studied bacterial strains Cd^{2+} is predominantly complexed at one binding site their use in bioremediation might be more cost-effective than organisms binding Cd^{2+} to two or more binding sites. The more binding sites involved, the more acid and base would be necessary to adsorb and to desorb the metal ions efficiently.

Investigations of microbial biosorption can deepen the insight of metal mobilization in the environment (such as contamination of drinking water) and can be used to improve waste treatment of metal-polluted water and soil. Further studies should evaluate the adsorption of other metals by the species studied here and by other thermophiles, for example to determine if systematic metal affinity series exist as have been observed for mesophiles (Borrok *et al.*, 2005; Yee & Fein, 2001).

3.6 Acknowledgements

Research funding was provided by the New Zealand Foundation of Research, Science & Technology (Contract Number C05X0303: Extremophilic

Microorganisms for Metal Sequestration from Aqueous Solutions). Special thanks are due to Hannah Heinrich for the TEM micrographs and the electrophoretic mobility measurements at the University of Otago, Dunedin, and Marshall Muller and Moya Appleby for their assistance at the Institute of Geological and Nuclear Sciences in Wairakei. The editor and the two anonymous reviewers offered comments and suggestions that greatly improved this manuscript.

Chapter 4: Champagne Pool

Microbial life in Champagne Pool, a geothermal spring in Waiotapu, New Zealand

(A condensed version of this chapter has been published in
Extremophiles, July 2007)

4.1 Abstract

Surveys of Champagne Pool, one of New Zealand's largest terrestrial hot springs and rich in arsenic ions and compounds, have been restricted to geological and geochemical descriptions, and a few microbiological studies applying culture-independent methods. In the current investigation, a combination of culture and culture-independent approaches were chosen to determine microbial density and diversity in Champagne Pool. Recovered total DNA and adenosine 5'-triphosphate (ATP) content of spring water revealed relatively low values compare to other geothermal springs within New Zealand and are in good agreement with low cell numbers of 5.6 ± 0.5 x10^6 cells per ml obtained for Champagne Pool water samples by 4',6-diamidino-2-phenylindole (DAPI) staining. Denaturing Gradient Gel Electrophoresis (DGGE) and 16S rRNA (small-subunit ribosomal nucleic acid) gene clone library analyses of environmental DNA indicated the abundance of *Sulfurihydrogenibium*, *Sulfolobus* and *Thermofilum*-like populations in Champagne Pool. From these results, media were selected to target the enrichment of hydrogen-oxidizing and sulfur-dependent microorganisms. Three isolates were successfully obtained having 16S rRNA gene sequences with similarities of approximately 98% to *Thermoanaerobacter tengcongensis*, 94% to *Sulfuri-hydrogenibium azorense*, and 99% to *Thermococcus waiotapuensis*, respectively.

4.2 Introduction

The Waiotapu geothermal region covers a surface area of around 18 km^2 in the

North Island of New Zealand. It is one of the largest areas of terrestrial thermal activity in New Zealand with a predicted surface heat flow of approximately 540 MW (Hedenquist, 1986b). The largest geothermal feature within the Waiotapu region is Champagne Pool. The hot spring is approximately 65 m in diameter (Figure 16 A), has an estimated volume of 50,000 m^3 (Hedenquist & Henley, 1985) and was formed over 900 years ago by hydrothermal eruption (Lloyd, 1959). Many terrestrial thermal springs often exhibit sulfur- and iron-rich systems with pH values of approximately 1.8 to 2.2 or carbonate-bicarbonate systems with pH values of around pH 7.5 to 9.0 (Brock, 1986), reflecting the capacity of the main buffers in natural geothermal habitat: sulfuric acid (pK=1.8), carbonate (pK=6.3), and bicarbonate (pK=10.2). However, Champagne Pool does not follow any of the described bimodal distribution schemas. The alkali chloride spring water (pH 5.5 at around 75° C) consists of high concentrations of silica and metalloid ion-sulfide complexes (Jones *et al.*, 2001) which form the white terrestrial silica sinter rim that rises 50 cm above the pool surface and the orange subaqueous sediments around the margin of Champagne Pool (Figure 16 B). Champagne Pool fluid is oversaturated with orpiment (As_2S_3), stibnite (Sb_2S_3) and carlinite (Tl_2S) leading to precipitation of those sulfide minerals (Pope *et al.*, 2005). The hot spring discharges about 10 liter per s of undiluted geothermal water and around 7 liter per s of vapor (Hedenquist & Henley, 1985) providing a mean residence time of the order of 34 days. The pool name derives from its effervescence due to the copious amounts of gas evolved, mainly 73.0% (v/v) CO_2, 16.2% (v/v) N_2, 6.4% (v/v) CH_4, 2.3% H_2, 1.7% (v/v) H_2S, and traces of O_2 (Jones *et al.*, 2001), which simultaneously serves to mix the water and maintain an even temperature of around 75° C by convection and pH values of approximately 5.5 by buffering due CO_2.

The Waiotapu geothermal region has been subject to many geological and geochemical investigations (Giggenbach *et al.*, 1994; Hedenquist & Henley, 1985; Hedenquist, 1986a; Lloyd, 1959; Pope *et al.*, 2005). Only a few studies have recently described microbial activity in Champagne Pool restricted to electron microscopy examinations (Jones *et al.*, 2001; Mountain *et al.*, 2003; Phoenix *et al.*, 2005) and PCR studies (Ellis *et al.*, 2005). Micrographs have shown filamentous, rod- and coccoid structures to be similar to morphologies of

Chloroflexus, *Thermothrix*, *Thermus*, *Thermoproteus*, *Pyrobaculum* or *Thermofilum* species (Jones *et al.*, 2001) and S-layer patterns to be similar to *Clostridium thermohydrosulfuricum* or *Desulfotomaculum nigrifacans* (Phoenix *et al.*, 2005). Ellis *et al.* (2005) identified a 16S rRNA gene sequence closely related to *Thermofilum pendens*. However, successful attempts to culture microorganisms from Champagne Pool have not been reported.

Previous studies showed that the standing biomass and microbial diversity in the pool is low (Ellis *et al.*, 2005; Jones *et al.*, 2001) with reasons suggested being: 1) Lack of substrate due to long residence time of spring water; 2) Toxic levels of arsenic or other pool components; 3) Silica deposition precludes bacterial growth due to nucleation. Niederberger (2005) reported a dissolved organic carbon concentration for Champagne Pool water of 700 mg per m^3, which was at least on a par with many other thermal pools, which contained greater biomass. Additionally, there would appear to be ample CO_2 and H_2 for methanogenesis and other reduced inorganics to support autotrophy**.** Champagne Pool is supersaturated in silica and evaporative cooling (or bacterial nucleation) enhances precipitation. Exposed glass slides build up a silica sinter at a rate of 0.023 mm per day to form mini-stromatolites in 2 to 3 months due to wave action. The sinter contains fossilized bacteria and has a complex chemistry (Mountain *et al.*, 2003). However, sinter does not form as readily in anaerobic zones and is unlikely to explain the low biomass throughout the pool.

In this investigation, culture-dependent and culture-independent approaches are applied to describe microbial density and diversity in Champagne Pool. Microbial density assessed by microscopy, DNA extraction and determination of the adenosine 5'-triphosphate (ATP) content of spring water and colonized slides revealed relatively low values compared to other hot springs in New Zealand (Niederberger, 2005). The culture-independent studies involved Denaturing Gradient Gel Electrophoresis (DGGE) analysis and construction of 16S rRNA (small-subunit ribosomal nucleic acid) gene clone libraries of environmental DNA obtained from Champagne Pool. On account of the results culture medium was selected and designed. Isolates phylogenetically related to strains of the genera *Sulfurihydrogenibium*, *Thermoanaerobacter* and *Thermococcus* were successfully

obtained.

4.3 Materials and Methods

4.3.1 Sampling and ATP measurements

Water and sediment samples were taken in 2004, 2005 and 2006 from the north-east side of the terrestrial hot spring Champagne Pool in Waiotapu, New Zealand (Figure 15, Figure 16 A). Samples were obtained from 10 cm, 30 cm, and 100 cm below water surface level (Figure 16 B) immediately transferred into sterile 2-liter glass flasks (Schott) and hermetically closed. By two hours at the latest, the samples were processed in the laboratory.

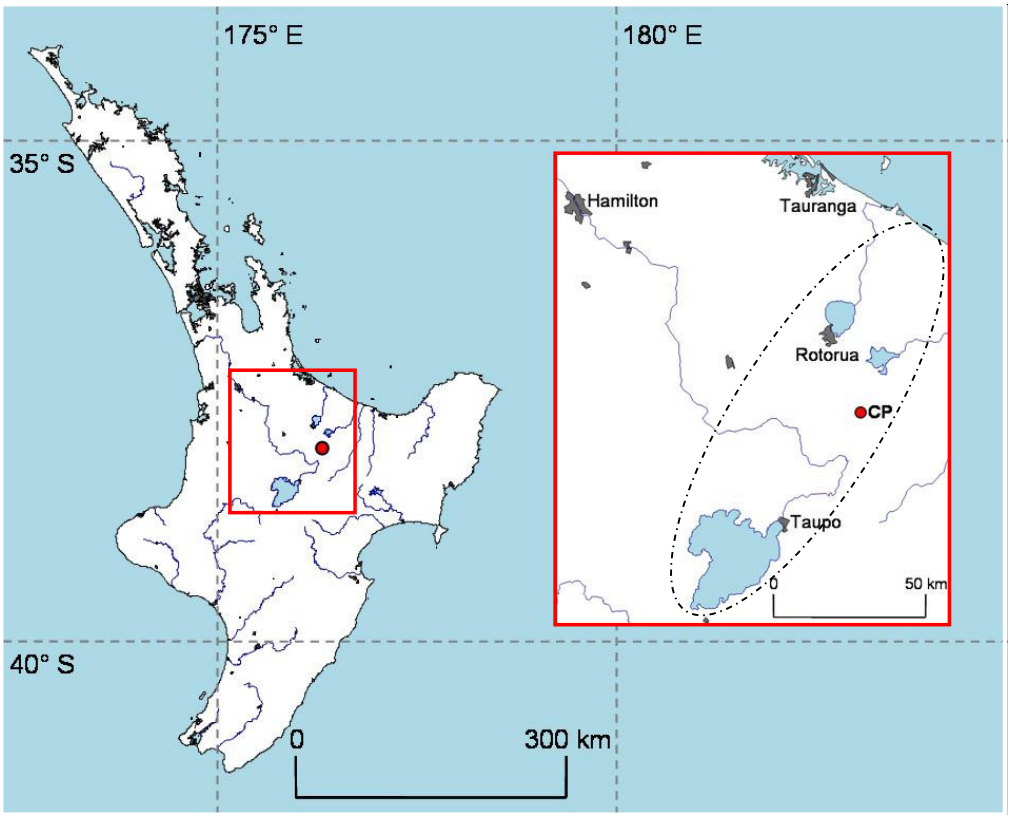

Figure 15. Map of New Zealand North Island and enlargement of the central part. Dotted lines within the enlargement outline the Taupo Volcanic Zone, which exhibit many geothermally active features such as the hot spring Champagne Pool (CP, red circle). Mapping was performed using the New Zealand Freshwater Fish Database Assistant software developed by I. G. Jowett (National Institute of Water & Atmospheric Research).

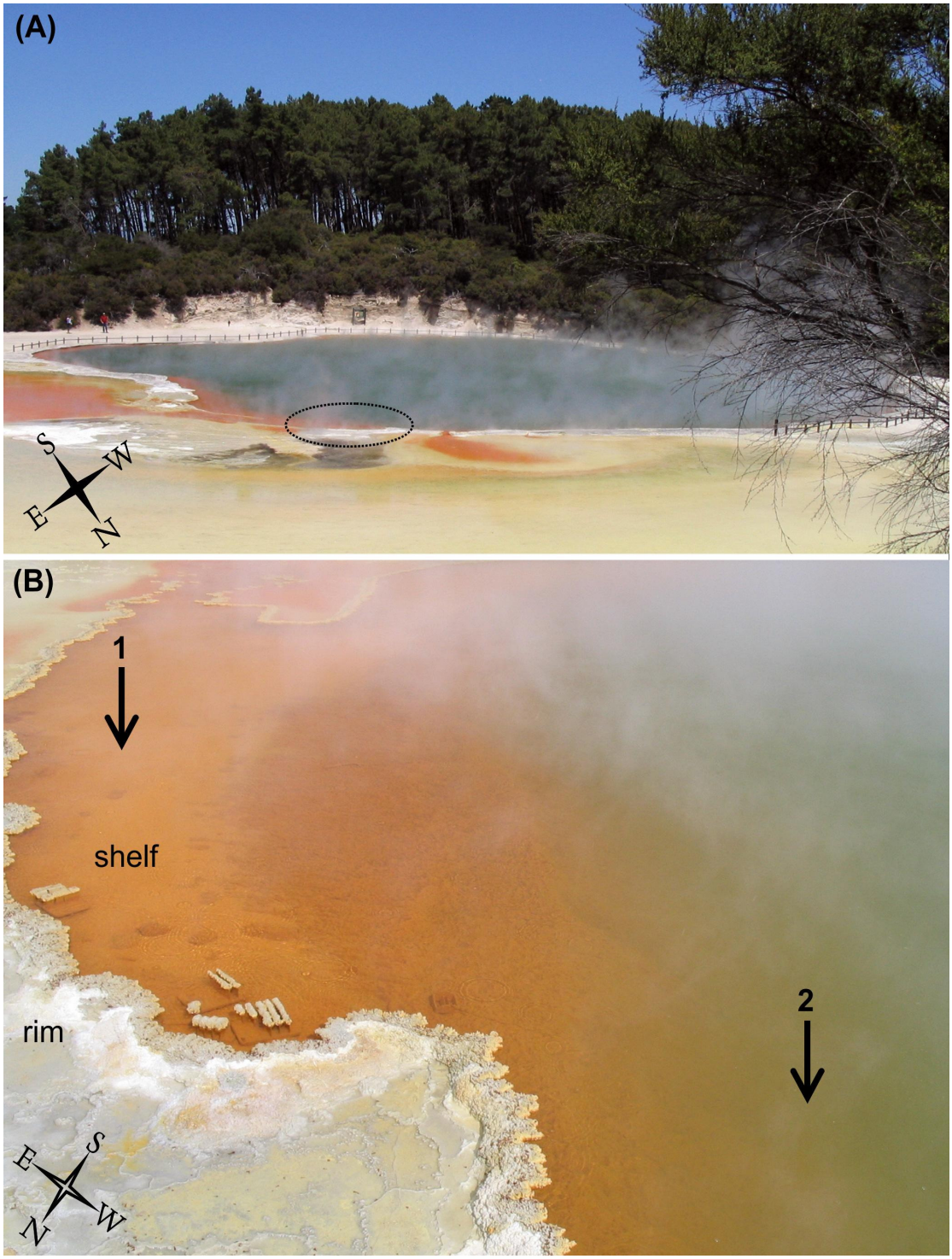

Figure 16. Champagne Pool hot spring surrounded by a grey-white rim of silica. (A) Sampling area is indicated by a dotted ellipse. (B) Sampling site 1 was located within the shallow water area (approximately 10 cm below surface water level) on the subaqueous shelf covered by orange-colored sediment. Site 2 was off the shelf within the deep-water area (maximum depth approximately 150 m) and samples were obtained from 30 to 100 cm below surface water level.

To estimate biomass in Champagne Pool the content of ATP was determined. Intracellular ATP is closely related to metabolic activity. Therefore, glass slides were immersed in Champagne Pool and incubated for several days. The glass slides provided a surface for potential microbial colonization (Niederberger, 2005). ATP measurements were carried out by wiping defined areas of the glass slides using a commercially available Clean Trace swab device (Biotrace). The swab system extracts ATP from microbial cells, and released ATP reacts with luciferin and luciferase reflected by light emission. Light production was measured using a portable ATP meter (Uni-Lite, Biotrace) and recorded as Relative Light Units (RLU). Fresh spring water samples were measured by dispensing a standard volume of sample directly onto a swab device and RLU were determined as described.

4.3.2 DNA extraction

Nucleic acids were extracted by enzymatic digestion (Marmur, 1961) and by a fast method based on physical disruption and cationic detergents modified from the protocol of Dempster *et al.* (1999). Approximately 500 ml spring water was filtered through a cellulose acetate filter (pore size 0.22 µm, diameter 47 mm). The filter was then placed in CTAB extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) cetyltrimethylammoniumbromide (CTAB), 1% (w/v) polyvinylpyrrolidone (PVP) (360,000, pH 8.0) and freshly made 0.4% (w/v) 2-mercaptoethanol and incubated at 100° C for 20 min. Next, nucleic acids were extracted with an equal volume of phenol, followed by extraction with chloroform-isoamyl alcohol (24:1 (v/v)). To the aqueous phase, an equal volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.5) were added. After overnight incubation at -20° C, the solution was centrifuged. The pellets were then washed twice in 70% ethanol, air-dried and resuspended in TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA).

4.3.3 PCR amplification of 16S rRNA gene and gene cloning experiments

Microbial 16S rRNA genes were amplified in PCR mixtures consisting of PCR buffer (10 mM Tris-HCI, 50 mM KCI; pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTP

(dATP, dCTP, dGTP, and dTTP), 1.0 µM of each primer, 5.0% (w/v) dimethyl sulfoxide (DMSO) and 1.0 unit of *Taq* polymerase (Roche). For DGGE-PCR reaction buffer (10 mM Tris-HCI, 50 mM KCI; pH 8.3), 1.25 mM $MgCl_2$, 0.2 mM dNTP (dATP, dCTP, dGTP, and dTTP), 0.2 µM of each primer, and 1.25 units of *Taq* polymerase (Roche) were used. Sequences of PCR primers are shown in Table 7. For amplification 20 to 100 ng of DNA template was used. The *Taq* polymerase was added to the reaction mixtures after the initial denaturation step to minimize the possibility of nonspecific DNA amplification. PCR conditions using archaeal and bacterial primers were an initial denaturation step at 94° C for 210 s, 33 cycles annealing for 30 s, extension at 72° C for 120 s and denaturation at 94° C for 30 s, followed by a final extension step for 360 s. For DGGE-PCRs the initial denaturation was extended to 300 s, annealing and extension steps were applied for 60 s and the total cycle number was 36. The annealing temperatures for each primer set are enlisted in Table 7. Archaea-specific primers often lack specificity for Nanoarchaea. Therefore an additional primer set was used which was specific for Nanoarchaea. PCRs amplifying the nanoarchaeal 16S rRNA gene (primer 9bF and 511mcR) were performed according to Huber *et al.* (2002b).

Amplified 16 rRNA genes were purified by agarose gel electrophoresis and used in cloning experiments with pGEM-T Easy vector (Promega). After electrophoresis of PCR products in 1% agarose gel and TAE buffer, the gel was stained in ethidium bromide (0.5 mg per liter) for 10 min and observed under UV light with a wavelength of 302 nm. A slice of the agarose gel containing the DNA fragment of interest was excised and transferred into a sterile tube. An equal volume of phenol was added. Then the mix was snap-frozen in liquid N_2 and centrifuged at 12000×*g* (Eppendorf Centrifuge 5415D) for 15 min. DNA from the aqueous phase was extracted with an equal volume chloroform-isoamyl alcohol (24:1 (v/v)). To the aqueous phase 2.5 volume of 100% ethanol and 0.1 volume of 4 M LiCl were added. After overnight incubation at -20° C, the solution was centrifuged at 12000×*g* for 5 min. The pellets were then washed twice in 70% ethanol, air-dried and resuspended in TE buffer.

The appropriate amount of purified PCR product (insert) to be added in the ligation reactions was calculated by

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert - to - vector ratio} = \text{ng of insert} \qquad (11)$$

in which assuming an equal insert-to-vector ratio and a PCR product size of the 16S rRNA gene of 1.5 kb the following result was obtained

$$\frac{50 \text{ ng vector} \times 1.5 \text{ kb insert}}{3.0 \text{ kb vector}} \times \frac{1}{1} = 25 \text{ ng insert} \qquad (12)$$

Ligation reactions consisting of rapid ligation buffer (60 mM Tris-HCl, 20 mM $MgCl_2.6H_2O$, 20 mM DTT, 2 mM ATP; pH 7.8), 10% polyethylene glycol, 50 ng of pGEM-T easy vector, 25 ng of purified PCR product, and 3 Weiss units of T4 DNA ligase were incubated overnight at 4° C.

For transformation, competent cells were prepared by growing *E. coli* JM 109 cells in 1 liter LB medium (10 g of trypticase peptone, 5 g of yeast extract, 5 g of NaCl; pH 7.5) at 37° C and 200 rpm until optical density at 600 nm reached around 0.600. Cells were harvested by centrifugation at 4000×*g* and 4° C (Sorvall RC 26 Plus) for 15 min. Pellets were washed in 1000 ml, 500 ml, and then 100 ml ice-cold, sterile 10% glycerol. The final cell pellet was resuspended in 10% glycerol to a final concentration of approximately $1x10^{10}$ cells per ml. Aliquots of 400 µl were frozen in liquid N_2 and stored at -70° C until further use.

The insert was transformed into competent cells by electroporation. A 2.0 µl aliquot of the overnight ligation reaction containing the vector was gently mixed with 80 µl thawed competent cell suspension and transferred to a chilled 0.1 cm gap Gene Pulser cuvette (Bio-Rad) on ice. Electroporation settings were 200 Ω (Ohm) resistance, 25 µFd (Faraday) capacitance, and 1.8 kV voltage using a Gene Pulser (Bio-Rad). Immediately following transformation, cells were transferred to 920 µl 37° C-prewarmed SOC medium (containing per liter: 20.0 g of trypticase, 5.0 g of yeast extract, 10 mM NaCl, 10 mM KCl, 10 mM $MgCl_2.6H_2O$, 10 mM $MgSO_4.7H_2O$, 20 mM glucose; pH 7.0). After incubation at 37° C and 150 rpm for 2 h cell suspension was harvested by centrifugation at 800×*g* (Eppendorf Centrifuge 5415D) for 2.5 min and cell pellets were resuspended in 200 µl SOC

medium. 100 µl aliquots of the transformed cells were then spread onto LB medium (which has been solidified by using 8 g of agarose per liter) supplemented with 100 mg of sodium ampicillin, 120 mg of IPTG, and 80 mg of X-gal per liter, respectively.

E. coli cells containing the plasmid and the insert were selected by blue-white screening of bacterial colonies. The vector used contains a *lacZ* gene with an internal multi-cloning site (Figure 17). The *lacZ* gene codes for the subunit of the enzyme β-galactosidase and gene expression is induced by IPTG. The enzyme β-galactosidase catalyzes the conversion of X-gal to a hardly soluble indigo dye. Cells containing a plasmid without an insert form blue-colored colonies due the activity of the β-galactosidase. In contrast, a successful ligation of a DNA fragment (PCR product) into the multi-cloning site inactivates the enzyme β-galactosidase due to the location of the insert within the *lacZ* gene (Figure 17). Colorless bacterial colonies indicate disrupted enzyme activity. However, cells without a plasmid might also form colorless colonies. To exclude this option, the vector used contains furthermore an *amp(R)* gene conferring ampicillin resistance (Figure 17). Ampicillin, a beta-lactam antibiotic, inhibits bacterial cell wall synthesis causing cell lysis. Only *E. coli* cells containing the plasmid are able to grow in the presence of ampicillin.

Plasmids were isolated as previously described in Chapter 2 (2.2.3 Small-scale plasmid isolation) and then categorized by restriction fragment length polymorphism (RFLP). The enzymatic digestions of the plasmids with *Eco*R I (cut site: G↕AATTCC), *Rsa* I (cut site: GT↕AC), and *Bam*H I (cut site: G↕GATCC) (Roche) followed electrophoresis in 3% agarose gel and TBE buffer (89 mM Tris borate and 2 mM EDTA, pH 8.3). Restriction reactions contained SuRE/ cut buffer A (Roche; 33 mM Tris acetate, 10 mM Mg-acetate, 66 mM K-acetate, 0.5 mM DDT; pH 7.9 at 37° C), 1% BSA, and 10 units of all three restriction enzymes used were incubated overnight at 37° C following 20 min at 80° C. Representatives of each distinct restriction enzyme fingerprint were selected for further sequence analyses.

Table 7. Target, position, specificity, sequences, and annealing temperature of oligonucleotide primers used for PCR studies.

Target	Position[a]	Oligonucleotide primer sequence (5' → 3')	T [° C]
Bacterial 16S rRNA gene	27F	AGAGTTTGATCCTGGCTCAG	50[d]
(Johnson, 1994)	1522R	AAGGAGGTGATCCA(A/G)CCGCA	
Bacterial 16S rRNA gene (DGGE)	338F	**GC**[b]**-**CTCCTACGGGAGGCAGCAG	65[e]
(Lane, 1991)	519R	ATTACCGCGGCTGCTGG	
Archaeal 16S rRNA gene	347F	CCAGGCCCTACGGGGCGCA	60
(Niederberger *et al.*, 2006)	1335R	GTGTGCAAGGAGCAGGGAC	
Archaeal 16S rRNA gene (DGGE)	915F	**GC**[c]**-**AGGAATTGGCGGGGGAGCAC	60
(Niederberger *et al.*, 2006)	1335R	TGTGCAAGGAGCAGGGACG	
Nanoarchaeal 16S rRNA gene	9F	G(A/G)GTTTGATCCTGGCTCAG	65[e]
(Huber *et al.*, 2002b)	511R	CTTGCCCACCGCTT	

[a] Corresponding 16S rRNA gene sequence position in *Escherichia coli*.

[b] GC-clamp: **CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCC**.

[c] GC-clamp: **CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC** (Ferris *et al.*, 1996).

[d] Annealing temperature was 50° C for two cycles and 48° C for 31 cycles.

[e] Annealing temperature was decreased from 65° C to 54° C in intervals of 0.5° C per cycle.

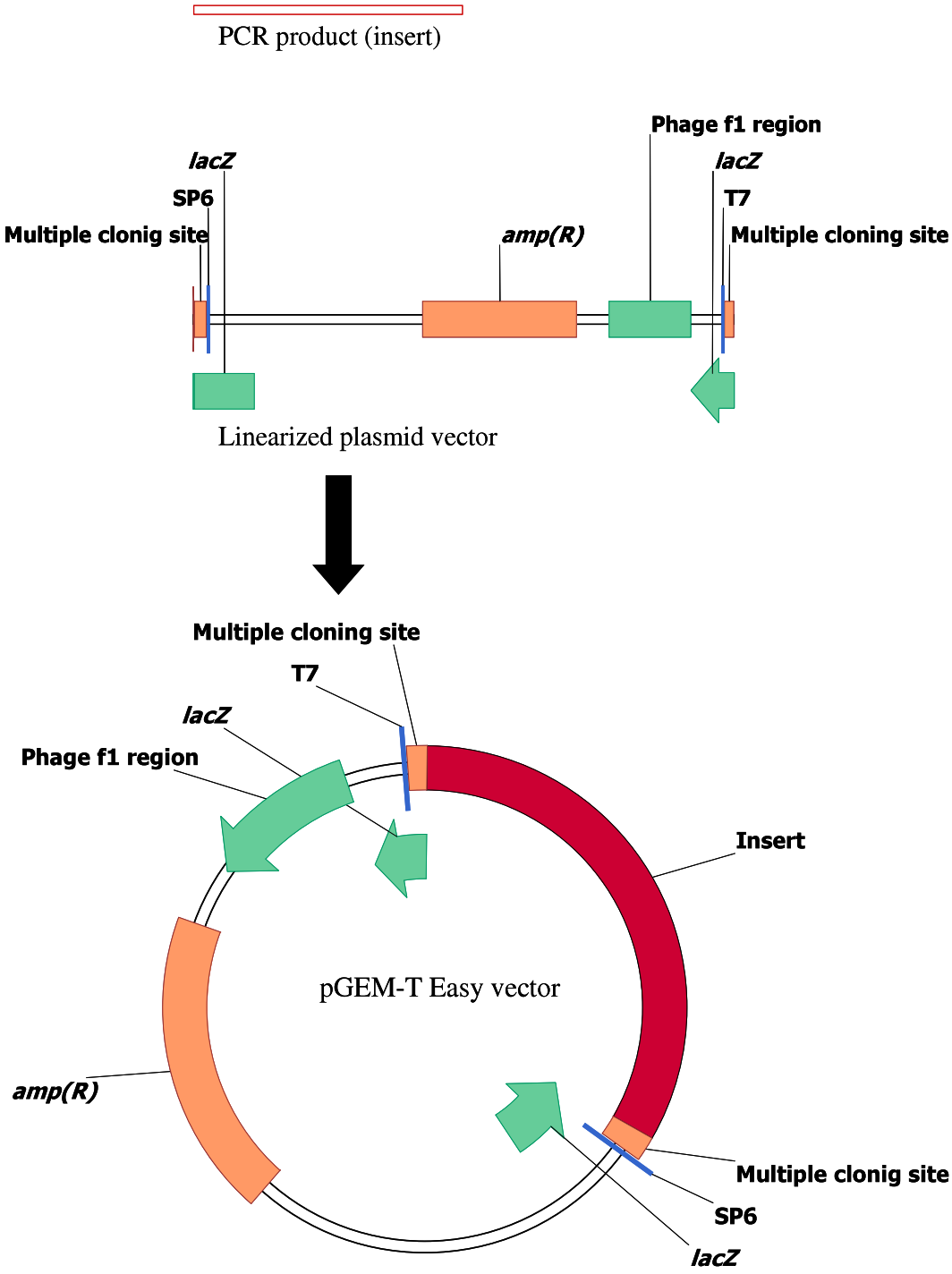

Figure 17. Scheme of ligation of a PCR product into a vector.

DNA sequencing of clone inserts were carried out by the Waikato DNA Sequencing Facility at the University of Waikato, Hamilton, New Zealand based on MegaBACE DNA analysis system (Amersham Biosciences). In sequencing

reactions the oligonucleotide primers M13pUC forward (5'- CCC AGT CAC GAC GTT GTA AAA CG -3'), M13pUC reverse (5'- AGC GGA TAA CAA TTT CAC ACA GG -3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG -3') were used.

4.3.4 DGGE analysis of 16S rRNA gene fragments

DGGE profiles were obtained by separation of the DGGE-PCR products obtained from environmental DNA in polyacrylamide gels with denaturing gradients (Muyzer *et al.*, 1993) ranging from 40% to 80% and 45% to 65% for bacterial and archaeal samples, respectively (100% denaturant is defined as 7 M urea and 40% (v/v) deionized formamide). Electrophoresis was conducted in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) at constant voltage of 140 V for 5 hours using a DCode system (Bio-Rad). After electrophoresis, gels were stained with ethidium bromide (0.5 mg per liter) for 10 min, washed in distilled water for 5 min and then photographed (AlphaImager System, AlphaInnotech) under UV light with a wavelength of 302 nm. DNA fragments were excised from polyacrylamide gels, reamplified in DGGE-PCRs, and verified by a second DGGE analysis to ensure purity. Representative DNA fragments were sequenced.

4.3.5 Enrichment experiments

Based on the results of DGGE analysis and gene cloning experiments media for hydrogen-oxidizing (modified MSH medium), sulfur-oxidizing (*Sulfolobus* medium) and sulfur-reducing (V24N medium) microorganisms were used in enrichment experiments. Medium V24N (Stetter, 1986) and *Sulfolobus* medium (Brock *et al.*, 1972) were prepared as previously described, except that the final pH was adjusted to a value of 5.5.

The V24N medium contained per liter: 1.30 g of $(NH_4)_2SO_4$, 0.28 g of K_2HPO_4, 0.25 g of $MgSO_4.7H_2O$, 0.07 g of $CaCl_2.2H_2O$, 0.02 g of $FeCl_3.6H_2O$, 1.80 mg of $MnCl_2.4H_2O$, 4.50 mg of $Na_2B_4O_7.10H_2O$, 0.22 mg of $ZnSO_4.7H_2O$, 0.05 mg of $CuCl.2H_2O$, 0.03 mg of $Na_2MoO_4.2H_2O$, 0.03 mg of $VOSO_4.2H_2O$, 0.01 mg of

$CoSO_4$, 1.00 g of powdered sulfur, 0.20 g of yeast extract, 2.0 g of starch, and 0.2 mg of resazurin. Medium was prepared and distributed under 100% N_2 atmosphere and sterilized by heating 2 h at 90° C on three consecutive days.

Sulfolobus medium for lithotrophic growth consisted of the V24N medium without the starch and only 0.10 g of yeast extract per liter. The medium was prepared anaerobically. After autoclaving the initial CO_2 gas phase was replaced with 80% H_2/ 20% CO_2. *Sulfolobus* medium composition for heterotrophic growth was the V24N medium without sulfur, starch, resazurin, and with 1.00 g of yeast extract per liter. Medium was autoclaved and cultivation was carried out under aerobic conditions.

MSH medium (Aguiar *et al.*, 2004) was modified and contained the following components (per liter of anaerobic water): 0.15 g of NaOH, 0.50 g of KCl, 1.36 g of $MgCl_2.6H_2O$, 7.00 g of $MgSO_4.7H_2O$, 2.00 g of $Na_2S_2O_3.5H_2O$, 0.40 g of $CaCl_2.2H_2O$, 0.20 g of NH_4Cl, 0.25 g of K_2HPO_4, 1.95 g of MES and trace minerals containing final concentrations per liter medium: 5.00 mg of Na-EDTA.$2H_2O$, 1.50 mg of $CoCl_2.6H_2O$, 1.00 mg of $MnCl_2.4H_2O$, 1.00 mg of $FeSO_4.7H_2O$, 1.00 mg of $ZnCl_2$, 0.40 mg of $AlCl_3.6H_2O$, 0.30 mg of $Na_2WO_4.2H_2O$, 0.20 mg of CuCl, 0.20 mg of $Ni_2SO_4.6H_2O$, 0.10 mg of $NaSeO_3$, 0.10 mg of H_3BO, 0.10 mg of $Na_2MoO_4.2H_2O$. The pH value of the medium was adjusted to 5.5. Aliquots of 40 ml and 8 ml were autoclaved under CO_2 atmosphere in 160 ml serum bottles and 30 ml test tubes, respectively. After inoculation using 5% inocula, the initial gas phase was exchanged with 79% H_2/ 16% CO_2/ 5% O_2 at 170 kPa.

In addition, growth medium to enrich carbon monoxide-utilizing microorganisms (Svetlichny *et al.*, 1991) was made (per liter): 0.33 g of KCl, 0.50 g of $MgCl_2.6H_2O$, 0.33 g of $CaCl_2.2H_2O$, 0.33 g of NH_4Cl, 0.33 g of KH_2PO_4, 1.95 g of MES, 0.05 g of yeast extract, 0.5 mg of resazurin, and trace minerals containing final concentrations per liter medium: 15.00 mg of nitrilotriacetic acid, 30.00 mg of $MgSO_4.7H_2O$, 6.00 mg of $MnSO_4.4H_2O$, 10.00 mg of NaCl, 1.00 mg of $FeSO_4.7H_2O$, 1.80 mg of $CoSO_4.7H_2O$, 1.00 mg of $CaCl_2.2H_2O$, 1.80 mg of $ZnSO_4.7H_2O$, 0.70 mg of $CuSO_4$, 0.20 mg of $KAl(SO_4)_2.12H_2O$, 0.10 mg of

H_3BO_3, 0.10 mg of $Na_2MoO_4.2H_2O$, 0.25 mg of $Ni_2SO_4.6H_2O$, and 0.30 mg of $Na_2SeO_3.5H_2O$. The pH was adjusted to a value of 5.4. Aliquots of 40 ml were autoclaved under N_2 atmosphere in 160 ml serum bottles. After autoclaving the following heat-labile components were added from sterile stock solutions (per liter of medium): 1.00 g of $NaHCO_3$, 0.35 g of $Na_2S.9H_2O$, and vitamin solution containing 2.00 mg of biotin (vitamin B_7), 5.00 mg of nicotinic acid (vitamin B_3), 5.00 mg of thiamine-HCl.$2H_2O$ (vitamin B_1), 5.00 mg of p-aminobenzoic acid (vitamin B_{10}), 5.00 mg of D-Ca-pantothenate (vitamin B_5), 10.00 mg of pyridoxine-HCl (vitamin B_6), 1.00 mg of cyanocobalamine (vitamin B_{12}), 2.00 mg of folic acid (vitamin B_9), 5.00 mg of riboflavin (vitamin B_2), and 5.00 mg of lipoic acid. The initial gas phase of inoculated vessels was exchanged with CO and 80% CO/ 20% H_2 at 170 kPa.

Pure cultures were obtained by dilution series continued over eight 10-fold steps. Cells from the highest dilution step were inoculated in medium solidified by 0.6% (w/v) gel rite gellan gum and 0.06% (w/v) $MgSO_4.7H_2O$. Individual colonies were picked from the solidified medium for new dilution series. This process was repeated at least twice. The first pure cultures were designated as strains CP.B1, CP.B2, and CP.B3.

Culture experiments were performed with *Bacillus caldotenax* cultures using untreated, N_2 or CO_2 sparged, sterile-filtered (filter pore size of 0.22 µm), and autoclaved (20 min at 121° C and 104 kPa) Champagne Pool water supplemented with tryptic soy broth (TSB; Difco) to final 1x and 0.1x concentrations. Lower TSB concentrations were used as higher concentrations might alter spring water compounds in the culture medium (e.g. precipitation) due to the autoclaving process. *Bacillus caldotenax* was selected due to its simple culture requirements and its ability to grow under thermophilic and slightly acidic conditions as found in Champagne Pool. Incubations were performed at 70° C at pH 6.0.

4.3.6 Light and electron microscopy

Microbial cells were subsequently observed under a phase-contrast microscope (Olympus BH-2). Microbial enumeration was performed by staining DNA with

4',6-diamidino-2-phenylindole (DAPI). Formalin-fixed water samples obtained from Champagne Pool were filtered through black polycarbonate nucleopore filters (Osmonics Inc., pore size 0.22 µm). Filter-bound cells were stained with DAPI using standard procedures (Porter & Feig, 1980; Wetzel & Likens, 2000) and observed under a Leica DMR research microscope (excitation filter BP 340-380 nm, dichromic mirror RKP 400 nm, suppression filter LP 425 nm) at 100x objective magnification. Images were taken with an Olympus DP70 digital camera.

For scanning electron microscopy (SEM), cells were captured onto a 0.22 µm filter by filtration and fixed using 2.5% glutaraldehyde. The filter was exposed to four changes of 0.1 M sodium cacodylate buffer, rinsed in water and dehydrated in an increasing concentration series of ethanol, 50%, 75% and 90%, respectively, then four changes of absolute ethanol. The filter was critical point dried, sputtered with platinum, and viewed using a Hitachi S-4100 field emission SEM.

4.3.7 Phylogenetic analyses

The partial 16S rRNA gene sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997) and the sequences were aligned using the ARB software package (Ludwig *et al.*, 2004). The phylogenetic position of the 16S rRNA gene sequences were determined using the PHYLIP package within ARB with analysis of sequences undertaken using DNADIST, DNAML, DNAPARS, FITCH, NEIGHBOR, and SEQBOOT programs (Felsenstein, 1993).

4.3.8 Nucleotide sequence accession numbers

16S rRNA gene sequences (Appendix E) have been deposited in the GenBank database under accession numbers as follows: EF101533 (DGGE fragment a), EF101534 (DGGE fragment b), EF101535 (DGGE fragment c), EF101536 (DGGE fragment d), EF101537 (OTU Arc03), EF101538 (OTU Bac03), EF101539 (OTU Bac04), EF101540 (OTU Bac12), EF101541 (CP.B3), EF101542 (CP.B1), and DQ989208 (CP.B2).

4.3.9 Eh-pH diagram

The stability of arsenic compounds in Champagne Pool spring water as a function of the activity of hydrogen ions (pH) and the activity of electrons (Eh) were calculated from chemical species and concentrations listed in Table 8. Eh-pH diagram was generated using the Act2 module of the Geochemist's Workbench software package (RockWare).

Table 8. Chemical composition of water samples collected from Champagne Pool (T = 75.2° C, pH = 5.17) according to Jones *et al.* (2001).

Compound		Concentration	
Chlorine	Cl	1915.0	ppm
Sodium	Na	1150.0	ppm
Silica	SiO_2	472.0	ppm
Sulfate	SO_4^{2-}	255.0	ppm
Carbonate	CO_3^{2-}	195.0	ppm
Calcium	Ca	34.5	ppm
Lithium	Li	8.5	ppm
Arsenic	As	5.2	ppm
Fluorine	F	3.8	ppm
Aluminum	Al	375.0	ppb
Strontium	Sr	295.0	ppb
Tungsten	W	130.0	ppb
Manganese	Mn	84.0	ppb
Iron	Fe	50.0	ppb
Magnesium	Mg	40.0	ppb
Barium	Ba	10.0	ppb
Titanium	Ti	6.6	ppb
Zinc	Zn	1.7	ppb
Lead	Pb	0.2	ppb
Potassium	K	0.2	ppb
Silver	Ag	0.1	ppb
Gold	Au	0.1	ppb
Vanadium	V	0.1	ppb

4.4 Results

4.4.1 Physico- and biochemical properties

No seasonal variability is expected for the geothermal site and during sampling from 2004 to 2006 spring water from Champagne Pool displayed only slight fluctuations in temperature (74.2 ± 0.8° C) and pH (5.5 ± 0.1) values (Table 9). Although some hot springs in the Rotorua area (ca. 30 km away from Champagne Pool) showed significant changes in water levels in the past 3 years, the water level of Champagne Pool remained stable.

Table 9. Date, time, temperature, and pH values of water samples collected from 30 cm below water surface level of Champagne Pool in 2004, 2005, and 2006. The pH values were measured at the corresponding pool temperature.

Date	Time	T [° C]	pH	Weather
02.06.2004	12.00	74.0	5.6	cloudy
12.02.2005	14.00	74.5	5.5	sunny
24.02.2005	12.00	75.5	5.5	sunny, windy
16.05.2005	13.00	73.5	5.5	cloudy
18.05.2005	10.45	74.0	5.5	cloudy
30.05.2005	12.00	74.0	5.5	cloudy, windy
22.06.2005	12.00	74.0	5.5	rainy
05.09.2005	11.30	75.0	5.5	cloudy
29.09.2005	13.30	75.0	5.5	sunny
05.05.2006	11.30	75.5	5.6	rainy
23.06.2006	12.00	73.0	5.4	cloudy, windy
11.07.2006	12.30	73.0	5.5	cloudy
24.10.2006	15.30	74.0	5.5	cloudy, windy
13.12.2006	11.30	74.0	5.5	sunny

Sediment and water samples collected from different depths revealed low concentrations of ATP (Table 10, Figure 18). Inhibitory effects of Champagne Pool water on the ATP assay can be excluded as measurements of ATP standards

of defined concentrations in sterile water and in spring water samples showed similar values. The highest ATP levels in Champagne Pool were detected in the orange-colored sediment indicating a higher biomass in the sediments than in the water column. Similar results were obtained by measurements on the surface of glass slides that had been immersed in Champagne Pool for 24 days and had been colonized by microorganisms.

Table 10. Summary of ATP content determined for Champagne Pool samples. ATP values for the hot springs KP1 (75° C, pH 7.5) and AQ1 (95° C, pH 7.5) are listed as reference. Values are given as Relative Light Units (RLU) and ATP concentrations.

Sample	Depth [m]	RLU	ATP [ng per ml]
25 µl of water sample	0.00	677	1.8
25 µl of sediment suspension	0.10	4800	12.4
25 µl of water sample	0.10	740	1.9
25 µl of water sample	1.00	875	2.3
Glass slides (1976 mm²) immersed in sediment	0.10	8900	[a]
Glass slides (1976 mm²) immersed in water	0.30	2300	[a]
25 µl of 1:1 mix of sediment suspension and 100 ng/ml ATP	0.10	17051	44.0
25 µl of 1:1 mix of sterile water and 100 ng/ml ATP	-	18389	47.4
Glass slides immersed in KP1 (Kuirau Park, Rotorua, NZ)[b]		57,826	[a]
Glass slides immersed in AQ1 (Kuirau Park, Rotorua, NZ)[b]		>180,000	[a]

[a] Data were obtained from slide surfaces and cannot be expressed per volume.

[b] Niederberger *et al.*, 2005.

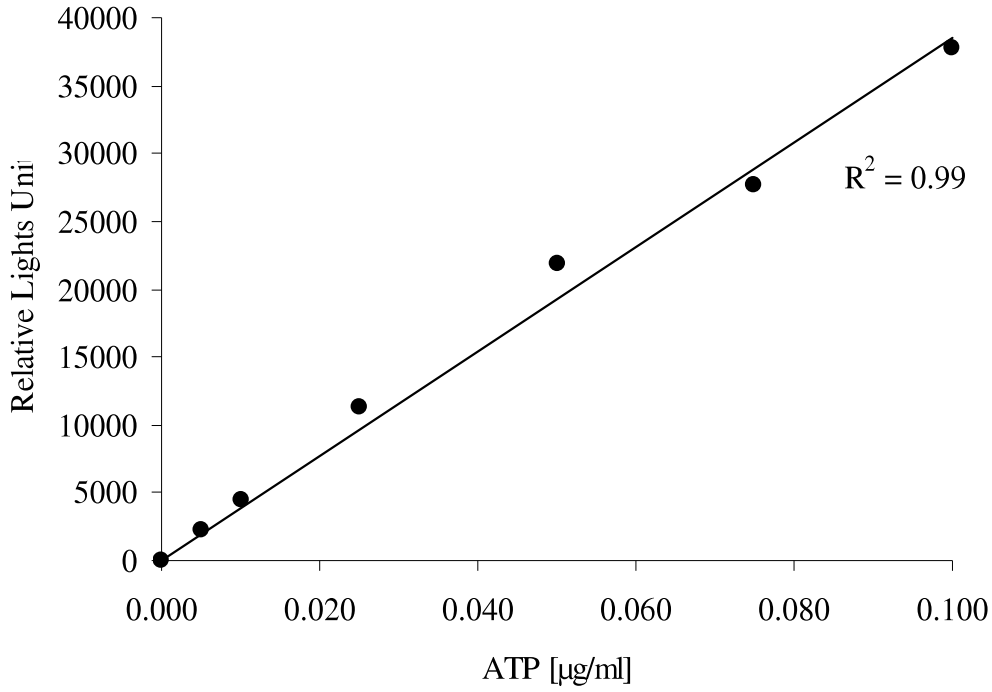

Figure 18. Calibration curve for ATP concentration. The coefficient of determination (R^2) value is 99%.

Nucleic acid concentrations of up to 6.4 µg per l could be extracted from mixed spring water and sediment samples. Values were significantly lower than for DNA concentrations obtained from enrichment cultures isolated from Champagne Pool (3.9 mg per l). To exclude possible inhibition of the applied DNA isolation method by Champagne Pool compounds, DNA from *Escherichia coli* cells was extracted with and without pool sample water. No inhibition of the assay could be detected.

4.4.2 Microscopy

Microscopic observation of water and sediment samples and glass slides incubated in Champagne Pool revealed coccoid, various rod-shaped and predominately filamentous cell structures (Figure 19). SEM micrographs of the isolates CP.B1 and CP.B2 showed different morphologies to each other (Figure 25).

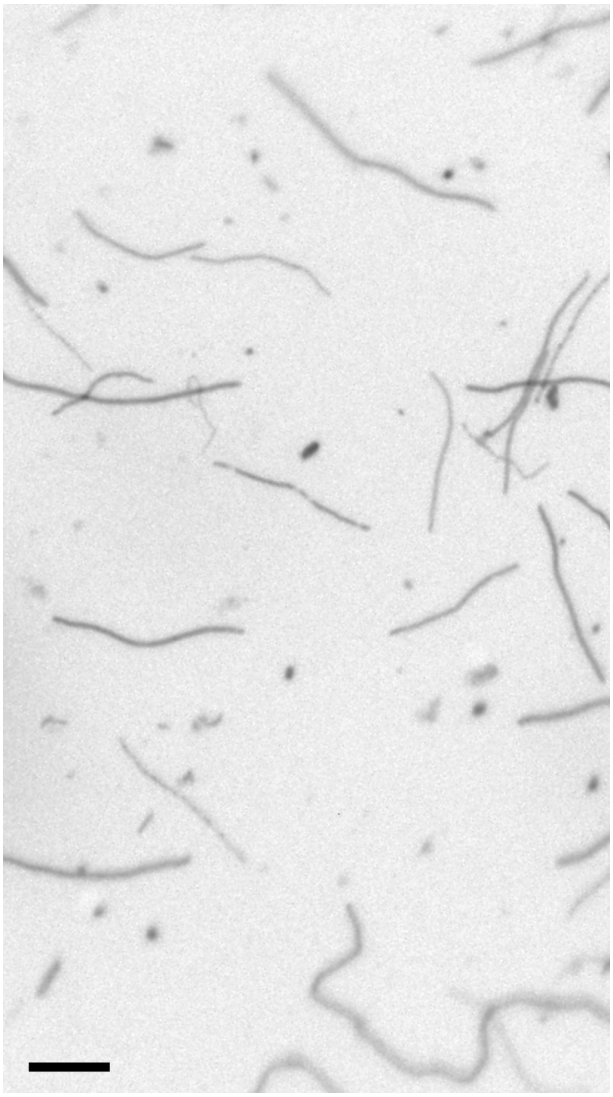

Figure 19. Inverse image of DAPI-stained Champagne Pool water samples. Bar 5 µm.

Total cell numbers by direct counting of DAPI-stained cells using epifluorescence microscopy were 5.6 ± 0.5 x10^6 cells per ml of mixed Champagne Pool sediment and water samples.

4.4.3 Molecular analysis

Archaeal and bacterial 16S rRNA genes were successfully amplified by PCR, whereas amplification of nanoarchaeal 16S rRNA genes remained negative (Figure 20). DGGE profiles derived from the archaeal and bacterial PCR products, displayed two (Figure 21 A) and five distinct bands (Figure 21 B), respectively. Sequences were obtained for four of the excised DNA fragments. Phylogenetic

analysis of both the archaeal DNA sequences and two of the bacterial DNA sequences revealed relationships to species of the genera *Sulfolobus*, *Thermofilum*, *Nevskia*, and *Sulfurihydrogenibium* (Table 11, Figure 23, and Figure 24).

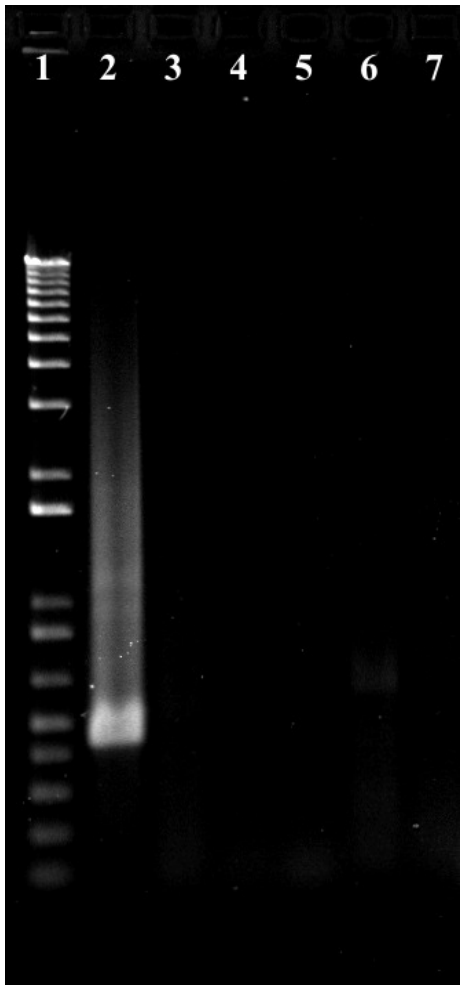

Figure 20. Image of an ethidium bromide-stained 1.0% agarose gel showing different Champagne Pool samples after PCR amplification of nanoarchaeal 16S rRNA genes. Lanes: 1, 1 Kb Plus DNA ladder (Invitrogen); 2, positive control; 3, glass slide immersed in the spring; 4, sediment suspension; 5, sediment suspension, diluted 1 to 20; 6, enrichment cultures growing in V24N medium; 7, negative control.

Clone libraries consisting of 40 archaeal and 90 bacterial 16S rRNA genes amplified by PCR were generated. Analysis of the clone libraries (Appendix D: Figure 39, Figure 40) indicated species of the genera *Sulfolobus*, *Sulfurihydrogenibium* and *Paracoccus* (Table 11, Figure 23, Figure 24) to be present in Champagne Pool partially confirming the results of the DGGE analysis.

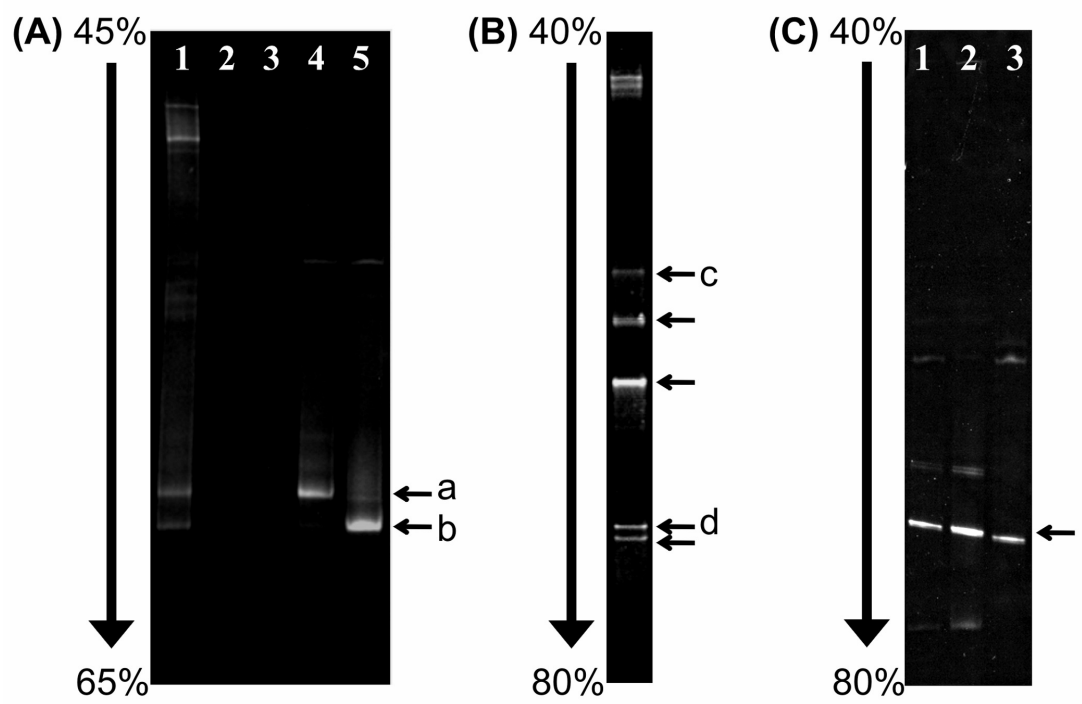

Figure 21. DGGE profiles of archaeal and bacterial 16S rRNA gene fragments amplified by PCR from Champagne Pool water samples in polyacrylamide gels with corresponding denaturing gradients. (A) Archaeal DGGE profile. Lanes: 1, Champagne Pool; 2, no sample; 3, negative control; 4, reamplificated DNA fragment a from lane 1; 5, reamplificated DNA fragment b from lane 1. DNA fragments revealed phylogenetic relationship to species of the genera *Thermofilum* (a) and *Sulfolobus* (b). (B) Bacterial DGGE profile obtained in April 2004. Lane: 1, Champagne Pool. DNA fragments showed phylogenetic relationship to species of the genera *Nevskia* (c) and *Sulfurihydrogenibium* (d). (C) Bacterial DGGE profile obtained in October 2004. Lanes: 1 and 2, Champagne Pool; 3, Isolate CP.B2. The arrow indicates the position of DNA fragment obtained for isolate CP.B2.

Operational Taxonomic Units (OTUs) were defined as groups of 16S rRNA gene sequences in which nucleotide sequences differed by not more than 1%. Alignment of the 16S rRNA gene sequences of the most abundant bacterial OTUs Bac12 and Bac04 with *Sulfurihydrogenibium azorense* revealed a gap of 258 base pairs between both OTUs indicating that OTUs Bac12 and Bac04 were the same inserts but with different orientation within the cloning vector (Figure 22). Although OTU Bac03 had close database matches to species of *Paracoccus*, the highest sequence similarities were for several uncultured bacteria and a thiosulfate-oxidizing bacterium from a hydrothermal vent. For OTU Bac86 no sequence similarity to known 16S rRNA gene sequences could be established.

Table 11. Summary of 16S rRNA gene sequences identified by culture and culture-independent approaches in samples obtained from Champagne Pool. The lengths of the DNA fragments and the sequence identities to the closest relatives are displayed.

Sample	Species	[bp]	[%]
DGGE			
Fragment a	*Thermofilum pendens*	330	92
Fragment b	*Sulfolobus acidocaldarius*	411	95
Fragment c	*Nevskia ramosa*	146	91
Fragment d	Uncultured *Sulfurihydrogenibium sp.*	64	92
Clone libraries			
OTU Arc03 (100%[a])	*Sulfolobus tokodaii*	699	94
OTU Bac03 (17%[b])	*Paracoccus thiocyanatus*	1,426	90
OTU Bac04 (61%[b])	*Sulfurihydrogenibium azorense*	715	95
OTU Bac12 (20%[b])	*Sulfurihydrogenibium azorense*	536	95
OTU Bac86 (2%[b])	Non-specific amplicon	600	
Cultures			
CP.B3	*Thermococcus waiotapuensis*	962	99
CP.B1	*Thermoanaerobacter tengcongensis*	1,310	98
CP.B2	*Sulfurihydrogenibium azorense*	1,506	94

[a] Percentage of clones containing archaeal inserts.

[b] Percentages of clones containing bacterial inserts.

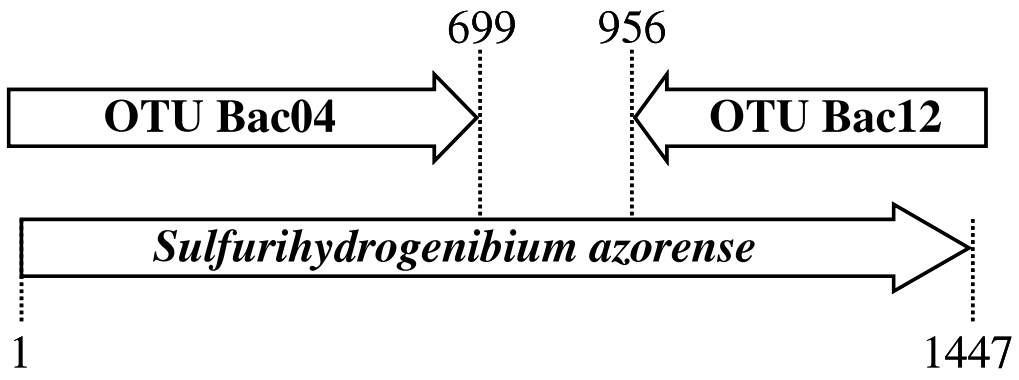

Figure 22. The position of the partial 16S rRNA gene sequences of OTU Bac04 and Bac12 in relation to the 16 rRNA gene of *S. azorense*. Numbers indicate the nucleotide position within the *S. azorense* sequence.

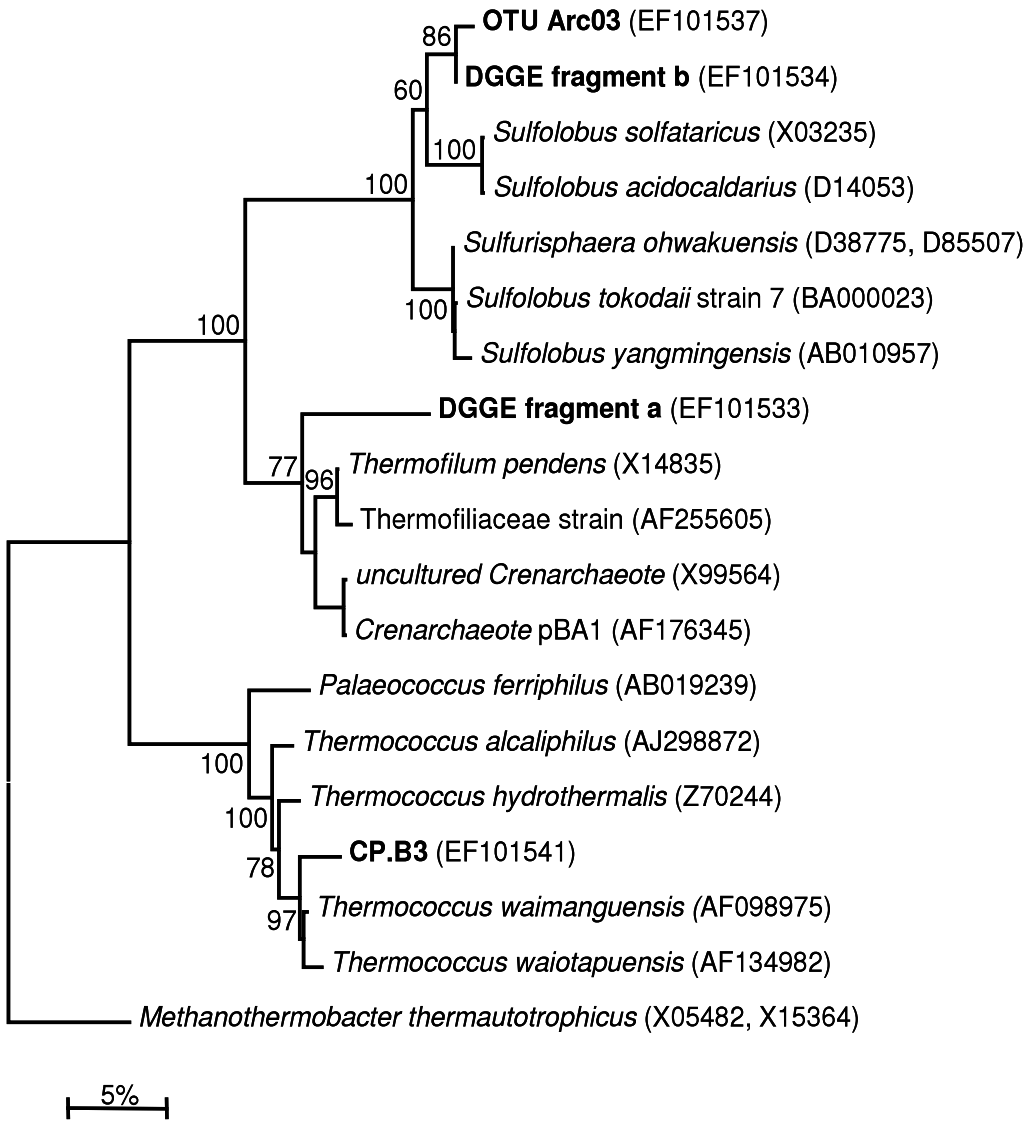

Figure 23. Phylogenetic tree demonstrating relationships of archaeal 16S rRNA gene sequences obtained from Champagne Pool spring samples. The dendogram was produced with ARB (Neighbor joining) and bootstrap values were determined using PHYLIP. Bootstrap values at the nodes are from 1,000 iterations (only bootstrap values >60% are indicated). The numbers in parentheses are accession numbers. Sequences obtained in this study are indicated in bold. The scale bars represents an estimated sequence divergence of 5%.

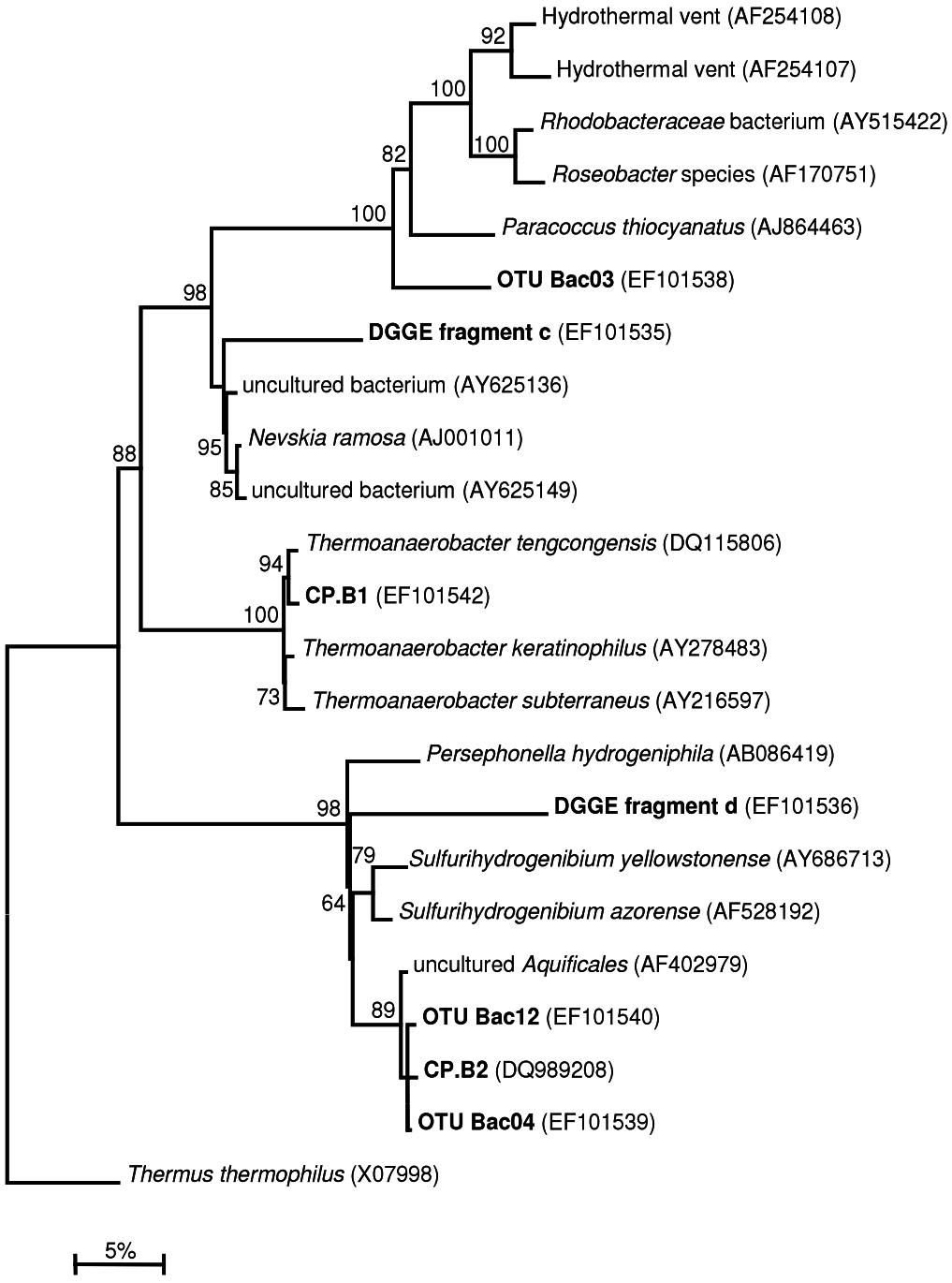

Figure 24. Phylogenetic tree demonstrating relationships of bacterial 16S rRNA gene sequences obtained from Champagne Pool spring samples. The dendogram was produced with ARB (Neighbor joining) and bootstrap values were determined using PHYLIP. Bootstrap values at the nodes are from 1,000 iterations (only bootstrap values >60% are indicated). The numbers in parentheses are accession numbers. Sequences obtained in this study are indicated in bold. The scale bars represents an estimated sequence divergence of 5%.

Differences between DGGE and clone library results might be due to possible DNA extraction and PCR biases (Suzuki & Giovannoni, 1996; von Wintzingerode *et al.*, 1997) and/ or changes in the microbial community. DGGE and clone library analyses have been conducted separately using DGGE samples obtained from different sampling periods. Bacterial DGGE profiles (Figure 21 B and C) varied over time showing fewer bands and changes in band intensities. While DNA fragment d (Figure 21 B) assigned to *Sulfurihydrogenibium*, showed only a relatively weak band intensity in April 2004; in October 2006 a DNA fragment of the isolate CP.B2 (closest related to *Sulfurihydrogenibium*) was the most intense band (Figure 21 C). Although band intensities might not reflect the degree of abundance of species in an environment due to PCR biases, the latter DGGE profile is in good agreement with the clone library results, in which *Sulfurihydrogenibium*-like cells are the most abundant bacterial species.

According to the results obtained from DGGE analysis and the gene cloning experiments growth media were designed to isolate species closely related to members of the genera *Sulfolobus*, *Thermofilum*, and *Sulfurihydrogenibium*. As *Nevskia* and *Paracoccus* strains are both mesophilic bacteria and the 16S rRNA gene sequences obtained shared low sequence similarities of approximately 91% to *Nevskia ramosa* and 90% to *Paracoccus thiocyanatus*, medium was not selected to enrich those species.

4.4.4 Enrichment

To enrich hydrogen-oxidizing microorganisms modified MSH medium as described was used. After inoculation with Champagne Pool spring water (inoculum size 5%) microbial growth was observed by changes in turbidity within two days. Cells appeared to have a slightly curved rod-shaped morphology (Figure 25 B). The first pure culture was designated as strain CP.B2.

In V24N growth medium (Stetter, 1986) selected to enrich sulfur-reducing microorganisms different morphologies could be distinguished. A rod-shaped bacterium (isolate CP.B1, Figure 25 A) and a coccoid archaeon (strain CP.B3 was isolated by Hugh Morgan) were successfully obtained.

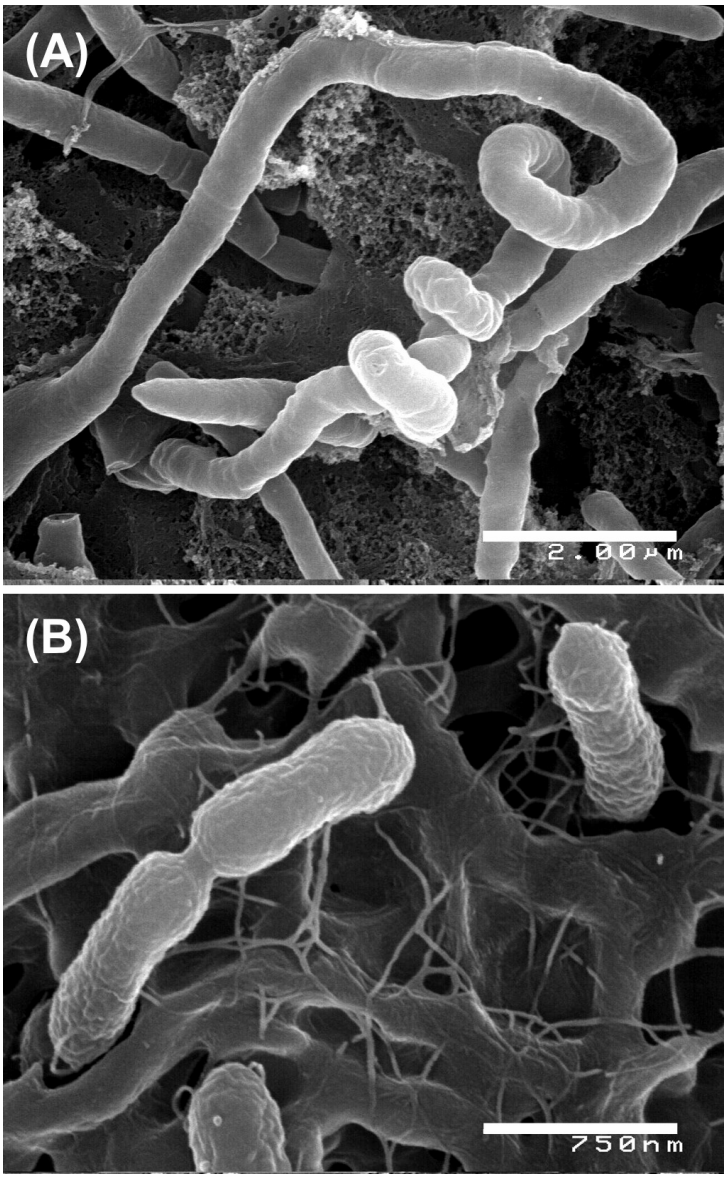

Figure 25. Scanning electron micrographs of isolate CP.B1 (A) and CP.B2. (B). (A) Bar 2 µm. (B) Bar 750 nm.

Enrichment attempts in the *Sulfolobus* medium (Brock *et al.*, 1972) under chemolithothrophic and heterotrophic conditions and in the medium for CO-utilizing microorganisms were negative.

DNA isolated from the three novel isolates and successive analysis of the amplified 16S rRNA gene revealed a distant phylogenetic relationship of isolate CP.B1 to *Thermoanaerobacter tengcongensis* (Xue *et al.*, 2001), of CP.B2 to *Sulfurihydrogenibium azorense* (Aguiar *et al.*, 2004), and of CP.B3 to

Thermococcus waiotapuensis (Gonzalez *et al.*, 1999) (Table 11). All isolates grew under temperature and pH conditions as found in Champagne Pool (Table 12).

Table 12. Growth temperature and pH ranges for microbial strains isolated from Champagne Pool. Values in brackets are the optima.

Isolate	Energy metabolism	T [° C]	pH
CP.B1	Sulfur reduction	45-80 (65)	5.5 (N. D.[a])
CP.B2	Hydrogen oxidation	45-75 (70)	4.8-5.8 (5.4)
CP.B3	Sulfur reduction	70-85 (N. D.)	4.5-8.0 (7.0-7.5)

[a] N.D., not determined.

4.4.5 Growth experiments using Champagne Pool water

Growth of *B. caldotenax* cells could not be detected when inoculated into untreated or sterile-filtered Champagne Pool water. However, when the spring water was purged with N_2 or CO_2, or autoclaved prior to inoculation, microbial growth was observed. These results suggest that a volatile component inhibiting microbial life might have been removed from Champagne Pool water during the sparging or autoclaving process.

4.4.6 Stability of arsenic compounds

Calculation of the distribution of arsenic species revealed the predominance of $As(OH)_3$ (As^{3+}, arsenite) under the chemophysical conditions (T = 75.2° C; pH = 5.5; Eh = -40 mV, Hirner *et al.*, 1998) present in Champagne Pool spring water (Figure 26). In less reducing conditions at Eh values of approximately above 100 mV, which might be found at the hot spring-atmosphere interface, where atmospheric oxygen dissolves into the top water layer of Champagne Pool, the pentavalent arsenic compound $H_2AsO_4^-$ (As^{5+}, arsenate) appears to be more dominant. Although oxygen solubility at the hot spring temperature is relatively low (oxygen saturation concentration is around 3 mg per liter at 75.2° C and 400 m altitude; http://www.pointfour.com/cgi/pfscalc.cgi), traces of oxygen have been

reported for the upper water layer of Champagne Pool (Giggenbach *et al.*, 1994; Jones *et al.*, 2001).

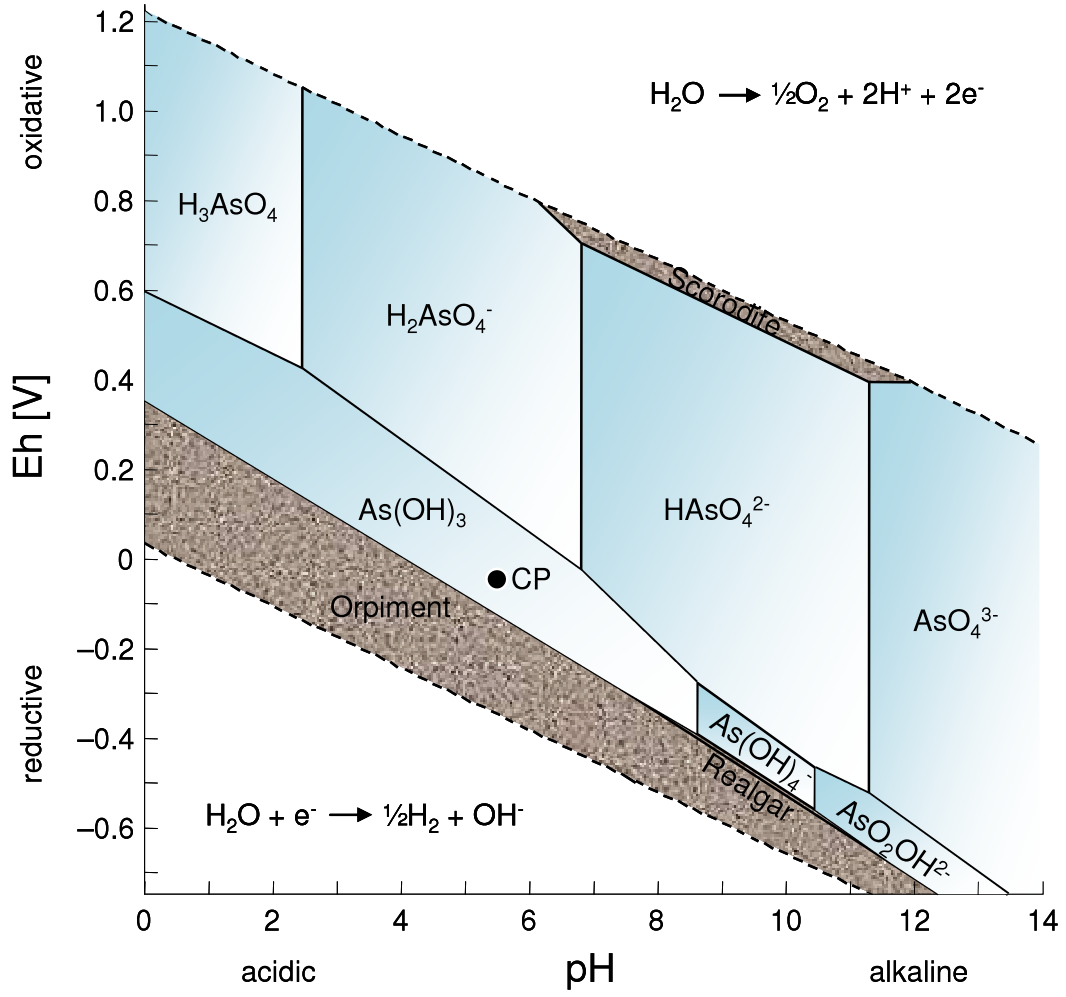

Figure 26. Eh-pH diagram displays predominance fields of arsenic species in spring water of Champagne Pool at a temperature of 75.2° C and a pressure of 101.3 kPa. Dotted lines indicate the stability area of water, where the upper dotted line represents the oxidation line of water and the lower dotted line the reduction line of water, respectively. Stability areas of aqueous arsenic species are displayed in blue-white and of the solid minerals orpiment (As_2S_3), realgar (As_4S_4), and scorodite ($FeAsO_4.2H_2O$) in grey, respectively. The black circle indicates pH and Eh values found in Champagne Pool water (CP).

4.5 Discussion

Both archaeal (Figure 23) and bacterial representatives (Figure 24) but no Nanoarchaea were detected in the terrestrial hot spring Champagne Pool. While

culture-independent methods indicate the abundance of strains phylogenetically related to species of the genera *Sulfurihydrogenibium*, *Nevskia*, *Paracoccus, Sulfolobus* and *Thermofilum*, pure cultures were isolated from Champagne Pool distantly related to *Sulfurihydrogenibium azorense* (Aguiar *et al.*, 2004), *Thermoanaerobacter tengcongensis* (Xue *et al.*, 2001), and *Thermococcus waiotapuensis* (Gonzalez *et al.*, 1999). Those findings (Table 11) are in agreement with the physiochemical properties of Champagne Pool. Dissolved gases in the spring water contain CO_2, H_2, and O_2 in the shallow areas (Giggenbach *et al.*, 1994; Jones *et al.*, 2001) providing chemolithotrophic conditions for hydrogen-oxidizing microorganisms such as *Sulfurihydrogenibium* species. Available sulfur compounds in Champagne Pool are essential for growth of sulfur-oxidizing (*Sulfolobus* species), sulfur-reducing (*Thermoanaerobacter* and *Thermofilum*), and sulfur-dependent (*Thermococcus* species) microorganisms. The detection of the novel isolates CP.B2 and CP.B3 are in good accordance with results by Richard Pancost (personal communication), where the structural differences of biomembrane lipids on samples from Champagne Pool were investigated (Figure 27). The abundance of archaeol (Figure 27 A), an archaeal biomarker, and non-isoprenoidal ether lipids (Figure 27 B) characteristic for *Aquificales*, *Ammonifex*, and *Thermodesulfobacterium* strains were detected.

Other than *Nevskia* and *Paracoccus*, all detected microbial representatives are thermophilic. However, the low sequence similarities of approximately 91% between the DNA fragment c and *Nevskia ramosa*, and 90% among the cloned bacterial 16S rRNA genes and *Paracoccus thiocyanatus* may indicate that the sequences represent organisms phenotypically different from *Nevskia* and *Paracoccus* strains able to grow under thermophilic conditions. The detection of a *Thermofilum* signature is in agreement with previous reports based on electron microscopy examinations (Jones *et al.*, 2001) and PCR studies (Ellis *et al.*, 2005). However, in microscopic examinations of Champagne Pool water and sediment samples only filamentous, coccoid, and rod structures were present, while “golf club”- and “lobe”-shaped morphologies, distinct characteristics for members of the genera *Thermofilum* and *Sulfolobus*, respectively, could not be observed.

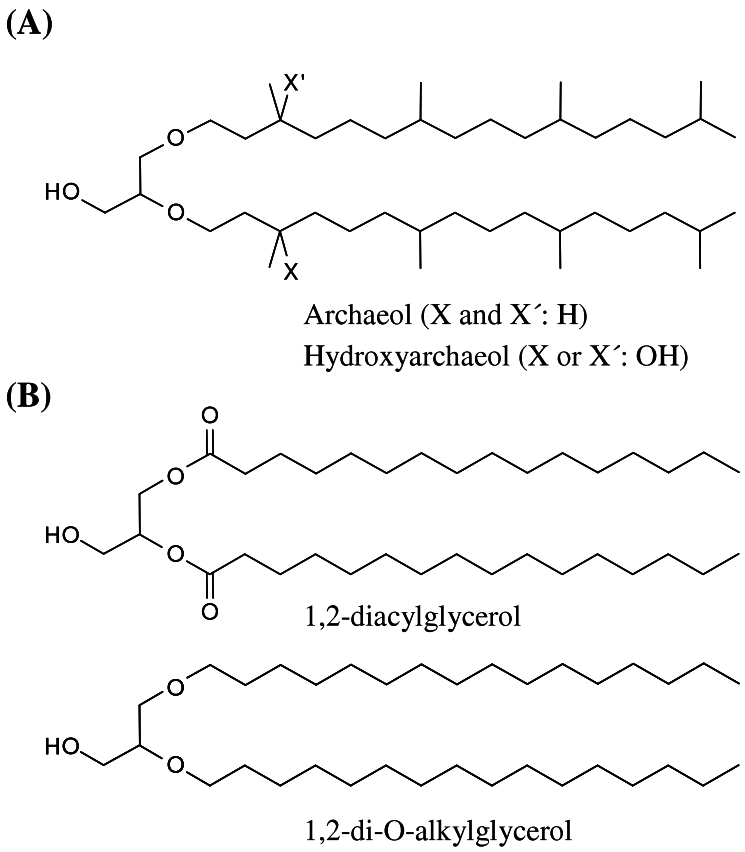

Figure 27. Structures of microbial membrane lipids found in samples obtained from Champagne Pool. (A) Archaeole, (B) bacterial diester and diether lipid structures.

DGGE profiles, nucleic acid yields, cell counts, and ATP measurements indicate low diversity and biomass in Champagne Pool. ATP determinations (Table 10) showed differences between sediment and water samples, with 6x (suspension) and 4x (glass slides) more ATP in the sediment, suggesting an accumulation of biomass within the sediment. However, the highest values obtained for glass slides incubated in Champagne Pool (8,900 RLU) were much lower than reported for other hot springs (>180,000 RLU) in New Zealand (Niederberger, 2005). Those findings are consistent with relatively low cell numbers of 5.6 ± 0.5 $x10^6$ cells per ml determined for Champagne Pool water samples by DAPI staining.

This evidence raises the question: what is the limiting factor for microbial diversity and biomass in Champagne Pool besides the thermal conditions in the spring? There may be several factors responsible. Geothermal systems of New Zealand are known to contain high concentrations of metalloids (Hirner *et al.*,

1998). Arsenic ions and compounds are found in Champagne Pool water to a concentration up to 5.6 mg per liter (Giggenbach *et al.*, 1994). Arsenic toxicity is mediated by arsenite reacting with thiol functional groups in enzymes thus inhibiting their activities and by arsenate through substitution of phosphate (Stolz & Oremland, 1999). It has also been shown that trivalent species of arsenic, predominately found in anaerobic environments such as Champagne Pool (Figure 26), were highly inhibitory to methanogenic microorganisms (Sierra-Alvarez *et al.*, 2004). Therefore, only metalloid ion-tolerant or metalloid ion-resistant microorganisms might survive in Champagne Pool. In the present study, culture experiments with *B. caldotenax* in medium supplemented with Champagne Pool water suggest that the spring water might contain volatile compounds toxic to some microorganisms. Possible candidates might be H_2S and toxic volatile methyl and hydride derivatives of arsenic and antimony, such as AsH_3 (arsine), CH_3AsH_2 (monomethylarsine), $(CH_3)_2AsH$ (dimethylarsine), $(CH_3)_3As$ (trimethylarsine), SbH_3 (stibine), CH_3SbH_2 (monomethylstibine), $(CH_3)_2SbH$ (dimethylstibine), and $(CH_3)_3Sb$ (trimethylstibine). In Champagne Pool geothermal water concentrations of 4.95 ppm AsH_3, 1.90 ppb CH_3AsH_2, 0.10 ppb $(CH_3)_2AsH$, 5 ppb SbH_3, and 0.05 ppb CH_3SbH_2 have been detected (Hirner *et al.*, 1998). Previously published investigations (Jones *et al.*, 1999; Mountain *et al.*, 2003; Phoenix *et al.*, 2005) also considered that microbial cells in Champagne Pool may be subject to biomineralization and bioimmobilisation by rapid silicification and deposition in the sinter.

Champagne Pool seems to be an extreme environment for life forms in terms of its thermal and unique chemical properties. In this study, low microbial diversity and biomass in Champagne Pool and the isolation of three novel strains species was accomplished. The characterization and description of the novel isolates may bring deeper insights into the ecology of the thermal spring.

4.6 Acknowledgements

Research funding was provided by the New Zealand Foundation of Research, Science & Technology (Contract Number C05X0303: Extremophilic Microorganisms for Metal Sequestration from Aqueous Solutions). Special thanks

are due to Richard Fulton for permission to collect samples from Champagne Pool and Helen M. Turner who helped taking SEM micrographs.

Chapter 5: *Venenivibrio stagnispumantis*

***Venenivibrio stagnispumantis* gen. nov., sp. nov., a thermophilic hydrogen-oxidizing bacterium isolated from Champagne Pool, Waiotapu, New Zealand**

(A condensed version of this chapter has been accepted for publication in International Journal of Systematic and Evolutionary Microbiology)

5.1 Abstract

A novel thermophilic, hydrogen-oxidizing bacterium, designated strain CP.B2^T, was isolated from a terrestrial hot spring in Waiotapu, New Zealand. Cells were motile, slightly rod-shaped, non-spore-forming, and Gram-negative. Isolate CP.B2^T was an obligate chemolithothroph growing by utilizing H_2 as electron donor and O_2 as corresponding electron acceptor. Elemental sulfur (S^0) or thiosulfate ($S_2O_3^{2-}$) was essential for growth. Microbial growth occurred under microaerophilic conditions in the O_2 range of 1% to 10.0% (v/v) O_2 (optimum 4% to 8% (v/v) O_2), between 45° C and 75° C (optimum 70° C), and at pH values 4.8 to 5.8 (optimum pH 5.4). The G+C content was 29.3 mol%. 16S rRNA gene sequence similarity demonstrated strain CP.B2^T to belong to the order *Aquificales*, with a close phylogenetic relationship to *Sulfurihydrogenibium azorense* (94% sequence identity). However, genotypic and metabolic characteristics differentiated the novel isolate from previously described genera of the *Aquificales*. CP.B2^T therefore represents a novel genus and a new species, for which the name *Venenivibrio stagnispumantis* gen. nov., sp. nov. is proposed. The type strain is CP.B2^T (=JCM 14244^T = DSM 18763^T).

5.2 Introduction

The Taupo Volcanic Zone in New Zealand is the terrestrial extension of the Tonga-Kermadec subduction system (Hedenquist, 1986a) exhibiting geothermal activities such as fumaroles, steaming grounds, mud pools, geysers and hot springs. Within the Taupo Volcanic Zone Champagne Pool is the largest hot spring. It is around 65 m in diameter and is located in a 900 year old hydrothermal

eruption crater (Lloyd, 1959). The name Champagne Pool derives from the constant flow of CO_2 visible as gas bubbles buffering the spring water at a pH value of around pH 5.5. Champagne Pool discharges siliceous geothermal fluid at approximately 75° C, rich in arsenic and antimony (Jones *et al.*, 2001). Although, a few studies (Ellis *et al.*, 2005; Jones *et al.*, 2001; Mountain *et al.*, 2003; Phoenix *et al.*, 2005) have recently described microbial activity in Champagne Pool, the successful isolation of a microorganism has not yet been reported.

Previous investigations (Chapter 4) applying culture-independent approaches, such as Denaturing Gradient Gel Electrophoresis (DGGE) analysis and construction of clone libraries based on the bacterial small-subunit ribosomal nucleic acid (16S rRNA) gene, indicated that hydrogen-oxidizing members of the *Aquificales* order were some of the few dominant microorganisms present in Champagne Pool. These findings are in good agreement with results of phylogenetic studies on five geothermal springs above 70° C in Yellowstone National Park, USA showing hydrogen-metabolizing organisms to dominate these hot spring communities. 77% of all obtained archaeal and bacterial 16S rRNA genes were assigned to species of the order *Aquificales* (Spear *et al.*, 2005). Skirnisdottir and colleagues (2000) reported that 68% of all obtained bacterial 16S rRNA genes obtained from a sulfide-rich hot spring in south-western Iceland belonged to the order *Aquificales*.

The order *Aquificales* comprise currently the genera *Aquifex* (Huber *et al.*, 1992), *Hydrogenivirga* (Nakagawa *et al.*, 2004), *Hydrogenobacter* (Kawasumi *et al.*, 1984), *Hydrogenobaculum* (Stöhr *et al.*, 2001b), and *Thermocrinis* (Huber *et al.*, 1998) of the *Aquificaceae* family; the genera *Hydrogenothermus* (Stöhr *et al.*, 2001b), *Persephonella* (Götz *et al.*, 2002b), *Sulfurihydrogenibium* (Takai *et al.*, 2003a) of the *Hydrogenothermaceae* family, and the genera *Balnearium* (Takai *et al.*, 2003c), *Desulfurobacterium* (L'Haridon *et al.*, 1998), and *Thermovibrio* (Huber *et al.*, 2002a) of the *Desulfurobacteriaceae* family (Euzeby, 1997). Representatives are thermophilic to hyperthermophilic bacteria commonly colonizing geothermally heated environments. Most *Aquificales* have in common the ability to use the Knallgas reaction ($2\ H_2 + O_2 \rightarrow 2\ H_2O$) to yield energy for biosynthesis, therefore using H_2 as electron donor and O_2 as electron acceptor. On

account of their chemolithothrophic metabolism these organisms are regarded as primary producers of bacterial biomass within high temperature ecosystems (Huber & Eder, 2001).

Although members of the order *Aquificales* are regarded as representing the earliest branching lineage within the phylogenetic domain *Bacteria* based on 16 rRNA gene sequences (Figure 1) (Burggraf *et al.*, 1992; Reysenbach *et al.*, 1994), signature protein sequences (e.g. heat shock protein 60 and 70, alanine-tRNA synthetase, CTP synthase, inorganic pyrophosphatase, and RNA polymerase subunit) support a later divergence of the order and a phylogenetic placing between the δ-/ ε-*Proteobacteria* and the *Chlamydiae* groups (Griffiths & Gupta, 2004).

In the present study, the novel thermophilic bacterium CP.B2^T isolated from Champagne Pool is characterized and it is shown that it differs from previously described strains of the order *Aquificales*. *Venenivibrio stagnispumantis* gen. nov., sp. nov. is the proposed name.

5.3 Materials and Methods

5.3.1 Collection of samples

Geothermal fluid (30 cm below spring water surface) and sediment (10 cm below water surface) were sampled in September 2005 at the north-east side of Champagne Pool, Waiotapu (Figure 4, Figure 16) located in the Taupo Volcanic Zone in New Zealand. The samples were collected in sterile 2-liter glass bottles (Schott) carefully avoiding trapping any air. Within two hours samples were transferred into enrichment medium.

5.3.2 Enrichment and isolation

Enrichments were performed in a modified version of MSH medium (4.4.4 Enrichment). The pH value of the medium was adjusted to 5.5. Aliquots of 40 ml and 8 ml were autoclaved under a CO_2 atmosphere in 160 ml serum bottles and 30

ml test tubes, respectively. After inoculation using a 5% inoculum, the initial gas phase was exchanged with 79% H_2/ 16% CO_2/ 5% O_2 at 170 kPa. Enrichment cultures were incubated at 70° C.

The enrichment contained only cells having a slightly curved, rod-shaped morphology. To ensure purity, dilution series were continued over eight 10-fold steps. Cells from the highest dilution step were subsequently inoculated in modified MSH medium solidified by 0.6% (w/v) gel rite gellan gum and 0.06% (w/v) $MgSO_4.7H_2O$. Individual colonies isolated from the solidified medium were used as inocula for new dilution series. This process was repeated at least twice. The first pure culture was designated as strain CP.B2^T.

5.3.3 Culture conditions

The novel isolate was routinely cultivated in modified MSH medium as described. Microbial growth was determined by measuring changes in turbidity at 450 nm and by direct cell counts using a Thoma counting chamber (depth, 0.02 mm).

The influence of pH on growth was determined at 70° C in the range of 4.5 to 6.5 using 10 mM MES, PIPES, MOPS, and HEPES. The pH value of the medium was checked at the incubation temperature prior to the inoculation and after the growth experiments. NaCl and O_2 requirement and tolerance were determined at 70° C and pH 5.5 in the range of 0% to 2% (w/v) NaCl and 0% to 20% (v/v) O_2, respectively.

The ability of CP.B2^T to metabolize different substrates was investigated using a basal MSH medium without $Na_2S_2O_3.5H_2O$ and with only 4.00 g of $MgSO_4.7H_2O$ to provide a sulfur source for assimilation. The basal medium was supplemented with either 80% (v/v) H_2, 10 mM $Na_2S_2O_3$, or 1% (w/v) S^0 as electron donor and either 10 mM $NaNO_2$, 10 mM $NaNO_3$, 10 mM Na_2SO_3, 10 mM Na_2SO_4, 5 mM Na_2SeO_3, 5 mM Na_2SeO_4, 5 mM $NaAsO_2$, 5 mM Na_2HAsO_4, or O_2 as complementary electron acceptor. Further experiments were performed using 1 mM and 5 mM Na_2HAsO_4 and $NaAsO_2$ as electron acceptor and donor, respectively. As carbon source either casamino acids, sodium pyruvate, starch,

trypticase peptone, yeast extract (final concentration 0.2% (w/v)), or CO_2 were provided (Appendix F: Table 26).

In toxicity experiments, the novel bacterium was tested for tolerance to elevated arsenic and antimony ion levels. Modified MSH medium supplemented with defined concentrations of $Na_2HAsO_4.7H_2O$, $NaAsO_2$, and $KSbOC_4H_4O_6.0.5H_2O$ was inoculated using a 2.5% inoculum of a fresh grown culture. The MIC was defined as the absence of growth of the species in 30 ml test tubes after 7 days.

Spectrophotometric determination of H_2S (Cline, 1969; Gilboa-Garber, 1971) after 24 h and 48 h growth was performed. A 100 µl aliquot of cell suspension was transferred to 500 µl 4% (w/v) zinc acetate solution (per liter: 40.0 g of $Zn(C_2H_3O_2)_2.2H_2O$ and 20.0 ml acetic acid glacial) and then 100 µl 0.4% (w/v) N,N-dimethyl-p-phenylenediamine dichloride solution (per liter: 4.0 g of N,N-dimethyl-p-phenylenediamine dichloride and 400 ml H_2SO_4) and 100 µl 2% (w/v) ammonium iron sulfate solution (per liter: 20.0 g of $NH_4Fe(SO_4)_2.12H_2O$) were added. After incubation in the dark for 25 min, the absorbance of the sample at 670 nm was spectrophotometrically determined. Sulfide is initially fixed with zinc solution and reacts with N,N-dimethyl-p-phenylenediamine to colorless leucomethylene blue, which then in the presence of Fe^{3+} is oxidized to methylene blue. Methylene blue shows maximum absorbance at 670 nm.

5.3.4 Light and electron microscopy

Cells were routinely examined under a phase-contrast microscope (Olympus BH-2) equipped with a digital camera (Nikon Coolpix 4500). Gram-staining was carried out using standard methods (Hucker & Conn, 1927) with *Escherichia coli* cells (strain JM109) as a control. Cells were heat-fixed onto a microscope glass slide and then flooded with filtered crystal violet solution (per liter: 20.0 g of crystal violet, 200 ml 95% ethanol, 8.0 g of ammonium oxalate) for 30 s. Then the glass slide was rinsed gently with running water and covered with fresh iodine solution (per liter: 3.3 g of iodine crystals, 6.6 g of potassium iodine) for 30 s. The sample was rinsed with 96% ethanol until run-off became clear but not for more than 10 s to ensure Gram-positive cells were not decolorized. Ethanol is used to

remove the crystal violet-iodine complex from Gram-negative cells by dissolving lipids incorporated into the outer membrane, whereas the multi-layered peptidoglycan of Gram-positive cells is dehydrated by ethanol and the violet stain is retained due to closure of the pores as the cells shrink. Thus, Gram-positive cells appear violet. To further differentiate Gram-positive and Gram-negative cells samples were counterstained with the dye carbol fuchsin (per liter: 1.0 g of basic fuchsin, 5.0 g of phenol, 10 ml of 90% ethanol) staining Gram-negative cells red.

For scanning electron microscopy (SEM), cells were captured onto a 0.22 µm filter by filtration and fixed using 2.5% glutaraldehyde. The filter was exposed to four changes of 0.1 M sodium cacodylate buffer, rinsed in water and dehydrated in increasing concentrations of ethanol, 50%, 75%, and 90%, respectively, then four changes of absolute ethanol. The filter was critical point dried, sputtered with platinum, and viewed using a Hitachi S-4100 field emission SEM.

5.3.5 DNA isolation and base composition

Genomic DNA of CP.B2^T was isolated using a modification of the CTAB method described previously (Dempster *et al.*, 1999). A 40 ml bacterial suspension was harvested by centrifugation at 1,850×*g* (JouanCR4.11) for 10 min. The cell pellet was resuspended in 1 ml of CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) cetyltrimethylammoniumbromide (CTAB), 1% (w/v) polyvinylpyrrolidone (PVP) (360,000; pH 8.0) and freshly prepared 0.4% (w/v) 2-mercaptoethanol. The mixture was incubated at 100° C for 20 min followed by extractions with an equal volume of phenol and then with chloroform-isoamyl alcohol (24:1 by volume). Nucleic acids were precipitated with an equal volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.5) at -20° C overnight, then washed twice in 70% ethanol and air-dried.

The G+C content of the DNA was determined by the Identification Service of the German Collection of Microorganisms (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) according to the method of Mesbah (1989).

5.3.6 PCR amplification and sequencing

PCR amplification of the 16S rRNA gene sequence between the corresponding *E. coli* position 9 and 1522 was performed using a forward primer 5'- AGA GTT TGA TCC TGG CTC AG -3' and a reverse primer 5'- AAG GAG GTG ATC CAR CCG CA -3' (Johnson, 1994). PCR mixtures consisted of PCR buffer (10 mM Tris-HCI, 50 mM KCI, pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTP (dATP, dCTP, dGTP, dTTP), 1.0 µM of each amplification primer, 5.0% (w/v) dimethyl sulfoxide (DMSO) and 1.0 unit of *Taq* polymerase (Roche). The following PCR conditions were applied: an initial denaturation step at 94° C for 210 s, two cycles annealing at 50° C for 30 s, extension at 72° C for 120 s and denaturation at 94° C for 30 s, followed by 31 cycles applying an annealing temperature of 48° C for 120 s. The final extension step was 300 s.

PCR products purified by agarose gel electrophoresis were used in cloning experiments applying the pGEM-T Easy vector system (Promega) as recommended by the manufacturer (4.3.3 PCR amplification of 16S rRNA gene and gene cloning experiments). DNA sequencing of the cloned inserts was undertaken by the Waikato DNA Sequencing Facility based at the University of Waikato in Hamilton, New Zealand using the MegaBACE capillary analysis system (Amersham Biosciences). Sequences of the inserts were obtained by using primer set M13pUC forward 5'- CCC AGT CAC GAC GTT GTA AAA CG -3' and M13pUC reverse 5'- AGC GGA TAA CAA TTT CAC ACA GG -3'. Sequences were checked by the Chimera Detection computer program (Cole *et al.*, 2003) of the Ribosomal Database Project II to identify the presence of possible chimeric artefacts (http://rdp8.cme.msu.edu/cgis/chimera.cgi).

PCR experiments were carried out targeting the DNA polymerase I (PolA) gene distinctive of species from the *Aquificales* order using forward primer 5'- ASA YCC WAA TWT VCA RAA YAT HC -3' and reverse primer 5'- CCC GTN CCY TGW ATN GGR TAR TT -3' (Griffiths & Gupta, 2006). DNA isolated from *Sulfurihydrogenibium azorense*, a member of the *Aquificales* order, acted as a control.

To determine, if *ars* genes were present in CP.B2^T and contribute to arsenic resistance, a nested PCR approach was employed to amplify *arsA*, *arsB*, and *arsC*, according to Ford and colleagues (2005). Conditions for PCR reactions using outer primers following PCR using inner primers (Table 13) were an initial denaturation step at 94° C for 300 s, 30 cycles annealing at 50° C for 60 s, extension at 72° C for 60 s, and denaturation at 94° C for 60 s, followed by a final extension step for 600 s.

Table 13. Target and sequences of oligonucleotide primers used in PCR studies. Abbreviations: OF, outer forward primer; OR, outer reverse primer; IF, inner forward primer; IR, inner reverse primer.

Primer	Target	Oligonucleotide primer sequence (5' → 3')[a]
arsA OF	ATPase	TATTTCCTGCGCCACGGCGAT
arsA OR	ATPase	GAAGGCGAATGGTGTGAC
arsA IF	ATPase	CTGCTGGTCAGTACCGAT
arsA IR	ATPase	GATATGGTCAAACGTCAG
arsB OF	Arsenite efflux pump	CCGGTGGTGTGGAATATTGT
arsB OR	Arsenite efflux pump	ACTCCGTGAATCCCAGTT
arsB IF	Arsenite efflux pump	GTTGCTGGATGAGTCAGGCT
arsB IR	Arsenite efflux pump	GTATCGGAAATACCGGC
arsC OF	Arsenate reductase	CTGATATGAGCAACATCACTATTT
arsC OR	Arsenate reductase	ATTTCAGCCGTTTTCCTGCTTCA
arsC IF	Arsenate reductase	ATCATAACCCAGCCTGC
arsC IR	Arsenate reductase	CTGCGCATCCTGTAGGATARC

[a] Primer sequence according to Ford *et al.*, 2005.

Randomly amplified polymorphic DNA (RAPD) testing was undertaken on DNA extracted from the CP.B2^T isolate and *S. azorense* to determine the similarity using primer OPR13 5'- GGA CGA CAA G -3' (Williams *et al.*, 1990). After an initial denaturation step at 94° C for 90 s, 42 cycles of annealing at 36° C for 30 s, extension at 72° C for 120 s and denaturation at 94° C for 30 s, and a final extension step for 240 s were applied.

5.3.7 Phylogenetic analyses

The computer algorithm Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997) was used to search the sequence database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) for 16S rRNA gene sequence similarities. Phylogenetic analysis and alignment of 16S rRNA gene sequences was performed using the ARB software package (Ludwig *et al.*, 2004). The phylogenetic position of the sequences were determined using the PHYLIP package with analysis of sequences undertaken using DNADIST, DNAML, DNAPARS, FITCH, NEIGHBOR, and SEQBOOT programs (Felsenstein, 1993).

5.4 Results

5.4.1 Isolation

To enrich for hydrogen-oxidizing thermophilic microorganisms, 40 ml modified MSH medium was inoculated with 2 ml Champagne pool spring water and a gas atmosphere of 79% H_2/ 16% CO_2/ 5% O_2 pressurized to 170 kPa. After two days incubation at 70° C without agitation the medium became turbid due to microbial growth. Serial dilution series on solidified medium led to the isolation of CP.B2^T. The purity of the novel isolate was routinely checked by microscopy and sequence analysis of the 16S rRNA gene.

5.4.2 Morphology

Cells of isolate CP.B2^T appeared to have a slightly curved, rod-shaped morphology (Figure 28 b–d), with a mean length of 1.30 ± 0.26 µm and a mean width of 0.37 ± 0.04 µm. The cells were motile and stained Gram-negative. Sporulation could not be observed during growth, in 6 months-old cultures, or at temperatures above the maximum growth temperature. The bacterial cells occurred predominate singly or in pairs. Large cell aggregates were formed in the late-stationary growth phase, macroscopically visible as whitish flocks (Figure 28 a).

5.4.3 Physiological characteristics

Strain CP.B2^T was able to grow between 45° C and 75° C with an optimum of around 70° C, whereas growth below 40° C and above 80° C was not observed even after one-week incubation. The pH range for growth was 4.8 to 5.8 with an optimum around 5.4. CP.B2^T grew without NaCl and NaCl concentration up to 0.8% (w/v) with an optimum of 0.4% (w/v) NaCl. Growth occurred only under microaerophilic conditions in the O_2 range of 1% to 10.0% (v/v) with an optimum of approximately 4% to 8% (v/v) O_2.

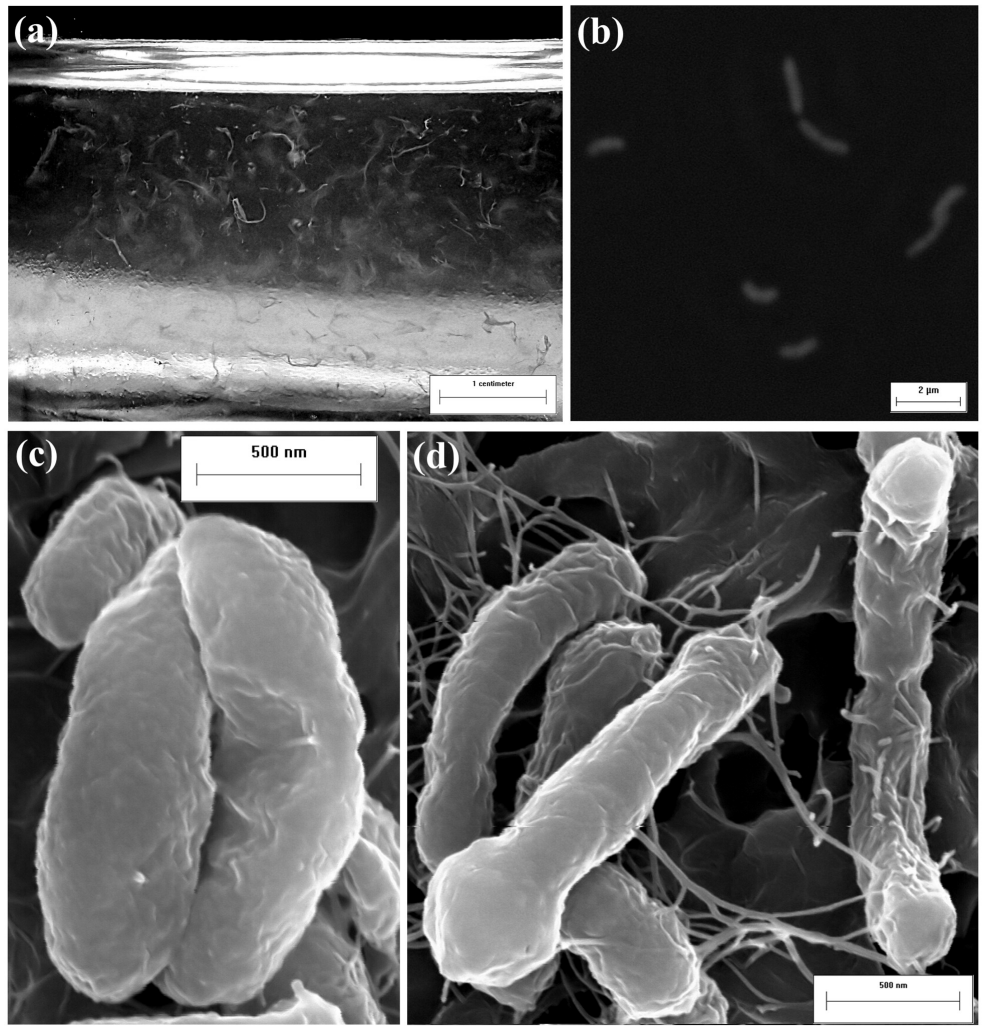

Figure 28. Macroscopic (a), phase-contrast, (b) and scanning electron (c-d) micrographs of isolate CP.B2^T. (a) Large cell aggregates were formed after two days incubation in serum bottles. Bar 1 cm. (b) Cells predominately occurred as single cells or in pairs. Bar 2 µm. (c-d) Electron micrographs showing vibrio (c) and biofilm-like (d) structures. Bars 500 nm.

5.4.4 Metabolic characteristics

Isolate CP.B2^T grew chemolithotrophically under microaerobic conditions utilizing H_2, sulfur compounds, and CO_2 as sole carbon source up to maximum concentration of 10^9 cells per ml (Figure 29), with a doubling time of 230 min (Figure 30). H_2 could not be replaced by other electron donors such as S^0 and $S_2O_3^{2-}$ under anaerobic (either 100% N_2 supplemented with organic substrates or 80% N_2/ 20% CO_2) or microaerobic (either 94% N_2/ 6% O_2 with organic carbon sources or 79% N_2/ 16% CO_2/ 5% O_2) conditions as energy source. However, growth of CP.B2^T was dependent on either S^0 or $S_2O_3^{2-}$ in the medium. Reduction of the sulfur compounds could not be detected in significant quantities (< 9 µM sulfide in comparison to > 4 mM sulfide obtained for CP.B1 cultures) indicating that S^0 and $S_2O_3^{2-}$ were not involved in dissimilative metabolism. The strain was not capable of using any of the nine tested electron acceptors in sulfur-limited basal MSH medium providing around 16 mM SO_4^{2-} for assimilation. None of the tested organic compounds was utilized (Appendix F: Table 26). In all growth experiments, *S. azorense* cultures were used as control to verify that all solutions and methods were working correctly.

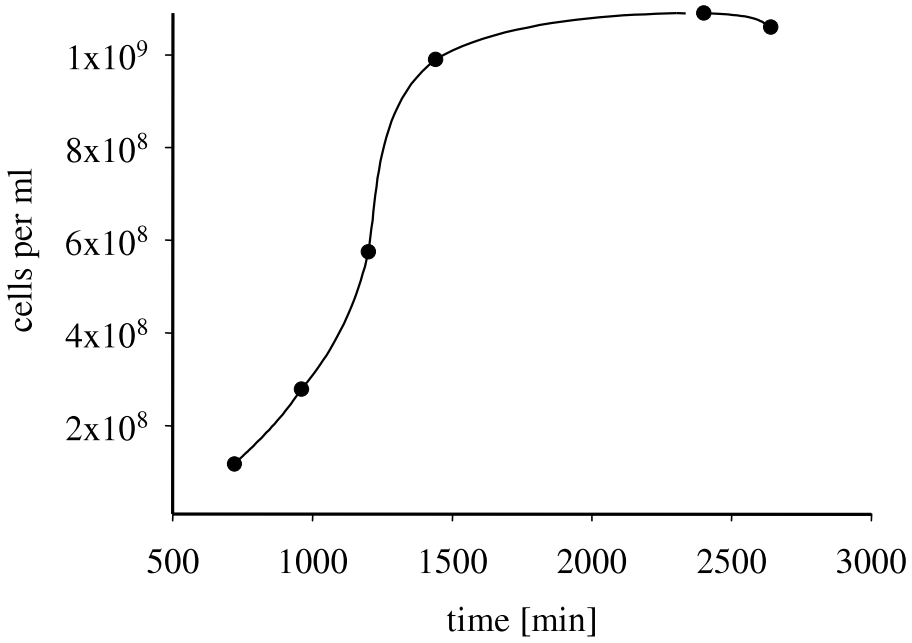

Figure 29. Growth curve observed for CP.B2^T cells.

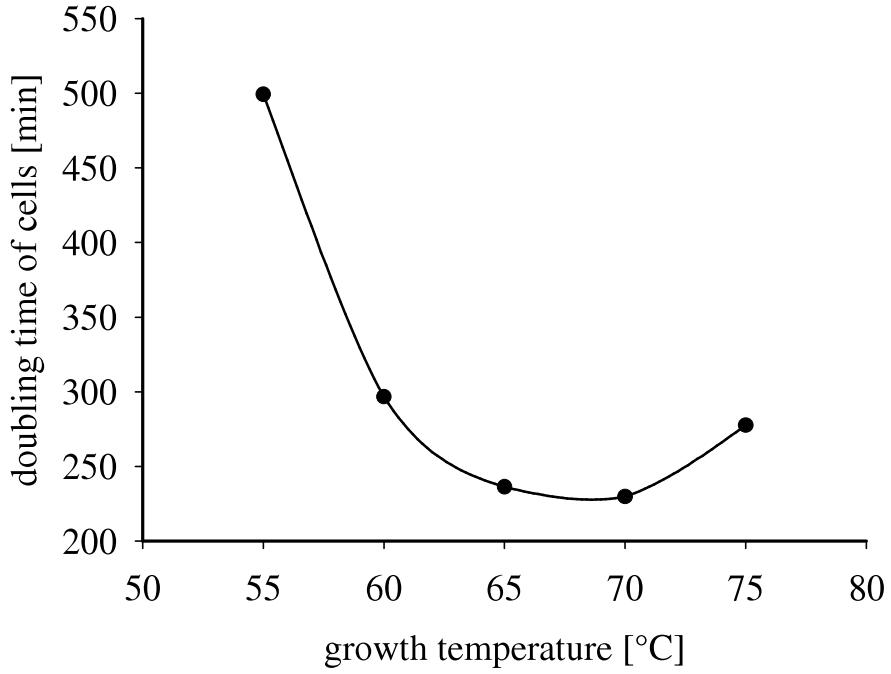

Figure 30. Effect of temperature on the growth of CP.B2^T cells. Doubling times were calculated from the slopes of the corresponding growth curves after 21 hours incubation. Microbial growth at 45° C and 50° C was only observed after one week.

5.4.5 Arsenic and antimony ion susceptibility tests

The novel strain was tested for tolerance to elevated arsenic and antimony ion levels. The isolate displayed growth in the presence of up to 8 mM $NaAsO_2$ and 15 mM $KSbOC_4H_4O_6.0.5H_2O$, respectively, and in the presence of more than 20 mM $Na_2HAsO_4.7H_2O$. However, growth was not observed when $NaAsO_2$ and Na_2HAsO_4 were provided as the sole electron donor and acceptor pair.

Amplification of the genes *arsA* and *arsB* were positive for the isolates CP.B2^T and CP.B3, whereas no amplification in CP.B2^T and non-specific amplification in CP.B3 occurred using the *arsC* primers (Figure 31). None of the studied *ars* genes could be detected in CP.B1 by PCR. The result obtained for CP.B1 either indicates that genes confer arsenic resistance, which have different sequences from those *ars* genes, which have been described to date, or that another mechanisms as described in Chapter 1 might be responsible for arsenic resistance and tolerance. However, in initial arsenic ion susceptibility assays no growth was

observed for CP.B1 cells in the presence of 500 µM $NaAsO_2$ and $Na_2HAsO_4.7H_2O$ reflecting that the absence of an efflux system might be responsible for the observed sensitivity to arsenic species.

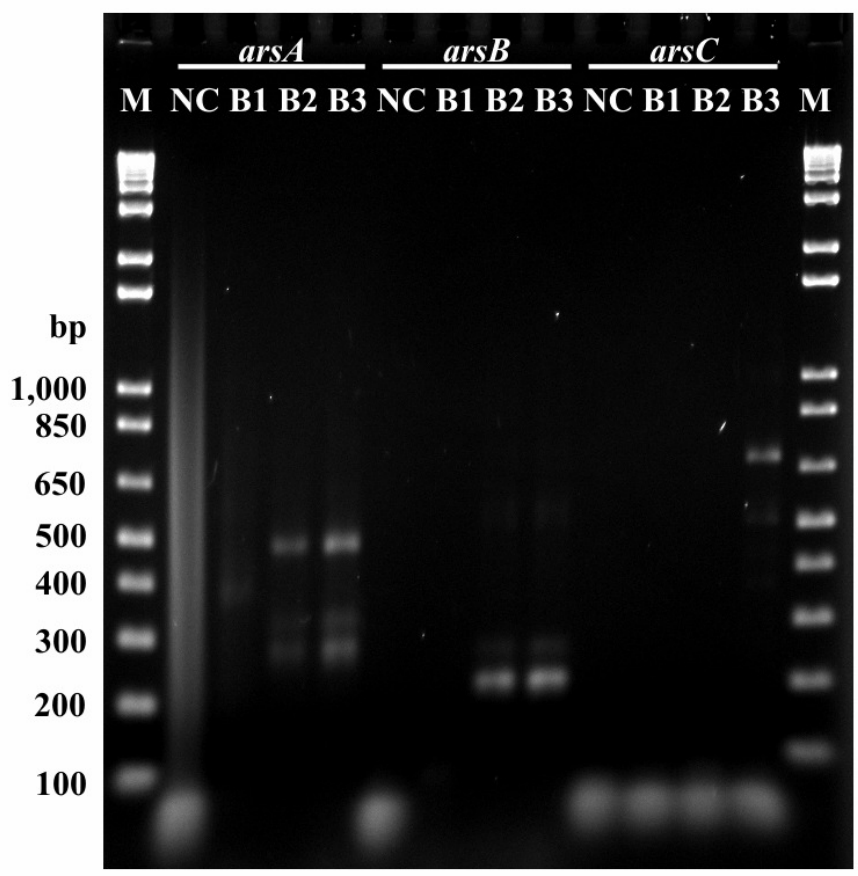

Figure 31. Image of ethidium bromide-stained 1.75% agarose gels displaying *ars* gene fragments from CP.B1, CP.B2^T and CP.B3. Abbreviations: M, 1 Kb Plus DNA ladder (Invitrogen); NC, negative control; B1, CP.B1; B2, CP.B2; and B3, CP.B3. Expected amplicon sizes were 300 bp (*arsA*), 259 bp (*arsB*), and 341 (*arsC*).

5.4.6 Genotypic characteristics

The G+C content of DNA isolated from strain CP.B2^T was 29.3 mol%. The amplified 16S rRNA gene sequence for CP.B2^T consisting of 1506 bases had a G+C content of 57.5 mol%.

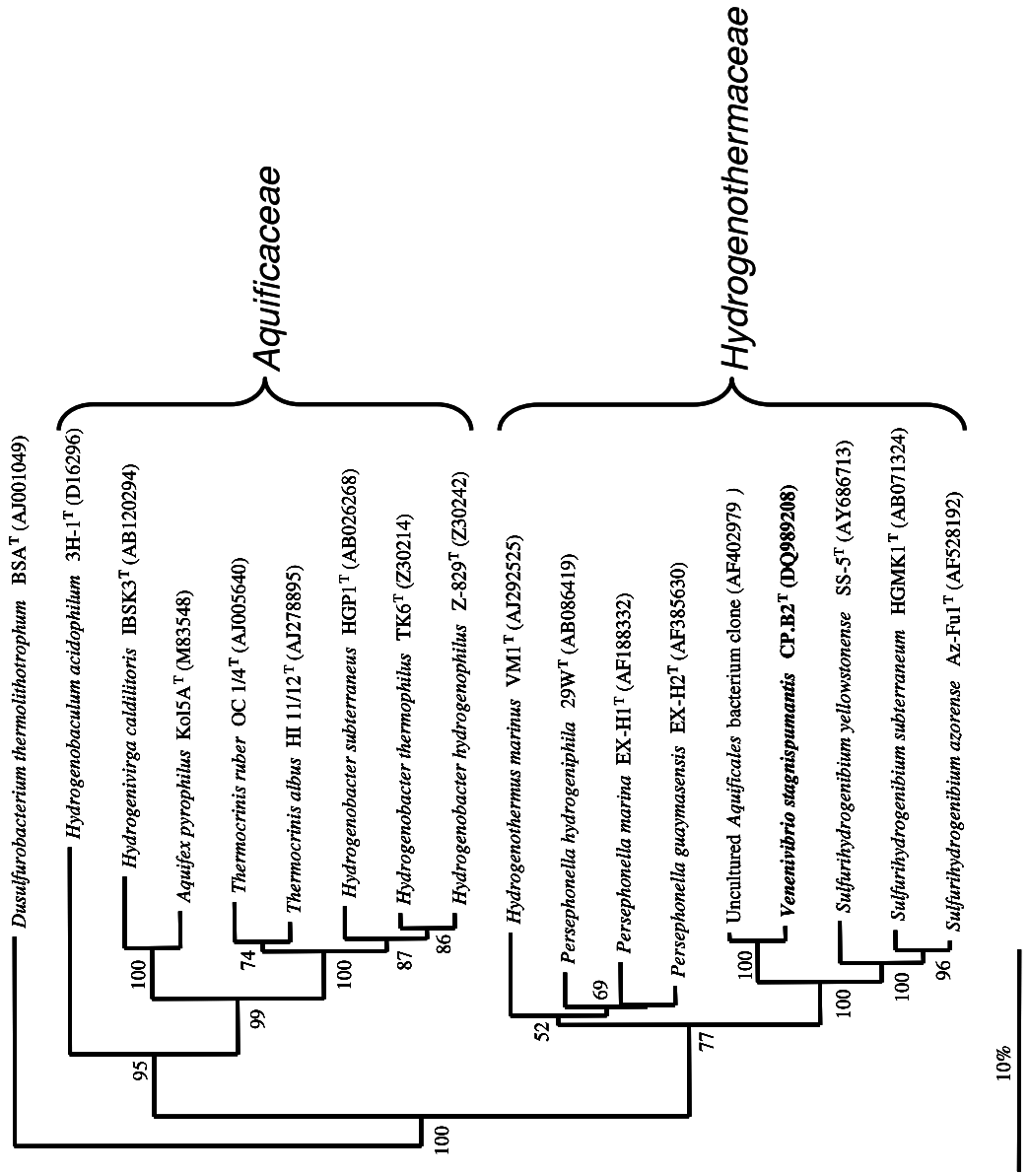

Figure 32. Phylogenetic tree based on analysis of the 16S rRNA gene sequences of strain CP.B2^T, and closely related species of the *Aquificales* showing the position of the novel isolate. The dendogram was produced with DNADIST (Neighbor joining) analysis using 1158 base pairs of aligned sequence. The phylogenetic tree was rooted to *Methanococcus jannaschii* JAL-1^T (M59126). Bootstrap values above 65% are shown from 1000 replicates. Bar, 10% sequence divergence.

Phylogenetic analysis based on 16S rRNA gene sequence indicates that CP.B2^T is closely related to previously described species of the order *Aquificales* (Figure 32). This evidence was supported by amplification of the DNA polymerase I (PolA) gene distinctive of members of the *Aquificales* by PCR (Figure 33 A). Within the order *Aquificales*, CP.B2^T displayed the greatest 16S rRNA gene

sequence similarity (94%) with *S. azorense* (Aguiar *et al.*, 2004). However, RAPD profiles generated for both of the related strains were able to differentiate among the species (Figure 33 B). The results presented indicate that CP.B2^T represents a novel genus within the *Aquificales* order.

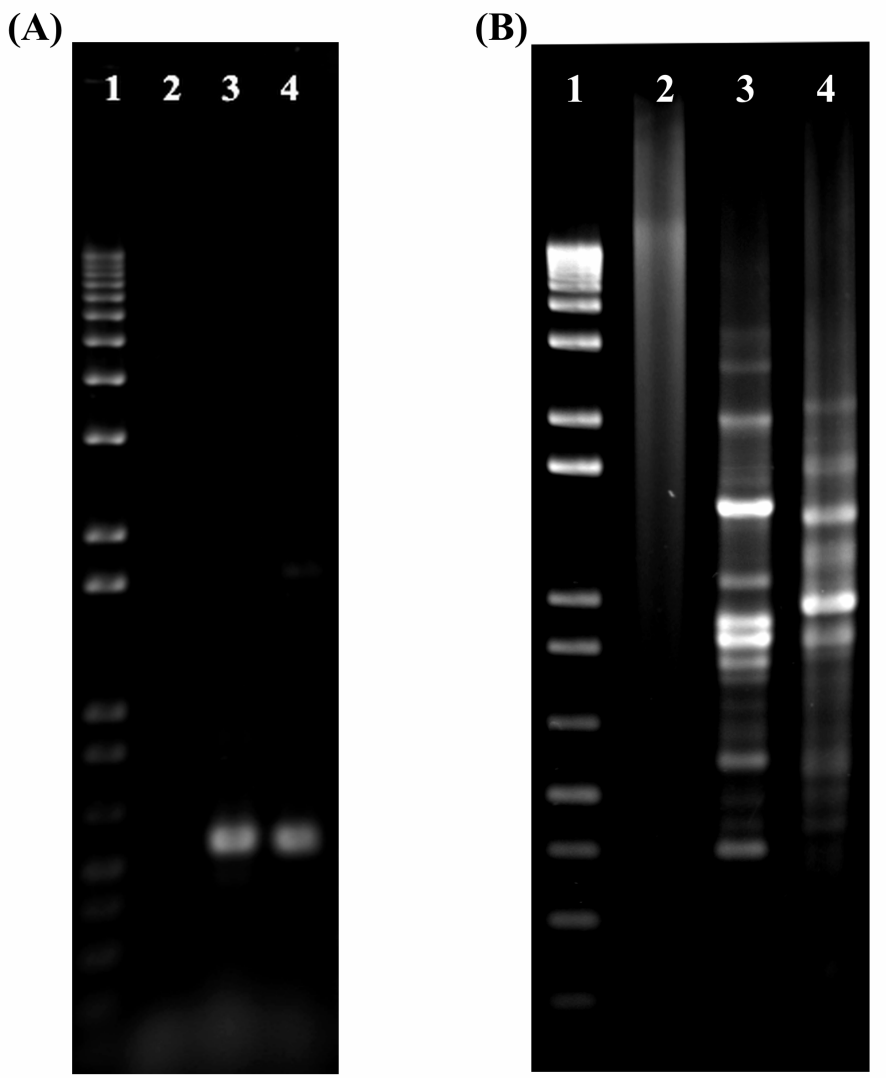

Figure 33. Image of ethidium bromide-stained 1.5% agarose gels. (A) displays the 500 bp gene fragment of *polA* amplified from CP.B2^T and *S. azorense*. Lanes: 1, 1 Kb Plus DNA ladder (Invitrogen); 2, negative control; 3, CP.B2^T; 4, *S. azorense* as positive control. (B) RAPD profiles generated from genomic DNA of CP.B2^T and *S. azorense* cells using primer OPR13. Lanes: 1, 1 Kb Plus DNA ladder; 2, negative control; 3, CP.B2^T; 4, *S. azorense*.

5.5 Discussion and proposal of a novel genus

A novel thermophilic bacterium was isolated from the geothermal Champagne Pool in Waiotapu, New Zealand. The new isolate CP.B2^T grew chemolithotrophically under microaerophilic conditions with H_2 as electron donor and O_2 as electron acceptor. Sulfur compounds, such as S^0 or $S_2O_3^{2-}$, were required for growth. Because of its 16S rRNA gene sequence and the molecular signature of the DNA polymerase I, the isolate belongs to the *Aquificales* order.

Phylogenetic analysis based on 16S rRNA gene sequences (Figure 32) indicates that it is closely related to the genus *Sulfurihydrogenibium*. However, the new isolate differs markedly in genotypic and metabolic properties from members of the genus *Sulfurihydrogenibium* and other genera of the order *Aquificales*. Although the physiological properties of the novel isolate largely resemble those of the genus *Sulfurihydrogenibium* (Table 14), the pH range is slightly limited and the optimal pH value is lower. This might be explained due to adaptation to its environment. Champagne Pool spring water is buffered by a constant flow of CO_2 keeping the pH value stable at around pH 5.5.

The low G+C content distinguishes it from previously described species of the genera *Sulfurihydrogenibium*, *Persephonella*, and *Hydrogenothermus.* This is the lowest G+C content reported for a species belonging to the *Aquificales* order. Strain CP.B2^T grew chemolithotrophically under microaerobic conditions utilizing H_2 as electron donor, O_2 as electron acceptor, and CO_2 as carbon source. Growth was dependent on S^0 or $S_2O_3^{2-}$, but no growth occurred when the sulfur compounds replaced H_2 as potential electron donor or O_2 as potential electron acceptor. *Sulfurihydrogenibium* species (Aguiar *et al.*, 2004; Nakagawa *et al.*, 2005; Takai *et al.*, 2003b), *Persephonella marina* and *P. guaymasensis* (Götz *et al.*, 2002a) are able to use sulfur compounds either as electron donors or acceptors. The hydrogen-oxidizing bacterium *H. marinus* requires elemental sulfur for growth for biosynthesis of sulfur-containing compounds (Stöhr *et al.*, 2001a), but like CP.B2^T cannot use it as potential electron donor. However, *H. marinus* can be clearly separated from CP.B2^T. As the name indicates, *H. marinus* is of marine origin, therefore adapted to higher NaCl concentrations (Table 14).

The novel isolate displayed tolerance to relatively high concentrations of arsenic and antimony compounds. CP.B2 cells grew in the presence of up to 8 mM $NaAsO_2$, 15 mM $KSbOC_4H_4O_6.0.5H_2O$ and more than 20 mM $Na_2HAsO_4.7H_2O$. Those concentrations are considerably higher compared to levels found in Champagne Pool spring water but lower compared to levels found in the sediment (Jones *et al.*, 2001): 5.3 ppm (70 µM) As and 3.5 ppb (29 nM) Sb in the pool water and 16,700 ppm (223 mM) As and 15,600 ppm Sb (128 mM) in the orange sediment. PCR studies revealed the presence of *arsA* and *arsB* genes in $CB.B2^T$ (Figure 31) suggesting arsenic resistance was determined by an energy-dependent efflux system mediated by an ATPase.

The occurrence of the novel isolate might not be limited to Champagne Pool in Waiotapu. A 16S rRNA gene sequence identity of 98% was obtained between $CP.B2^T$ and a 16S rRNA gene clone (AF402979) amplified from an environmental DNA sample. The DNA sample was extracted from a hot spring in Rotorua, New Zealand (Sunna & Bergquist, 2003). Rotorua and Waiotapa are both located in the geothermally active Taupo Volcanic Zone indicating that $CP.B2^T$ and close relatives might be distributed within this zone. Water samples obtained from a hot spring on Fountain Flat (N 44° 33.671, W 110° 49.989) in Yellowstone National Park, USA, featuring similar chemophysical properties (75 °C, pH 6.2) as Champagne Pool were used for enrichment experiments in modified MSH medium. The enrichment cultures had different morphological characteristics compared to $CP.B2^T$ cells indicating that $CP.B2^T$ was not present. However, it cannot be ruled out that the novel isolate might be abundant outside New Zealand.

Taken together, the results indicate that $CP.B2^T$ represents a novel genus within the *Aquificales* order, for which the name *Venenivibrio stagnispumantis* gen. nov., sp. nov. is proposed.

Table 14. Comparison of properties of *Venenivibrio stagnispumantis* gen. nov., sp. nov. CP.B2^T with the related species *Sulfurihydrogenibium yellowstonense* (Nakagawa *et al.*, 2005), *S. azorense* (Aguiar *et al.*, 2004), *S. subterraneum* (Takai *et al.*, 2003b), *Persephonella guaymasensis* (Götz *et al.*, 2002a), and *Hydrogenothermus marinus* (Stöhr *et al.*, 2001a). In brackets are the optimal growth conditions.

Strain	*Venenivibrio stagnispumantis*	*Sulfurihydrogenibium yellowstonense*	*Sulfurihydrogenibium azorense*	*Sulfurihydrogenibium subterraneum*	*Persephonella guaymasensis*	*Hydrogenothermus marinus*
Source	Terrestrial hot spring	Terrestrial hot spring	Terrestrial hot spring	Subsurface gold mine	Hydrothermal vent	Hydrothermal area
T [° C]	45-75 (70)	55-78 (70)	50-73 (68)	40-70 (65)	60-80 (75)	45-80 (65)
pH	4.80-5.8 (5.4)	6.0-8.0 (7.5)	5.5-7.0 (6.0)	6.4-8.8 (7.5)	4.7-7.5 (6.0)	5.0-7.0
NaCl [% (w/v)]	0.0-0.8 (0.4)	0.0-0.6 (0.0)	0.0-0.25 (0.1)	0.0-4.8 (0.5)	1.0-4.5 (2.5)	0.5-6.0 (2-3)
Electron donor	H_2	S^0, $S_2O_3^{2-}$	H_2, S^0, $S_2O_3^{2-}$, SO_3^{2-}, Fe^{2+}, AsO_3^{3-}	H_2, S^0, $S_2O_3^{2-}$	H_2, S^0, $S_2O_3^{2-}$	H_2
Electron acceptor	O_2 (up to 10%),	O_2 (up to 18%)	O_2 (up to 9%), S^0, SO_3^{2-}, $Fe3^+$, SeO_4^{2-}, $AsO4^{3-}$	O_2, NO^{3-} Fe^{3+}, SeO_3^{2-} SeO_4^{2-}, $AsO4^{3-}$	O_2 (up to 11%), NO_3^-	O_2 (up to 8%)
G+C content [mol%]	29.3	32	33.6	31.3	37.4	43

5.5.1 Description of the genus *Venenivibrio* gen. nov.

Venenivibrio (Ve.ne.ni.vi'bri.o. L. neut. n. venenum, poison; N.L. masc. n. vibrio, that which vibrates; N.L. masc. n. Venenivibrio, the vibrio of poison).

Cells are slightly curved rods, motile and stain Gram-negative. No sporulation. Microaerophilic. Thermophilic. Strictly chemolithothrophic. Able to utilize molecular hydrogen as electron donor and oxygen as electron acceptor. Either elemental sulfur or thiosulfate is essential growth requirements. NaCl is not required for growth. G+C content of genomic DNA is around 29 mol%. On the basis of 16S rRNA gene sequence analysis the genus *Venenivibrio* is closely related to the genus *Sulfurihydrogenibium*. Species of the genus *Venenivibrio* occur in terrestrial geothermally heated freshwater systems.

The type species is *Venenivibrio stagnispumantis*.

5.5.2 Description of the species *Venenivibrio stagnispumantis* sp. nov.

Venenivibrio stagnispumantis (stag.ni.spu.man'tis. L. neut. n. stagnum, pool, L. pres. gen. part. spumantis of the bubbling one, N.L. stagnispumantis from the bubbling pool, referring to Champagne Pool).

Exhibits the following properties in addition to those described for the genus. Strain was isolated from the terrestrial hot spring, Champagne Pool, in Waiotapu, New Zealand. Slightly curved rods with a mean length of 1.30 ± 0.26 µm and a mean width of 0.37 ± 0.04 µm. Temperature range for growth is 45° C to 75° C with an optimum of 70° C. pH range is 4.8 to 5.8 with an optimum of 5.4. Sodium chloride concentration range is 0% (w/v) to 0.8% (w/v) and is not required for growth, optimum growth occurs at 0.4% (w/v). Tolerates oxygen in the range of 1% to 10% (v/v) and is essential for growth. The base composition of genomic DNA is 29.3 G+C mol%. The 16S rRNA gene exhibits 94% similarity to the sequence from *S. azorense*.

The type strain is CP.B2^T and has been deposited in the Japan Collection of

Microorganisms (JCM) and the German Collection of Microorganisms (DSM) under accession numbers JCM 14244^T and DSM 18763^T, respectively.

5.6 Acknowledgements

Research funding was provided by the New Zealand Foundation of Research, Science & Technology (Contract Number C05X0303: Extremophilic Microorganisms for Metal Sequestration from Aqueous Solutions). Special thanks to Richard Fulton for permission to take spring water and sediment samples from Champagne Pool, to Helen M. Turner for the electron microscopy preparation, to Anna-Louise Reysenbach for providing samples of *S. azorense* and to Craig Cary for spring water samples obtained from Yellowstone National Park (Permit issued by the National Park Service with support from the National Geographic Society). The help of Hans G. Trüper with naming the novel isolate is greatly appreciated.

Chapter 6: General discussion

Results of this study sought to provide insights into how thermophilic bacteria interact with metal and metalloid ions and to characterize microbial communities in a terrestrial hot spring rich in arsenic ions and compounds.

Initial cadmium ion susceptibility experiments determining minimal inhibition concentrations (MIC) for 46 thermophilic bacterial and archaeal representatives of to the genera *Aneurinibacillus*, *Anoxybacillus*, *Bacillus*, *Brevibacillus*, *Geobacillus*, and *Thermus* revealed relatively high MIC values for members of the genus *Geobacillus* and two different patterns of growth responses depending on the *Geobacillus* strain (Chapter 2). An increasing concentration of Cd^{2+} resulted either in a decrease of the maximum optical density (e.g. *G. thermocatenulatus*) or alternatively, in a delay of the onset of growth (e.g. *G. stearothermophilus). G. stearothermophilus* and *G. thermocatenulatus* displaying the same MIC value of 600 µM Cd^{2+}, but having different growth responses in the presence of Cd^{2+}, showed similar results in subsequent surface complexation models (SCMs) experiments. However, the variations in model parameters were too small to relate the different growth pattern observed at elevated concentrations of Cd^{2+} to different biosorption behaviors.

Experimental data from electrophoretic mobility, acid-base titration, and cadmium ion adsorption experiments obtained for the thermophilic bacteria *G. stearothermophilus* and *G. thermocatenulatus* were used to quantify the concentrations and deprotonation constants of binding sites and to determine the thermodynamic stability constants for the metal ion-bacteria complexes (Chapter 3). The developed SCMs incorporating the Donnan electrostatic model, which considers the cell wall as an ion-penetrable volume, whereas other electrostatic models only account for ion-impenetrable planar surfaces, predicted successfully proton and cadmium ion biosorption for *G. stearothermophilus* and *G. thermocatenulatus* as a function of pH and different bacteria-to-metal ion concentration ratios, which is a prerequisite for potential bioremediation

applications. In comparison to recently reported SCMs parameters obtained for mesophilic bacteria (Borrok *et al.*, 2004a; Borrok & Fein, 2005; Daughney & Fein, 1998; Daughney *et al.*, 2001; Yee & Fein, 2001), the results indicate different binding capacities for cadmium ions for both studied thermophilic *Geobacillus* strains. The developed SCMs implied cation complexation reactions dominantly occurring at one type of functional group with a deprotonation constants p*K* value of approximately 3.8. Consequently, it has been demonstrated that discrete one-binding site models could fully interpret the analytical data, which were in contrast to multi-binding models obtained for mesophilic species that have been studied to date.

Microbial biosorption influences the distribution and availability of metal ions in some natural and anthropogenic environments and might have potential to be utilized in waste management of metal- and metalloid-polluted water and soil. A better understanding of metal mobilization and demobilization in thermal settings must rely on more studies of metal ion surface complexation by bacterial and archaeal thermophiles in order to extend the knowledge that is solely based on SCMs obtained from mesophilic bacteria. It would be interesting to investigate if more thermophilic bacterial strains than the two studied *Geobacillus* strains differ from the generalized multi-site adsorption model which was proposed for mesophilic bacteria (Borrok *et al.*, 2005; Yee & Fein, 2001). Microorganisms which predominantly complex metal or metalloid ions at one binding site would be favored in potential bioremediation applications since for adsorption and desorption reactions less acid and base might be used, and therefore the process would be more time- and cost-effective. For large-scale applications microorganisms, which act as biosorbent, should permit simple cultivation in inexpensive available medium compounds, e.g. industrial waste products such as yeast, a by-product from fermentation processes.

Surveys of Champagne Pool, one of New Zealand's largest terrestrial hot springs exhibiting high concentrations of arsenic ions and compounds, have been mainly restricted to geological and geochemical descriptions as previously microbial culturing experiments failed. By a combination of first molecular analyses followed by culturing experiments, novel bacterial and archaeal species were

successfully isolated from Champagne Pool (Chapter 4). Low biomass concentration was indicated by relatively low recovered ATP and DNA yields and relatively low cell numbers ($5.6 \pm 0.5 \times 10^6$ cells per ml) obtained for Champagne Pool compared to other terrestrial hot springs in New Zealand. In toxicity assays using Champagne Pool water samples inoculated with *Bacillus caldotenax*, bacterial growth was only observed when the spring water was purged with N_2 or CO_2, or autoclaved prior to inoculation. These results suggest that a volatile component might be present within the hot spring inhibiting microbial growth. Toxic volatile methyl and hydride derivatives of arsenic and antimony (Hirner *et al.*, 1998) and H_2S have been previously reported for Champagne Pool.

Denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone library analyses revealed few dominant species, which were related to members of the genera *Nevskia*, *Paracoccus*, *Sulfurihydrogenibium*, *Sulfolobus*, and *Thermofilum*, and indicated *Sulfurihydrogenibium*-like populations to be highly abundant in Champagne Pool. These findings are in good agreement with previous reports which indicate the dominance of hydrogen-oxidizing bacteria in geothermal ecosystems in USA and Iceland (Skirnisdottir *et al.*, 2000; Spear *et al.*, 2005). Future work might involve further constructions of archaeal and bacterial 16S rRNA gene-based clone libraries of DNA obtained from Champagne Pool water, which could be compared to previous molecular results. This approach would establish if there are changes in microbial community structure, e.g. due to seasonal variability (this is unlikely, given the constancy of the pool environment, but cannot comprehensively be ruled out until further data is obtained) or contamination of the sampling site with material that was not originally from the pool (Champagne Pool has a large surface; and as a major tourist attraction, it is visited by many tourists). If 16S rRNA gene sequences distantly related to *Nevskia* and *Paracoccus* are still present in the terrestrial hot spring, appropriate media could be chosen to enrich these microorganisms.

From results of molecular analyses, media were selected to enrich hydrogen-oxidizing and sulfur-dependent microorganisms. Three novel species were isolated and analysis of the amplified 16S rRNA gene showed a close phylogenetic relationship of isolate CP.B1 to *Thermoanaerobacter*, of CP.B2 to

Sulfurihydrogenibium, and of CP.B3 to *Thermococcus*. Isolate CP.B2 represents a novel genus within the *Aquificales* order, for which the name *Venenivibrio stagnispumantis* gen. nov., sp. nov. is proposed (Chapter 5). The novel isolate displayed tolerance to relatively high concentrations of arsenic and antimony compounds, but growth was not observed when As^{3+} and As^{5+} were provided as the sole electron donor and acceptor pair. *V. stagnispumantis* grew in the presence of up to 8 mM As^{3+}, 15 mM Sb^{3+}, and more than 20 mM As^{5+}, which was considerably higher compared to concentrations found within the water column of the thermal spring Champagne Pool. Arsenic resistance was mediated by a two-component energy-dependent efflux system (ArsA and ArsB).

If time had permitted, Fluorescent *In Situ* Hybridization (FISH) or quantitative Polymerase Chain Reaction (qPCR) experiments would have been conducted in order to detect and enumerate particular phylogenetic groups within Champagne Pool. Fluorescently labelled oligonucleotide probes specific for *V. stagnispumantis* could support the molecular results, which suggest that this isolate is one of the more dominant species in the spring. Additionally it could be used to screen other geothermally active areas for the presence of *V. stagnispumantis* cells. The advantage of the FISH technique over other quantitative methods is the visualization of a specific target, which permits prediction of its spatial distribution, e.g. planktonic or sediment-associated. However, FISH experiment might be troublesome since sediment samples obtained from Champagne Pool cause strong background fluorescence. The qPCR technique allows faster procedure times than the FISH method and has been widely used to enumerate gene and transcript numbers within environmental samples. The reliability of this method is sensitive to biases during DNA extraction and PCR amplification, which may result in gene and transcript number variability. It has been also reported that quantification of low-copy genes was less accurate resulting in a higher portion of false-positive signals (Smith *et al.*, 2006), which might provide only semi-quantitative results for Champagne Pool, where it is expected the bacterium *V. stagnispumantis* dominates the microbial community. Therefore, less abundant microorganisms might be overrepresented in pPCR analysis.

Experiments are currently underway to characterize further isolate CP.B1 phenotypically and phylogenetically. Due to the absence of 'classical' *ars* genes in CP.B1, further studies might address changes at the transcriptome level (e.g. by differential display PCR) or the proteome level (e.g. by 2-dimensional difference electrophoresis) when CP.B1 cells are exposed to arsenic ions to determine the mode of resistance mechanisms.

References

Aguiar, P., Beveridge, T. J. & Reysenbach, A. L. (2004). *Sulfurihydrogenibium azorense*, sp. nov., a thermophilic hydrogen-oxidizing microaerophile from terrestrial hot springs in the Azores. *International Journal of Systematic and Evolutionary Microbiology* **54**, 33-39.

Ahmad, S. A., Sayed, S. U., Barua, S., Khan, M. H., Jalil, A., Hadi, S. A. & Talukder, H. K. (2001). Arsenic in drinking water and pregnancy outcomes. *Environmental Health Perspectives* **109**, 629-631.

Allison, J. D., Brown, D. S. & Novo-Gradac, K. J. (1991). MINTEQA2/PRODEFA2, A geochemical assessment model for environmental systems: Version 3.0 User's Manual, pp. EPA/600/603-691/021, p.106. Athens, Georgia: Environmental Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402.

Ash, C., Farrow, J. A. E., Wallbanks, S. & Collins, M. D. (1991). Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Letters in Applied Microbiology* **13**, 202-206.

Ballatori, N. (2002). Transport of toxic metals by molecular mimicry. *Environmental Health Perspectives* **110**, 689-694.

Benson, A. A. & Cooney, R. A. (1988). Antimony metabolites in marine algae. In *Organometallic Compounds in the Environment Principles and Reactions*, pp. 135-137. Edited by P. J. Craig & F. Glockling. Harlow: Longmans.

Beveridge, T. J. (1989). Role of cellular design in bacterial metal accumulation

and mineralization. *Annual Review of Microbiology* **43**, 147-171.

Beveridge, T. J. (1990). Mechanism of Gram variability in select bacteria. *Journal of Bacteriology* **172**, 1609-1620.

Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513-1523.

Bissen, M. & Frimmel, F. H. (2003). Arsenic – a Review. Part I: Occurrence, Toxicity, Speciation, Mobility. *Acta Hydrochimica et Hydrobiologica* **31**, 9–18.

Borrok, D., Fein, J. B. & Kulpa, C. F. (2004a). Proton and Cd adsorption onto natural bacterial consortia: testing universal adsorption behavior. *Geochimica et Cosmochimica Acta* **68**, 3231-3238.

Borrok, D., Fein, J. B., Tischler, M., O'Loughlin, E., Meyer, H., Liss, M. & Kemner, K. M. (2004b). The effect of acidic solutions and growth conditions on the adsorptive properties of bacterial surfaces. *Chemical Geology* **209**, 107-119.

Borrok, D., Turner, B. F. & Fein, A. B. (2005). A universal surface complexation framework for modeling proton binding onto bacterial surfaces in geologic settings. *American Journal of Science* **305**, 826-853.

Borrok, D. M. & Fein, J. B. (2005). The impact of ionic strength on the adsorption of protons, Pb, Cd, and Sr onto the surfaces of Gram negative bacteria: testing non-electrostatic, diffuse, and triple-layer models. *Journal of Colloid and Interface Science* **286**, 110-126.

Boyanov, M. I., Kelly, S. D., Kemner, K. M., Bunker, B. A., Fein, J. B. & Fowle, D. A. (2003). Adsorption of cadmium to *Bacillus subtilis* bacterial cell walls: A pH-dependent X-ray absorption fine structure spectroscopy study. *Geochimica et Cosmochimica Acta* **67**, 3299-3311.

Brock, T. D., Brock, K. M., Belly, R. T. & Weiss, R. L. (1972). *Sulfolobus* -

New genus of sulfur-oxidizing bacteria living at low pH and high-temperature. *Archiv für Mikrobiologie* **84**, 54-68.

Brock, T. D. (1986). Introduction: An overview of the thermophiles. In *Thermophiles: General, molecular and applied microbiology*, pp. 1-16. Edited by T. D. Brock. New York: Wiley & Sons.

Bruins, M. R., Kapil, S. & Oehme, F. W. (2000). Microbial resistance to metals in the environment. *Ecotoxicology and Environmental Safety* **45**, 198-207.

Bruins, M. R., Kapil, S. & Oehme, F. W. (2003). Characterization of a small plasmid (pMBCP) from bovine *Pseudomonas pickettii* that confers cadmium resistance. *Ecotoxicology and Environmental Safety* **54**, 241-248.

Burggraf, S., Olsen, G. J., Stetter, K. O. & Woese, C. R. (1992). A phylogenetic analysis of *Aquifex pyrophilus*. *Systematic and Applied Microbiology* **15**, 352 - 356.

Burnett, P.-G. G., Heinrich, H., Peak, P., Bremer, P. J., McQuillan, A. J. & Daughney, C. J. (2006). The effect of pH and ionic strength on proton adsorption by the thermophilic bacterium *Anoxybacillus flavithermus*. *Geochimica et Cosmochimica Acta* **70**, 1914-1927.

Chang, I. S., Groh, J. L., Ramsey, M. M., Ballard, J. D. & Krumholz, L. R. (2004). Differential expression of *Desulfovibrio vulgaris* genes in response to Cu(II) and Hg(II) toxicity. *Applied and Environmental Microbiology* **70**, 1847–1851.

Chatellier, X. & Fortin, D. (2004). Adsorption of ferrous ions onto *Bacillus subtilis* cells. *Chemical Geology* **212**, 209-228.

Cheeseman, K. H. & Slater, T. F. (1993). An introduction to free radical biochemistry. *British Medical Bulletin* **49**, 481-493.

Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B. L. & Hughson, F. M. (2002). Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**, 545-549.

Chien, A., Edgar, D. B. & Trela, J. M. (1976). Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *Journal of Bacteriology* **127**, 1550-1557.

Claessens, J., Behrends, T. & Van Cappellen, P. (2004). What do acid-base titrations of live bacteria tell us? A preliminary assessment. *Aquatic Sciences* **66**, 19-26.

Cline, J. D. (1969). Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnology and Oceanography* **14**, 454-458.

Cole, J. R., Chai, B., Marsh, T. L. & other authors (2003). The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Research* **31**, 442-443.

Corliss, J. B., Dymond, J., Gordon, L. I. & other authors (1979). Submarine thermal springs on the Galapagos Rift. *Science* **203**, 1073-1083.

Croal, L. R., Gralnick, J. A., Malasarn, D. & Newman, D. K. (2004). The genetics of geochemistry. *Annual Review of Genetics* **38**, 175-202.

Crowley, J. D., Traynor, D. A. & Weatherburn, D. C. (2000). Enzymes and proteins containing manganese: an overview. *Metal Ions in Biological Systems* **37**, 209-278.

Daughney, C. J. & Fein, J. B. (1998). The effect of ionic strength on the adsorption of H^+, Cd^{2+}, Pb^{2+}, and Cu^{2+} by *Bacillus subtilis* and *Bacillus licheniformis*: A surface complexation model. *Journal of Colloid and Interface Science* **198**, 53-77.

Daughney, C. J. & Fortin, D. (2001). Mineral adsorption by bacteria. In *Encyclopaedia of Surface and Colloid Science*, pp. 3430-3446. Edited by A. T. Hubbard. New York: Marcell Dekker Inc.

Daughney, C. J., Fowle, D. A. & Fortin, D. E. (2001). The effect of growth phase on proton and metal adsorption by *Bacillus subtilis*. *Geochimica et Cosmochimica Acta* **65**, 1025-1035.

de Ronde, C. E. J., Baker, E. T., Massoth, G. J., Lupton, J. E., Wright, I. C., Feely, R. A. & Greene, R. R. (2001). Intra-oceanic subduction-related hydrothermal venting, Kermadec volcanic arc, New Zealand. *Earth and Planetary Science Letters* **193**, 359-369.

de Ronde, C. E. J., Faure, K., Bray, C. J., Chappell, D. A. & Wright, I. C. (2003). Hydrothermal fluids associated with seafloor mineralization at two southern Kermadec arc volcanoes, offshore New Zealand. *Mineralium Deposita* **38**, 217-233.

Deckert, G., Warren, P. V., Gaasterland, T. & other authors (1998). The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* **392**, 353-358.

Degens, E. T. & Ross, D. A. (1969). *Hot brines and recent heavy metal deposits in the Red Sea*. New York: Springer Verlag.

Dempster, E. L., Pryor, K. V., Francis, D., Young, J. E. & Rogers, H. J. (1999). Rapid DNA extraction from ferns for PCR-based analyses. *Biotechniques* **27**, 66-68.

Donk, P. J. (1920). A highly resistant thermophilic organism. *Journal of Bacteriology* **5**, 373-374.

Duffus, J. H. (2002). "Heavy metals" - A meaningless term? (IUPAC technical report). *Pure and Applied Chemistry* **74**, 793-807.

Edwards, C. (1990). Thermophiles. In *Microbiology of extreme environments*. Edited by C. Edwards. New York: McGraw-Hill.

Ehrlich, L. E. (1995). *Geomicrobiology*, 3 edn. New York: Marcel Dekker.

Ellis, D. G., Bizzoco, R. L. W., Maezato, Y., Baggett, J. N. & Kelley, S. T. (2005). Microscopic examination of acidic hot springs of Waiotapu, North Island, New Zealand. *New Zealand Journal of Marine and Freshwater Research* **39**, 1001-1011.

Emerson, D. & Moyer, C. L. (2002). Neutrophilic Fe-oxidizing bacteria are abundant at the Loihi Seamount hydrothermal vents and play a major role in Fe oxide deposition. *Applied and Environmental Microbiology* **68**, 3085-3093.

Euzeby, J. P. (1997). List of bacterial names with standing in nomenclature: A folder available on the Internet. *International Journal of Systematic Bacteriology* **47**, 590-592.

Fein, J. B., Daughney, C. J., Yee, N. & Davis, T. A. (1997). A chemical equilibrium model for metal adsorption onto bacterial surfaces. *Geochimica et Cosmochimica Acta* **61**, 3319-3328.

Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Seattle, University of Washington, Department of Genetics.

Ferris, M. J., Muyzer, G. & Ward, D. M. (1996). Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Applied and Environmental Microbiology* **62**, 340-346.

Ford, T., Jay, J., Patel, A., Kile, M., Prommasith, P., Galloway, T., Sanger, R., Smith, K. & Depledge, M. (2005). Use of ecotoxicological tools to evaluate the health of New Bedford Harbor sediments: A microbial biomarker approach.

Environmental Health Perspectives **113**, 186-191.

Gadd, G. M. & Griffiths, A. J. (1978). Microorganisms and heavy metal toxicity. *Microbial Ecology* **4**, 303–317.

Gadd, G. M. (1988). Accumulation of metals by microorganisms and algae. In *Biotechnology - A Comprehensive Treatise in 8 Volumes*, pp. 401-433. Edited by H.-J. Rehm & G. Reed. Weinheim: VCH Publishers.

Gadd, G. M. (1990). Metal tolerance. In *Microbiology of extreme environments*, pp. 178-210. Edited by C. Edwards. New York: McGraw-Hill.

Gadd, G. M. (2000). Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. *Current Opinion in Biotechnology* **11**, 271-279.

Gadd, G. M. (2004). Microbial influence on metal mobility and application for bioremediation. *Geoderma* **122**, 109-119.

Gage, J. D. & Tyler, P. A. (1991). *Deep-seabiology: A natural history of organisms at the deep-sea floor*. Cambridge: Cambridge University Press.

Giggenbach, W. F., Sheppard, D. S., Robinson, B. W., Stewart, M. K. & Lyon, G. L. (1994). Geochemical structure and position of the Waiotapu geothermal field, New Zealand. *Geothermics* **23**, 599-644.

Gihring, T. M. & Banfield, J. F. (2001). Arsenite oxidation and arsenate respiration by a new *Thermus* isolate. *FEMS Microbiology Letters* **204**, 335-340.

Gilboa-Garber, N. (1971). Direct spectrophotometric determination of inorganic sulfide in biological materials and in other complex mixtures. *Analytical Biochemistry* **43**, 129-133.

Golovacheva, R. S., Loginova, L. G., Salikhov, T. A., Kolesnikov, A. A. & Zaitseva, G. N. (1975). A new thermophilic species, *Bacillus thermocatenulatus*

nov. spec. *Mikrobiologiya* **44**, 265-268.

Gonzalez, J. M., Sheckells, D., Viebahn, M., Krupatkina, D., Borges, K. M. & Robb, F. T. (1999). *Thermococcus waiotapuensis* sp. nov., an extremely thermophilic archaeon isolated from a freshwater hot spring. *Archives of Microbiology* **172**, 95-101.

Götz, D., Banta, A., Beveridge, T. J., Rushdi, A. I., Simoneit, B. R. T. & Reysenbach, A. (2002a). *Persephonella marina* gen. nov., sp. nov. and *Persephonella guaymasensis* sp nov., two novel, thermophilic, hydrogen-oxidizing microaerophiles from deep-sea hydrothermal vents. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1349-1359.

Götz, D., Banta, A., Beveridge, T. J., Rushdi, A. I., Simoneit, B. R. T. & Reysenbach, A. (2002b). *Persephonella marina* gen. nov., sp nov and *Persephonella guaymasensis* sp nov., two novel, thermophilic, hydrogen-oxidizing microaerophiles from deep-sea hydrothermal vents. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1349-1359.

Griffiths, E. & Gupta, R. S. (2004). Signature sequences in diverse proteins provide evidence for the late divergence of the order *Aquificales*. *International Microbiology* **7**, 41-52.

Griffiths, E. & Gupta, R. S. (2006). Molecular signatures in protein sequences that are characteristics of the phylum *Aquificae*. *International Journal of Systematic and Evolutionary Microbiology* **56**, 99-107.

Haas, J. R. (2004). Effects of cultivation conditions on acid-base titration properties of *Shewanella putrefaciens*. *Chemical Geology* **209**, 67-81.

Hall, J. L. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* **53**, 1-11.

Handley, P. S., Hargreaves, J. & Harty, D. W. (1988). Ruthenium red staining

reveals surface fibrils and a layer external to the cell wall in *Streptococcus salivarius* HB and adhesion deficient mutants. *Journal of General Microbiology* **134**, 3165-3172.

Harden, V. P. & Harris, J. O. (1953). The isoelectric point of bacterial cells. *Journal of Bacteriology* **65**, 198-202.

Hedenquist, J. W. & Henley, R. W. (1985). Hydrothermal eruptions in the Waiotapu geothermal system, New Zealand: Their origin, associated breccias and relation to precious metal mineralization. *Economic Geology* **80**, 1640-1668.

Hedenquist, J. W. (1986a). Geothermal systems in the Taupo Volcanic Zone: Their characteristics and relation to volcanism and mineralisation. *Bulletin of the Royal Society of New Zealand* **23**, 134-168.

Hedenquist, J. W. (1986b). Waiotapu geothermal field. In *Guide to the active epithermal (geothermal) systems and precious metal deposits of New Zealand, Monograph Series on Mineral Deposits*, pp. 65-79. Edited by R. W. Henley, J. W. Hedenquist & P. J. Roberts. Berlin-Stuttgart: Gebrüder Borntraeger.

Hirner, A. V., Feldmann, J., Krupp, E., Grumping, R., Goguel, R. & Cullen, W. R. (1998). Metal(loid)organic compounds in geothermal gases and waters. *Organic Geochemistry* **29**, 1765-1778.

Huber, H., Diller, S., Horn, C. & Rachel, R. (2002a). *Thermovibrio ruber* gen. nov., sp nov., an extremely thermophilic, chemolithoautotrophic, nitrate-reducing bacterium that forms a deep branch within the phylum *Aquificae*. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1859-1865.

Huber, H., Hohn, M. J., Rachel, R., Fuchs, T., Wimmer, V. C. & Stetter, K. O. (2002b). A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature* **417**, 63-67.

Huber, R., Wilharm, T., Huber, D. & other authors (1992). *Aquifex pyrophilus*

gen. nov., sp. nov. represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. *Systematic and Applied Microbiology* **15**, 340-351.

Huber, R., Eder, W., Heldwein, S., Wanner, G., Huber, H., Rachel, R. & Stetter, K. O. (1998). *Thermocrinis ruber* gen. nov., sp. nov., a pink-filament-forming hyperthermophilic bacterium isolated from Yellowstone National Park. *Applied and Environmental Microbiology* **64**, 3576-3583.

Huber, R. & Eder, W. (2001). *Aquificales*. In *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*. Edited by M. Dworkin. New York, http://link.springer-ny.com/link/service/books/10125/: release 3.8, Springer-Verlag.

Hucker, G. J. & Conn, H. J. (1927). Further studies on the methods of Gram staining. *New York state Agricultural Experiment Station Technical Bulletin No 128.*

Hudson, J. A. (1986). The taxonomy and ecology of the genus *Thermus*. Thermophile Research Unit, University of Waikato, Hamilton, New Zealand. PhD thesis.

Islam, F. S., Pederick, R. L., Gault, A. G., Adams, L. K., Polya, D. A., Charnock, J. M. & Lloyd, J. R. (2005). Interactions between the Fe(III)-reducing bacterium *Geobacter sulfurreducens* and arsenate, and capture of the metalloid by biogenic Fe(II). *Applied and Environmental Microbiology* **71**, 8642-8648.

Jeanthon, C. (2000). Molecular ecology of hydrothermal vent microbial communities. *Antonie van Leeuwenhoek* **77**, 117-133.

Jenkins, R. O., Forster, S. N. & Craig, P. J. (2002). Formation of methylantimony species by an aerobic prokaryote: *Flavobacterium sp. Archives of Microbiology* **178**, 274-278.

Johnson, J. L. (1994). Similarity analysis of rRNAs. In *Methods for general and molecular bacteriology*, pp. 683-700. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, D.C: American Society for Microbiology.

Jones, B., Renaut, R. W. & Rosen, M. R. (1999). Actively growing siliceous oncoids in the Waiotapu geothermal area, North Island, New Zealand. *Journal of the Geological Society* **156**, 89-103.

Jones, B., Renaut, R. W. & Rosen, M. R. (2001). Biogenicity of gold- and silver-bearing siliceous sinters forming in hot (75 degrees C) anaerobic spring-waters of Champagne Pool, Waiotapu, North Island, New Zealand. *Journal of the Geological Society* **158**, 895-911.

Karl, D. M. (1995). Ecology of free-hydrothermal vent microbial communities. In *The microbiology of deep-sea hydrothermal vents*, pp. 35-124. Edited by D. M. Karl. Boca Raton: CRC Press.

Kashefi, K. & Lovley, D. R. (2003). Extending the upper temperature limit for life. *Science* **301**, 934.

Kawasumi, T., Igarashi, Y., Kodama, T. & Minoda, Y. (1984). *Hydrogenobacter thermophilus* gen. nov., sp. nov. an extremely thermophilic, aerobic, hydrogen-oxidizing cacterium. *International Journal of Systematic Bacteriology* **34**, 5-10.

Kelly, R. M. & Adams, M. W. W. (1994). Metabolism In hyperthermophilic microorganisms. *Antonie van Leeuwenhoek* **66**, 247-270.

Kisker, C., Schindelin, H. & Rees, D. C. (1997). Molybdenum-cofactor-containing enzymes: structure and mechanism. *Annual Review of Biochemistry* **66**, 233-267.

Knauer, K., Ahner, B., Xue, H. B. & Sigg, L. (1998). Metal and phytochelatin content in phytoplankton from freshwater lakes with different metal

concentrations. *Environmental Toxicology and Chemistry* **17**, 2444-2452.

Kobayashi, M. & Shimizu, S. (1999). Cobalt proteins. *European Journal of Biochemistry* **261**, 1-9.

Köhler, M. & Völsgen, F. (1997). *Geomikrobiologie: Grundlagen und Anwendung*. Weinheim: Wiley-VCH.

Konings, W. N., Albers, S. V., Koning, S. & Driessen, A. J. M. (2002). The cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. *Antonie van Leeuwenhoek* **81**, 61-72.

Kroll, R. G. & Booth, I. R. (1983). The relationship between intracellular pH, the pH gradient and potassium transport in *Escherichia coli*. *Biochemical Journal* **216**, 709-716.

L'Haridon, S., Cilia, V., Messner, P., Raguenes, G., Gambacorta, A., Sleytr, U. B., Prieur, D. & Jeanthon, C. (1998). *Desulfurobacterium thermolithotrophum* gen. nov., sp. nov., a novel autotrophic, sulphur-reducing bacterium isolated from a deep-sea hydrothermal vent. *International Journal Of Systematic Bacteriology* **48**, 701-711.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic acid techniques in bacterial systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. New York, N.Y.: John Willy and Sons.

Lane, T. W. & Morel, F. M. M. (2000). A biological function for cadmium in marine diatoms. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 4627-4631.

Langworthy, T. A. & Pond, J. L. (1986). Membranes and lipids of thermophiles. In *Thermophiles: General, molecular and applied microbiology*. Edited by T. D. Brock. New York: John Wiley & Sons.

Levitus, S. & Boyer, T. (1994). World Ocean Atlas, vol. 4: Temperature. Washington, D. C.: U.S. Department of Commerce.

Li, X. Z., Nikaido, H. & Williams, K. E. (1997). Silver-resistant mutants of *Escherichia coli* display active efflux of Ag+ and are deficient in porins. *Journal of Bacteriology* **179**, 6127-6132.

Lindsay, W. L. (1979). *Chemical equilibria in soils*. New York: John Wiley & Sons.

Llanos, J., Capasso, C., Parisi, E., Prieur, D. & Jeanthon, C. (2000). Susceptibility to heavy metals and cadmium accumulation in aerobic and anaerobic thermophilic microorganisms isolated from deep-sea hydrothermal vents. *Current Microbiology* **41**, 201-205.

Lloyd, E. F. (1959). The hot springs and hydrothermal eruptions of Waiotapu. *New Zealand Journal of Geology and Geophysics* **2**, 141-176.

Lovley, D. R. (1993). Dissimilatory metal reduction. *Annual Review of Microbiology* **47**, 263-290.

Ludwig, W., Strunk, O., Westram, R. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Research* **32**, 1363-1371.

Madigan, M. T., Martinko, J. M. & Parker, J. (2000). Brock: Biology of Microorganisms. Upper Saddle River, NJ: Prentice Hall.

Malik, A. & Jaiswal, R. (2000). Metal resistance in *Pseudomonas* strains isolated from soil treated with industrial wastewater. *World Journal of Microbiology and Biotechnology* **16**, 177-182.

Malouin, F., Chamberland, S., Brochu, N. & Parr Jr, T. R. (1991). Influence of growth media on *Escherichia coli* cell composition and ceftazidime susceptibility. *Antimicrobial Agents and Chemotherapy* **35**, 477–483.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Journal of Molecular Biology* **3**, 208-218.

Martin, W. (1999). Mosaic bacterial chromosomes: a challenge on route to a tree of genomes. *Bioessays* **21**, 99-104.

Matin, A. (1990). Keeping a neutral cytoplasm; the bioenergetics of obligate acidophiles. *FEMS Microbiology Review* **75**, 307-318.

McSwain, B. S., Irvine, R. L., Hausner, M. & Wilderer, P. A. (2005). Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. *Applied and Environmental Microbiology* **71**, 1051-1057.

Mendoza-Cozatl, D., Loza-Tavera, H., Hernandez-Navarro, A. & Moreno-Sanchez, R. (2005). Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, protists and plants. *FEMS Microbiology Review* **29**, 653-671.

Mesbah, M., Premachandran, U. & Whitman, W. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *International Journal of Systematic Bacteriology* **39**, 159-167.

Morita, R. Y. (1975). Psychrophilic bacteria. *Bacteriological Reviews* **39**, 144-167.

Mountain, B. W., Benning, L. G. & Boerema, J. A. (2003). Experimental studies on New Zealand hot spring sinters: rates of growth and textural development. *Canadian Journal of Earth Sciences* **40**, 1643-1667.

Mulks, M. H., Souza, K. A. & Boylen, C. W. (1980). Effect of restrictive temperature on cell wall synthesis in a temperature-sensitive mutant of *Bacillus stearothermophilus*. *Journal of Bacteriology* **144**, 413-421.

Muyzer, G., Dewaal, E. C. & Uitterlinden, A. G. (1993). Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. *Applied and Environmental Microbiology* **59**, 695-700.

Nakagawa, S., Nakamura, S., Inagaki, F., Takai, K., Shirai, N. & Sako, Y. (2004). *Hydrogenivirga caldilitoris* gen. nov., sp nov., a novel extremely thermophilic, hydrogen- and sulfur-oxidizing bacterium from a coastal hydrothermal field. *International Journal of Systematic and Evolutionary Microbiology* **54**, 2079-2084.

Nakagawa, S., Shtaih, Z., Banta, A., Beveridge, T. J., Sako, Y. & Reysenbach, A. L. (2005). *Sulfurihydrogenibium yellowstonense* sp. nov., an extremely thermophilic, facultatively heterotrophic, sulfur-oxidizing bacterium from Yellowstone National Park, and emended descriptions of the genus *Sulfurihydrogenibium*, *Sulfurihydrogenibium subterraneum* and *Sulfurihydrogenibium azorense*. *International Journal of Systematic and Evolutionary Microbiology* **55**, 2263-2268.

Nazina, T. N., Tourova, T. P., Poltaraus, A. B. & other authors (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrific*. *International Journal of Systematic and Evolutionary Microbiology* **51**, 433-446.

Niederberger, T. D. (2005). The microbial ecology and colonisation of surfaces in selected hot springs - a molecular- and culture-based approach. Thermophile Research Unit, University of Waikato, Hamilton, New Zealand. PhD thesis.

Niederberger, T. D., Gotz, D. K., McDonald, I. R., Ronimus, R. S. & Morgan, H. W. (2006). *Ignisphaera aggregans* gen. nov., sp. nov., a novel hyperthermorphilic crenarchaeote isolated from hot springs in Rotorua and Tokaanu, New Zealand. *International Journal of Systematic and Evolutionary Microbiology* **56**, 965-971.

Nies, D. H. (1999). Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology* **51**, 730-750.

Nies, D. H. (2003). Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiology Review* **27**, 313-339.

Oger, C., Mahillon, J. & Petit, F. (2003). Distribution and diversity of a cadmium resistance (CadA) determinant and occurrence of IS257 insertion sequences in staphylococcal bacteria isolated from a contaminated estuary (Seine, France). *FEMS Microbiology Ecology* **43**, 173-183.

Palmiter, R. D. (1998). The elusive function of metallothioneins. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8428-8430.

Pask-Hughes, R. A. & Williams, R. A. D. (1978). Cell envelope components of strains belonging to the genus *Thermus*. *Journal of General Microbiology* **107**, 65-72.

Paulsen, I. T. & Saier, M. H. (1997). A novel family of ubiquitous heavy metal ion transport proteins. *Journal of Membrane Biology* **156**, 99-103.

Phoenix, V. R., Renaut, R. W., Jones, B. & Ferris, F. G. (2005). Bacterial S-layer preservation and rare arsenic-antimony-sulphide bioimmobilization in siliceous sediments from Champagne Pool hot spring, Waiotapu, New Zealand. *Journal of the Geological Society* **162**, 323-331.

Pope, J. G., Brown, K. L. & McConchie, D. M. (2005). Gold concentrations in springs at Waiotapu, New Zealand: Implications for precious metal deposition in

geothermal systems. *Economic Geology* **100**, 677-687.

Porter, K. G. & Feig, Y. S. (1980). The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography* **25**, 943-948.

Price, R. C., Gamble, J. A., Smith, I. E. M., Stewart, R. B., Eggins, S. & Wnight, I. C. (2005). An integrated model for the temporal evolution of andesites and rhyolites and crustal development in New Zealand's North Island. *Journal of Volcanology and Geothermal Research* **140**, 1-24.

Rawlings, D. E. (2002). Heavy metal mining using microbes. *Annual Review of Microbiology* **56**, 65-91.

Reysenbach, A. L., Wickham, G. S. & Pace, N. R. (1994). Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. *Applied and Environmental Microbiology* **60**, 2113–2119.

Reysenbach, A. L. & Gotz, D. (2001). Methods for the study of hydrothermal vent microbes. In *Methods in Microbiology, Vol 30*, pp. 639-656.

Robinson, N. J., Whitehall, S. K. & Cavet, J. S. (2001). Microbial metallothioneins. *Annual Review of Biochemistry* **44**, 183-213.

Ronimus, R. (1993). Investigation into the control of genome topology in the archaeal isolate AN1. Thermophile Research Unit, University of Waikato, Hamilton, New Zealand. PhD thesis.

Rosen, B. P. (1996). Bacterial resistance to heavy metals and metalloids. *Journal of Biological Inorganic Chemistry* **1**, 273-277.

Saier, M. H. (2000). A functional-phylogenetic classification system for transmembrane solute transporters. *Microbiology and Molecular Biology Reviews* **64**, 354-411.

Schär-Zammaretti, P., Dillmann, M. L., D'Amico, N., Affolter, M. & Ubbink, J. (2005). Influence of fermentation medium composition on physicochemical surface properties of *Lactobacillus acidophilus*. *Applied and Environmental Microbiology* **71**, 8165-8173.

Scheffers, D. J. & Pinho, M. G. (2005). Bacterial cell wall synthesis: New insights from localization studies. *Microbiology and Molecular Biology Reviews* **69**, 585-607.

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological Reviews* **36**, 407-477.

Schultze-Lam, S., Thompson, J. B. & Beveridge, T. J. (1993). Metal ion immobilization by bacterial surfaces in freshwater environments. *Water Pollution Research Journal of Canada* **28**, 51-81.

Shumate, S. E. & Strandberg, G. W. (1985). Accumulation of metals by microbial cells. In *Comprehensive Biotechnology*, pp. 235-247. Edited by M. Moo-Young, C. N. Robinson & J. A. Howell. New York: Pergamon Press.

Sierra-Alvarez, R., Cortinas, I., Yenal, U. & Field, J. A. (2004). Methanogenic inhibition by arsenic compounds. *Applied and Environmental Microbiology* **70**, 5688-5691.

Silver, S. & Phung, L. T. (1996). Bacterial heavy metal resistance: New surprises. *Annual Review of Microbiology* **50**, 753-789.

Singh, S., Kayastha, A. M., Asthana, R. K., Srivastava, P. K. & Singh, S. P. (2001). Response of *Rhizobium leguminosarum* to nickel stress. *World Journal of Microbiology and Biotechnology* **17**, 667-672.

Skinner, F. A. (1968). The limits of microbial existence. *Proceedings of the Royal Society of London Series B* **171**, 77-89.

Skirnisdottir, S., Hreggvidsson, G. O., Hjorleifsdottir, S., Marteinsson, V. T., Petursdottir, S. K., Holst, O. & Kristjansson, J. K. (2000). Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. *Applied and Environmental Microbiology* **66**, 2835-2841.

Smith, C. J., Nedwell, D. B., Dong, L. F. & Osborn, A. M. (2006). Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environmental Microbiology* **8**, 804-815.

Smith, R. L. & Maguire, M. E. (1998). Microbial magnesium transport: unusual transporters searching for identity. *Molecular Microbiology* **28**, 217-226.

Sonnenfeld, E. M., Beveridge, T. J. & Doyle, R. J. (1985a). Discontinuity of charge on cell wall poles of *Bacillus subtilis*. *Canadian Journal of Microbiology* **31**, 875-877.

Sonnenfeld, E. M., Beveridge, T. J., Koch, A. L. & Doyle, R. J. (1985b). Asymmetric distribution of charge on the cell wall of *Bacillus subtilis*. *Journal of Bacteriology* **163**, 1167–1171.

Spada, S., Pembroke, J. T. & Wall, J. G. (2002). Isolation of a novel *Thermus thermophilus* metal efflux protein that improves *Escherichia coli* growth under stress conditions. *Extremophiles* **6**, 301-308.

Spear, J. R., Walker, J. J. & Pace, N. R. (2005). Hydrogen and primary production: Inference of biogeochemistry from phylogeny in a geothermal ecosystem. In *Geothermal biology and geochemistry in Yellowstone National Park*, pp. 113-127. Edited by W. P. Inskeep & T. R. McDermontt: Thermal Biology Institute, Montana State University.

Stetter, K. O. (1986). Diversity of extremely thermophilic archaebacteria. In *Thermophiles: General, molecular and applied microbiology*, pp. 39-74. Edited

by T. D. Brock. New York: Wiley & Sons.

Stetter, K. O. (1996). Hyperthermophilic prokaryotes. *FEMS Microbiology Review* **18**, 149-158.

Stöhr, R., Waberski, A., Volker, H., Tindall, B. J. & Thomm, M. (2001a). *Hydrogenothermus marinus* gen. nov., sp. nov., a novel thermophilic hydrogen-oxidizing bacterium, recognition of *Calderobacterium hydrogenophilum* as a member of the genus *Hydrogenobacter* and proposal of the reclassification of *Hydrogenobacter acidophilus* as *Hydrogenobaculum acidophilum* gen. nov., comb. nov., in the phylum '*Hydrogenobacter/Aquifex*'. *International Journal of Systematic and Evolutionary Microbiology* **51**, 1853-1862.

Stöhr, R., Waberski, A., Volker, H., Tindall, B. J. & Thomm, M. (2001b). *Hydrogenothermus marinus* gen. nov., sp nov., a novel thermophilic hydrogen-oxidizing bacterium, recognition of *Calderobacterium hydrogenophilum* as a member of the genus *Hydrogenobacter* and proposal of the reclassification of *Hydrogenobacter acidophilus* as *Hydrogenobaculum acidophilum* gen. nov., comb. nov., in the phylum '*Hydrogenobacter/Aquifex*'. *International Journal of Systematic and Evolutionary Microbiology* **51**, 1853-1862.

Stolz, J. E., Basu, P., Santini, J. M. & Oremland, R. S. (2006). Arsenic and selenium in microbial metabolism. *Annual Review of Microbiology* **60**, 107-130.

Stolz, J. F. & Oremland, R. S. (1999). Bacterial respiration of arsenic and selenium. *FEMS Microbiology Review* **23**, 615-627.

Straub, K. L., Benz, M. & Schink, B. (2001). Iron metabolism in anoxic environments at near neutral pH. *FEMS Microbiology Ecology* **34**, 181-186.

Sundaram, T. K. (1986). Physiology and growth of thermophilic bacteria. In *Thermophiles: General, molecular and applied microbiology*. Edited by T. D. Brock. New York: John Wiley & Sons.

Sunna, A. & Bergquist, P. (2003). A gene encoding a novel extremely thermostable 1,4-beta-xylanase isolated directly from an environmental DNA sample. *Extremophiles* **7**, 63-70.

Suzuki, M. T. & Giovannoni, S. J. (1996). Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied and Environmental Microbiology* **62**, 625-630.

Svetlichny, V. A., Sokolova, T. G., Zavarzin, G. A., Gerhardt, M. & Ringpfeil, T. (1991). *Carboxydothermus hydrogenoformans* gen. nov., sp. nov., a CO - utilizing thermophilic anaerobic bacterium from hydrothermal environments of Kunashir Island. *Systematics and Applied Microbiology* **14**, 254-260.

Takai, K., Kobayashi, H., Nealson, K. H. & Horikoshi, K. (2003a). *Sulfurihydrogenibium subterraneum* gen. nov., sp nov., from a subsurface hot aquifer. *International Journal of Systematic and Evolutionary Microbiology* **53**, 823-827.

Takai, K., Kobayashi, H., Nealson, K. H. & Horikoshi, K. (2003b). *Sulfurihydrogenibium subterraneum* gen. nov., sp. nov., from a subsurface hot aquifer. *International Journal of Systematic and Evolutionary Microbiology* **53**, 823-827.

Takai, K., Nakagawa, S., Sako, Y. & Horikoshi, K. (2003c). *Balnearium lithotrophicum* gen. nov., sp nov., a novel thermophilic, strictly anaerobic, hydrogen-oxidizing chemolithoautotroph isolated from a black smoker chimney in the Suiyo Seamount hydrothermal system. *International Journal of Systematic and Evolutionary Microbiology* **53**, 1947-1954.

Tansey, M. R. & Brock, T. D. (1972). The upper temperature limit for eukaryotic organisms. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 2426–2428.

Tivey, M. K. (1998). How to build a black smoker chimney: The formation of

mineral deposits at mid-ocean ridges. In *Oceanus*, pp. 22-26.

Udo, E. E., Jacob, L. E. & Mathew, B. (2000). A cadmium resistance plasmid, pXU5, in *Staphylococcus aureus*, strain ATCC25923. *FEMS Microbiology Letters* **189**, 79-80.

Unz, R. F. & Shuttleworth, K. L. (1996). Microbial mobilization and immobilization of heavy metals. *Current Opinion in Biotechnology* **7**, 307-310.

van der Wal, A., Minor, M., Norde, W., Zehnder, A. J. B. & Lyklema, J. (1997). Electrokinetic potential of bacterial cells. *Langmuir* **13**, 165-171.

Vecchio, A., Finoli, C., Di Simine, D. & Andreoni, V. (1998). Heavy metal biosorption by bacterial cells. *Fresenius Journal of Analytical Chemistry* **361**, 338-342.

Vig, K., Megharaj, M., Sethunathan, N. & Naidu, R. (2003). Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. *Advances in Environmental Research* **8**, 121-135.

Volesky, B. & Holan, Z. R. (1995). Biosorption of heavy metals. *Biotechnology Progress* **11**, 235-250.

von Wintzingerode, F., Gobel, U. B. & Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Review* **21**, 213-229.

Watt, R. K. & Ludden, P. W. (1999). Nickel-binding proteins. *Cellular and Molecular Life Sciences* **56**, 604-625.

Westall, J. C. (1982). FITEQL: A computer program for determination of chemical equilibrium constants from experimental data: Version 2.0: Department of Chemistry, Oregon State University.

Wetzel, R. G. & Likens, G. E. (2000). *Limnological Analysis*. New York: Springer-Verlag.

WHO (2004). *Guidelines for drinking-water quality: Volume 1 - Recommendations*, 3 edn. Geneva: World Health Organization.

Wightman, P. G., Fein, J. B., Wesolowski, D. J., Phelps, T. J., Benezeth, P. & Palmer, D. A. (2001). Measurement of bacterial surface protonation constants for two species at elevated temperatures. *Geochimica et Cosmochimica Acta* **65**, 3657-3669.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalsk, J. A. & Tingly, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535.

Woese, C. R., Kandler, O. & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4576–4579.

Xue, Y. F., Xu, Y., Liu, Y., Ma, Y. H. & Zhou, P. J. (2001). *Thermoanaerobacter tengcongensis* sp. nov., a novel anaerobic, saccharolytic, thermophilic bacterium isolated from a hot spring in Tengcong, China. *International Journal of Systematic and Evolutionary Microbiology* **51**, 1335-1341.

Yee, N. & Fein, J. (2001). Cd adsorption onto bacterial surfaces: A universal adsorption edge? *Geochimica et Cosmochimica Acta* **65**, 2037-2042.

Yee, N., Fowle, D. A. & Ferris, F. G. (2004). A Donnan potential model for metal sorption onto *Bacillus subtilis*. *Geochimica et Cosmochimica Acta* **68**, 3657-3664.

Yilmaz, E. I. (2003). Metal tolerance and biosorption capacity of *Bacillus*

circulans strain EB1. *Research in Microbiology* **154**, 409-415.

Zeigler, D. R. (2001). *Bacillus Genetic Stock Center, Catalog of Strains*, 7 edn.

Zheng, Z. W., Fang, W., Lee, H. Y. & Yang, Z. Y. (2005). Responses of *Azorhizobium caulinodans* to cadmium stress. *FEMS Microbiology Ecology* **54**, 455-461.

Zinoni, F., Birkmann, A., Stadtman, T. C. & Bock, A. (1986). Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 4650-4654.

Zobrist, J., Dowdle, P. R., Davis, J. A. & Oremland, R. S. (2000). Mobilization of arsenite by dissimilatory reduction of adsorbed arsenate. *Environmental Science and Technology* **34**, 4747-4753.

Appendix A: Growth curves

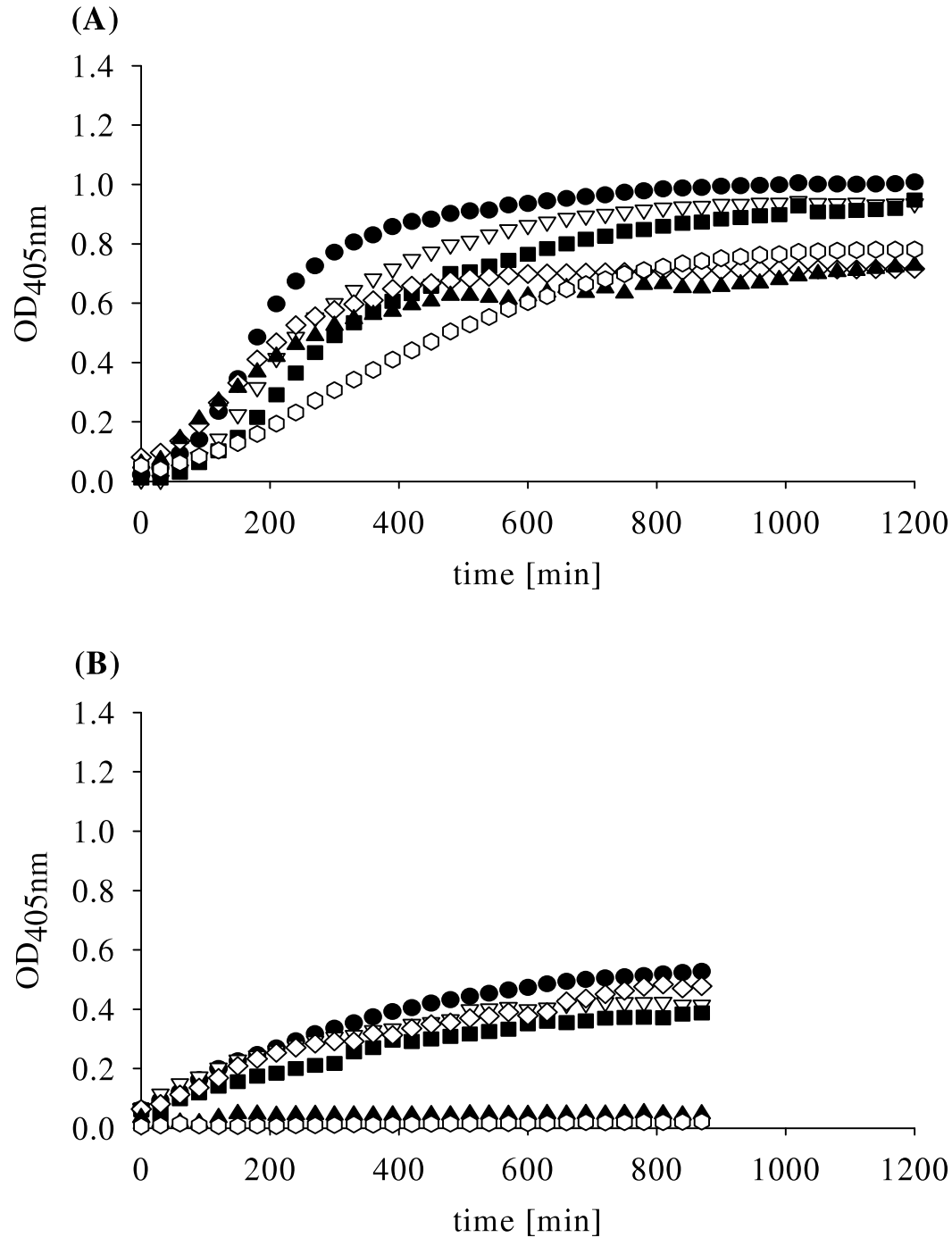

Figure 34. Microbial growth monitored spectrophotometrically at 405 nm as a function of concentration of Cd^{2+} (black circles: 0 µM, white triangles: 100 µM, black squares: 200 µM, white diamonds: 400 µM, black triangles: 600 µM, white hexagons: 800 µM). (A) Growth curves for *B. mycoides* (DSM 299) and (B) growth curves for *G. thermocatenulatus* (DSM 730). For clarity, only every second data point is shown.

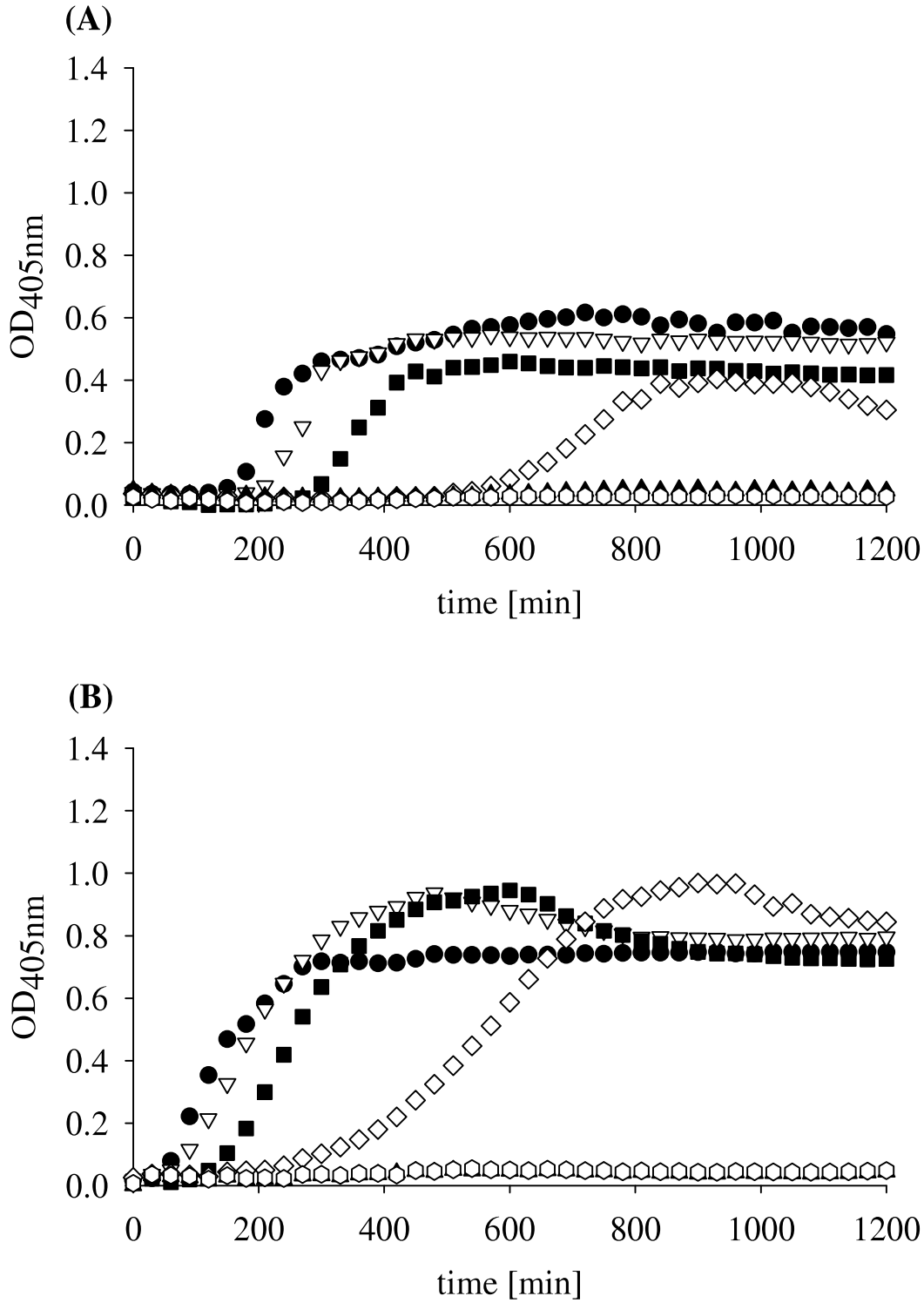

Figure 35. Microbial growth monitored spectrophotometrically at 405 nm as a function of concentration of Cd^{2+} (black circles: 0 µM, white triangles: 100 µM, black squares: 200 µM, white diamonds: 400 µM, black triangles: 600 µM, white hexagons: 800 µM). (A) Growth curves for *G. thermoglucosidasius* (DSM 2543) and (B) growth curves for TOK13.A4. For clarity, only every second data point is shown.

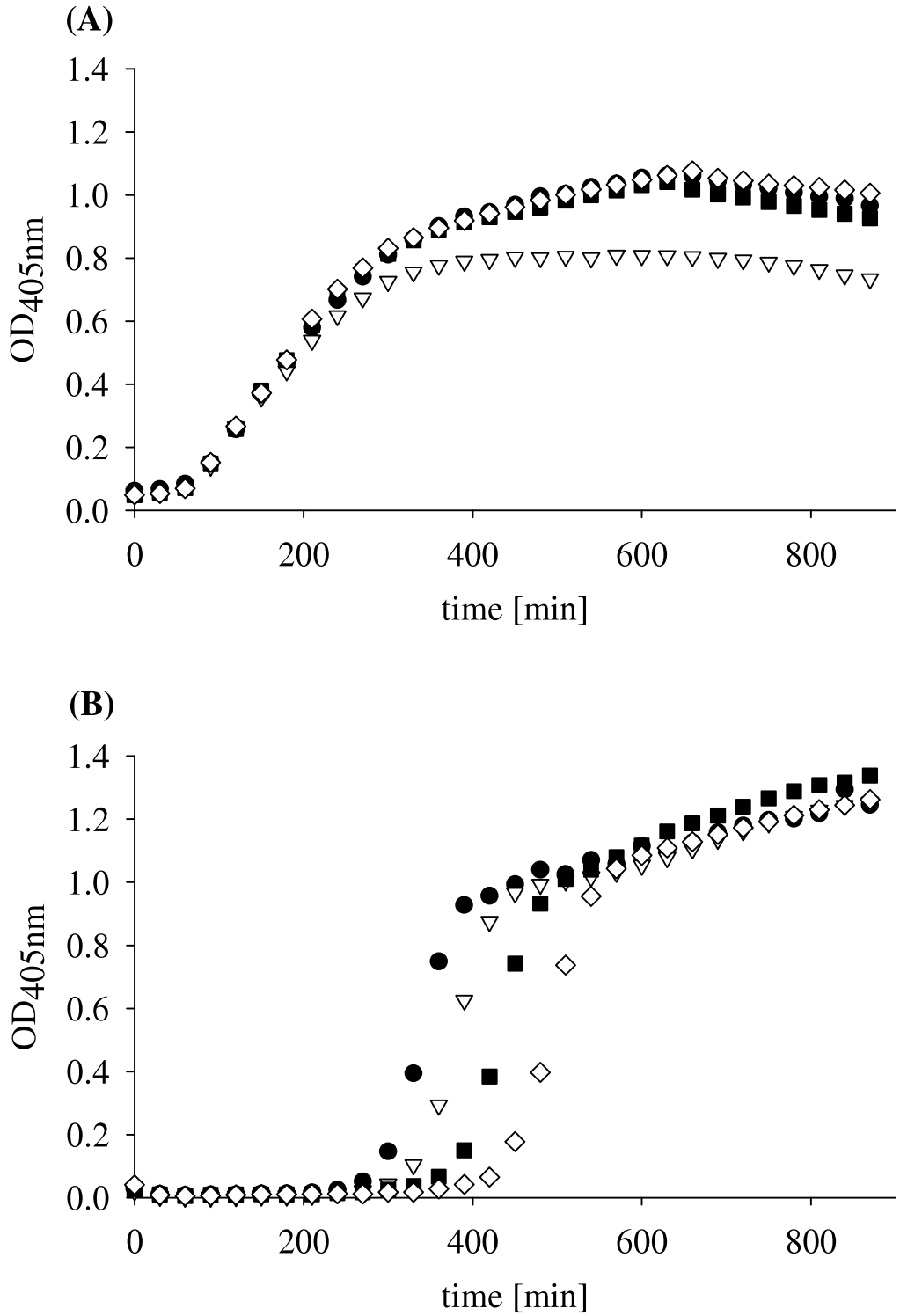

Figure 36. Microbial growth monitored spectrophotometrically at 405 nm as a function of concentration of As^{5+} (black circles: 0 µM, white triangles: 2 µM, black squares: 4 µM, white diamonds: 8 µM). (A) Growth curves for *G. thermoleovorans* (DSM 5366) and (B) growth curves for *G. stearothermophilus* (DSM 6790). For clarity, only every second data point is shown.

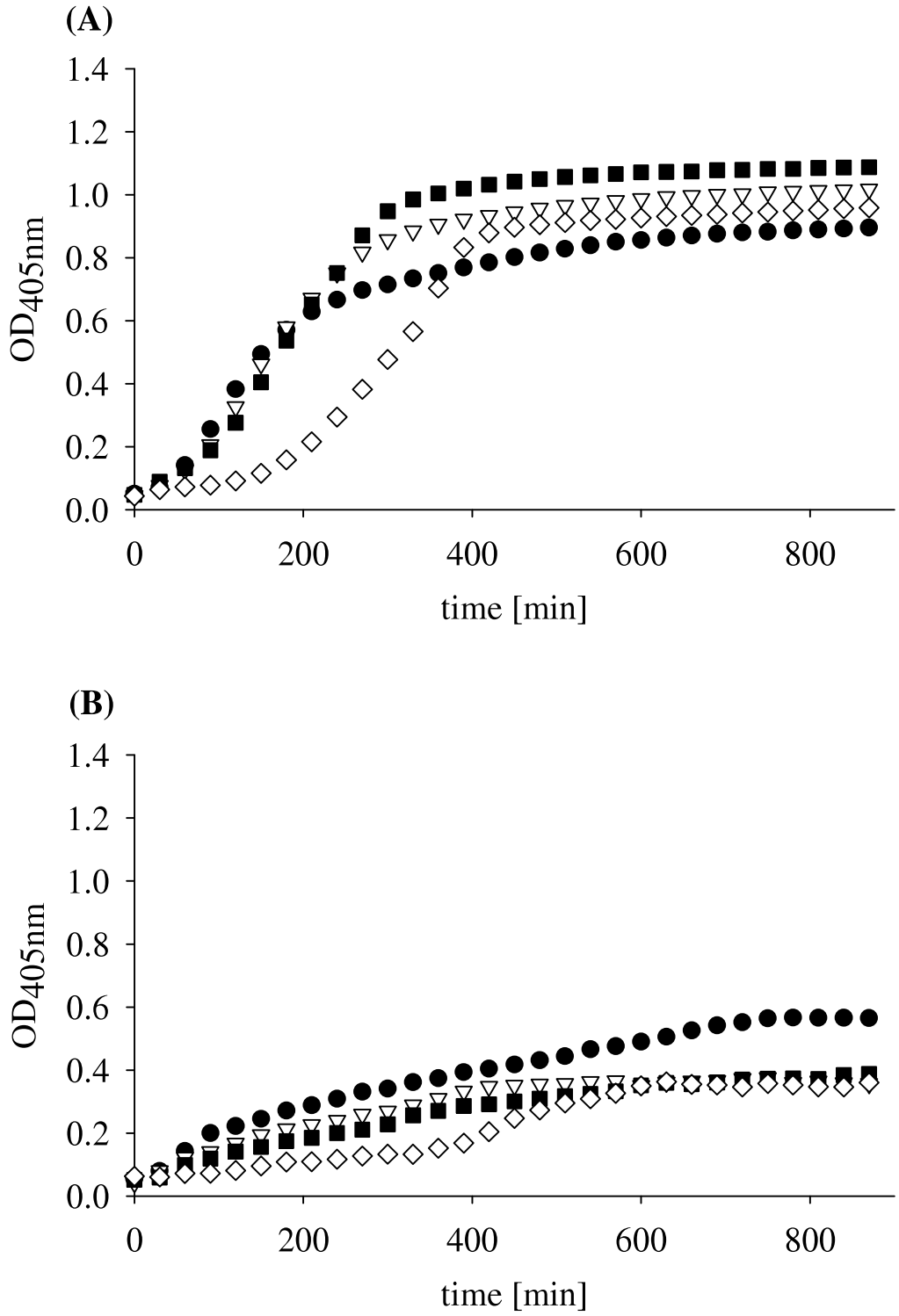

Figure 37. Microbial growth monitored spectrophotometrically at 405 nm as a function of concentration of As^{5+} (black circles: 0 µM, white triangles: 2 µM, black squares: 4 µM, white diamonds: 8 µM). (A) Growth curves for *B. mycoides* (DSM 299) and (B) growth curves for *G. thermocatenulatus* (DSM 730). For clarity, only every second data point is shown.

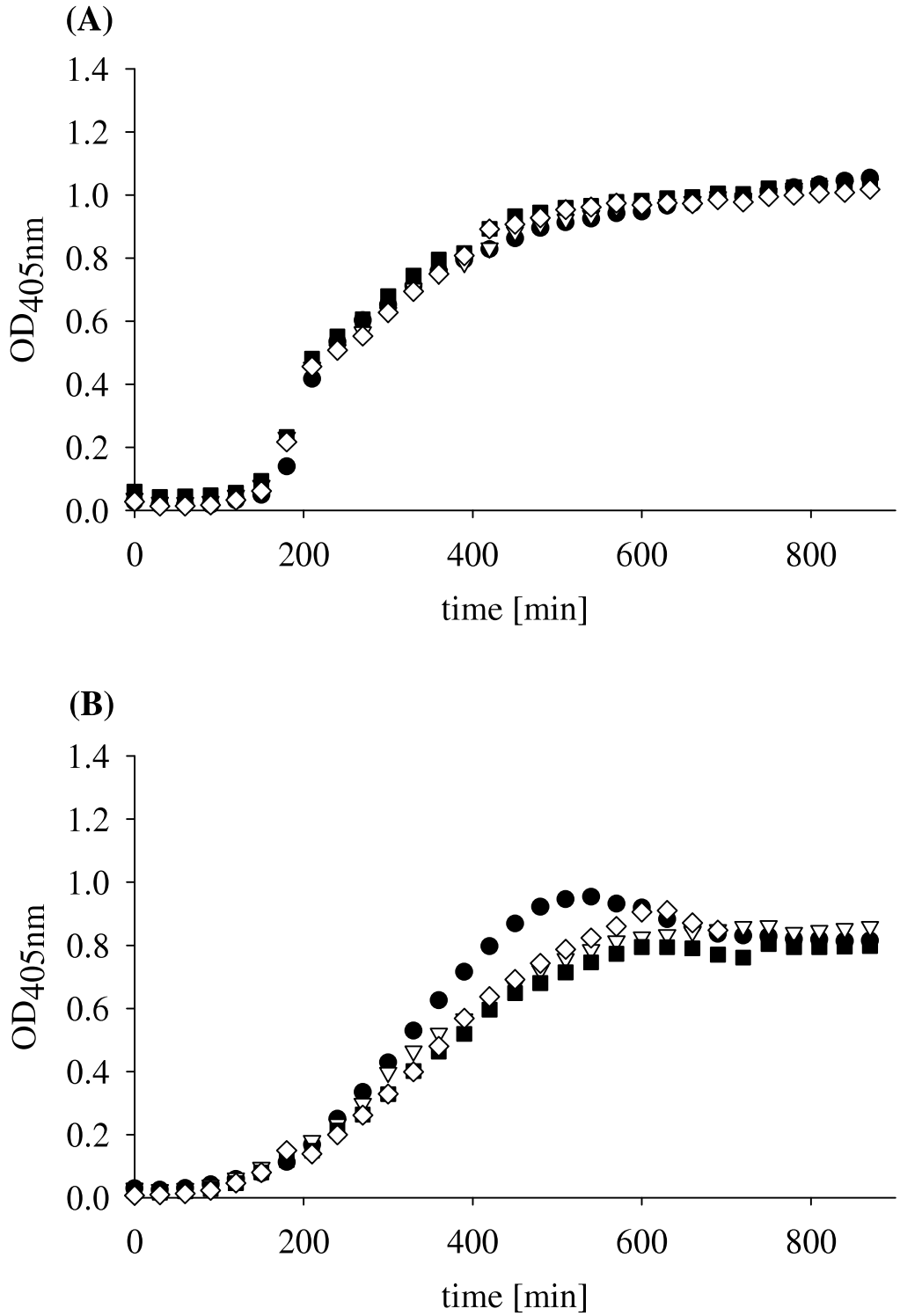

Figure 38. Microbial growth monitored spectrophotometrically at 405 nm as a function of concentration of As^{5+} (black circles: 0 µM, white triangles: 2 µM, black squares: 4 µM, white diamonds: 8 µM). (A) Growth curves for *G. thermoglucosidasius* (DSM 2543) and (B) growth curves for TOK13.A4. For clarity, only every second data point is shown.

Appendix B: One-site and two-site models

Table 15. One-site SCMs calculated for each individual dataset obtained from Cd^{2+} adsorption experiments using *G. thermocatenulatus* cells (DSM 730).

Dataset	pK_1	pK_2	pK_3	*V(Y)*
Set 1, subset A	1.608	-	-	0.077
Set 1, subset A	-	1.712	-	1.074
Set 1, subset A	-	-	2.123	1.193
Set 1, subset B	1.523	-	-	0.111
Set 1, subset B	-	1.506	-	2.876
Set 1, subset B	-	-	1.948	3.221
Set 1, subset C	1.295	-	-	0.235
Set 1, subset C	-	1.017	-	7.803
Set 1, subset C	-	-	1.438	1.127
Set 2, subset A	1.564	-	-	0.166
Set 2, subset A	-	1.352	-	1.218
Set 2, subset A	-	-	1.761	1.278
Set 2, subset B	1.221	-	-	1.855
Set 2, subset B	-	0.737	-	5.999
Set 2, subset B	-	-	1.168	7.575
Set 2, subset C	1.227	-	-	0.698
Set 2, subset C	-	0.756	-	3.183
Set 2, subset C	-	-	1.316	9.612
Set 3, subset A	1.154	-	-	0.996
Set 3, subset A	-	0.632	-	2.045
Set 3, subset A	-	-	1.025	2.141
Set 3, subset B	0.984	-	-	3.872
Set 3, subset B	-	0.408	-	6.548
Set 3, subset B	-	-	0.807	7.662
Set 3, subset C	1.137	-	-	1.569
Set 3, subset C	-	0.618	-	4.901
Set 3, subset C	-	-	1.056	6.982

Table 15. Continued.

Dataset	pK_1	pK_2	pK_3	*V(Y)*
Set 1	1.342	-	-	0.458
Set 1	-	1.091	-	4.114
Set 1	-	-	1.525	5.302
Set 2	1.231	-	-	1.076
Set 2	-	0.750	-	3.533
Set 2	-	-	1.237	6.261
Set 3	1.071	-	-	2.409
Set 3	-	0.520	-	4.778
Set 3	-	-	0.932	5.916
Set 1, 2 and 3	1.185	-	-	1.807
Set 1, 2 and 3	-	0.672	-	4.776
Set 1, 2 and 3	-	-	1.122	6.682

Table 16. One-site SCMs calculated for each individual dataset obtained from Cd^{2+} adsorption experiments using *G. stearothermophilus* cells (DSM 6790).

Dataset	pK_1	pK_2	pK_3	*V(Y)*
Set 1, subset A	2.458	-	-	0.742
Set 1, subset A	-	2.852	-	8.138
Set 1, subset A	-	-	2.904	8.457
Set 1, subset B	2.262	-	-	1.516
Set 1, subset B	-	2.367	-	17.930
Set 1, subset B	-	-	2.416	18.660
Set 1, subset C	2.280	-	-	0.949
Set 1, subset C	-	3.174	-	10.920
Set 1, subset C	-	-	3.302	14.830
Set 2, subset A	1.933	-	-	6.157
Set 2, subset A	-	1.544	-	9.526
Set 2, subset A	-	-	1.590	9.554
Set 2, subset B	2.221	-	-	2.112
Set 2, subset B	-	2.017	-	10.060
Set 2, subset B	-	-	2.067	10.270
Set 2, subset C	2.327	-	-	1.102
Set 2, subset C	-	2.573	-	14.010
Set 2, subset C	-	-	2.636	14.980
Set 3, subset A	2.120	-	-	3.357
Set 3, subset A	-	1.828	-	7.344
Set 3, subset A	-	-	1.876	7.387
Set 3, subset B	1.992	-	-	5.270
Set 3, subset B	-	1.664	-	11.260
Set 3, subset B	-	-	1.713	11.530
Set 3, subset C	2.234	-	-	2.278
Set 3, subset C	-	2.197	-	14.210
Set 3, subset C	-	-	2.250	14.820

Table 16. Continued.

Dataset	pK_1	pK_2	pK_3	*V(Y)*
Set 1	2.467	-	-	1.892
Set 1	-	2.883	-	18.190
Set 1	-	-	2.951	20.080
Set 2	2.278	-	-	6.973
Set 2	-	1.991	-	18.350
Set 2	-	-	2.038	18.720
Set 3	2.199	-	-	5.735
Set 3	-	1.909	-	13.180
Set 3	-	-	1.959	13.490
Set 1, 2 and 3	2.303	-	-	5.905
Set 1, 2 and 3	-	2.065	-	20.090
Set 1, 2 and 3	-	-	2.104	20.510

Table 17. Two-site SCMs generated for each individual dataset obtained from Cd^{2+} adsorption experiments using *G. thermocatenulatus* cells (DSM 730).

Dataset	pK_1	pK_2	pK_3	*V(Y)*
Set 1, subset A	N. C.[a]	N. C.	-	
Set 1, subset A	N. C.	-	N. C.	
Set 1, subset A	-	N. C.	N. C.	
Set 1, subset B	N. C.	N. C.	-	
Set 1, subset B	N. C.	-	N. C.	
Set 1, subset B	-	N. C.	N. C.	
Set 1, subset C	N. C.	N. C.	-	
Set 1, subset C	N. C.	-	N. C.	
Set 1, subset C	-	N. C.	N. C.	
Set 2, subset A	N. C.	N. C.	-	
Set 2, subset A	N. C.	-	N. C.	
Set 2, subset A	-	N. C.	N. C.	
Set 2, subset B	N. C.	N. C.	-	
Set 2, subset B	N. C.	-	N. C.	
Set 2, subset B	-	N. C.	N. C.	
Set 2, subset C	N. C.	N. C.	-	
Set 2, subset C	N. C.	-	N. C.	
Set 2, subset C	-	N. C.	N. C.	
Set 3, subset A	N. C.	N. C.	-	
Set 3, subset A	N. C.	-	N. C.	
Set 3, subset A	-	N. C.	N. C.	
Set 3, subset B	N. C.	N. C.	-	
Set 3, subset B	N. C.	-	N. C.	
Set 3, subset B	-	N. C.	N. C.	
Set 3, subset C	N. C.	N. C.	-	
Set 3, subset C	N. C.	-	N. C.	
Set 3, subset C	-	N. C.	N. C.	

Table 17. Continued.

Dataset	pK_1	pK_2	pK_3	*V(Y)*
Set 1	N. C.	N. C.	-	
Set 1	N. C.	-	N. C.	
Set 1	-	N. C.	N. C.	
Set 2	N. C.	N. C.	-	
Set 2	N. C.	-	N. C.	
Set 2	-	N. C.	N. C.	
Set 3	N. C.	N. C.	-	
Set 3	N. C.	-	N. C.	
Set 3	-	N. C.	N. C.	
Set 1, 2 and 3	N. C.	N. C.	-	
Set 1, 2 and 3	N. C.	-	N. C.	
Set 1, 2 and 3	-	N. C.	N. C.	

[a] N. C., data which does not converge.

Table 18. Two-site SCMs generated for each individual dataset obtained from Cd^{2+} adsorption experiments using *G. stearothermophilus* cells (DSM 6790).

Dataset	pK_1	pK_2	pK_3	*V(Y)*
Set 1, subset A	N. C.[a]	N. C.	-	
Set 1, subset A	N. C.	-	N. C.	
Set 1, subset A	-	N. C.	N. C.	
Set 1, subset B	N. C.	N. C.	-	
Set 1, subset B	N. C.	-	N. C.	
Set 1, subset B	-	N. C.	N. C.	
Set 1, subset C	2.373	4.284	-	0.501
Set 1, subset C	2.348	-	4.535	0.557
Set 1, subset C	-	N. C.	N. C.	
Set 2, subset A	N. C.	N. C.	-	
Set 2, subset A	N. C.	-	N. C.	
Set 2, subset A	-	N. C.	N. C.	
Set 2, subset B	N. C.	N. C.	-	
Set 2, subset B	N. C.	-	N. C.	
Set 2, subset B	-	N. C.	N. C.	
Set 2, subset C	2.337	4.573	-	1.202
Set 2, subset C	2.337	-	4.604	1.200
Set 2, subset C	-	N. C.	N. C.	
Set 3, subset A	N. C.	N. C.	-	
Set 3, subset A	N. C.	-	N. C.	
Set 3, subset A	-	N. C.	N. C.	
Set 3, subset B	N. C.	N. C.	-	
Set 3, subset B	N. C.	-	N. C.	
Set 3, subset B	-	N. C.	N. C.	
Set 3, subset C	N. C.	N. C.	-	
Set 3, subset C	N. C.	-	N. C.	
Set 3, subset C	-	N. C.	N. C.	

Table 18. Continued.

Dataset	pK_1	pK_2	pK_3	*V(Y)*
Set 1	2.460	5.205	-	1.953
Set 1	2.464	-	5.060	1.958
Set 1	-	N. C.	N. C.	
Set 2	N. C.	N. C.	-	
Set 2	N. C.	-	N. C.	
Set 2	-	N. C.	N. C.	
Set 3	N. C.	N. C.	-	
Set 3	N. C.	-	N. C.	
Set 3	-	N. C.	N. C.	
Set 1, 2 and 3	N. C.	N. C.	-	
Set 1, 2 and 3	N. C.	-	N. C.	
Set 1, 2 and 3	-	N. C.	N. C.	

[a] N. C., data which does not converge.

Appendix C: Surface complexation model data

Table 19. Dataset 1 obtained from titration experiments using *G. thermocatenulatus* cells (24.70 g wet cells). Proton concentration (H^+) in mol per liter.

Added H^+	pH	Dilution factor	Added H^+	pH	Dilution factor
-6.72E-04	4.03	0.985	-1.88E-03	7.58	0.959
-7.38E-04	4.14	0.983	-1.89E-03	7.72	0.959
-8.02E-04	4.26	0.982	-1.91E-03	7.86	0.958
-8.56E-04	4.29	0.981	-1.93E-03	8.01	0.958
-9.74E-04	4.50	0.979	-1.94E-03	8.09	0.958
-1.04E-03	4.63	0.977	-1.95E-03	8.21	0.958
-1.08E-03	4.73	0.976	-1.95E-03	8.27	0.957
-1.13E-03	4.84	0.975	-1.96E-03	8.37	0.957
-1.18E-03	4.94	0.974	-1.97E-03	8.47	0.957
-1.23E-03	5.05	0.973	-1.98E-03	8.60	0.957
-1.29E-03	5.18	0.972	-2.00E-03	8.73	0.956
-1.33E-03	5.27	0.971	-2.01E-03	8.87	0.956
-1.37E-03	5.38	0.970	-2.03E-03	9.00	0.956
-1.42E-03	5.49	0.969	-2.04E-03	9.12	0.955
-1.47E-03	5.62	0.968	-2.06E-03	9.25	0.955
-1.51E-03	5.75	0.967	-2.09E-03	9.37	0.955
-1.55E-03	5.87	0.966	-2.11E-03	9.49	0.954
-1.59E-03	6.00	0.965	-2.14E-03	9.60	0.953
-1.61E-03	6.09	0.965	-2.18E-03	9.72	0.953
-1.65E-03	6.21	0.964	-2.22E-03	9.83	0.952
-1.68E-03	6.35	0.964	-2.27E-03	9.92	0.951
-1.70E-03	6.43	0.963	-2.33E-03	10.04	0.950
-1.72E-03	6.55	0.963	-7.04E-04	4.10	0.940
-1.73E-03	6.58	0.962	-7.22E-04	4.13	0.939
-1.73E-03	6.59	0.962	-7.81E-04	4.23	0.938
-1.77E-03	6.82	0.961	-8.42E-04	4.35	0.937
-1.80E-03	6.98	0.961	-9.01E-04	4.47	0.936
-1.81E-03	7.03	0.961	-9.51E-04	4.57	0.935
-1.81E-03	7.05	0.961	-1.00E-03	4.68	0.934
-1.83E-03	7.25	0.960	-1.06E-03	4.80	0.933
-1.85E-03	7.37	0.960	-1.11E-03	4.91	0.932
-1.86E-03	7.42	0.960	-1.16E-03	5.03	0.931

Table 19. Continued.

Added H^+	pH	Dilution factor
-1.21E-03	5.14	0.930
-1.26E-03	5.26	0.929
-1.30E-03	5.38	0.928
-1.35E-03	5.50	0.927
-1.39E-03	5.62	0.926
-1.43E-03	5.64	0.925
-1.55E-03	6.02	0.922
-1.59E-03	6.17	0.922
-1.61E-03	6.27	0.921
-1.62E-03	6.31	0.921
-1.63E-03	6.36	0.921
-1.65E-03	6.43	0.921
-1.67E-03	6.57	0.920
-1.68E-03	6.64	0.920
-1.70E-03	6.75	0.919
-1.71E-03	6.82	0.919
-1.73E-03	6.97	0.919
-1.74E-03	7.00	0.919
-1.74E-03	7.02	0.919
-1.76E-03	7.17	0.918
-1.77E-03	7.28	0.918
-1.79E-03	7.41	0.918
-1.80E-03	7.58	0.917
-1.81E-03	7.62	0.917
-1.81E-03	7.65	0.917
-1.83E-03	7.81	0.917
-1.85E-03	7.95	0.916
-1.86E-03	8.10	0.916
-1.87E-03	8.20	0.916
-1.88E-03	8.34	0.916
-1.89E-03	8.43	0.915
-1.90E-03	8.55	0.915
-1.91E-03	8.62	0.915
-1.92E-03	8.75	0.915
-1.93E-03	8.88	0.914
-1.95E-03	9.01	0.914
-1.97E-03	9.13	0.914
-1.99E-03	9.25	0.913
-2.01E-03	9.37	0.913
-2.04E-03	9.49	0.912
-2.07E-03	9.60	0.912
-2.11E-03	9.72	0.911
-2.15E-03	9.82	0.910
-2.20E-03	9.92	0.910
-2.26E-03	10.03	0.909

Table 20. Dataset 2 obtained from titration experiments using *G. thermocatenulatus* cells (24.70 g wet cells). Proton concentration (H^+) in mol per liter.

Added H^+	pH	Dilution factor	Added H^+	pH	Dilution factor
-5.72E-04	4.04	0.963	-1.86E-03	7.65	0.936
-6.39E-04	4.14	0.961	-1.88E-03	7.80	0.936
-7.06E-04	4.25	0.960	-1.89E-03	7.89	0.935
-7.70E-04	4.36	0.959	-1.90E-03	8.04	0.935
-8.34E-04	4.46	0.958	-1.91E-03	8.11	0.935
-8.97E-04	4.57	0.956	-1.92E-03	8.18	0.935
-9.58E-04	4.68	0.955	-1.92E-03	8.22	0.935
-1.02E-03	4.78	0.954	-1.94E-03	8.40	0.934
-1.08E-03	4.87	0.953	-1.94E-03	8.42	0.934
-1.15E-03	5.00	0.951	-1.96E-03	8.63	0.934
-1.21E-03	5.10	0.950	-1.98E-03	8.74	0.933
-1.27E-03	5.22	0.949	-1.99E-03	8.88	0.933
-1.32E-03	5.33	0.947	-2.01E-03	9.00	0.933
-1.38E-03	5.45	0.946	-2.03E-03	9.12	0.932
-1.42E-03	5.56	0.945	-2.05E-03	9.24	0.932
-1.47E-03	5.68	0.944	-2.08E-03	9.36	0.931
-1.51E-03	5.81	0.943	-2.10E-03	9.48	0.931
-1.54E-03	5.91	0.943	-2.14E-03	9.58	0.930
-1.58E-03	6.02	0.942	-2.18E-03	9.69	0.930
-1.61E-03	6.16	0.941	-2.22E-03	9.80	0.929
-1.64E-03	6.26	0.941	-2.27E-03	9.90	0.928
-1.66E-03	6.35	0.940	-2.33E-03	10.00	0.927
-1.69E-03	6.49	0.940	-6.17E-04	4.12	0.915
-1.71E-03	6.60	0.939	-6.82E-04	4.23	0.914
-1.73E-03	6.74	0.939	-7.42E-04	4.33	0.913
-1.75E-03	6.85	0.938	-8.04E-04	4.44	0.912
-1.77E-03	7.00	0.938	-8.66E-04	4.54	0.910
-1.78E-03	7.08	0.938	-9.27E-04	4.65	0.909
-1.80E-03	7.18	0.937	-9.85E-04	4.76	0.908
-1.81E-03	7.29	0.937	-1.04E-03	4.86	0.907
-1.82E-03	7.38	0.937	-1.10E-03	4.97	0.906
-1.84E-03	7.52	0.936	-1.16E-03	5.09	0.905

Table 20. Continued.

Added H^+	pH	Dilution factor
-1.21E-03	5.19	0.904
-1.26E-03	5.26	0.902
-1.34E-03	5.45	0.901
-1.39E-03	5.57	0.900
-1.43E-03	5.69	0.899
-1.47E-03	5.81	0.898
-1.50E-03	5.93	0.898
-1.53E-03	6.05	0.897
-1.56E-03	6.17	0.897
-1.58E-03	6.27	0.896
-1.61E-03	6.41	0.896
-1.63E-03	6.56	0.895
-1.64E-03	6.61	0.895
-1.65E-03	6.68	0.895
-1.66E-03	6.73	0.894
-1.68E-03	6.89	0.894
-1.69E-03	6.95	0.894
-1.70E-03	7.00	0.894
-1.71E-03	7.06	0.893
-1.72E-03	7.21	0.893
-1.73E-03	7.29	0.893
-1.74E-03	7.35	0.893
-1.76E-03	7.51	0.892
-1.77E-03	7.63	0.892
-1.79E-03	7.77	0.892
-1.80E-03	7.89	0.892
-1.81E-03	8.02	0.891
-1.82E-03	8.12	0.891
-1.83E-03	8.22	0.891
-1.84E-03	8.33	0.891
-1.86E-03	8.45	0.890
-1.87E-03	8.59	0.890
-1.88E-03	8.71	0.890
-1.90E-03	8.84	0.889
-1.92E-03	8.96	0.889
-1.94E-03	9.08	0.889
-1.96E-03	9.20	0.888
-1.99E-03	9.31	0.888
-2.02E-03	9.43	0.887
-2.05E-03	9.54	0.887
-2.09E-03	9.64	0.886
-2.14E-03	9.75	0.885
-2.19E-03	9.85	0.884
-2.25E-03	9.95	0.883
-2.31E-03	10.04	0.882

Table 21. Dataset 3 obtained from titration experiments using *G. thermocatenulatus* cells (24.70 g wet cells). Proton concentration (H^+) in mol per liter.

Added H^+	pH	Dilution factor	Added H^+	pH	Dilution factor
-6.95E-04	4.01	0.970	-1.78E-03	7.40	0.947
-7.50E-04	4.11	0.968	-1.83E-03	7.80	0.946
-8.12E-04	4.22	0.967	-1.85E-03	7.97	0.945
-8.67E-04	4.33	0.966	-1.85E-03	8.00	0.945
-9.24E-04	4.45	0.965	-1.86E-03	8.01	0.945
-9.80E-04	4.56	0.964	-1.88E-03	8.28	0.945
-1.04E-03	4.68	0.963	-1.89E-03	8.37	0.944
-1.09E-03	4.79	0.962	-1.89E-03	8.38	0.944
-1.14E-03	4.90	0.961	-1.90E-03	8.40	0.944
-1.19E-03	5.02	0.959	-1.92E-03	8.62	0.944
-1.24E-03	5.13	0.958	-1.93E-03	8.72	0.943
-1.29E-03	5.25	0.957	-1.94E-03	8.73	0.943
-1.34E-03	5.38	0.956	-1.94E-03	8.74	0.943
-1.38E-03	5.47	0.955	-1.96E-03	8.94	0.943
-1.42E-03	5.58	0.955	-1.98E-03	9.03	0.942
-1.45E-03	5.69	0.954	-1.98E-03	9.05	0.942
-1.47E-03	5.75	0.953	-1.99E-03	9.07	0.942
-1.50E-03	5.86	0.953	-2.02E-03	9.24	0.942
-1.52E-03	5.95	0.952	-2.04E-03	9.34	0.941
-1.56E-03	6.08	0.952	-2.05E-03	9.36	0.941
-1.59E-03	6.24	0.951	-2.10E-03	9.53	0.940
-1.60E-03	6.27	0.951	-2.14E-03	9.65	0.939
-1.60E-03	6.30	0.951	-2.18E-03	9.74	0.939
-1.64E-03	6.50	0.950	-2.23E-03	9.84	0.938
-1.66E-03	6.62	0.949	-2.29E-03	9.94	0.937
-1.68E-03	6.74	0.949	-2.36E-03	10.03	0.935
-1.70E-03	6.87	0.949	-6.35E-04	4.03	0.917
-1.71E-03	6.96	0.948	-6.97E-04	4.14	0.916
-1.73E-03	7.06	0.948	-7.58E-04	4.26	0.915
-1.74E-03	7.16	0.948	-8.14E-04	4.36	0.914
-1.76E-03	7.31	0.947	-8.72E-04	4.47	0.913
-1.77E-03	7.38	0.947	-9.29E-04	4.58	0.912

Table 21. Continued.

Added H^+	pH	Dilution factor
-9.85E-04	4.69	0.911
-1.04E-03	4.80	0.910
-1.09E-03	4.89	0.909
-1.16E-03	5.02	0.907
-1.21E-03	5.14	0.906
-1.25E-03	5.25	0.905
-1.30E-03	5.36	0.904
-1.34E-03	5.48	0.904
-1.38E-03	5.61	0.903
-1.41E-03	5.73	0.902
-1.44E-03	5.84	0.902
-1.47E-03	5.95	0.901
-1.50E-03	6.08	0.900
-1.53E-03	6.25	0.900
-1.54E-03	6.28	0.900
-1.55E-03	6.33	0.899
-1.56E-03	6.38	0.899
-1.59E-03	6.57	0.899
-1.59E-03	6.59	0.899
-1.60E-03	6.61	0.898
-1.63E-03	6.86	0.898
-1.64E-03	6.97	0.897
-1.65E-03	7.02	0.897
-1.67E-03	7.18	0.897
-1.68E-03	7.22	0.897
-1.69E-03	7.32	0.897
-1.70E-03	7.42	0.896
-1.72E-03	7.58	0.896
-1.72E-03	7.63	0.896
-1.73E-03	7.67	0.896
-1.75E-03	7.85	0.895
-1.76E-03	7.98	0.895
-1.77E-03	8.12	0.895
-1.79E-03	8.27	0.894
-1.80E-03	8.39	0.894
-1.82E-03	8.53	0.894
-1.83E-03	8.65	0.894
-1.85E-03	8.78	0.893
-1.87E-03	8.91	0.893
-1.89E-03	9.03	0.892
-1.91E-03	9.15	0.892
-1.94E-03	9.26	0.891
-1.97E-03	9.38	0.891
-2.00E-03	9.48	0.890
-2.05E-03	9.60	0.889
-2.09E-03	9.70	0.889
-2.14E-03	9.80	0.888
-2.20E-03	9.90	0.887
-2.26E-03	9.99	0.886
-2.34E-03	10.08	0.884

Table 22. Dataset 1 obtained from titration experiments using *G. stearothermophilus* cells (12.96 g wet cells). Proton concentration (H^+) in mol per liter.

Added H^+	pH	Dilution factor	Added H^+	pH	Dilution factor
-2.76E-04	4.01	0.896	-1.21E-03	7.53	0.877
-3.31E-04	4.12	0.895	-1.21E-03	7.56	0.877
-3.79E-04	4.22	0.894	-1.23E-03	7.70	0.877
-4.28E-04	4.34	0.893	-1.25E-03	7.84	0.877
-4.72E-04	4.46	0.892	-1.26E-03	8.00	0.876
-5.17E-04	4.58	0.891	-1.27E-03	8.08	0.876
-5.59E-04	4.70	0.890	-1.28E-03	8.21	0.876
-6.00E-04	4.83	0.890	-1.29E-03	8.30	0.876
-6.39E-04	4.94	0.889	-1.30E-03	8.39	0.876
-6.79E-04	5.07	0.888	-1.32E-03	8.55	0.875
-7.17E-04	5.19	0.887	-1.33E-03	8.69	0.875
-7.55E-04	5.31	0.887	-1.34E-03	8.82	0.875
-7.94E-04	5.42	0.886	-1.36E-03	8.94	0.874
-8.35E-04	5.53	0.885	-1.37E-03	9.07	0.874
-8.80E-04	5.68	0.884	-1.39E-03	9.19	0.874
-9.16E-04	5.83	0.883	-1.41E-03	9.32	0.873
-9.36E-04	5.89	0.883	-1.44E-03	9.43	0.873
-9.48E-04	5.93	0.883	-1.47E-03	9.55	0.872
-9.88E-04	6.09	0.882	-1.50E-03	9.66	0.872
-1.02E-03	6.24	0.881	-1.54E-03	9.77	0.871
-1.04E-03	6.32	0.881	-1.58E-03	9.87	0.871
-1.06E-03	6.45	0.881	-1.64E-03	9.97	0.870
-1.07E-03	6.50	0.880	-1.70E-03	10.07	0.869
-1.09E-03	6.60	0.880	-2.50E-04	4.08	0.856
-1.10E-03	6.66	0.880	-3.08E-04	4.21	0.855
-1.12E-03	6.81	0.879	-3.53E-04	4.31	0.855
-1.13E-03	6.89	0.879	-4.02E-04	4.44	0.854
-1.15E-03	6.98	0.879	-4.44E-04	4.55	0.853
-1.16E-03	7.14	0.878	-4.87E-04	4.67	0.852
-1.17E-03	7.16	0.878	-5.30E-04	4.80	0.851
-1.17E-03	7.17	0.878	-5.70E-04	4.91	0.851
-1.19E-03	7.42	0.878	-6.11E-04	5.04	0.850

Table 22. Continued.

Added H^+	pH	Dilution factor
-6.51E-04	5.16	0.849
-6.91E-04	5.28	0.848
-7.31E-04	5.40	0.848
-7.71E-04	5.53	0.847
-8.09E-04	5.60	0.846
-8.66E-04	5.80	0.845
-9.01E-04	5.96	0.844
-9.18E-04	6.03	0.844
-9.43E-04	6.15	0.844
-9.59E-04	6.22	0.843
-9.85E-04	6.38	0.843
-9.96E-04	6.42	0.843
-1.00E-03	6.46	0.843
-1.01E-03	6.49	0.842
-1.02E-03	6.60	0.842
-1.03E-03	6.65	0.842
-1.05E-03	6.76	0.842
-1.06E-03	6.82	0.842
-1.07E-03	6.96	0.841
-1.08E-03	7.01	0.841
-1.09E-03	7.06	0.841
-1.11E-03	7.26	0.841
-1.12E-03	7.37	0.840
-1.13E-03	7.43	0.840
-1.14E-03	7.59	0.840
-1.16E-03	7.73	0.839
-1.17E-03	7.89	0.839
-1.18E-03	7.97	0.839
-1.19E-03	8.08	0.839
-1.20E-03	8.19	0.839
-1.22E-03	8.31	0.838
-1.23E-03	8.44	0.838
-1.24E-03	8.57	0.838
-1.25E-03	8.71	0.838
-1.27E-03	8.83	0.837
-1.29E-03	8.96	0.837
-1.30E-03	9.08	0.837
-1.33E-03	9.20	0.836
-1.35E-03	9.32	0.836
-1.38E-03	9.44	0.835
-1.41E-03	9.55	0.835
-1.45E-03	9.66	0.834
-1.49E-03	9.75	0.834
-1.54E-03	9.87	0.833
-1.60E-03	9.97	0.832
-1.66E-03	10.06	0.831

Table 23. Dataset 2 obtained from titration experiments using *G. stearothermophilus* cells (12.96 g wet cells). Proton concentration (H^+) in mol per liter.

Added H^+	pH	Dilution factor	Added H^+	pH	Dilution factor
-3.72E-04	4.09	0.987	-1.29E-03	7.39	0.967
-4.25E-04	4.20	0.986	-1.32E-03	7.60	0.967
-4.76E-04	4.32	0.985	-1.33E-03	7.72	0.966
-5.24E-04	4.44	0.984	-1.34E-03	7.78	0.966
-5.68E-04	4.55	0.983	-1.35E-03	7.83	0.966
-6.15E-04	4.67	0.982	-1.36E-03	8.00	0.966
-6.57E-04	4.78	0.981	-1.37E-03	8.03	0.966
-7.02E-04	4.90	0.980	-1.37E-03	8.04	0.966
-7.45E-04	5.02	0.979	-1.39E-03	8.30	0.965
-7.88E-04	5.13	0.978	-1.40E-03	8.40	0.965
-8.31E-04	5.25	0.977	-1.41E-03	8.41	0.965
-8.74E-04	5.38	0.977	-1.41E-03	8.42	0.965
-9.14E-04	5.50	0.976	-1.45E-03	8.88	0.964
-9.53E-04	5.62	0.975	-1.47E-03	8.98	0.963
-9.89E-04	5.73	0.974	-1.47E-03	8.99	0.963
-1.03E-03	5.85	0.973	-1.48E-03	9.00	0.963
-1.05E-03	5.97	0.973	-1.51E-03	9.21	0.963
-1.07E-03	6.03	0.972	-1.53E-03	9.29	0.962
-1.09E-03	6.13	0.972	-1.53E-03	9.30	0.962
-1.11E-03	6.18	0.971	-1.59E-03	9.53	0.961
-1.12E-03	6.24	0.971	-1.63E-03	9.64	0.960
-1.13E-03	6.30	0.971	-1.67E-03	9.73	0.959
-1.16E-03	6.48	0.970	-1.69E-03	9.77	0.959
-1.17E-03	6.50	0.970	-1.75E-03	9.88	0.958
-1.17E-03	6.51	0.970	-1.81E-03	9.98	0.957
-1.21E-03	6.77	0.969	-1.87E-03	10.08	0.956
-1.23E-03	6.93	0.969	-3.01E-04	4.02	0.944
-1.24E-03	6.98	0.969	-3.58E-04	4.14	0.943
-1.25E-03	7.00	0.968	-4.10E-04	4.26	0.942
-1.26E-03	7.11	0.968	-4.59E-04	4.37	0.941
-1.27E-03	7.27	0.968	-5.07E-04	4.49	0.940
-1.29E-03	7.34	0.967	-5.54E-04	4.61	0.940

Table 23. Continued.

Added H^+	pH	Dilution factor
-5.98E-04	4.72	0.939
-6.43E-04	4.84	0.938
-6.87E-04	4.96	0.937
-7.30E-04	5.07	0.936
-7.73E-04	5.19	0.935
-8.15E-04	5.31	0.934
-8.56E-04	5.44	0.933
-8.91E-04	5.55	0.933
-9.26E-04	5.62	0.932
-9.38E-04	5.66	0.932
-9.73E-04	5.81	0.931
-9.97E-04	5.91	0.930
-1.02E-03	6.04	0.930
-1.05E-03	6.15	0.929
-1.07E-03	6.29	0.929
-1.09E-03	6.40	0.928
-1.12E-03	6.55	0.928
-1.13E-03	6.65	0.927
-1.16E-03	6.80	0.927
-1.17E-03	6.88	0.927
-1.18E-03	6.95	0.927
-1.20E-03	7.13	0.926
-1.20E-03	7.15	0.926
-1.21E-03	7.16	0.926
-1.24E-03	7.48	0.925
-1.26E-03	7.65	0.925
-1.26E-03	7.69	0.925
-1.27E-03	7.70	0.925
-1.29E-03	7.97	0.924
-1.30E-03	8.07	0.924
-1.31E-03	8.08	0.924
-1.31E-03	8.14	0.924
-1.33E-03	8.33	0.923
-1.34E-03	8.44	0.923
-1.35E-03	8.45	0.923
-1.38E-03	8.72	0.922
-1.39E-03	8.81	0.922
-1.41E-03	8.96	0.922
-1.43E-03	9.04	0.921
-1.45E-03	9.19	0.921
-1.48E-03	9.29	0.920
-1.51E-03	9.41	0.920
-1.55E-03	9.51	0.919
-1.59E-03	9.62	0.918
-1.64E-03	9.72	0.917
-1.69E-03	9.82	0.916
-1.75E-03	9.92	0.915
-1.81E-03	10.01	0.914

Table 24. Data obtained from Cd^{2+} adsorption experiments using *G. thermocatenulatus* cells (19.71 g wet cells).

Dataset	Initial Cd^{2+} [mol/l]	Adsorbed Cd^{2+} [mol/l]	Final pH	Dilution factor
Set 1, subset A	4.19E-05	3.86E-05	5.55	0.796
Set 1, subset A	4.19E-05	3.92E-05	6.04	0.796
Set 1, subset A	4.19E-05	3.93E-05	6.23	0.796
Set 1, subset A	4.19E-05	3.94E-05	6.37	0.796
Set 1, subset A	4.19E-05	3.93E-05	6.51	0.796
Set 1, subset A	4.19E-05	3.93E-05	6.57	0.796
Set 1, subset A	4.19E-05	3.93E-05	6.72	0.796
Set 1, subset A	4.19E-05	3.94E-05	6.94	0.796
Set 1, subset A	4.19E-05	3.95E-05	7.15	0.796
Set 1, subset A	4.19E-05	3.94E-05	7.33	0.795
Set 1, subset B	4.42E-05	3.72E-05	5.42	0.462
Set 1, subset B	4.42E-05	3.96E-05	6.33	0.462
Set 1, subset B	4.42E-05	3.96E-05	6.08	0.462
Set 1, subset B	4.42E-05	4.00E-05	6.63	0.462
Set 1, subset B	4.42E-05	4.03E-05	6.89	0.462
Set 1, subset B	4.42E-05	4.04E-05	6.96	0.462
Set 1, subset B	4.42E-05	4.05E-05	7.00	0.462
Set 1, subset B	4.42E-05	4.05E-05	7.03	0.461
Set 1, subset B	4.42E-05	4.06E-05	7.40	0.461
Set 1, subset B	4.42E-05	4.03E-05	7.57	0.461
Set 1, subset C	4.32E-05	1.56E-05	4.45	0.233
Set 1, subset C	4.32E-05	2.98E-05	5.30	0.233
Set 1, subset C	4.32E-05	3.22E-05	5.58	0.233
Set 1, subset C	4.32E-05	3.48E-05	6.08	0.233
Set 1, subset C	4.32E-05	3.57E-05	6.33	0.233
Set 1, subset C	4.32E-05	3.67E-05	6.66	0.233
Set 1, subset C	4.32E-05	3.64E-05	6.71	0.233
Set 1, subset C	4.32E-05	3.72E-05	7.04	0.233
Set 1, subset C	4.32E-05	3.80E-05	7.34	0.233
Set 1, subset C	4.31E-05	3.79E-05	7.35	0.233

Table 24. Continued.

Dataset	Initial Cd^{2+} [mol/l]	Adsorbed Cd^{2+} [mol/l]	Final pH	Dilution factor
Set 2, subset A	3.99E-05	3.49E-05	5.06	0.886
Set 2, subset A	3.99E-05	3.64E-05	5.41	0.886
Set 2, subset A	3.99E-05	3.66E-05	5.61	0.886
Set 2, subset A	3.99E-05	3.68E-05	5.71	0.886
Set 2, subset A	3.99E-05	3.68E-05	5.79	0.886
Set 2, subset A	3.99E-05	3.71E-05	5.93	0.886
Set 2, subset A	3.99E-05	3.72E-05	6.04	0.886
Set 2, subset A	3.99E-05	3.72E-05	6.26	0.886
Set 2, subset A	3.99E-05	3.73E-05	6.35	0.886
Set 2, subset A	3.98E-05	3.72E-05	6.50	0.886
Set 2, subset A	3.98E-05	3.73E-05	6.66	0.886
Set 2, subset A	3.98E-05	3.73E-05	6.81	0.886
Set 2, subset B	4.35E-05	1.15E-05	3.99	0.386
Set 2, subset B	4.35E-05	1.73E-05	4.23	0.385
Set 2, subset B	4.35E-05	1.98E-05	4.35	0.385
Set 2, subset B	4.35E-05	2.48E-05	4.59	0.385
Set 2, subset B	4.35E-05	2.71E-05	4.70	0.385
Set 2, subset B	4.35E-05	2.93E-05	4.84	0.385
Set 2, subset B	4.35E-05	3.21E-05	5.06	0.385
Set 2, subset B	4.35E-05	3.38E-05	5.24	0.385
Set 2, subset B	4.35E-05	3.55E-05	5.43	0.385
Set 2, subset B	4.35E-05	3.67E-05	5.76	0.385
Set 2, subset B	4.35E-05	3.79E-05	6.06	0.385
Set 2, subset B	4.35E-05	3.84E-05	6.25	0.385
Set 2, subset C	4.34E-05	3.10E-06	3.72	0.176
Set 2, subset C	4.34E-05	4.23E-06	3.85	0.176
Set 2, subset C	4.34E-05	4.54E-06	3.93	0.176
Set 2, subset C	4.34E-05	5.86E-06	4.00	0.176
Set 2, subset C	4.34E-05	7.26E-06	4.13	0.176
Set 2, subset C	4.34E-05	8.56E-06	4.21	0.176
Set 2, subset C	4.34E-05	1.30E-05	4.47	0.176
Set 2, subset C	4.34E-05	1.94E-05	4.80	0.176
Set 2, subset C	4.34E-05	2.19E-05	4.96	0.176
Set 2, subset C	4.34E-05	2.34E-05	5.10	0.176
Set 2, subset C	4.34E-05	3.08E-05	5.80	0.176
Set 2, subset C	4.34E-05	3.21E-05	6.06	0.176

Table 24. Continued.

Dataset	Initial Cd^{2+} [mol/l]	Adsorbed Cd^{2+} [mol/l]	Final pH	Dilution factor
Set 3, subset A	4.21E-05	2.37E-05	4.09	0.999
Set 3, subset A	4.21E-05	2.88E-05	4.29	0.999
Set 3, subset A	4.21E-05	3.29E-05	4.53	0.999
Set 3, subset A	4.21E-05	3.54E-05	4.76	0.999
Set 3, subset A	4.21E-05	3.67E-05	4.95	0.999
Set 3, subset A	4.21E-05	3.73E-05	5.09	0.999
Set 3, subset A	4.21E-05	3.85E-05	5.49	0.999
Set 3, subset A	4.20E-05	3.90E-05	5.62	0.999
Set 3, subset A	4.20E-05	3.92E-05	5.92	0.999
Set 3, subset A	4.20E-05	3.94E-05	6.32	0.999
Set 3, subset A	4.20E-05	3.96E-05	6.55	0.999
Set 3, subset A	4.20E-05	3.96E-05	6.85	0.999
Set 3, subset B	4.44E-05	5.76E-06	3.44	0.444
Set 3, subset B	4.44E-05	6.57E-06	3.48	0.444
Set 3, subset B	4.44E-05	7.20E-06	3.59	0.444
Set 3, subset B	4.44E-05	9.45E-06	3.73	0.444
Set 3, subset B	4.44E-05	1.24E-05	3.88	0.444
Set 3, subset B	4.44E-05	1.42E-05	3.97	0.444
Set 3, subset B	4.44E-05	2.00E-05	4.25	0.444
Set 3, subset B	4.44E-05	3.14E-05	4.82	0.444
Set 3, subset B	4.44E-05	3.53E-05	5.10	0.444
Set 3, subset B	4.44E-05	3.84E-05	5.56	0.444
Set 3, subset B	4.44E-05	3.96E-05	5.99	0.444
Set 3, subset B	4.44E-05	4.06E-05	6.48	0.444
Set 3, subset C	4.35E-05	1.68E-06	3.27	0.223
Set 3, subset C	4.35E-05	1.85E-06	3.32	0.223
Set 3, subset C	4.35E-05	2.20E-06	3.40	0.223
Set 3, subset C	4.35E-05	2.85E-06	3.48	0.223
Set 3, subset C	4.35E-05	3.61E-06	3.58	0.223
Set 3, subset C	4.35E-05	4.66E-06	3.75	0.223
Set 3, subset C	4.35E-05	6.32E-06	3.88	0.223
Set 3, subset C	4.35E-05	1.08E-05	4.18	0.223
Set 3, subset C	4.35E-05	1.66E-05	4.49	0.223
Set 3, subset C	4.35E-05	3.00E-05	5.34	0.223
Set 3, subset C	4.35E-05	3.55E-05	6.20	0.223
Set 3, subset C	4.35E-05	3.65E-05	6.62	0.223

Table 25. Data obtained from Cd^{2+} adsorption experiments using *G. stearothermophilus* cells (10.56 g wet cells).

Dataset	Initial Cd^{2+} [mol/l]	Adsorbed Cd^{2+} [mol/l]	Final pH	Dilution factor
Set 1, subset A	4.31E-05	3.43E-05	5.79	0.608
Set 1, subset A	4.31E-05	3.49E-05	6.04	0.608
Set 1, subset A	4.31E-05	3.49E-05	6.24	0.608
Set 1, subset A	4.31E-05	3.52E-05	6.35	0.608
Set 1, subset A	4.31E-05	3.53E-05	6.54	0.608
Set 1, subset A	4.31E-05	3.49E-05	6.69	0.608
Set 1, subset A	4.31E-05	3.52E-05	6.88	0.608
Set 1, subset A	4.31E-05	3.57E-05	7.19	0.608
Set 1, subset A	4.31E-05	3.54E-05	7.35	0.608
Set 1, subset B	4.41E-05	5.85E-06	4.13	0.346
Set 1, subset B	4.41E-05	1.59E-05	4.83	0.346
Set 1, subset B	4.41E-05	2.47E-05	5.40	0.346
Set 1, subset B	4.41E-05	2.75E-05	5.69	0.346
Set 1, subset B	4.41E-05	3.02E-05	6.02	0.346
Set 1, subset B	4.41E-05	3.19E-05	6.25	0.346
Set 1, subset B	4.41E-05	3.29E-05	6.45	0.346
Set 1, subset B	4.41E-05	3.39E-05	6.76	0.346
Set 1, subset B	4.41E-05	3.51E-05	6.88	0.346
Set 1, subset B	4.41E-05	3.54E-05	7.12	0.346
Set 1, subset B	4.41E-05	3.62E-05	7.86	0.346
Set 1, subset C	4.33E-05	6.23E-06	4.59	0.199
Set 1, subset C	4.33E-05	1.95E-05	5.89	0.199
Set 1, subset C	4.33E-05	2.48E-05	6.56	0.199
Set 1, subset C	4.33E-05	2.48E-05	6.55	0.199
Set 1, subset C	4.33E-05	2.63E-05	6.79	0.199
Set 1, subset C	4.33E-05	2.79E-05	6.94	0.199
Set 1, subset C	4.33E-05	2.93E-05	7.16	0.199
Set 1, subset C	4.33E-05	3.22E-05	7.59	0.199
Set 1, subset C	4.33E-05	3.19E-05	7.59	0.199
Set 1, subset C	4.33E-05	3.30E-05	7.75	0.199

Table 25. Continued.

Dataset	Initial Cd^{2+} [mol/l]	Adsorbed Cd^{2+} [mol/l]	Final pH	Dilution factor
Set 2, subset A	4.13E-05	1.59E-05	4.28	0.999
Set 2, subset A	4.13E-05	2.63E-05	4.49	0.999
Set 2, subset A	4.13E-05	2.90E-05	4.63	0.999
Set 2, subset A	4.13E-05	3.11E-05	4.96	0.999
Set 2, subset A	4.13E-05	3.36E-05	5.30	0.999
Set 2, subset A	4.13E-05	3.42E-05	5.46	0.999
Set 2, subset A	4.13E-05	3.44E-05	5.64	0.999
Set 2, subset A	4.13E-05	3.41E-05	5.73	0.999
Set 2, subset A	4.13E-05	3.41E-05	5.94	0.999
Set 2, subset A	4.13E-05	3.36E-05	6.00	0.999
Set 2, subset A	4.13E-05	3.41E-05	6.17	0.999
Set 2, subset A	4.13E-05	3.34E-05	6.31	0.999
Set 2, subset B	4.00E-05	4.81E-06	3.95	0.511
Set 2, subset B	4.00E-05	9.89E-06	4.38	0.511
Set 2, subset B	3.99E-05	1.71E-05	4.76	0.511
Set 2, subset B	3.99E-05	2.02E-05	4.90	0.511
Set 2, subset B	3.99E-05	2.43E-05	5.18	0.511
Set 2, subset B	3.99E-05	2.48E-05	5.27	0.511
Set 2, subset B	3.99E-05	2.76E-05	5.47	0.511
Set 2, subset B	3.99E-05	2.95E-05	5.76	0.511
Set 2, subset B	3.99E-05	3.09E-05	6.04	0.511
Set 2, subset B	3.99E-05	3.22E-05	6.25	0.511
Set 2, subset B	3.99E-05	3.22E-05	6.61	0.511
Set 2, subset C	3.98E-05	1.37E-06	3.65	0.255
Set 2, subset C	3.98E-05	2.59E-06	3.99	0.255
Set 2, subset C	3.98E-05	3.50E-06	4.14	0.255
Set 2, subset C	3.98E-05	2.60E-06	4.05	0.255
Set 2, subset C	3.98E-05	1.10E-05	4.89	0.255
Set 2, subset C	3.98E-05	1.46E-05	5.16	0.255
Set 2, subset C	3.98E-05	1.93E-05	5.55	0.255
Set 2, subset C	3.98E-05	2.21E-05	5.97	0.255
Set 2, subset C	3.98E-05	2.39E-05	6.12	0.255
Set 2, subset C	3.98E-05	2.72E-05	6.56	0.255
Set 2, subset C	3.98E-05	2.73E-05	6.61	0.255
Set 2, subset C	3.98E-05	2.73E-05	6.72	0.255

Table 25. Continued.

Dataset	Initial Cd^{2+} [mol/l]	Adsorbed Cd^{2+} [mol/l]	Final pH	Dilution factor
Set 3, subset A	4.16E-05	2.44E-05	4.62	0.946
Set 3, subset A	4.16E-05	3.02E-05	4.89	0.946
Set 3, subset A	4.16E-05	3.26E-05	5.05	0.946
Set 3, subset A	4.16E-05	3.37E-05	5.20	0.946
Set 3, subset A	4.16E-05	3.48E-05	5.37	0.946
Set 3, subset A	4.16E-05	3.56E-05	5.42	0.946
Set 3, subset A	4.16E-05	3.48E-05	5.61	0.946
Set 3, subset A	4.16E-05	3.42E-05	5.79	0.946
Set 3, subset A	4.16E-05	3.40E-05	5.92	0.946
Set 3, subset A	4.16E-05	3.42E-05	6.05	0.946
Set 3, subset A	4.16E-05	3.47E-05	6.19	0.946
Set 3, subset A	4.16E-05	3.38E-05	6.48	0.946
Set 3, subset B	4.55E-05	2.98E-06	3.65	0.477
Set 3, subset B	4.55E-05	4.84E-06	3.80	0.477
Set 3, subset B	4.55E-05	6.02E-06	3.88	0.477
Set 3, subset B	4.55E-05	6.19E-06	3.97	0.477
Set 3, subset B	4.55E-05	8.23E-06	4.11	0.477
Set 3, subset B	4.55E-05	9.20E-06	4.20	0.477
Set 3, subset B	4.55E-05	1.30E-05	4.38	0.477
Set 3, subset B	4.55E-05	1.48E-05	4.50	0.477
Set 3, subset B	4.55E-05	1.78E-05	4.63	0.477
Set 3, subset B	4.55E-05	2.54E-05	5.01	0.477
Set 3, subset B	4.55E-05	2.84E-05	5.35	0.477
Set 3, subset B	4.55E-05	3.17E-05	5.53	0.477
Set 3, subset C	4.14E-05	2.52E-06	3.76	0.272
Set 3, subset C	4.14E-05	2.75E-06	3.91	0.272
Set 3, subset C	4.14E-05	2.29E-06	3.83	0.272
Set 3, subset C	4.14E-05	5.40E-06	4.22	0.272
Set 3, subset C	4.14E-05	5.98E-06	4.34	0.272
Set 3, subset C	4.14E-05	9.50E-06	4.63	0.272
Set 3, subset C	4.14E-05	1.54E-05	5.03	0.272
Set 3, subset C	4.14E-05	1.62E-05	5.11	0.272
Set 3, subset C	4.14E-05	2.19E-05	5.52	0.272
Set 3, subset C	4.14E-05	2.42E-05	5.92	0.272
Set 3, subset C	4.14E-05	2.82E-05	6.39	0.272
Set 3, subset C	4.14E-05	2.81E-05	6.44	0.272

Appendix D: Clone library analyses

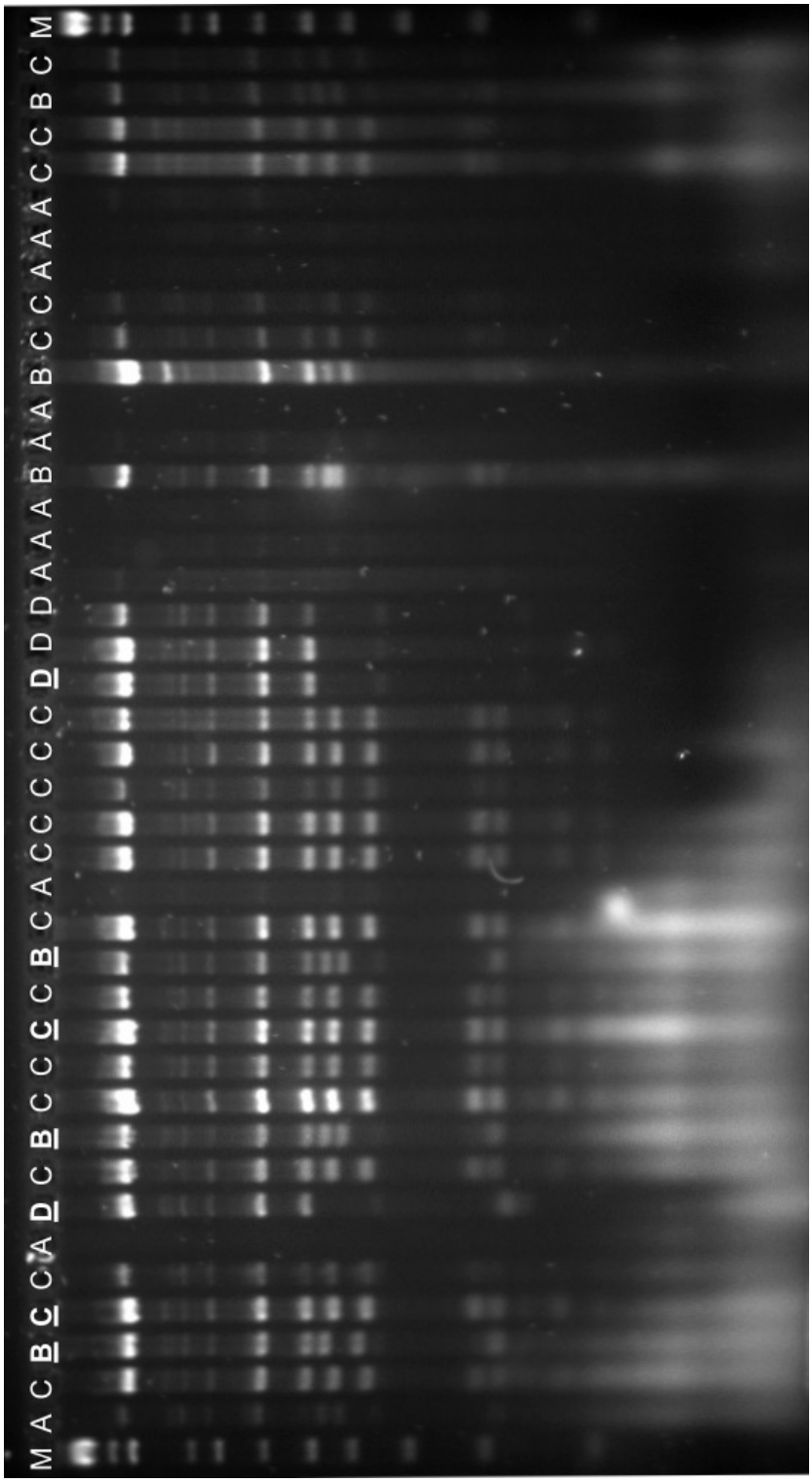

Figure 39. RFLP profiles of bacterial 16S rRNA genes. Continued on next page.

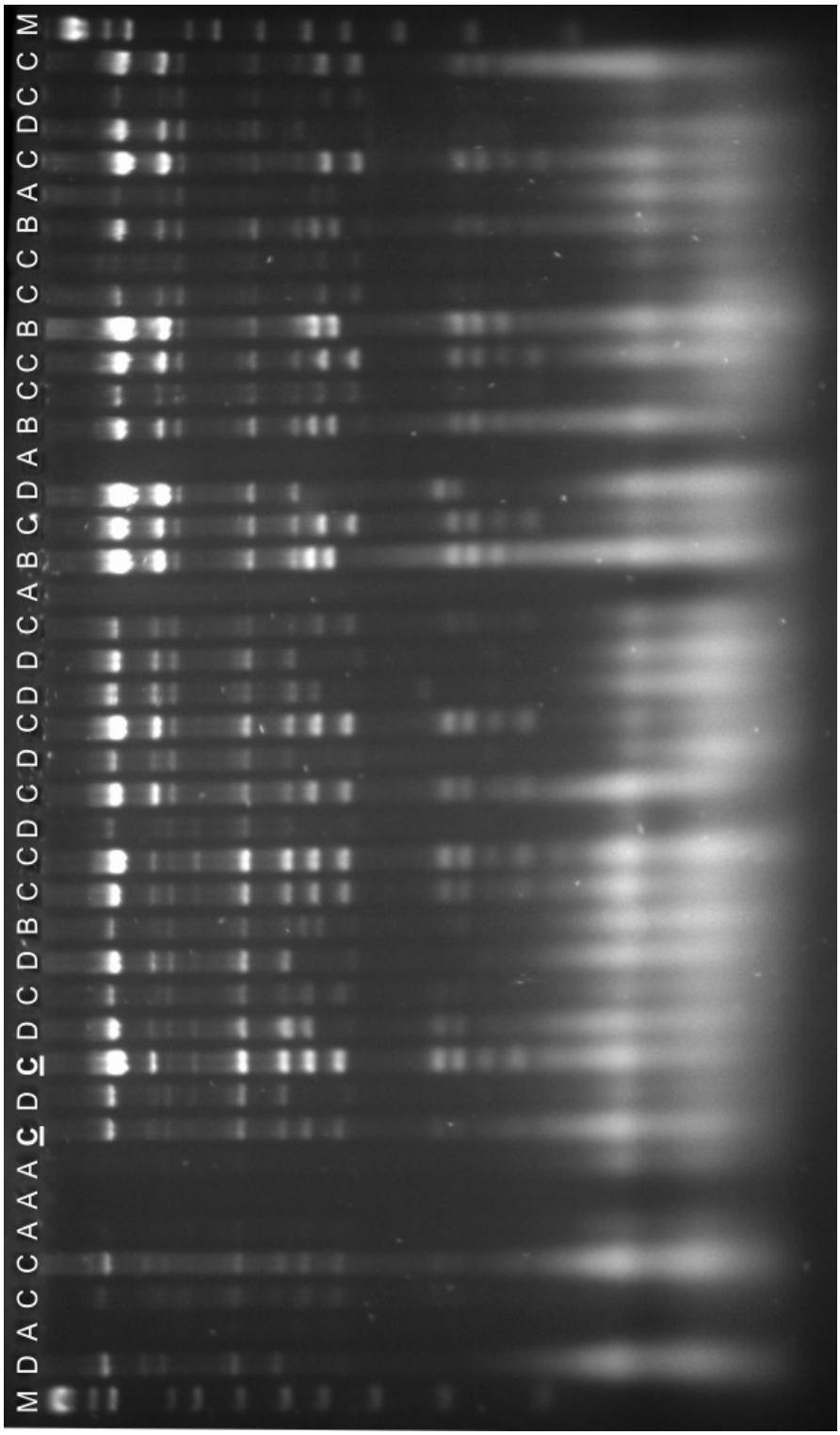

Figure 39. RFPL profiles of bacterial 16S rRNA genes. Continued on next page.

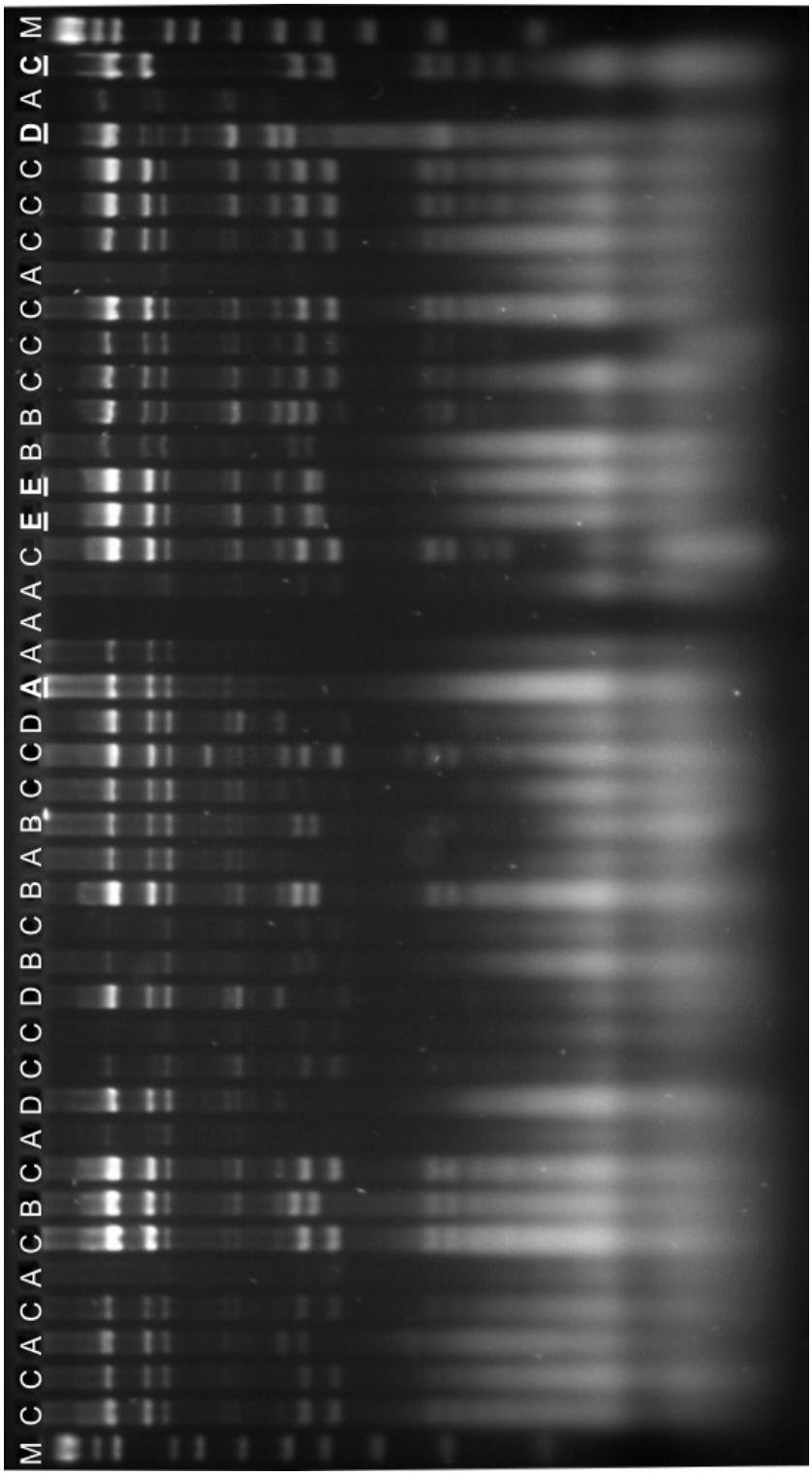

Figure 39. Restriction fragment length polymorphism (RFLP) profiles of bacterial 16S rRNA genes obtained from samples of Champagne Pool after enzymatic digestions with *Eco*R I, *Rsa* I, and *Bam*H I. M, 1 Kb Plus DNA ladder (Invitrogen); A, no sample or vector without insert; B, OTU Bac03; C, OTU Bac04; D, OTU Bac12; E, OTU Bac86. Bold and underlined letters indicate samples that have been subject to further sequence analyses.

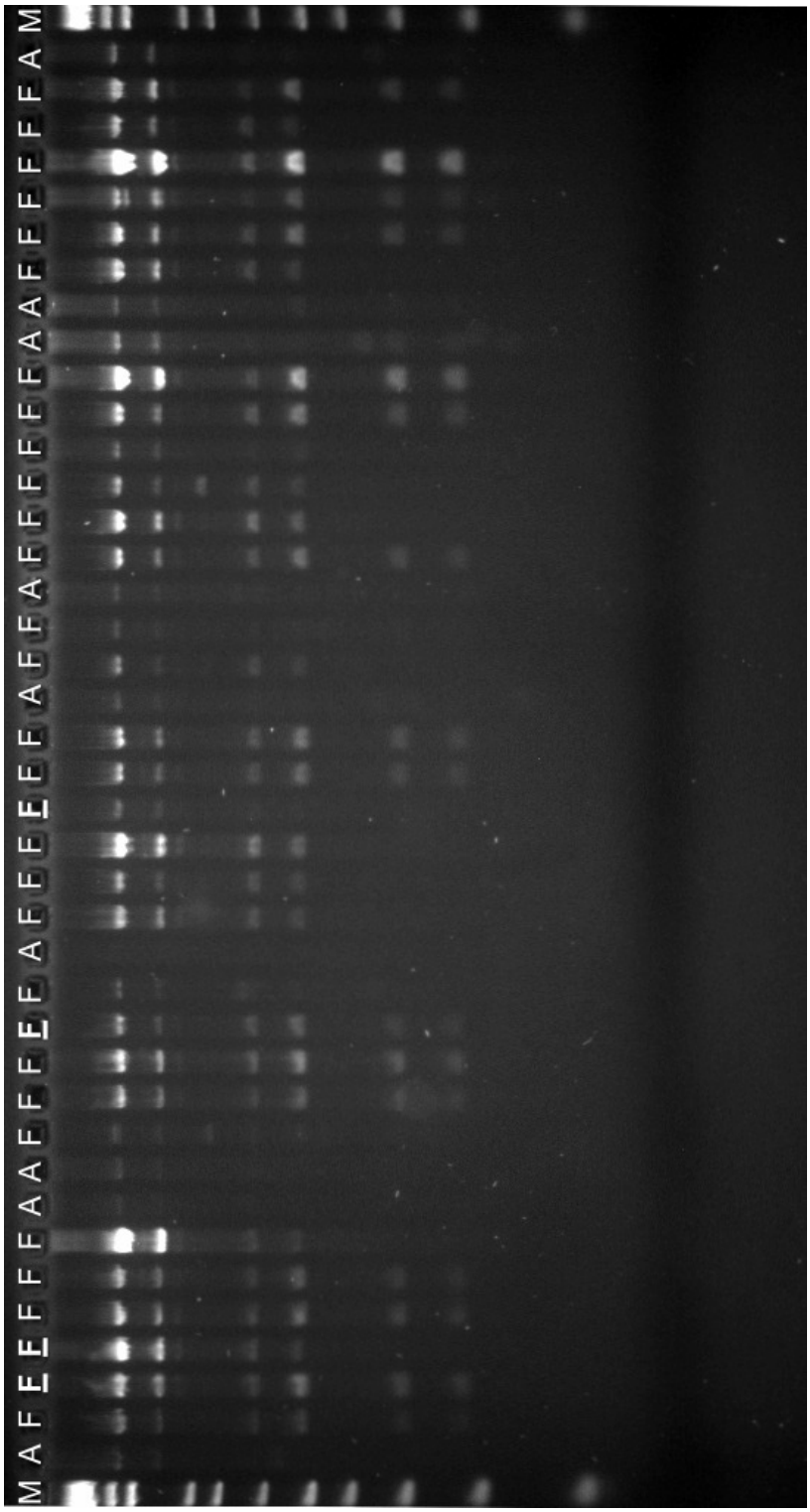

Figure 40. RFPL profiles of archaeal 16S rRNA genes. Continued on next page.

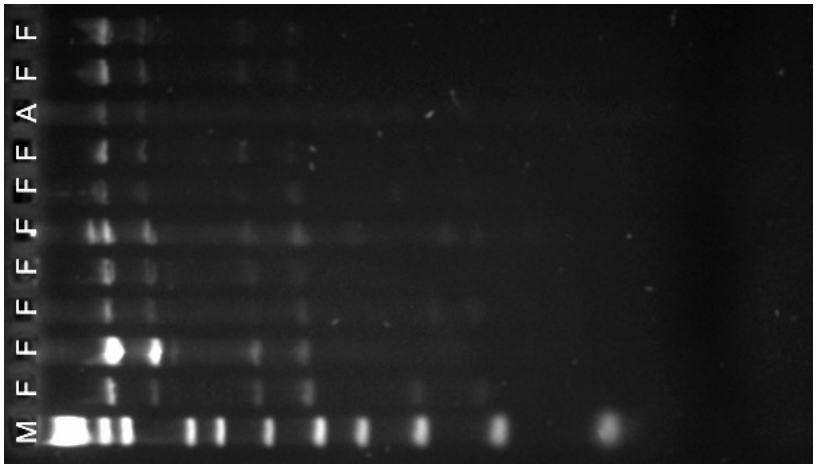

Figure 40. Restriction fragment length polymorphism (RFLP) profiles of archaeal 16S rRNA genes obtained from samples of Champagne Pool after enzymatic digestions with *Eco*R I, *Rsa* I, and *Bam*H I. M, 1 Kb Plus DNA ladder (Invitrogen); A, no sample or vector without insert; F, OTU Arc03. Bold and underlined letters indicate samples that have been subject to further sequence analyses.

Appendix E: Nucleotide sequences and alignments

Nucleotide sequences of 16S rRNA gene fragments amplified from DNA extracted from samples obtained from Champagne Pool in GenBank format. Single letter code: a, adenosine; c, cytidine; g, guanosine; t, thymidine.

LOCUS **DQ989208** 1506 bp DNA linear BCT 01-NOV-2006
DEFINITION Venenivibrio stagnispumantis strain CP.B2 16S ribosomal RNA gene, complete sequence.
ACCESSION DQ989208
VERSION DQ989208.1 GI:116518485
FEATURES Location/Qualifiers
source 1..1506
/organism="Venenivibrio stagnispumantis"
/mol type="genomic DNA"
/strain="**CP.B2**"
/db xref="taxon:407998"
/note="type strain of Venenivibrio stagnispumantis"
rRNA 1..1506
/product="16S ribosomal RNA"
ORIGIN

```
      1 agagtttgat cctggctcag cgcgaacgtt ggcggcgtgc ctaacacatg caagtcgtgg
     61 ggcagcaggc cttaccttcg ggtaaggtgc tggcgaccgg caaacgggtg agtaacacgt
    121 agcttaccta cccataggtt ggggataact gcccgaaagg gcagctaata cccaataatg
    181 agaaaactcc aaagccgcaa ggcgcctatg gatggggctg cgtcccatca gcttgttggt
    241 gaggtaatgg ctcaccaagg ctacgacggg tagccggtct gagaggatga ccggccacag
    301 tgggactgag acacggcccg cacccctacg gggggcagca gtggggaata ttgggcaatg
    361 gccgaaaggc tgacccagca acgccgcgtg gtggatgaag cccttcgggg tgtaaacacc
    421 ttttctgggg gaagattatg acggtacccc aggaataagg gacggctaac tacgtgccag
    481 cagccgcggt aatacgtagg tcccgaacgt tgcgcgaaat tactgggcgt aaagggtccg
    541 tagcggtctg gtaagtggta ggtgaaagcc cacgggttaa ccgtggaatt gccttccaaa
    601 ctgccggact tgaggtggaa gaggtcggcg gaattcccgg tgtacggtga aatgcgtaga
    661 tatcgggagg aacaccagta gcgaagcggc gactgggaca ttcctgacgc tgagggacga
    721 aagctaggga gcaaaccgga ttagataccc gggtagtcct agccgtaaac gatggatgct
    781 tgatgtgacc tgggatacca ggtcgtgtcg taagctaacg cgttaagcat cccgcctggg
    841 gagtacggcc gcaaggttga aactcaaagg aattgacggg gacccgcaca accggtggag
    901 cgtctggttt aattcgatgc taaccgaaaa acctcaccag ggtttgacat ccggtgtgtc
```

```
      961 gtatccgaaa gggtactagc ctatgatttc tcataggtgc gccggacagg tggtgcatgg
     1021 tcgtcgtcag ctcgtgtcgt gagatgttgg gttaagtccc gcaacgagcg caacccttgc
     1081 cctgtgttac cagcgggtta tgccggggac tcacagggga ctgccggcga taagtcggag
     1141 gaaggagggg atgacgtcag atcagtatgc cctttatgcc ctgggctaca caggcgctac
     1201 agtggcaggg acaatgggat gcaatgcagc aatgcggagc taaccccaaa aaccctgtcg
     1261 tggtgcggat tgggggttgc aactcacccc catgaaggcg gaatcggtag taatggcgaa
     1321 tcagcaatgt cgccgtgaat acgttcccgg gtcttgtaca caccgcccgt cacgccatgg
     1381 gagtcgggtt catcggaagt ccccgagcta accgcaagga ggcaggggcc gatgatgggc
     1441 ccgatgactg gggcgaagtc gtaacaaggt agccctaggg gaacctgcgg ctggatcacc
     1501 tcctta
```

//

LOCUS **EF101533** 330 bp DNA linear ENV 10-DEC-2006

DEFINITION Uncultured archaeon isolate DGGE fragment a 16S ribosomal RNA gene, partial sequence.

ACCESSION EF101533

VERSION EF101533.1 GI:118723383

FEATURES Location/Qualifiers

source 1..330

/organism="uncultured archaeon"

/mol type="genomic DNA"

/isolate="**DGGE fragment a**"

/isolation source="geothermal hot spring water"

/db xref="taxon:115547"

/environmental sample

/country="New Zealand: Waiotapu, Champagne Pool"

rRNA <1..>330

/product="16S ribosomal RNA"

ORIGIN

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        1 aggatgtgca tgccgtcgcc ggctcgtgct cgtaggtgtc ctgttaagtc aggcaacgag
       61 cgagaccccc acctcctagt tgcgtactcc aggcgctttc gggagccgtg ggcactctag
      121 ggaggactgc cggcgataag ccggaggaag gtgggggcta cggcaggttc agtatgcccc
      181 gaaaatcccc gggctacacg cgagtctgca agtggcgggg aacagcggga ttcctgaccc
      241 cgaaagaggg gaggtaatct cccgtaaacc ccgcctcatt aggaatcgag ggtctgacaa
      301 ctcgccctcg ttaaacgtag aaatccctag
```

//

LOCUS **EF101534** 411 bp DNA linear ENV 10-DEC-2006
DEFINITION Uncultured archaeon isolate DGGE fragment b 16S ribosomal RNA gene, partial sequence.
ACCESSION EF101534
VERSION EF101534.1 GI:118723384
FEATURES Location/Qualifiers
source 1..411
/organism="uncultured archaeon"
/mol type="genomic DNA"
/isolate="**DGGE fragment b**"
/isolation source="geothermal hot spring water"
/db xref="taxon:115547"
/environmental sample
/country="New Zealand: Waiotapu, Champagne Pool"
rRNA <1..>411
/product="16S ribosomal RNA"
ORIGIN

```
        1 ctgcggctca attggagtca acgcctggaa tcttaccggg ggagaccgca gtatgacggc
       61 cagcttacga ccttgcctga ctcgcggaga ggaggtgcat ggccgtcgcc agctcgtgtt
      121 gtgaaatgtc ctgttaagtc aggcaacgag cgagaccccc actcctagtt ggtattacgg
      181 tctccggacc gtgaccacac taggaggact gccgccgtaa ggcggaggaa ggaggggcc
      241 acggcaggtc agcatgcccc gaaactcccg ggccgcacgc gggttacaat ggcagggaca
      301 acgggaagct acctcgaaag ggggagccaa tccctaaacc tgccgcagtt gggatcgagg
      361 gctgaaaccc gccctcgtga acgaggatcc ctataaccgc gggtcaacaa c
```

//

LOCUS **EF101535** 146 bp DNA linear ENV 10-DEC-2006
DEFINITION Uncultured bacterium isolate DGGE fragment c 16S ribosomal RNA gene, partial sequence.
ACCESSION EF101535
VERSION EF101535.1 GI:118723385
FEATURES Location/Qualifiers
source 1..146
/organism="uncultured bacterium"

/mol_type="genomic DNA"
/isolate="**DGGE fragment c**"
/isolation_source="geothermal hot spring water"
/db_xref="taxon:77133"
/environmental_sample
/country="New Zealand: Waiotapu, Champagne Pool"

rRNA <1..>146
/product="16S ribosomal RNA"

ORIGIN

```
        1 ccagcaatac tgcgtgtttg atgaaggcct tcgggttgta aagcacttta acttgggaag
       61 aaaatgcttc cctacctacg tgaagccttg actgtaccag acagcaataa gcaccggcta
      121 acactgtgcc agcagccgcg gtaata
```

//

LOCUS **EF101536** 64 bp DNA linear ENV 10-DEC-2006
DEFINITION Uncultured bacterium isolate DGGE fragment d 16S ribosomal RNA gene, partial sequence.
ACCESSION EF101536
VERSION EF101536.1 GI:118723386
FEATURES Location/Qualifiers
source 1..64
/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolate="**DGGE fragment d**"
/isolation_source="geothermal hot spring water"
/db_xref="taxon:77133"
/environmental_sample
/country="New Zealand: Waiotapu, Champagne Pool"

rRNA <1..>64
/product="16S ribosomal RNA"

ORIGIN

```
        1 ccagcgacgc tgcgtggtgg atgaaggcct tcgggttgta acccctttc tgggggacga
       61 tcct
```

//

LOCUS **EF101537** 699 bp DNA linear ENV 10-DEC-2006
DEFINITION Uncultured archaeon 16S ribosomal RNA gene, partial sequence.
ACCESSION EF101537
VERSION EF101537.1 GI:118723387
FEATURES Location/Qualifiers
source 1..699
/organism="uncultured archaeon"
/mol type="genomic DNA"
/isolation source="geothermal hot spring water"
/db xref="taxon:115547"
/clone="**OTU Arc03**"
/environmental sample
/country="New Zealand: Waiotapu, Champagne Pool"
rRNA <1..>699
/product="16S ribosomal RNA"
ORIGIN

```
      1 ccaggcccta cggtggcgca ccaggcgcgc aacgtcccca atgcgcgcaa gcgtgagggc
     61 gctaccccga gtgcctcctt ttggaggctt ttctccggct ctaaaaaggc ggaggaataa
    121 gcgggggggca agtctggtgt cagccgccgc ggtaacacca gctccgcgag tggtcgggac
    181 gtttactggg cctaaagcgc ctgtagccgg ctcactaagt tgctccttaa agccccgggc
    241 tcaacccggg gactgggagc aatactagtg agctaggggg cgggagaggc caggggtact
    301 cccggaggag gggcgaaatc cgtagatcct gggaggacca ccagtggcgg aggcgcctgg
    361 ctagaacgcg cccgacggtg agaggcgaaa gccggggcag caaacaggat tagataccct
    421 ggtagtcccg gctgtaaacg atgcgggcta ggtgtcacgt gggcttagag ctcacgtggt
    481 gccgcaagga agccgttaag cccgccgcct ggggagtacg gtcgcaagac tgaaacttaa
    541 aggaattggc gggggagcac cacaaggggt ggaacctgcg gctcaattgg agtcaacgcc
    601 tggaatctta ccgggggaga ccgcagtatg acggccaggc ttacgacctt gcctgactcg
    661 cggagaggag gtgcatggcc gtcgccagct cgtgttgtg
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//

LOCUS **EF101538** 1426 bp DNA linear ENV 10-DEC-2006
DEFINITION Uncultured bacterium 16S ribosomal RNA gene, partial sequence.
ACCESSION EF101538
VERSION EF101538.1 GI:118723388
FEATURES Location/Qualifiers
source 1..1426

/organism="uncultured bacterium"
/mol_type="genomic DNA"
/isolation_source="geothermal hot spring water"
/db_xref="taxon:77133"
/clone="**OTU Bac03**"
/environmental_sample
/country="New Zealand: Waiotapu, Champagne Pool"

rRNA <1..>1426
/product="16S ribosomal RNA"

ORIGIN

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      1 ggcggcagtg cttaacacat gcaagtcgag cgggcgcttc gggcgtcagc ggcggacggg
     61 ttagtaacgc gtgggaacgt gccctttgct ctggaatagc cctgggaaac tgggggtaat
    121 accggatgtg ccctgagggg gaaagattta tcggcaaggg atcggcccgc gtctgattag
    181 gtagttggtg aggtaacggc tcaccaagcc gacgatcggt agtggtttga gaggatgatc
    241 agcaacactg ggactgagac acggcccaga ctcctacggg aggcagcagt ggggaatctt
    301 agacaatggg cgcaagcctg atctagccat gccgcgtggg tgatgaaggc cttagggtcg
    361 taaagccctt tcgccgggga agataatgac ggtacccggt aaagaaaccc cggctaactc
    421 cgtgccagca gccgcggtaa tacggagggg gttagcgttg ttcggaatga ctgggcgtaa
    481 agcgcgcgta ggcggatcat caagtcgggg gtgaaatccc ggggctcaac cccggaatgg
    541 ccttcgagac tggtggtctt gagttcgaga gaggtgagtg gaattccgag tgtagaggtg
    601 aaattcgtag atattcggag gaacaccagt ggcgaaggcg gctcactggc tcgacactga
    661 cgctgaggtg cgaaagcgtg gggagcaaac aggattagat accctggtag tccacgcccg
    721 taaacgatga atgccagacg tcgggggggct tgctcttcgg tgtcacacct aacggattaa
    781 gcattccgcc ctggggagta cggtcgcaag attaaactca aaggaattga cgggggcccg
    841 acaacggtgg agcatgtggt ttaattcgaa gcaacgcgca gaaccttacc aacccttgac
    901 atcgcctgac cggcctggag aaggtctttc ccgcaaggga caggtggaca ggtgctgcat
    961 ggctgtcgtc agctcgtgtc gtgagatgtt cggttaagtc cggcaacgag cgcaacccac
   1021 acccttggtt gccagcacgt aatggtgggc actttagggg aactgcccgt gataagcggg
   1081 aggaaggtgt ggatgacgtc aagtcctcat ggcccttacg ggttgggcta cacacgtgct
   1141 acaatggtgg tgacagaggg ttcatcccta aaagccatct cagttcggat tgtcgtctgc
   1201 aactcgacgg catgaagtcg gaatcgctag taatcgcgca tcagctcggc gcggtgaata
   1261 cgttcccggg ccttgtacac accgcccgtc acaccatggg agttgggttg acccgaaggt
   1321 ggtgcgccga cccgtaaggg gggcagccaa ccacggtcag ttcagcgact ggggtgaagt
   1381 cgtaacaagg tagccgtagg ggaacctgcg gctggatcac ctcctt
```

//

LOCUS **EF101539** 715 bp DNA linear ENV 10-DEC-2006
DEFINITION Uncultured bacterium 16S ribosomal RNA gene, partial sequence.
ACCESSION EF101539

VERSION EF101539.1 GI:118723389
FEATURES Location/Qualifiers
source 1..715
/organism="uncultured bacterium"
/mol type="genomic DNA"
/isolation source="geothermal hot spring water"
/db xref="taxon:77133"
/clone="**OTU Bac04**"
/environmental sample
/country="New Zealand: Waiotapu, Champagne Pool"
rRNA <1..>715
/product="16S ribosomal RNA"
ORIGIN

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      1 agagtttgat cctggctcag cgcgaacgtt ggcggcgtgc ctaacacatg caagtcgtgg
     61 ggcagcaggc cttaccttcg ggtaaggtgc tggcgaccgg caaacgggtg agtaacacgt
    121 agcttaccta cccataggtt ggggataact gcccgaaagg gcagctaata cccaataatg
    181 aggaaactcc aaagccgcaa ggcgcctatg gatggggctg cgtcccatca gcttgttggt
    241 gaggtaatgg ctcaccaagg ctacgacggg tagccggtct gagaggatga ccggccacag
    301 tgggactgag acacggcccg cacccctacg gggggcagca gtggggaata ttgggcaatg
    361 gccgaaaggc tgacccagca acgccgcgtg gtggatgaag cccttcgggg tgtaaacacc
    421 ttttctgggg gaagataatg acggtacccc aggaataagg gacggctaac tacgtgccag
    481 cagccgcggt aatacgtagg tcccgaacgt tgcgcgaaat tactgggcgt aaagggtccg
    541 taggcggtct ggtaagtgga aggtgaaagc ccacggctta accgtggaat tgccttccaa
    601 actgccggac ttgaggatgg aagaggtcgg cggaattccc ggttgtagcg gttgaaatgc
    661 gtagatatcg ggaggaacac cagtagcgaa ggcggcgact gggacattcc tgacg
```

//

LOCUS **EF101540** 536 bp DNA linear ENV 10-DEC-2006
DEFINITION Uncultured bacterium 16S ribosomal RNA gene, partial sequence.
ACCESSION EF101540
VERSION EF101540.1 GI:118723390
FEATURES Location/Qualifiers
source 1..536
/organism="uncultured bacterium"
/mol type="genomic DNA"
/isolation source="geothermal hot spring water"

/db_xref="taxon:77133"
/clone="**OTU Bac12**"
/environmental_sample
/country="New Zealand: Waiotapu, Champagne Pool"
rRNA <1..>536
/product="16S ribosomal RNA"
ORIGIN

```
        1 gaaagggcac tagcctatga tttctcatag gtgcgccgga cagtggtgca tggtcgtcgt
       61 cagctcgtgt cgtgagatgt tgggttaagt cccgcaacga gcgcaaccct tgccctgtgt
      121 taccagcggg ttatgccggg gactcacagg ggactgccgg cgataagtcg gaggaaggag
      181 gggatgacgt cagatcagta tgcccttttat gccctgggct acacaggcgc tacagtggca
      241 gggacaatgg gatgcaatgc agcaatgcgg agctaacccc aaaccctgtc gtggtgcgga
      301 ttgggggttg caactcaccc ccatgaaggc ggaatcggta gtaatggcga atcagcaatg
      361 tcgccgtgaa tacgttcccg ggtcttgtac acaccgcccg tcacgccatg ggagtcgggt
      421 tcatcggaag tccccgagct aaccgcaagg aggcaggggc cgatgatggg cccgatgact
      481 ggggcgaagt cgtaacaagg tagccctagg ggaacctgcg gctggatcac ctcctt
```

//

LOCUS **EF101541** 962 bp DNA linear BCT 10-DEC-2006
DEFINITION Archaeon CP.B3 16S ribosomal RNA gene, partial sequence.
ACCESSION EF101541
VERSION EF101541.1 GI:118723391
FEATURES Location/Qualifiers
source 1..962
/organism="archaeon CP.B3"
/mol_type="genomic DNA"
/strain="**CP.B3**"
/isolation_source="geothermal hot spring water"
/db_xref="taxon:413980"
/country="New Zealand: Waiotapu, Champagne Pool"
rRNA <1..>962
/product="16S ribosomal RNA"
ORIGIN

```
        1 cgcaatgcgg gaaacgcgac ggggggaccc cagtgccgtg gcaatgccac ggcttttccg
       61 gagtgtaagg agctccggga ataagggctg ggcaaggccg gtggcagccg ccgcggtaat
      121 accggcggcc cgagtggtgg ccactattat tgggcctaaa gcgtccgtag ccgggcccgt
```

```
      181 aagtccctgg cgaaatctca cggctcaacc gtggggctcg ctggggatac tgcgggcctt
      241 gggaccggga gaggcggagg gtacccctgg ggtaggggtg aaatcctata atcccagggg
      301 gaccgccagt ggcgaaggcg ctccgctgga acgggtccga cggtgaggga cgaaggccag
      361 gggagcaaac cggattagat acccgggtag tcctggctgt aaaggatgcg ggctaggtgt
      421 cgggtgagct tcgggctcgc ccggtgccgg agggaagccg ttaagcccgc cgcctgggga
      481 gtacggccgc aaggctgaaa cttaaaggaa ttggcggggg agcaccacaa ggggtggagc
      541 gtgcggttta attggattca acgccgggaa cctcaccggg ggcgacggca ggatgaaggc
      601 caggctgaag gtcttgccgg acacgccgag aggaggtgca tggccgccgt cagctcgtac
      661 cgtgaggcgt ccacttaagt gtggtaacga gcgagacccg tgcccccagt tgccagtcct
      721 ccccgctggg gaggaggcac tctgggggga ccgccggcga taagccggag gaaggagcgg
      781 gcgacggtag gtcagtatgc cccgaaaccc ccgggctaca cgcgcgctac aatgggcggg
      841 acaatgggat ccgaccccga aaggggaagg aaatccccta aacccgcccc cagttcggat
      901 tgcgggctgc aactcgcccg catgaagctg gaatccctag tacccgcggt catcatcgcg
      961 cg
```

//

LOCUS **EF101542** 1310 bp DNA linear BCT 10-DEC-2006

DEFINITION Bacterium CP.B1 16S ribosomal RNA gene, partial sequence.

ACCESSION EF101542

VERSION EF101542.1 GI:118723392

FEATURES Location/Qualifiers

source 1..1310

/organism="bacterium CP.B1"

/mol type="genomic DNA"

/strain="**CP.B1**"

/isolation source="geothermal hot spring water"

/db xref="taxon:413981"

/country="New Zealand: Waiotapu, Champagne Pool"

rRNA <1..>1310

/product="16S ribosomal RNA"

ORIGIN

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      121 ccgctcaaga tgggctcgcg tcccatcagc tagttggtga ggtaacggct caccaaggct
      181 acgacgggta gccggcctga gagggtggtc ggccacactg ggactgagac acggcccaga
      241 ctcctacggg aggcagcagt ggggaatctt ccgcaatggg cgaaagcctg acggagcgac
      301 gccgcgtgag cgaagaaggc cttcgggtcg taaagctcga tagtgtggga agaaggtatg
      361 acggtaccac acgaaagccc cggctaacta cgtgccagca gccgcggtaa gacgtagggg
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 421 cgagcgttgt ccggagttac tgggcgtaaa gggcgcgtag cggtttagca agtcaggtgt
 481 aaaaggccac ggctcaaccg tggatatgca tctgaaactg ctgagctaga gggcaggaga
 541 ggggagtgga attcccggtg tagcggtgaa atgcgtagat atcgggagga ataccagtgg
 601 cgaaagcgac tcctggactg gccctgacgc tgaggcgcga aagcgtgggg agcaaacagg
 661 attagatacc ctggtagtcc acgctgtaaa cgatgggtgc taggtgtggg gagcggaagc
 721 tttccgtgcc gtaggaaacc caataagcac cccgcctggg agtacggccg caaggctgaa
 781 actcaaagga attgacgggg gcccgcacaa gcggtggagc atgtggttta attcgaagca
 841 acgcgaagaa ccttaccagg gcttgacatg caggtagtag cgagccgaaa ggtgagcgac
 901 cctctcttat gggagggagc ctgcacaggt ggtgcatggt tgtcgtcagc tcgtgtcgtg
 961 agatgttggg ttaagtcccg caacgagcgc aacccctgcc tctagttgcc agcgggtaaa
1021 gccgggcact ctagagggac tgccgtcgat aagacggagg aaggtgggga tgacgtcaaa
1081 tcatcatgcc ctatatgccc tgggccacac acgtgctaca atggccggta cagagggaag
1141 cgaagccgcg agccggagcg aatcccaagc cggtctcagt tcggattgca ggctgcaact
1201 cgcctgcatg aaggcggaat cgctagtaat cgcggatcag catgcccggt gaatgcgttc
1261 ccgggccttg tacacaccgc ccgtcacacc acgagagtct gcaacacccg
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Alignments of archaeal sequences of 16S rRNA gene fragments obtained from Champagne Pool and closest relatives. Abbreviations: Thf.pend, *Thermofilum pendens*; Sulf.acid, *Sulfolobus acidocaldarius*; Sulfo.toko, *Sulfolobus tokodaii*; Thc.waio, *Thermococcus waiotapuensis*. Positions without nucleotide variability are indicated in bold.

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           1          11         21         31         41         51         61         71         80
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   GACTCCGGTT GATCTGCCGG ACCCGACCGC TATCGGGGTG GGGCTAACCC ATGGAAGTCT AGGAGCCTCG GCTCCGGCGG
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  .ATTCCGGTT GATCTGCCGG ACCCGACCGC TATCGGGGTA GGGATAAGCC ATGGGAGTCT TACACTCTAG AGTGTGGCGG
OTU Arc03  .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.toko  AATTCCGGTT GATCTGCCGG ACCCGACCGC TATCGGGGTA GCACTAAGCC ATGGGAGTCG TACACCCCAG GGTGTGGCGG
    CP.B3  .......... .......... .......... .......... .......... .......... .......... ..........
Thc.waio   .......... .......... .......... .......... .......... .......... .......... ..........

           81         91         101        111        121        131        141        151        160
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   ACGGCTCAGT AGCACGTGGC TAACCTACCC TCGGGAGGGG GATAACCCCG GGAAACTGGG GATAAACCCC CAAGGCGCGG
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  ACGGCTGAGT AACACGTGGC TAACCTACCC TCGGGACGGG GATAACCCCG GGAAACTGGG GATAATCCCC GAAGGGAAGG
OTU Arc03  .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.toko  ACGGCTGAGT AACACGTGGC TAACCTACCC TCGGGAGGGG GATAACCCCG GGAAACTGGG GATAATCCCC CAAGGGGAGG
    CP.B3  .......... .......... .......... .......... .......... .......... .......... ..........
Thc.waio   ..GGCTCAGT AACACGTCGG TAACCTACCC TCGGGAGGGG GATAACCCCG GGAAACTGGG GCTAATCCCC CAAGGTCTGA
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           161        171        181        191        201        211        221        231        240
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   ACACCTGGAA TGGGTCCGCG CTGAAAGATG CGCCCGAGGA TGGGGCTGCG CCCTATCAGG TAGTTGGCGG GGTAACGGCC
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  AGTCCTGGAA TGGTTCCTTC CCTAAAGTTC CGCCCGAGGA TGGGGCTACG GCCCATCAGG CTGTCGGTGG GGTAAGGCC
OTU Arc03  .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.toko  AGTCCTGGAA CGGTTCCTCC CTTAAAGTTC CGCCCGAGGA TGGGGCTGCG GCCCATCAGG CTGTTGGCGG GGTAACGGCC
    CP.B3  .......... .......... .......... .......... .......... .......... .......... ..........
Thc.waio   GGTACTGGAA GGTCCTCGGG CCGAAAGTCT CGCCCGAGGA TGGGCCGGCG GCCGATTAGG TAGTTGGTGG GGTAACGGCC

           241        251        261        271        281        291        301        311        320
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   CGCCAAGCCG ATAACGGGTG GGGGCCGTGA GAGCGGAGCC CGAGATGGGA GACAAGCCCA GGCTAACCAG GGGCGAACT
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  CACCGAACCT ATAACGGGTA GGGGCCGTGG AAGCGGAGCC CCAGTTGGGA GACAAGCCCA GGCTAACCAG GCGCGAACG
OTU Arc03  .......... .......... .......... .......... .......... .......CCA GGCTAACCAG GCGCGCAACG
Sulf.toko  CGCCAAACCT ATAACGGGTA GGGGCCGTGG GAGCGGAGCC CCAGTTGGGA GACAAGCCCA GGCTAACCAG GCGGGAAACG
    CP.B3  .......... .......... .......... .......... .......... .......... .......... ..........
Thc.waio   CACCAAGCCT AAGATCGGTA CGGGCCATGA GAGTGGAGCC GGAGATGGAA GACAAGTCCA GGCTAAGCAG GCGCGAAACC

           321        331        341        351        361        371        381        391        400
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   TCCGCAATGC GGGAAACCGT GACGGAGTCA CCCCGAGTAC CAAGGGTGGC TTTTGCCCGG TCTAAAAAGC CGGGGAATAA
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  TCCCCAATGC GCGAAAGCGT GAGGGCGCTA CCCCGAGTTC CGCAGGAGGC TTTTCCCCGC TCTAAAAAGG CGGGGAATAA
OTU Arc03  TCCCCAATGC GCGCAAGCGT GAGGGCGCTA CCCCGAGTTC CTTTGGAGGC TTTTCTCCGG TCTAAAAAGG CGGAGAATAA
Sulf.toko  TCCCCAATGC GCGAAAGCGT GAGGGCGCTA CCCCGAGTCC CGTAGGGGGC TTTTCCCCGC TCTACAAAGG CGGGGAATAA
    CP.B3  ....CGCAAT GCGGGAAACG CGACGGGGGG ACCCCAGTGT GAATCACGGC TTTTCCGGAG TGTAAGGAGC TCCGGAATAA
Thc.waio   TCCGCAATGC GGGAAACCGC GACGGGGGGA CCCCCAGTGT GAATCACGGC TTTTCCGGAG TGTAAGGAGC TCCGGAATAA
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           401        411        421        431        441        451        461        471        480
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   GCGGGGGGAA GCTGTGGCCG CCGCGGTAAT ACCACCCGCG AGTGGTCGGG ACGTTTATTG GGCCTAAAGC GTCCGTAGCC
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  GCGGGGGGAA GTTGTGGCCG CCGCGGTAAT ACCATCCGCG AGTGGTCGGG GTGATTACTG GGCCTAAAGC GCCTGTAGCC
OTU Arc03  GCGGGGGGAA GTTGTGGCCG CCGCGGTAAC ACCATCCGCG AGTGGTCGGG ACGTTTACTG GGCCTAAAGC GCCTGTAGCC
Sulf.toko  GCGGGGGGAA GTTGTGGCCG CCGCGGTAAC ACCATCCGCG AGTGGTCGGG ACGCTTACTG GGCCTAAAGC GCCTGTAGCC
   CP.B3   GGGCTGGGAA GGCGTGGCCG CCGCGGTAAT ACCGGGCCCG AGTGGTGGCC ACTATTATTG GGCCTAAAGC GTCCGTAGCC
Thc.waio   G.GCTGGGAA GCGGTGGCAG CCGCGGTAAT ACCGGGCCCG AGTGGTGGCC ACTATTATTG GGCCTAAAGC GTCCGTAGCC

           481        491        501        511        521        531        541        551        560
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   GGCCCGGTAA GTCCCTCCTT AAAGCCCACG GCTCAACCGT GGGGCGGAGG GATACTGCCG GGCTAGGGGG CGGAGAGGCC
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  GGCCCACCAA GTCGCCCCTT AAAGTCCCCG GCTCAACCGG GGATGGGGGC GATACTGGTG GGCTAGGGGG CGGAGAGGCG
OTU Arc03  GGCTCACTAA GTTGCTCCTT AAAGCCCCGG GCTCAACCCG GGGTGGGAGC AATACTAGTG AGCTAGGGGG CGGAGAGGCC
Sulf.toko  GGCCCACTAA GTCGCCCCTT AAAGACCCGG GCTCAACCCG GGATGGGGGC GATACTGGTG GGCTAGGGGG CGGAGAGGCT
   CP.B3   GGGCCCGTAA GTCCCTGGCG AAATCTCACG GCTCAACCGT GGGCGCTGGG GATACTGCGG GCCTTGGGAC CGGAGAGGCG
Thc.waio   GGGCCCGTAA GTCTCTGACG AAATCTCACG GCTCAACCGT GGGCGCTGGG GATACTGCGG GCCTTGGGAC CGGAGAGGCG

           561        571        581        591        601        611        621        631        640
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   GGGGGTACTC CTGGGGTAGG GGCGAAATCC TATAATCCCA GGAGGACCAC CAGTGGCGAA GGCGCCCGGC TAGCACGCGC
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  GGGGGTACTC CCGGAGTAGG GGCGAAATCC TTAGATACCG GGAGGACCAC CAGTGGCGGA AGCGCCCCGC TAGAACGCGC
OTU Arc03  AGGGGTACTC CCGGAGGAGG GGCGAAATCC GTAGATCCTG GGAGGACCAC CAGTGGCGGA GGCGCCTGGC TAGAACGCGC
Sulf.toko  AGGGGTACTC CCAGAGGAGG GGCGAAATCC GTAGATCCTG GGAGGACCAC CAGTGGCGGA GGCGCCTAGC TAGAACGCGC
   CP.B3   GAGGGTACCC CTGGGGTAGG GGTGAAATCC TATAATCCCA GGGGGACCGC CAGTGGCGAA GGCGCTCCGC TGGAACGGGT
Thc.waio   GATGGTACCC CTGGGGTAAG GGTGAAATCC TATAATCCCA GGGGGACCGC CAGTGGCGAA GGCGCTCCGC TGGAACGGGT
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           641        651        661        671        681        691        701        711        720
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   CGACGGTGAG GGACGAAAGC TGGGGGAGCA AAGGGGATTA GATACCCCCG TAGTCCCAGC TGTAAACGAT GCGGGCTAGG
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  CGACGGTGAG AGGCGAAAGC CGGGGCAGCA AACGGGATTA GATACCCCGG TAGTCCCGGC TGTAAACGAT GCGGGCTAGG
OTU Arc03  CGACGGTGAG AGGCGAAAGC CGGGGCAGCA AACAGGATTA GATACCCTGG TAGTCCCGGC TGTAAACGAT GCGGGCTAGG
Sulf.toko  CGACGGTGAG AGGCGAAAGC CGGGGCAGCA AATGGGATTA GATACCCCAG TAGTCCCGGC TGTAAACGAT GCGGGCTAGG
   CP.B3   CGACGGTGAG GGACGAAGGC CAGGGGAGCA AACCGGATTA GATACCCGGG TAGTCCTGGC TGTAAAGGAT GCGGGCTAGG
Thc.waio   CGACGGTGAG GGACGAAGGC CAGGGGAGCA AACCGGATTA GATACCCGGG TAGTCCTGGC TGTAAAGGAC GCGGGCTAGG

           721        731        741        751        761        771        781        791        800
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   TGTTGGGGCT TCGAGCCCGT CCAGTGCCGT AGGAAGCCGT TAAGCCCGCC GCCTGGGGAG TACGGCCGCA AGGCTGAAAC
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  TGTCGAGGCT TAGAGCCTAC TCGGTGCCGC AGGAAGCCGT TAAGCCCGCC GCCTGGGGAG TACGGTCGCA AGACTGAAAC
OTU Arc03  TGTCACGGCT TAGAGCTCAC GTGGTGCCGC AGGAAGCCGT TAAGCCCGCC GCCTGGGGAG TACGGTCGCA AGACTGAAAC
Sulf.toko  TGTCAGGGCT TAGAGCTCAC CTGGTGCCGC AGGAAGCCGT TAAGCCCGCC GCCTGGGGAG TACGGTCGCA AGACTGAAAC
   CP.B3   TGTCGGAGCT TCGGGCTCGC CCGGTGCCGG AGGAAGCCGT TAAGCCCGCC GCCTGGGGAG TACGGCCGCA AGGCTGAAAC
Thc.waio   TGTCGGAGCT TCGGGCTCGC CCGGTGCCGG AGGAAGCCGT TAAGCCCGCC GCCTGGGGAG TACGGCCGCA AGGCTGAAAC

           801        811        821        831        841        851        861        871        880
           |          |          |          |          |          |          |          |          |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   TTAAAGGAAT TGGCGGGGGA GCACCACAAG GGGTGAAGCT TGCGGTTTAA TTGGAGTCAA CGCCGGAAAC CTTACCGGGG
  DGGE b   .......... .......... .......... .........C TGCGGCTCAA TTGGAGTCAA CGCCTGGAAT CTTACCGGGG
Sulf.acid  TTAAAGGAAT TGGCGGGGGA GCACCACAAG GGGTGGAACC TGCGGCTCAA TTGGAGTCAA CGCCTGGAAT CTTACCGGGG
OTU Arc03  TTAAAGGAAT TGGCGGGGGA GCACCACAAG GGGTGGAACC TGCGGCTCAA TTGGAGTCAA CGCCTGGAAT CTTACCGGGG
Sulf.toko  TTAAAGGAAT TGGCGGGGGA GCACCACAAG GGGTGGAACC TGCGGCTCAA TTGGAGTCAA CGCCTGGAAT CTTACCGGGG
   CP.B3   TTAAAGGAAT TGGCGGGGGA GCACCACAAG GGGTGGAGCG TGCGGTTTAA TTGGATTCAA CGCCGGGAAC CTCACCGGGG
Thc.waio   TTAAAGGAAT TGGCGGGGGA GCACCACAAG GGGTGGAGCG TGCGGTTTAA TTGGATTCAA CGCCGGGAAC CTCACCGGGG
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           881         891         901         911         921         931         941         951         960
           |           |           |           |           |           |           |           |           |
  DGGE a   .......... .......... .......... .......... .......AGG ATGTGCATGC CGTCGCCGGC TCGTGCTCGT
Thf.pend   GCGACAGCAG GATGAAGGCC AGCTGCGACC TTGCCAGACG AGCTGAGAGG AGGTGCATGG CGTCGCCGGC TCGTGCCGTG
  DGGE b   GAGACCGCAG TATGACGGCC AGCTACGACC TTGCCTGACT CGCGGAGAGG AGGTGCATGG CGTCGCCAGC TCGTGTTGTG
Sulf.acid  GAGACCGCAG TATGACGGCC AGCTACGACC TTGCCTGACT CGCGGAGAGG AGGTGCATGG CGTCGCCAGC TCGTGTTGTG
OTU Arc03  GAGACCGCAG TATGACGGCC AGCTTCGACC TTGCCTGACT CGCGGAGAGG AGGTGCATGG CGTCGCCAGC TCGTGTTGTG
Sulf.toko  GAGACCGCAG GATGACGGCC AGCTACGACC TTGCCTGACT CGCGGAGAGG AGGTGCATGG CGTCGCCAGC TCGTGTTGTG
    CP.B3  GCGACGGCAG GATGAAGGCC AGCTGAGGTC TTGCCGGACA CGCCGAGAGG AGGTGCATGG CGCCGTCAGC TCGTACCGTG
 Thc.waio  GCGACGGCAG GATGAAGGCC AGCTGAGGTC TTGCCGGACA CGCCGAGAGG AGGTGCATGG CGCCGTCAGC TCGTACCGTG

           961         971         981         991         1001        1011        1021        1031        1040
           |           |           |           |           |           |           |           |           |
  DGGE a   AGGTGTCCTG TTAAGTCAGG CAACGAGCGA GACCCCCACC CCTAGTTGCT ACCCAGGGCT TCGAGCCGGC ACTCTAGGAG
Thf.pend   AGGTGTCCTG TTAAGTCAGG GAACGAGCGA GACCCCCGCC CCTAGTTGCT ACCCAGCCCT TCGGGCTGGC ACTCTAGGGG
  DGGE b   AAATGTCCTG TTAAGTCAGG CAACGAGCGA GACCCCCACT CCTAGTTGGT ATTACGGTCT CCGACCGTGC ACACTAGGAG
Sulf.acid  AAATGTCCGG TTAAGTCCGG CAACGAGCGA GACCCCCACC CCTAGTTGGT ATTCTGGACT CCGTCCAGAC ACACTAGGGG
OTU Arc03  .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.toko  AAATGTCCTG TTAAGTCAGG CAACGAGCGA GACCCCCACC CCTAGTTGGT ATCCCCATCT CCGATGGGGC ACACTAGGGG
    CP.B3  AGGCGTCCAC TTAAGTGTGG TAACGAGCGA GACCCGTGCC CCCAGTTGCC AGTCCTCCCG CTGGGAGGAC ACTCTGGGGG
 Thc.waio  AGGCGTCCAC TTAAGTGTGG TAACGAGCGA GACCCGTGCC CCCAGTTGCC AGTCTTCCCG TTGGGAGGAC ACTCTGGGGG

           1041        1051        1061        1071        1081        1091        1101        1111        1120
           |           |           |           |           |           |           |           |           |
  DGGE a   GACTGCCGGC GTAAGCCGAG GAAGGTGGGG GCTACGGCAG GTCAGTATGC CCCGAAATCC CCGGGCTACA CGCGAGCTGC
Thf.pend   GACTGCCGGC GTAAGCCGAG GAAGGTGGGG GCTACGGCAG GTCAGTATGC CCCGAAACCC CCGGGCTACA CGCGAGCTGC
  DGGE b   GACTGCCGCC GTAAGGCGAG GAAGGAGGGG GCCACGGCAG GTCAGCATGC CCCGAAACTC CCGGGCCGCA CGCGGGTTAC
Sulf.acid  GACTGCCGGC GTAAGCCGAG GAAGGAGGGG GCCACGGCAG GTCAGCATGC CCCGAAACTC CCGGGCCGCA CGCGGGTTAC
OTU Arc03  .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.toko  GACTGCCGCC GTAAGGCGAG GAAGGAGGGG GCCACGGCAG GTCAGCATGC CCCGAAACCC CCGGGCCGCA CGCGGGTTAC
    CP.B3  GACCGCCGGC GTAAGCCGAG GAAGGAGCGG GCGACGGTAG GTCAGTATGC CCCGAAACCC CCGGGCTACA CGCGCGCTAC
 Thc.waio  GACCGCCGGC GTAAGCCGAG GAAGGAGCGG GCGACGGTAG GTCAGTATGC CCCGAAACCC CCGGGCTACA CGCGCGCTAC
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           1121       1131       1141       1151       1161       1171       1181       1191      1200
           |          |          |          |          |          |          |          |         |
  DGGE a   AATGGCGGGG ACAGCGGGAT CCGACCCGA AAGAGGGAGG TAATCCCTAA ACCCGCCTCA TTAGGAATCG AGGGTTGAAA
Thf.pend   AATGGCGGGG ACAGCGGGTT CCGACCCGA AAGGGGGAGG TAATCCCTAA ACCCGCCTCA GTAGGAATCG AGGGCTGCAA
  DGGE b   AATGGCAGGG ACAACGGGAA GCTACCTCGA AAGGGGGAGC CAATCCCTAA ACCTGCCGCA GTTGGGATCG AGGGCTGAAA
Sulf.acid  AATGGCAGGG ACAACGGGAT GCTACCTCGA AAGGGGGAGC CAATCCTTAA ACCTGCCGCA GTTGGGATCG AGGGCTGAAA
OTU Arc03  .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.toko  AATGGCAGGG ACAGCGGGAT GCGACCCGA AAGGGGGAGC CAATCCCCAA ACCTGCCGCA GTTGGGATCG AGGGCTGAAA
    CP.B3  AATGGGCGGG ACAATGGGAT CCGACCCGA AAGGGGAAGG AAATCCCTAA ACCGCCCCCA GTTCGGATTG CGGGCTGCAA
 Thc.waio  AATGGGCGGG ACAATGGGAT CCGACCCGA AAGGGGAAGG GAATCCCTAA ACCGCCCCCA GTTCGGATTG CGGGCTGCAA

           1201       1211       1221       1231       1241       1251       1261       1271      1280
           |          |          |          |          |          |          |          |         |
  DGGE a   CTCGCCCTCG TTAACGTAGA TCCCTA.... .......... .......... .......... .......... ..........
Thf.pend   CTCGCCCTCG TGAACGTGGA TCCCTATAAC CGCGGTCACA ACGCGCGGTG AATACGTCCC TGCTCCTTGC ACACACCGCC
  DGGE b   CCCGCCCTCG TGAACGAGGA TCCCTATAAC CGCGGTCAAA AC......... .......... .......... ..........
Sulf.acid  CCCGCCCTCG TGAACGAGGA TCCCTATAAC CGCGGTCAAA ACCCGCGGTG AATACGTCCC TGCTCCTTGC ACACACCGCC
OTU Arc03  .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.toko  CCCGCCCTCG TGAACGAGGA TCCCTATAAC CGCGGTCAAA ACCCGCGGTG AATACGTCCC TGCTCCTTGC ACACACCGCC
    CP.B3  CTCGCCCGCA TGAAGCTGGA TCCCTATACC CGCGGTCATA TCGCGCG... .......... .......... ..........
 Thc.waio  CTCGCCCGCA TGAAGCTGGA TCCCTATACC CGCGGTCATA TCGCGCGGCG AATACGTCCC TGCTCCTTGC ACACACCGCC

           1281       1291       1301       1311       1321       1331       1341       1351      1360
           |          |          |          |          |          |          |          |         |
  DGGE a   .......... .......... .......... .......... .......... .......... .......... ..........
Thf.pend   CGTCGCTCCA CCCGAGGGAG GCCTAGGTGA GGCCTCCTGC GCGTGGGAGG TCGAACCTGG GCCTCCCAAG GGGGGAGAAT
  DGGE b   .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.acid  CGTCGCTCCA CCCGAGCGAG AAAGGGGTGA GGTCCCTTGC ATGTGGGGGA TCGAACTCCT TTCCCGCGAG GGGGGAGAAT
OTU Arc03  .......... .......... .......... .......... .......... .......... .......... ..........
Sulf.toko  CGTCGCTCCA CCCGAGAGAG GAAGGGGTGA GGTCCCCTGC ATGTGGGGGA TCGAACTCCT TCCTCTCGAG GGGGGAGAAT
    CP.B3  .......... .......... .......... .......... .......... .......... .......... ..........
 Thc.waio  CGTCACTCCA CCCGAGCGGG GTCCGGGTGA GGCTTGGTCT TTAGGCCGGG TCGAGCCCGG GCTCCGTGAG GGGGGAGAAT
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          1361       1371       1381       1391       1401
          |          |          |          |          |
   DGGE a ..........  ..........  ..........  ..........  .....
 Thf.pend CGTAACAAGG TGCCGTAGGG AACCTCGGCC GGATCACCTC CTTTC
   DGGE b ..........  ..........  ..........  ..........  .....
Sulf.acid CGTAACAAGG TAC.......  ..........  ..........  .....
OTU Arc03 ..........  ..........  ..........  ..........  .....
Sulf.toko CGTAACAAGG TACCGTAGGG AACCTCGGCT GGATCACCTC A....
    CP.B3 ..........  ..........  ..........  ..........  .....
 Thc.waio CGTAACAAGG TACCGTA...  ..........  ..........  .....
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Alignments of bacterial sequences of 16S rRNA gene fragments obtained from Champagne Pool and closest relatives. Abbreviations: Nev.ramo, *Nevskia ramosa*; Sfh.azor, *Sulfurihydrogenibium azorense*; Pac.thio, *Paracoccus thiocyanatus*; Tha.teng , *Thermoanaerobacter tengcongensis*.

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           1          11         21         31         41         51         61         71         80
           |          |          |          |          |          |          |          |          |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   .......... .......... .......... .......... .GCTAACACA TGCAAGTCGA ACGAGCTAGT GGCGGACGGG
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  ...AGAGTTT GATCCTGGCT CAGCGCGAAC GTTGGCGGCG TGCTAACACA TGCAAGTCGT GGGGTTCACC GGCAAACGGG
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
Sfh.azor   .......... .......... ...CGCGAAC GTTGGCGGCG TGCTAACACA TGCAAGTCGT GGGGTTCACC GGCAAACGGG
   CP.B2   ...AGAGTTT GATCCTGGCT CAGCGCGAAC GTTGGCGGCG TGCTAACACA TGCAAGTCGT GGGGTTCACC GGCAAACGGG
OTU Bac03  .......... .......... .......... ...GGCGGCA GGCTAACACA TGCAAGTCGA GCGGTTTAGC GGCGGACGGG
Pac.thio   .......... .......... .......... .......... GGCTAACACA TGCAAGTCGA GCGGTTTAGC GGCGGACGGG
   CP.B1   .......... .......... .......... .......... .......... .......... .........C GGCGGACGGG
Tha.teng   ATTAGAGTTT GATCCTGGCT CAGGACGAAC GCTGGCGGCG TGCTAACACA TGCAAGTCGA GCGGTTTAGC GGCGGACGGG

           81         91         101        111        121        131        141        151        160
           |          |          |          |          |          |          |          |          |

  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   TGAGGAATAC GTAGGGATCT GCCTTTAAGT GGGGGATAAC CCGGGGAAAC CCGGATTAAT ACCGCATGAT GCAACAAAGG
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  TGAGTAACAC GTAGCTACCT ACCCATAGGT TGGGGATAAC TGCCCGAAAG GGCAGCTAAT ACCCAATAAT GAAACAAAGG
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
Sfh.azor   TGAGTAACGC GTAGCTACCT ACCCATAGGT TGGGGATAAC CATCCGAAAG GGTGGCTAAT ACCCAATAAT GAGACAAAGT
   CP.B2   TGAGTAACAC GTAGCTACCT ACCCATAGGT TGGGGATAAC TGCCCGAAAG GGCAGCTAAT ACCCAATAAT AAAACAAAGG
OTU Bac03  TTAGTAACGC GTGGGAACGT GCCCTTTGCT CTGGAATAGC CCTGGGAAAC TGGGGGTAAT ACCGGATGTG TGAGGAAAGT
Pac.thio   TGAGTAACGC GTGGGAACGT GCCCTTTGCT ACGGAATAGT CCTGGGAAAC TGGGGGTAAT ACCGTATACG TATTGAAAGT
   CP.B1   TGAGTAACGC GTGAGCACCT ACCCTTGAGA CAGGGATAAC CCTGGGAAAC CGGGGCTAAT ACCTGATATA GCATGAAAGG
Tha.teng   TGAGTAACGC GTGAGCACCT ACCCTTGAGA CAGGGATAAC CCTGGGAAAC CGGGGCTAAT ACCTGATATA GCATGAAAGC
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           161        171        181        191        201        211        221        231        240
           |          |          |          |          |          |          |          |          |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   CATCGCTTTT AAGGAACCTA CGTCGGATTA GCTAGTTGGT GAGTAATGGC TCACCAAGGC GACGATCCGT AATGGTCTGA
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  CAGCGCCTAT GATGGGGCTG CGTCCCATCA GCTTGTTGGT GAGTAATGGC TCACCAAGGC TACGACGGGT AGCGGTCTGA
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
Sfh.azor   TTGCGCCTAT GATGGGGCTG CGTCCCATCA GCTAGTTGGT GAGTAATGGC TCACCAAGGC TACGACGGGT AACGGTCTGA
   CP.B2   CAGCGCCTAT GATGGGGCTG CGTCCCATCA GCTTGTTGGT GAGTAATGGC TCACCAAGGC TACGACGGGT AGCGGTCTGA
OTU Bac03  TTTCGGCAAG GATCGGCCCG CGTCTGATTA GGTAGTTGGT GAGTAACGGC TCACCAAGCC GACGATCGGT AGTGGTTTGA
Pac.thio   TTTCGGCGAA GATCGGCCCG CGTTGGATTA GGTAGTTGGT GGGTAATGGC TCACCAAGCC GACGATCCAT AGTGGTTTGA
   CP.B1   GGCCGCTCAA GATGGGCTCG CGTCCCATCA GCTAGTTGGT GAGTAACGGC TCACCAAGGC TACGACGGGT AGCGGCCTGA
Tha.teng   GACCGCCCAA GATGGGCTCG CGTCCCATCA GCTAGTTGGT GAGTAACGGC TCACCAAGGC GACGACGGGT AGCGGCCTGA

           241        251        261        271        281        291        301        311        320
           |          |          |          |          |          |          |          |          |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   GAGGATGATC AGTCACACCG GAACTGAGAC ACGGTCCGGA CTCCTACGGG AGGCAGCAGT GGGGAATATT GGACAATGGG
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  GAGGATGACC GGCCACAGTG GGACTGAGAC ACGGCCCGCA CCCCTACGGG GGGCAGCAGT GGGGAATATT GGGCAATGGC
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
Sfh.azor   GAGGATGATC GGTCACAGCG GGACTGAGAC ACGGCCCGCA CCCCTACGGG GGGCAGCAGT GGGGAATATT GGGCAATGGC
   CP.B2   GAGGATGACC GGCCACAGTG GGACTGAGAC ACGGCCCGCA CCCCTACGGG GGGCAGCAGT GGGGAATATT GGGCAATGGC
OTU Bac03  GAGGATGATC AGCAACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATCTT AGACAATGGG
Pac.thio   GAGGATGATC AGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATCTT AGACAATGGG
   CP.B1   GAGGGTGGTC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATCTT CCGCAATGGG
Tha.teng   GAGGGTGGTC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT GGGGAATCTT CCGCAATGGG
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           321        331        341        351        361        371        381        391        400
           |          |          |          |          |          |          |          |          |
  DGGE c   .......... ..CCAGCAAT ACTGCGTGTT TGATGAAGGC CTTCGGGTTG TAAAGACTTT ACTTGGGAAG AACCTGACTG
Nev.ramo   CGAAAGCCTG ATCCAGCAAT ACCGCGTGTG TGAAGAAGGC CTTCGGGTTG TAAAGACTTT AGTTGGGAAG ATAATGACGG
  DGGE d   .......... ..CCAGCGAC GCTGCGTGGT GGATGAAGGC CTTCGGGTTG TAACCCCTTT TTGGGGGACG ATCCT.....
OTU Bac04  CGAAAGGCTG ACCCAGCAAC GCCGCGTGGT GGATGAAGCC CTTCGGGGTG TAAACCCTTT TTGGGGGAAG ATAATGACGG
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
 Sfh.azor  CGAAAGGCTG ACCCAGCAAC GCCGCGTGGT GGATGAAGCC CTTCGGGGTG TAAACCCTTT TTGGGGGAAG ATAATGACGG
    CP.B2  CGAAAGGCTG ACCCAGCAAC GCCGCGTGGT GGATGAAGCC CTTCGGGGTG TAAACCCTTT TTGGGGGAAG ATTATGACGG
OTU Bac03  CGCAAGCCTG ATCTAGCCAT GCCGCGTGGG TGATGAAGGC CTTAGGGTCG TAAAGCCTTT CCCGGGGAAG ATAATGACGG
 Pac.thio  GGCAACCCTG ATCTAGCCAT GCCGCGTGAG TGATGAAGGC CCTAGGGTTG TAAAGTCTTT CGCTGGGAAG ATAATGACGG
    CP.B1  CGAAAGCCTG ACGGAGCGAC GCCGCGTGAG CGAAGAAGGC CTTCGGGTCG TAAAGTCGAT ATGTGGGAAG AAGGTGACGG
 Tha.teng  CGAAAGCCTG ACGGAGCGAC GCCGCGTGAG CGAAGAAGGC CTTCGGGTCG TAAAGTCGAT ATGTGGGAAG AAGGTGACGG

           401        411        421        431        441        451        461        471        480
           |          |          |          |          |          |          |          |          |
  DGGE c   TACCAACAGA ATGCACCGGC TAACACTGTG CCAGCAGCCG CGGTAATA.. .......... .......... ..........
Nev.ramo   TACCGACAGA ATGCACCGGC TAACTCTGTG CCAGCAGCCG CGGTAATACA GAGGGGCTAG CGTTAATCGG TTTACTGGGC
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  TACCCCAGGA ATGGGACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTCCGAA CGTTGCGCGA ATTACTGGGC
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
 Sfh.azor  TACCCCAGGA ATGGGACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTCCGAA CGTTGCGCGA ATTACTGGGC
    CP.B2  TACCCCAGGA ATGGGACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTCCGAA CGTTGCGCGA ATTACTGGGC
OTU Bac03  TACCCGGTAA AGACCCCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG GAGGGGTTAG CGTTGTTCGG ATGACTGGGC
 Pac.thio  TACCAGCAGA AGGCCCCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG GAGGGGCTAG CGTTGTTCGG ATTACTGGGC
    CP.B1  TACCACACGA AAGCCCCGGC TAACTACGTG CCAGCAGCCG CGGTAAGACG TAGGGGCGAG CGTTGTCCGG ATTACTGGGC
 Tha.teng  TACCACACGA AAGCCCCGGC TAACTACGTG CCAGCAGCCG CGGTAAGACG TAGGGGCGAG CGTTGTCCGG ATTACTGGGC
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           481        491        501        511        521        531        541        551        560
           |          |          |          |          |          |          |          |          |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   GTAAAGCGTG CGTAGCGGTT TGATAAGTGA TTGTGAAAGC CCCGGGCTCA ACCTGGGAAT TGCGGTCGAT ACTGTCAGAC
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  GTAAAGGGTC CGTAGCGGTC TGGTAAGTGA AGGTGAAAGC CCACGGCTTA ACCGTGGAAT TGCCTTCCAA ACTGCCGGAC
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
Sfh.azor   GTAAAGGGTC CGTAGCGGTC TGGTAAGTGA AGGTGAAATC CTACGGCTCA ACCGTAGAAT TGCCTTCCAA ACTGCTGGAC
   CP.B2   GTAAAGGGTC CGTAGCGGTC TGGTAAGTGT AGGTGAAAGC CCACGGGTTA ACCGTGGAAT TGCCTTCCAA ACTGCCGGAC
OTU Bac03  GTAAAGCGCG CGTAGCGGAT CATCAAGTGG GGGTGAAATC CCGGGGCTCA ACCCCGGAAT GGCCTTCGAG ACTGGTGGTC
Pac.thio   GTAAAGCGCA CGTAGCGGAC CTGAAAGTGG GGGTGAAATC CCGGGGCTCA ACCTCGGAAC TGCCTTCAAA ACTACGGGTC
   CP.B1   GTAAAGGGCG CGTAGCGGTT TAGCAAGTAG GTGTAAAAGG CCACGGCTCA ACCGTGGATA TGCATCTGAA ACTGCTGAGC
Tha.teng   GTAAAGGGCG CGTAGCGGTT TAGCAAGTAG GTGTAAAAGG CCACGGCTCA ACCGTGGATA TGCACCTGAA ACTGCTAGGC

           561        571        581        591        601        611        621        631        640
           |          |          |          |          |          |          |          |          |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   TCGAAACGGT AGAGGGAGGC GGAACTCCAG GTGTACGGTG AAATGCGTAG ATATCTGGAA GAACACCGAT GGCGAAGCAA
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  TTGAGATGGA AGAGGTCGGC GGAATTCCCG GTGTACGGTG AAATGCGTAG ATATCGGGAG GAACACCAGT AGCGAAGCGG
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
Sfh.azor   TTGAGCAGGG AGAGGTCGGC GGAATTCCCG GTGTACGGTG AAATGCGTAG ATATCGGGAG GAACACCAGT GGCGAAGCGG
   CP.B2   TTGAGGTGGA AGAGGTCGGC GGAATTCCCG GTGTACGGTG AAATGCGTAG ATATCGGGAG GAACACCAGT AGCGAAGCGG
OTU Bac03  TTGAGTCGAG AGAGGTGAGT GGAATTCCGA GTGTAAGGTG AAATTCGTAG ATATTCGGAG GAACACCAGT GGCGAAGCGG
Pac.thio   TGGAGTCGAG AGAGGTGAGT GGAATTCCGA GTGTAAGGTG AAATTCGTAG ATATTCGGAG GAACACCAGT GGCGAAGCGG
   CP.B1   TAGAGGCAGG AGAGGGGAGT GGAATTCCCG GTGTACGGTG AAATGCGTAG ATATCGGGAG GAATACCAGT GGCGAAACGA
Tha.teng   TAGAGGCAGG AGAGGGGAGT GGAATTCCCG GTGTACGGTG AAATGCGTAG ATATCGGGAG GAATACCAGT GGCGAAGCGA
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           641        651        661        671        681        691        701        711        720
           |          |          |          |          |          |          |          |          |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
 Nev.ramo  CCCTGGGCCT GTTTTGACGC TGAGGCACGA AAGCGTGGTA GCAAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  CACTGGGACA TTCCTGACG. .......... .......... .......... .......... .......... ..........
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
 Sfh.azor  CACTGGAACT GTCCTGACGC TGAGGGACGA AAGCTAGGGA GCAAACCGGA TTAGATACCC GGGTAGTCCT AGCCGTAAAC
   CP.B2   CACTGGGACA TTCCTGACGC TGAGGGACGA AAGCTAGGGA GCAAACCGGA TTAGATACCC GGGTAGTCCT AGCCGTAAAC
OTU Bac03  TACTGGCTCG ACACTGACGC TGAGGTGCGA AAGCGTGGGA GCAAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC
 Pac.thio  TACTGGCTCG ATACTGACGC TGAGGTGCGA AAGCGTGGGA GCAAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC
   CP.B1   TCCTGGACTG GCCCTGACGC TGAGGCGCGA AAGCGTGGGA GCAAACAGGA TTAGATACCC TGGTAGTCCA CGCTGTAAAC
 Tha.teng  TCCTGGACTG GCCCTGACGC TGAGGCGCGA GAGCGTGGGA GCGAACAGGA TTAGATACCC TGGTAGTCCA CGCTGTAAAC

           721        731        741        751        761        771        781        791        800
           |          |          |          |          |          |          |          |          |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
 Nev.ramo  ATGAGAACTT GACGTTGGTT TAAATCGGTG TCGTAGCTAA CGCGATAAGT TCTCCGCCTG GAGTACGGCC GCAAGGTTAA
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
 Sfh.azor  ATGGATGCTA GATGTGGCCT AAAGGCTGTG CCGCAGCTAA CGCGTTAAGC ATCCCGCCTG GAGTACGGCC GCAAGGTTAA
   CP.B2   ATGGATGCTT GATGTGACCT AAAGGTCGTG TCGTAGCTAA CGCGTTAAGC ATCCCGCCTG GAGTACGGCC GCAAGGTTAA
OTU Bac03  ATGAATGCCA GACGTCGGGG TGCTTCGGTG TCACACCTAA CGGATTAAGC ATTCCGCCTG GAGTACGGTC GCAAGATTAA
 Pac.thio  ATGAATGCCA GTCGTCGGGC AGGTTCGGTG ACACACCTAA CGGATTAAGC ATTCCGCCTG GAGTACGGTC GCAAGATTAA
   CP.B1   ATGGGTGCTA GGTGTGGGGA GATTTCCGTG CCGTAGGAAA CCCAATAAGC ACCCCGCCTG GAGTACGGCC GCAAGGCTAA
 Tha.teng  ATGGGTGCTA GGTGTGGGGA GATTTCCGTG CCGTAGGAAA CCCAATAAGC ACCCCGCCTG GAGTACGGCC GCAAGGCTAA
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           801        811        821        831        841        851        861        871        880
           |          |          |          |          |          |          |          |          |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   ACTCAAAGGA ATTGACGGGG GCCCGACAAC GGTGGAGTAT GTGGTTTAAT TCGATGCAAC GCGAAGAACC TTACCTGGTC
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac12  .......... .......... .......... .......... .......... .......... .......... ..........
Sfh.azor   ACTCAAAGGA ATTGACGGGG ACCCGACAAC GGTGGAGCGT CTGGTTTAAT TCGATGCTAA CCGAAAAACC TTACCAGGGT
   CP.B2   ACTCAAAGGA ATTGACGGGG ACCCGACAAC GGTGGAGCGT CTGGTTTAAT TCGATGCTAA CCGAAAAACC TCACCAGGGT
OTU Bac03  ACTCAAAGGA ATTGACGGGG GCCCGACAAC GGTGGAGCAT GTGGTTTAAT TCGAAGCAAC GCGCAGAACC TTACCAACCC
Pac.thio   ACTCAAAGGA ATTGACGGGG GCCCGACAAC GGTGGAGCAT GTGGTTTAAT TCGAAGCAAC GCGCAGAACC TTACCAACCC
   CP.B1   ACTCAAAGGA ATTGACGGGG GCCCGACAAC GGTGGAGCAT GTGGTTTAAT TCGAAGCAAC GCGAAGAACC TTACCAGGGC
Tha.teng   ACTCAAAGGA ATTGACGGGG GCCCGACAAC GGTGGAGCAT GTGGTTTAAT TCGAAGCAAC GCGAAGAACC TTACCAGGGC

           881        891        901        911        921        931        941        951        960
           |          |          |          |          |          |          |          |          |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   TTGACATGTC AAGGAGTCTG CAGAGATCGG ATGTGCCTTC GGGAACTTGA ACACAGGTGC TGCATGGCTG TCGTCAGCTC
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac12  .......... .......... ..GAAAGGCA CTAGCGATTT CTCGTGCGCC GGACAG-TGG TGCATGGTCG TCGTCAGCTC
Sfh.azor   TTGACATGGG TGTGTCGGGT ATGAAAGACC CTAGGGATTA TTCCCGCGCC GCACAGGTGG TGCATGGTCG TCGTCAGCTC
   CP.B2   TTGACATCGG TGTGTCGTAT CCGAAAGGTA CTAGCGATTT CTCGTGCGCC GGACAGGTGG TGCATGGTCG TCGTCAGCTC
OTU Bac03  TTGACATCGC CTGACCGGCC TGGAGAAGGT CTTTCCCGCA AGGGACAGGT GGACAGGTGC TGCATGGCTG TCGTCAGCTC
Pac.thio   TTGACATCGC AGGACCGCCC GAGAGATGGG TTTTCTCGTA AGAGACCTGT GGACAGGTGC TGCATGGCTG TCGTCAGCTC
   CP.B1   TTGACATGAG GTAGTAGCAG CCGAAAGTGG CGACCTCTTA TGGGGAGCCT GCACAGGTGG TGCATGGTTG TCGTCAGCTC
Tha.teng   TTGACATGAG GTAGTAGCAG CCGAAAGTGG CGACCTCGAA AGGGGAGCCT GCACAGGTGG TGCATGGTTG TCGCCAGCTC
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           961        971        981        991        1001       1011       1021       1031      1040
           |          |          |          |          |          |          |          |         |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCTTGCCCT TAGTTGCCAT CATTCATTGG GCACTCTAAG
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac12  GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCTTGCCCT GTGTTACCAG CGGTTACCGG GGACTCACAG
Sfh.azor   GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCTTGCCCT GTGTTACCAG CGGTAACCGG GTACTCACAG
   CP.B2   GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCTTGCCCT GTGTTACCAG CGGTTACCGG GGACTCACAG
OTU Bac03  GTGTCGTGAG ATGTTCGGTT AAGTCCGGCA ACGAGCGCAA CCCACACCCT TGGTTGCCAG CACTAAGTGG GCACTTTAGG
Pac.thio   GTGTCGTGAG ATGTTCGGTT AAGTCCGGCA ACGAGCGCAA CCCACGTCCC CAGTTGCCAG CATTCATTGG GCACTCTATG
   CP.B1   GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCCTGCCTC TAGTTGCCAG CGGTAACCGG GCACTCTAGA
Tha.teng   GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCCTGCCTC TAGTTGCCAG CGGTAACCGG GCACTCTAGA

           1041       1051       1061       1071       1081       1091       1101       1111      1120
           |          |          |          |          |          |          |          |         |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   GGGACCGCCG GTGAAAACCG GAGGAAGGTG GGGATGACGT CAAGTCATCA TGGCCCTTAT GACCAGGGCT ACACACGTAC
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac12  GGGACTGCCG GCGAAAGTCG GAGGAAGGAG GGGATGACGT CAGATCAGTA TGCCCTTTAT GCCCTGGGCT ACACAGGCGC
Sfh.azor   GGGACTGCCG GCGAAAGTCG GAGGAAGGAG GGGATGACGT CAGATCAGTA TGCCCTTTAT GCCCTGGGCT ACACAGGCGC
   CP.B2   GGGACTGCCG GCGAAAGTCG GAGGAAGGAG GGGATGACGT CAGATCAGTA TGCCCTTTAT GCCCTGGGCT ACACAGGCGC
OTU Bac03  GGAACTGCCC GTGAAAGCGG GAGGAAGGTG TGGATGACGT CAAGTCCTCA TGGCCCTTAC GGGTTGGGCT ACACACGTGC
Pac.thio   GAAACTGCCG ATGAAAGTCG GAGGAAGGTG TGGATGACGT CAAGTCCTCA TGGCCCTTAC GGGTTGGGCT ACACACGTGC
   CP.B1   GGGACTGCCG TCGAAAGACG GAGGAAGGTG GGGATGACGT CAAATCATCA TGCCCTATAT GCCCTGGGCC ACACACGTGC
Tha.teng   GGGACTGCCG TCGAAAGACG GAGGAAGGTG GGGATGACGT CAAATCATCA TGCCCTATAT GCCCTGGGCC ACACACGTGC
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           1121       1131       1141       1151       1161       1171       1181       1191      1200
           |          |          |          |          |          |          |          |         |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   TACAATGGCG GTACAGAGGG TAATCCCAAG CCGTCGTAGT CCGGATTGAG TCTGCAACTC GACTCCATGA AGTCGGAATC
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac12  TACAGTGGAG GGACAATGGG AAACCCCAAC CCTTCGTGGT GCGGATTGGG GTTGCAACTC ACCCCCATGA AGGCGGAATC
Sfh.azor   TACAGTGGAG GGACAATGGG AAATCCCAAC CCTTCGTGGT GCGGATTGGG GTTGCAACTC ACCCCCATGA AGGCGGAATC
   CP.B2   TACAGTGGAG GGACAATGGG AAACCCCAAC CCTTCGTGGT GCGGATTGGG GTTGCAACTC ACCCCCATGA AGGCGGAATC
OTU Bac03  TACAATGGGG TGACAGAGGG TCATCCCAAA GCCTCTCAGT TCGGATTGCG TCTGCAACTC GACGGCATGA AGTCGGAATC
Pac.thio   TACAATGGGG TGACAGTGGG TAATCCCAAA GCCTCTCAGT TCGGATTGGG TCTGCAACTC GACCCCATGA AGTTGGAATC
   CP.B1   TACAATGGCG GTACAGAGGG AAATCCCAAG CCGTCTCAGT TCGGATTGAG GCTGCAACTC GCCTGCATGA AGGCGGAATC
Tha.teng   TACAATGGCG GTACAGAGGG AAATCCCAAG CCGTCTCAGT TCGGATTGAG GCTGCAACTC GCCTGCATGA AGGCGGAATC

           1201       1211       1221       1231       1241       1251       1261       1271      1280
           |          |          |          |          |          |          |          |         |
  DGGE c   .......... .......... .......... .......... .......... .......... .......... ..........
Nev.ramo   GCTAGTAATC GCGGATCAGC TTGTGCGGTG AATACGTTCC CGAGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTGG
  DGGE d   .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac12  GGTAGTAATG GCGAATCAGC ATGTGCCGTG AATACGTTCC CGGGTCTTGT ACACACCGCC CGTCACGCCA TGGGAGTCGG
Sfh.azor   GGTAGTAATG GCGAATCAGC ATGTGCCGTG AATACGTTCC CGGGTCTTGT ACACACCGCC CGTCACGCCA TGGGAGTCGG
   CP.B2   GGTAGTAATG GCGAATCAGC ATGTGCCGTG AATACGTTCC CGGGTCTTGT ACACACCGCC CGTCACGCCA TGGGAGTCGG
OTU Bac03  GCTAGTAATC GCGCATCAGC TCGGGCGGTG AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTGG
Pac.thio   GCTAGTAATC GCGGAACAGC ATGCGCGGTG AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTGG
   CP.B1   GCTAGTAATC GCGGATCAGC ATGCCCGGTG AATGCGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA CGAGAGTCTG
Tha.teng   GCTAGTAATC GCGGATCAGC ATGCGCGGTG AATGCGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA CGAGAGTCTG
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           1281       1291       1301       1311       1321       1331       1341       1351      1360
           |          |          |          |          |          |          |          |         |
   DGGE c  .......... .......... .......... .......... .......... .......... .......... ..........
 Nev.ramo  CTGCACCAGA AGCC...... .......... .......... .......... .......... .......... ..........
   DGGE d  .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac04  .......... .......... .......... .......... .......... .......... .......... ..........
OTU Bac12  GTTCATCGGA AGTCCCCGAG AACCGCAAGC AGGGGCCGAT GATGGGCCCG ATGACTGGGG CGAAGTCGTA ACAAGGTAGC
 Sfh.azor  GTTCATCGGA AGTCCCCGAG AACCGCAAGC AGGGGCCGAT GATGGGCCTG ATGACTGGGG CG........ ..........
    CP.B2  GTTCATCGGA AGTCCCCGAG AACCGCAAGC AGGGGCCGAT GATGGGCCCG ATGACTGGGG CGAAGTCGTA ACAAGGTAGC
OTU Bac03  GTTGACCCGA AGGTGGTGCG GACCGTAAGC AGCCAACCAC GGTCAGTTCA GCGACTGGGG TGAAGTCGTA ACAAGGTAGC
 Pac.thio  GTCTACCCGA CGGCCGTGCG AACCGCAAGG CAGCGGACCA CGGTAGGCTC AG........ .......... ..........
    CP.B1  CAACACCCG. .......... .......... .......... .......... .......... .......... ..........
 Tha.teng  CAACACCCGA AGCCGGTGGC AACCGAAAGG AGCCGTCGAA GGTGGGGCAG ATGATTGGGG TGAAGTCGTA ACAAGGTAA.

           1361       1371       1381       1391
           |          |          |          |
   DGGE c  .......... .......... .......... ...
 Nev.ramo  .......... .......... .......... ...
   DGGE d  .......... .......... .......... ...
OTU Bac04  .......... .......... .......... ...
OTU Bac12  CCTAGGGGAA CCTGCGGCTG GATCACCTCC TT.
 Sfh.azor  .......... .......... .......... ...
    CP.B2  CCTAGGGGAA CCTGCGGCTG GATCACCTCC TTA
OTU Bac03  CGTAGGGGAA CCTGCGGCTG GATCACCTCC TT.
 Pac.thio  .......... .......... .......... ...
    CP.B1  .......... .......... .......... ...
 Tha.teng  .......... .......... .......... ...
```

Appendix F: Culture experiments

Table 26. Effect of potential electron donors, electron acceptors, and carbon sources on growth of strain CP.B2^T. Cultures were transferred at least three times on the same e^- donor / e^- acceptor combination to ensure the growth was not due to substrate carry-over from the inoculum. Abbreviations: -, no growth at all; +, growth after first transfer in fresh medium; +++, growth after third transfer in fresh medium, [1)] in presence of 8 mM $S_2O_3^{2-}$, [2)] in presence of 1% S^0. Negative controls are indicated in bold.

e^- donor	e^- acceptor	Carbon source	Gas phase 1.7 kPa	Growth
none	**10 mM $NaNO_2$**	**CO_2**	**N_2/CO_2 (80%/20%)**	-
none	**10 mM $NaNO_3$**	**CO_2**	**N_2/CO_2 (80%/20%)**	-
none	**10 mM Na_2SO_3**	**CO_2**	**N_2/CO_2 (80%/20%)**	-
none	**10 mM Na_2SO_4**	**CO_2**	**N_2/CO_2 (80%/20%)**	-
none	**5 mM Na_2SeO_3**	**CO_2**	**N_2/CO_2 (80%/20%)**	-
none	**5 mM Na_2SeO_4**	**CO_2**	**N_2/CO_2 (80%/20%)**	-
none	**5 mM $NaAsO_2$**	**CO_2**	**N_2/CO_2 (80%/20%)**	-
none	**5 mM**	**CO_2**	**N_2/CO_2 (80%/20%)**	-
H_2	**none**	**CO_2**	**H_2/CO_2 (80%/20%)**	**-**
H_2	O_2	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	-
H_2	O_2	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	[1)] +++
H_2	O_2	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	[2)] +++
H_2	10 mM $NaNO_2$	CO_2	H_2/CO_2 (80%/20%)	-
H_2	10 mM $NaNO_3$	CO_2	H_2/CO_2 (80%/20%)	-
H_2	10 mM Na_2SO_3	CO_2	H_2/CO_2 (80%/20%)	-
H_2	10 mM Na_2SO_4	CO_2	H_2/CO_2 (80%/20%)	-
H_2	5 mM Na_2SeO_3	CO_2	H_2/CO_2 (80%/20%)	-
H_2	5 mM Na_2SeO_4	CO_2	H_2/CO_2 (80%/20%)	-
H_2	5 mM $NaAsO_2$	CO_2	H_2/CO_2 (80%/20%)	-
H_2	5 mM Na_2HAsO_4	CO_2	H_2/CO_2 (80%/20%)	-
H_2	10 mM $NaNO_2$	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	-
H_2	10 mM $NaNO_3$	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	+
H_2	10 mM Na_2SO_3	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	-
H_2	10 mM Na_2SO_4	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	-
H_2	5 mM Na_2SeO_3	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	-
H_2	5 mM Na_2SeO_4	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	+
H_2	5 mM $NaAsO_2$	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	-
H_2	5 mM Na_2HAsO_4	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	+

Table 26. Continued.

e^- donor	e^- acceptor	Carbon source	Gas phase 1.7 kPa	Growth
H_2	1 mM $NaAsO_2$	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	+
H_2	1 mM Na_2HAsO_4	CO_2	$H_2/CO_2/O_2$ (79%/15%/6%)	+
H_2	10 mM $NaNO_2$	0.2% casa-amino acids	H_2 (100%)	-
H_2	10 mM $NaNO_2$	0.2% Na-pyruvate	H_2 (100%)	-
H_2	10 mM $NaNO_2$	0.2% starch	H_2 (100%)	-
H_2	10 mM $NaNO_2$	0.2% trypticase peptone	H_2 (100%)	-
H_2	10 mM $NaNO_2$	0.2% yeast extract	H_2 (100%)	-
H_2	10 mM $NaNO_3$	0.2% casa-amino acids	H_2 (100%)	-
H_2	10 mM $NaNO_3$	0.2% Na-pyruvate	H_2 (100%)	-
H_2	10 mM $NaNO_3$	0.2% starch	H_2 (100%)	-
H_2	10 mM $NaNO_3$	0.2% trypticase peptone	H_2 (100%)	-
H_2	10 mM $NaNO_3$	0.2% yeast extract	H_2 (100%)	-
H_2	10 mM Na_2SO_3	0.2% casa-amino acids	H_2 (100%)	-
H_2	10 mM Na_2SO_3	0.2% Na-pyruvate	H_2 (100%)	-
H_2	10 mM Na_2SO_3	0.2% starch	H_2 (100%)	-
H_2	10 mM Na_2SO_3	0.2% trypticase peptone	H_2 (100%)	-
H_2	10 mM Na_2SO_3	0.2% yeast extract	H_2 (100%)	-
H_2	10 mM Na_2SO_4	0.2% casa-amino acids	H_2 (100%)	-
H_2	10 mM Na_2SO_4	0.2% Na-pyruvate	H_2 (100%)	-
H_2	10 mM Na_2SO_4	0.2% starch	H_2 (100%)	-
H_2	10 mM Na_2SO_4	0.2% trypticase peptone	H_2 (100%)	-
H_2	10 mM Na_2SO_4	0.2% yeast extract	H_2 (100%)	-
H_2	5 mM Na_2SeO_3	0.2% casa-amino acids	H_2 (100%)	-
H_2	5 mM Na_2SeO_3	0.2% Na-pyruvate	H_2 (100%)	-
H_2	5 mM Na_2SeO_3	0.2% starch	H_2 (100%)	-
H_2	5 mM Na_2SeO_3	0.2% trypticase peptone	H_2 (100%)	-
H_2	5 mM Na_2SeO_3	0.2% yeast extract	H_2 (100%)	-
H_2	5 mM Na_2SeO_4	0.2% casa-amino acids	H_2 (100%)	-
H_2	5 mM Na_2SeO_4	0.2% Na-pyruvate	H_2 (100%)	-
H_2	5 mM Na_2SeO_4	0.2% starch	H_2 (100%)	-
H_2	5 mM Na_2SeO_4	0.2% trypticase peptone	H_2 (100%)	-
H_2	5 mM Na_2SeO_4	0.2% yeast extract	H_2 (100%)	-
H_2	5 mM $NaAsO_2$	0.2% casa-amino acids	H_2 (100%)	-
H_2	5 mM $NaAsO_2$	0.2% Na-pyruvate	H_2 (100%)	+

Table 26. Continued.

e^- donor	e^- acceptor	Carbon source	Gas phase 1.7 kPa	Growth
H_2	5 mM $NaAsO_2$	0.2% starch	H_2 (100%)	-
H_2	5 mM $NaAsO_2$	0.2% trypticase peptone	H_2 (100%)	+
H_2	5 mM $NaAsO_2$	0.2% yeast extract	H_2 (100%)	-
H_2	5 mM Na_2HAsO_4	0.2% casa-amino acids	H_2 (100%)	-
H_2	5 mM Na_2HAsO_4	0.2% Na-pyruvate	H_2 (100%)	+
H_2	5 mM Na_2HAsO_4	0.2% starch	H_2 (100%)	-
H_2	5 mM Na_2HAsO_4	0.2% trypticase peptone	H_2 (100%)	-
H_2	5 mM Na_2HAsO_4	0.2% yeast extract	H_2 (100%)	-
H_2	10 mM $NaNO_2$	0.2% casa-amino acids	H_2/O_2 (94%/6%)	-
H_2	10 mM $NaNO_2$	0.2% Na-pyruvate	H_2/O_2 (94%/6%)	-
H_2	10 mM $NaNO_2$	0.2% starch	H_2/O_2 (94%/6%)	-
H_2	10 mM $NaNO_2$	0.2% trypticase peptone	H_2/O_2 (94%/6%)	-
H_2	10 mM $NaNO_2$	0.2% yeast extract	H_2/O_2 (94%/6%)	-
H_2	10 mM $NaNO_3$	0.2% casa-amino acids	H_2/O_2 (94%/6%)	-
H_2	10 mM $NaNO_3$	0.2% Na-pyruvate	H_2/O_2 (94%/6%)	-
H_2	10 mM $NaNO_3$	0.2% starch	H_2/O_2 (94%/6%)	-
H_2	10 mM $NaNO_3$	0.2% trypticase peptone	H_2/O_2 (94%/6%)	-
H_2	10 mM $NaNO_3$	0.2% yeast extract	H_2/O_2 (94%/6%)	-
H_2	10 mM Na_2SO_3	0.2% casa-amino acids	H_2/O_2 (94%/6%)	-
H_2	10 mM Na_2SO_3	0.2% Na-pyruvate	H_2/O_2 (94%/6%)	-
H_2	10 mM Na_2SO_3	0.2% starch	H_2/O_2 (94%/6%)	-
H_2	10 mM Na_2SO_3	0.2% trypticase peptone	H_2/O_2 (94%/6%)	-
H_2	10 mM Na_2SO_3	0.2% yeast extract	H_2/O_2 (94%/6%)	-
H_2	10 mM Na_2SO_4	0.2% casa-amino acids	H_2/O_2 (94%/6%)	-
H_2	10 mM Na_2SO_4	0.2% Na-pyruvate	H_2/O_2 (94%/6%)	-
H_2	10 mM Na_2SO_4	0.2% starch	H_2/O_2 (94%/6%)	-
H_2	10 mM Na_2SO_4	0.2% trypticase peptone	H_2/O_2 (94%/6%)	-
H_2	10 mM Na_2SO_4	0.2% yeast extract	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2SeO_3	0.2% casa-amino acids	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2SeO_3	0.2% Na-pyruvate	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2SeO_3	0.2% starch	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2SeO_3	0.2% trypticase peptone	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2SeO_3	0.2% yeast extract	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2SeO_4	0.2% casa-amino acids	H_2/O_2 (94%/6%)	-

Table 26. Continued.

e^- donor	e^- acceptor	Carbon source	Gas phase 1.7 kPa	Growth
H_2	5 mM Na_2SeO_4	0.2% Na-pyruvate	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2SeO_4	0.2% starch	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2SeO_4	0.2% trypticase peptone	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2SeO_4	0.2% yeast extract	H_2/O_2 (94%/6%)	-
H_2	5 mM $NaAsO_2$	0.2% casa-amino acids	H_2/O_2 (94%/6%)	-
H_2	5 mM $NaAsO_2$	0.2% Na-pyruvate	H_2/O_2 (94%/6%)	-
H_2	5 mM $NaAsO_2$	0.2% starch	H_2/O_2 (94%/6%)	-
H_2	5 mM $NaAsO_2$	0.2% trypticase peptone	H_2/O_2 (94%/6%)	-
H_2	5 mM $NaAsO_2$	0.2% yeast extract	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2HAsO_4	0.2% casa-amino acids	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2HAsO_4	0.2% Na-pyruvate	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2HAsO_4	0.2% starch	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2HAsO_4	0.2% trypticase peptone	H_2/O_2 (94%/6%)	-
H_2	5 mM Na_2HAsO_4	0.2% yeast extract	H_2/O_2 (94%/6%)	-
8 mM $S_2O_3^{2-}$	**none**	**CO_2**	**N_2/CO_2 (80%/20%)**	-
8 mM $S_2O_3^{2-}$	10 mM $NaNO_2$	CO_2	N_2/CO_2 (80%/20%)	-
8 mM $S_2O_3^{2-}$	10 mM $NaNO_3$	CO_2	N_2/CO_2 (80%/20%)	-
8 mM $S_2O_3^{2-}$	10 mM Na_2SO_3	CO_2	N_2/CO_2 (80%/20%)	-
8 mM $S_2O_3^{2-}$	10 mM Na_2SO_4	CO_2	N_2/CO_2 (80%/20%)	-
8 mM $S_2O_3^{2-}$	5 mM Na_2SeO_3	CO_2	N_2/CO_2 (80%/20%)	-
8 mM $S_2O_3^{2-}$	5 mM Na_2SeO_4	CO_2	N_2/CO_2 (80%/20%)	-
8 mM $S_2O_3^{2-}$	5 mM $NaAsO_2$	CO_2	N_2/CO_2 (80%/20%)	-
8 mM $S_2O_3^{2-}$	5 mM Na_2HAsO_4	CO_2	N_2/CO_2 (80%/20%)	-
1% S^0	**none**	**CO_2**	**N_2/CO_2 (80%/20%)**	-
1% S^0	10 mM $NaNO_2$	CO_2	N_2/CO_2 (80%/20%)	-
1% S^0	10 mM $NaNO_3$	CO_2	N_2/CO_2 (80%/20%)	-
1% S^0	10 mM Na_2SO_3	CO_2	N_2/CO_2 (80%/20%)	-
1% S^0	10 mM Na_2SO_4	CO_2	N_2/CO_2 (80%/20%)	-
1% S^0	5 mM Na_2SeO_3	CO_2	N_2/CO_2 (80%/20%)	-
1% S^0	5 mM Na_2SeO_4	CO_2	N_2/CO_2 (80%/20%)	-
1% S^0	5 mM $NaAsO_2$	CO_2	N_2/CO_2 (80%/20%)	-
1% S^0	5 mM Na_2HAsO_4	CO_2	N_2/CO_2 (80%/20%)	-

Table 26. Continued.

e^- donor	e^- acceptor	Carbon source	Gas phase 1.7 kPa	Growth
1 mM $NaAsO_2$	1 mM Na_2HAsO_4	CO_2	N_2/CO_2 (80%/20%)	+
none	none	0.2% casa-amino acids	N_2 (100%)	[1)] -
none	none	0.2% Na-pyruvate	N_2 (100%)	[1)] -
none	none	0.2% starch	N_2 (100%)	[1)] -
none	none	0.2% trypticase peptone	N_2 (100%)	[1)] +
none	none	0.2% yeast extract	N_2 (100%)	[1)] +
organic	O_2	0.2% casa-amino acids	N_2/O_2 (94%/6%)	[1)] -
organic	O_2	0.2% Na-pyruvate	N_2/O_2 (94%/6%)	[1)] -
organic	O_2	0.2% starch	N_2/O_2 (94%/6%)	[1)] +
organic	O_2	0.2% trypticase peptone	N_2/O_2 (94%/6%)	[1)] +
organic	O_2	0.2% yeast extract	N_2/O_2 (94%/6%)	[1)] +
H_2	O_2	0.2% casa-amino acids	H_2/O_2 (94%/6%)	[1)] -
H_2	O_2	0.2% Na-pyruvate	H_2/O_2 (94%/6%)	[1)] -
H_2	O_2	0.2% starch	H_2/O_2 (94%/6%)	[1)] -
H_2	O_2	0.2% trypticase peptone	H_2/O_2 (94%/6%)	[1)] -
H_2	O_2	0.2% yeast extract	H_2/O_2 (94%/6%)	[1)] -

Appendix G: NZAPLUME III research cruise

Introduction

It is estimated that more than 90% of the entire sea floor has an average temperature below 4° C (Levitus & Boyer, 1994). However, the discovery of hot brines in the Red Sea in the 1960s (Degens & Ross, 1969) and hydrothermal vents in the Pacific Ocean in the late 1970s (Corliss *et al.*, 1979) showed the existence of submarine geothermal areas within the cold oceanic environment, and in the case of hydrothermal vents, the presence of thermophilic microorganisms in these habitats (Jeanthon, 2000; Stetter, 1996). The temperature of vent water varies from around 23° C (Galapagos vents) to around 350° C (vents of the East Pacific Rise) and is typically rich in dissolved metal ions (Gage & Tyler, 1991). When discharged hot vent fluid mixes with colder seawater metal sulfides and oxides such as zinc sulfide, iron sulfide, copper-iron sulfide, manganese oxide, and iron oxide are precipitated out and form a turbid hydrothermal vent plume (Tivey, 1998). The plume rises (up to 200 m) until the precipitates are neutral buoyant and is distributed by currents. In response to chemophysical conditions different from terrestrial hot springs (e.g. steeper temperature gradient, higher hydrostatic pressure, higher NaCl concentration), free-living microorganisms associated with hydrothermal vents (Karl, 1995) may have evolved unique strategies to interact with metal ions.

The geothermally active Kermadec arc lies in a subduction zone where the Pacific plate slides beneath the Indio-Australian plate and runs for approximately 1,200 km from the North Island of New Zealand northeast towards Tonga. The research cruises NZAPLUME I and II (New Zealand and American plume mapping expedition) surveyed several active submarine volcanoes and hydrothermal plumes in this area (de Ronde *et al.*, 2001; de Ronde *et al.*, 2003). The primary aim of this study during the NZAPLUME III cruise along the Kermadec arc started on 23rd September until 17th October 2004 was to identify and isolate novel microorganisms from hydrothermal vent plumes using molecular biological and microbiological approaches. Secondary aims, conducted in collaboration with Sean Langley and Danielle Fortin (University of Ottawa, Canada) included identification of minerals

within the plume samples by X-ray diffraction analyses, and evaluation of particulates (both mineral and biological) using electron microscopy.

Material and Methods

Teflon-coated 10-liter Niskin bottles were used to obtain discrete water samples (Figure 41). Samples were then collected directly from Niskin bottles for further processing.

Figure 41. Stainless steel stand accommodating 19 Niskin bottles. During sampling, the unit is connected to the mother ship via a tow-cable several thousands of meters in length and provides information on conductivity (salinity), pressure (depth), and optical light scattering (turbidity). The optical light scattering sensor (nephelometer) detects relative low concentrations of metal sulfides and oxides precipitated when discharged vent fluids mix with cold seawater.

In culture experiments, artificial seawater medium was used supplemented with various substrates (Table 27) to enrich sulfur-oxidizing (SO), sulfur-reducing (SR), manganese-oxidizing (MO), manganese-reducing (MR), arsenite-oxidizing (AO), and iron-oxidizing (IO) microorganisms. The basal medium contained (per liter):

27.5 g of NaCl, 5.38 g of $MgCl_2.6H_2O$, 6.78 g of $MgSO_4.7H_2O$, 0.72 g of KCl, 0.2 g of $NaHCO_3$, 1.4 g of $CaCl_2.2H_2O$, 1.0 g of NH_4Cl, and 0.05 g of $K_2HPO_4.3H_2O$ (Emerson & Moyer, 2002) and trace element solution adapted from Reysenbach and Gotz (2001) containing per liter medium: 15.0 mg of $MgSO_4.7H_2O$, 2.5 mg of $MnSO_4.2H_2O$, 5.0 mg of NaCl, 0.5 mg of $FeSO_4.7H_2O$, 0.5 mg of $CoCl_2$, 0.5 mg of $CaCl_2.2H_2O$, 0.5 mg of $ZnSO_4$, 0.5 mg of $SrCl_2.6H_2O$, 0.05 mg of $CuSO_4.5H_2O$, 0.05 mg of $AlK(SO_4)_2$, 0.05 mg of H_3BO_3, and 0.05 mg of $NaMoO_4.2H_2O$. Growth medium for iron-reducing microorganisms (IR) (Reysenbach & Gotz, 2001) consisted of (per liter): 2.52 g of $NaHCO_3$, 20.0 g of NaCl, 0.5 g of $MgSO_4.7H_2O$, 0.25 g of NH_4Cl, 0.05 g of $CaCl_2.2H_2O$, 0.05 g of K_2HPO_4, 7.0 mg of $FeSO_4.7H_2O$, 1.0 mg of $Na_2WO_4.7H_2O$, 1.0 mg of resazurin, 3.67 g of $Fe(C_6H_5O_7).xH_2O$ (Fe-citrate), and trace element solution as described above. Media inoculated with plume samples were incubated at least for five days at 50º C and at 75º C.

Table 27. Electron donors and acceptors pairs used in cultivation experiments. Abbreviations: SO, sulfur-oxidizing; SR, sulfur-reducing; MO, manganese-oxidizing; MR, manganese-reducing; AO, arsenite-oxidizing; IO, iron-oxidizing; IR, iron-reducing microorganisms.

Target	Electron donor	Electron acceptor
SO	10 mM NaSCN (S^0)	21% (v/v) O_2
SO	10 mM $K_2S_4O_6$ ($S^{2.5+}$; consists of S^{5+} and S^0)	21% (v/v) O_2
SR	10 mM Na-acetate	10 mM DMSO
MO	10 mM $MnSO_4.4H_2O$ (Mn^{2+})	1-2% (v/v) O_2
MR	10 mM Na-acetate	10 mM MnO_2 (Mn^{4+})
AO	2 mM $NaAsO_2$ (As^{3+})	1-2% (v/v) O_2
IO	10 mM $FeSO_4.7H_2O$ (Fe^{2+})	1-2% (v/v) O_2
IR	10 mM Na-acetate	15 mM Fe-citrate

Collected water samples were archived in 4% (v/v) formaldehyde for microbial DAPI enumeration. Water samples obtained with and without a plume signature (as background control) were captured on four different filter membranes (Table 28) for latter DNA extraction, X-ray diffraction, and scanning electron microscopy (SEM) analyses. Nucleic acid isolation was conducted according the CTAB method as described in Chapter 4 (4.3.2 DNA extraction). The mineralogy of the particulates

captured on the filters was assessed by X-ray diffraction at the Department of Earth Sciences, University of Ottawa in Canada, using a zero-background sample holder on a Philips X'Pert diffractometer, a monochromatic Cu K-α x-ray source operating at 45 kV and 40 mA, and a Kevex Si (Li) solid-state detector. All samples were run in a step-scan mode at 0.02° per second from 5° to 75° 2θ. SEM and energy dispersive X-ray spectroscopy (EDS) were performed at the Department of Earth Science, Carleton University in Ottawa, Canada. Filter discs of dried plume samples were fixed to a sample slide using carbon tape and subsequently coated with a 20 nm thick layer of gold and palladium. The samples were imaged in a JEOL JSM-6400 scanning electron microscope operating at 20 kV. The instrument was coupled to a Link Analytical Pentafet X-ray detector, which collected spectra for 80 seconds (live count time).

Table 28. Processing of water samples for further analyses.

Volume	Pore size	Preservation	Analyses
240 ml	0.22 µm	at -20° C	DNA extraction
120 ml	0.22 µm	at -20° C	DNA extraction
3000 ml	0.45 µm	at -20° C under N_2-atmosphere	X-ray diffraction
1000 ml	0.45 µm	at 4° C in filtered water sample and 2% glutaraldehyde	SEM

Results and Discussion

In total, 59 samples from different vent sites and different water depths were collected (Table 29). Microbial growth was not observed in any of the used media at either temperature. There is no obvious reason why this was the case. It might be that 1) the growth media compositions were not appropriate for the microorganisms present within the hydrothermal plume samples; 2) the organisms did not survive transport from the vent; or possibly 3) there were no or at relatively low concentration thermophilic microorganisms present in the collected plume samples.

A similar result was obtained for DNA extractions performed on five filters, where DNA yield was not sufficient and attempts to amplify archaeal and bacterial 16S

rRNA genes by PCR (4.3.3 PCR amplification) remained negative.

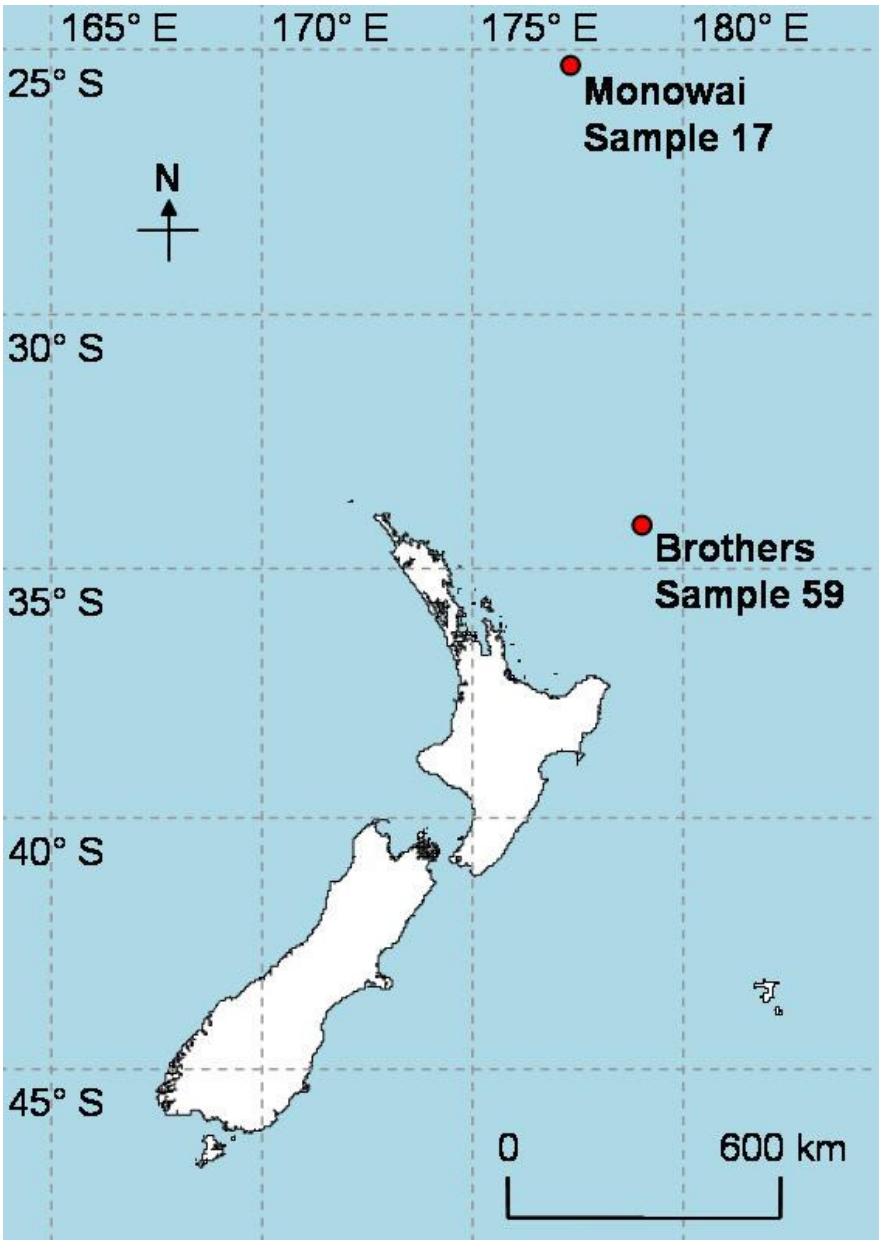

Figure 42. Relative position of the sample sites Monowai and Brothers Volcanoes (indicated by red circles) to the New Zealand mainland. Map was generated using the New Zealand Freshwater Fish Database Assistant software developed by I. G. Jowett (National Institute of Water & Atmospheric Research).

Figure 43 shows the X-ray diffraction pattern derived from a sample with a low nephelometer reading (0.009 nephelometry turbidity units, NTU) collected from Brothers Volcano (Sample 59, Figure 42, Table 29). The diffraction pattern included several sharp peaks, corresponding to peaks for the evaporite minerals halite (mineral form of NaCl), and sylvite (mineral form of KCl). The pattern was also dominated by

a large hump extending from approximately 5.5 to 3.0 Å. This feature is indicative of amorphous material. Although no exact identification could be made, the feature may represent amorphous silica or sulfur. Attempts were made to perform a longer, more-detailed scan on this region of the diffraction pattern, in hopes of extracting more useful data to permit a mineral identification, but no additional information could be gained.

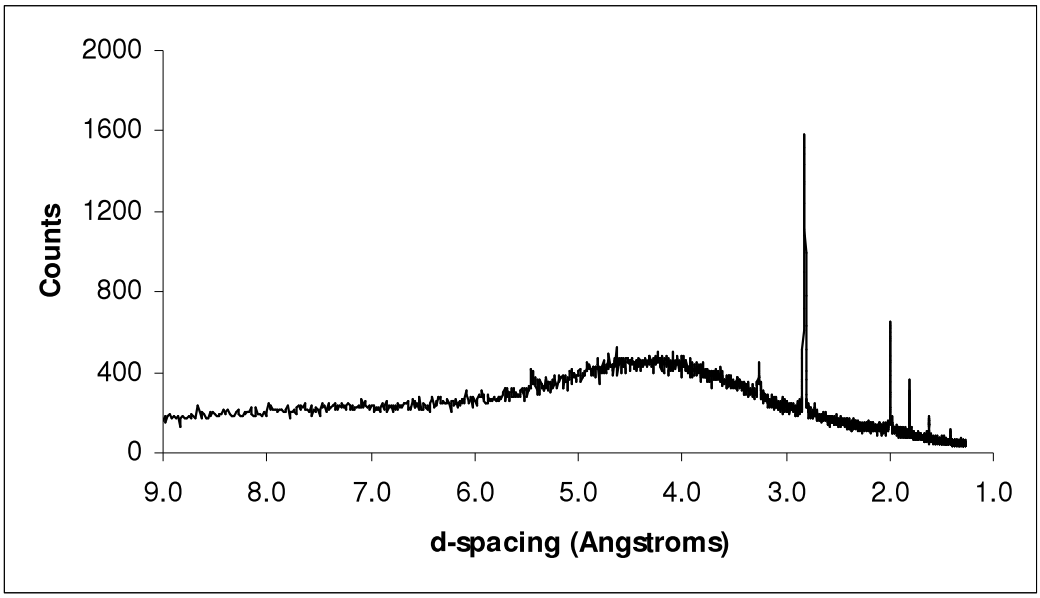

Figure 43. X-ray diffraction pattern obtained from the filtrate of the Brothers Volcano plume (Sample 59, NTU = 0.009). Peaks correspond to halite and sylvite. The broad hump is indicative of amorphous material, possibly silica or sulfur.

An X-ray diffraction pattern obtained from a sample with a much higher nephelometer reading (4.5 NTU) collected from Monowai Cone Volcano (Sample 17, Figure 42, Table 29) is depicted in Figure 44. Despite the higher nephelometry value, and the presence of visible rust-colored material on the filter, the pattern was virtually identical to that obtained from sample 59, again representing halite, sylvite, and unidentified amorphous material. The presence of rust-colored material in samples with high-nephelometry values suggested the presence of an iron oxide mineral, but the lack of corresponding peaks in the diffractograms would suggest that any iron oxide present was either amorphous or present in very small quantity with respect to the salt minerals. These results were consistent for all of the samples analyzed, regardless of plume source.

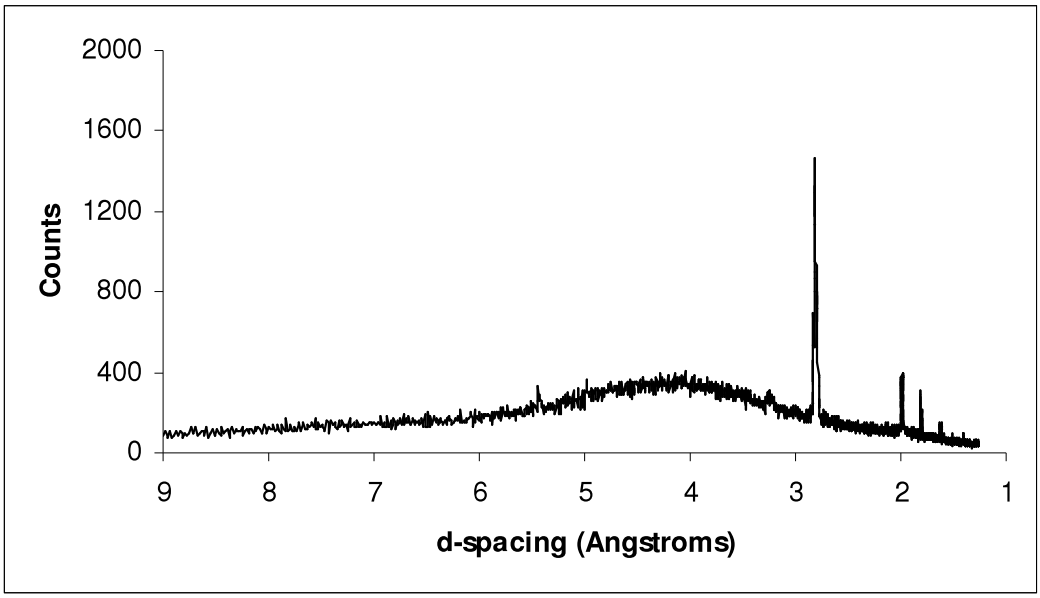

Figure 44. X-ray diffraction pattern for the sample 17 obtained from Monowai Volcano plume (NTU = 4.5). Peaks are representative for halite and sylvite. The broad hump is indicative of amorphous material, possibly silica or sulfur.

Scanning electron micrographs revealed that plume samples appeared nearly devoid of material other than occasional cubic crystals of halite (as determined by EDS). There was a general lack of identifiable organic matter in all samples, although diatom skeletons (Figure 45 A) and small spherical structures (Figure 45 B) were occasionally observed. In samples with high nephelometry values, the filters were nearly covered with similar cubic crystals ranging in size from less than one to several µm in length, and organic matter could not be distinguished (Figure 45 C and D). These results were essentially consistent for all of the samples analyzed, with the amount of halite observed increasing as the nephelometry values of the samples increased.

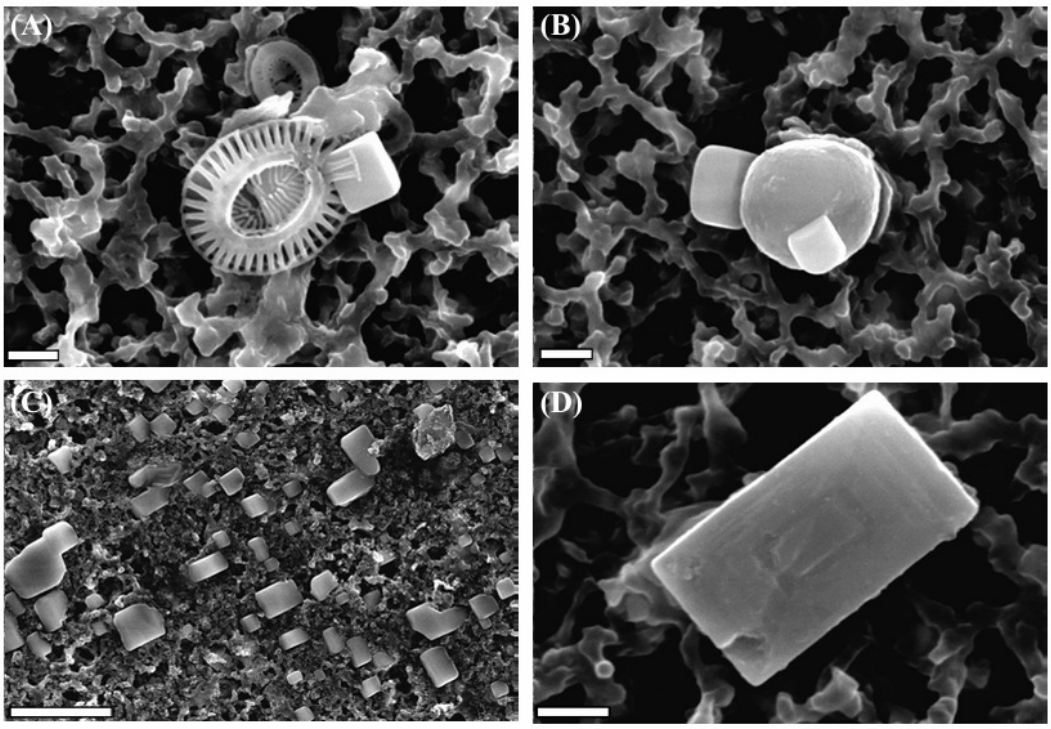

Figure 45. Scanning electron micrographs of plume samples showing (A) a diatom skeleton, and (B) a spherical structure (possibly a coccoid bacterium), both in close association with cubic crystals of halite. Bars 1 µm. (C) Abundance of halite crystals in samples 17, which displayed relatively high nephelometric turbidity units. Bar 10 µm. (D) Single crystals ranged in size from 1 to several µm in length. Bar 1µm.

The lack of growth observed in the prepared media, together with the evidence from DNA extraction and SEM / EDS, suggest that the obtained plume samples did not contain a significant quantity of thermophilic microorganisms.

Table 29. Location of sample sites specified in geographic coordinates and water depths. For each sample the measured nephelometric turbidity unit (NTU) is given. Volcanoes “U” and “V” lying within Tonga’s Economic Exclusive Zone have not been designated to names yet.

Sample	Site	Latitude			Longitude			Depth [m]	NTU
1	Ritchie Ridge	39°	58.382	’ S	178°	14.474	’ E	848	0.015
2	Putoto	27°	53.385	’ S	177°	35.782	’ W	589	0.120
3	Gamble - Rakahore	27°	01.730	’ S	177°	31.314	’ W	750	0.006
4	Gamble - Rakahore	27°	01.730	’ S	177°	31.314	’ W	1499	0.006
5	Hinepuia	26°	24.054	’ S	177°	15.383	’ W	352	0.125
6	Hinepuia	26°	24.567	’ S	177°	14.854	’ W	340	0.358
7	Hinepuia	26°	25.071	’ S	177°	14.122	’ W	301	0.450
8	Hinepuia	26°	24.116	’ S	177°	15.361	’ W	400	0.009
9	Hinepuia	26°	24.183	’ S	177°	15.319	’ W	283	0.007
10	Hinepuia	26°	24.639	’ S	177°	15.244	’ W	21	0.061
11	Hinepuia	26°	24.448	’ S	177°	15.183	’ W	148	0.020
12	Hinepuia	26°	24.381	’ S	177°	15.155	’ W	250	0.028
13	Hinepuia	26°	24.331	’ S	177°	15.131	’ W	320	0.040
14	Hinepuia	26°	24.195	’ S	177°	15.088	’ W	363	0.325
15	Hinepuia	26°	23.916	’ S	177°	15.032	’ W	317	0.200
16	Monowai	25°	50.395	’ S	177°	11.127	’ W	642	0.022
17	Monowai	25°	53.422	’ S	177°	11.101	’ W	129	4.500
18	Monowai	25°	53.422	’ S	177°	11.101	’ W	80	0.057
19	Monowai	25°	48.018	’ S	177°	9.474	’ W	1050	0.120
20	Volcano "U"	25°	25.836	’ S	177°	6.747	’ W	644	0.028
21	Volcano "U"	25°	25.939	’ S	177°	6.585	’ W	499	0.030
22	North of Volcano "V"	25°	58.582	’ S	177°	2.484	’ W	797	0.012
23	"U" - Monowai	25°	36.164	’ S	177°	6.264	’ W	1392	0.028
24	Monowai	25°	46.392	’ S	177°	10.090	’ W	1038	0.100
25	Monowai	25°	46.842	’ S	177°	9.772	’ W	1101	0.200
26	Monowai	25°	48.402	’ S	177°	9.976	’ W	1053	0.280
27	Monowai	25°	46.441	’ S	177°	9.990	’ W	1117	0.280
28	Monowai	25°	53.428	’ S	177°	11.089	’ W	137	4.300
29	Monowai	25°	53.428	’ S	177°	11.089	’ W	55	0.042

Table 29. Continued.

Sample	Site	Latitude			Longitude			Depth [m]	NTU
30	Monowai	25°	53.509	' S	177°	11.098	' W	133	4.895
31	Monowai	25°	53.509	' S	177°	11.098	' W	164	0.123
32	Monowai	25°	47.738	' S	177°	10.245	' W	1328	0.259
33	Volcano "U"	25°	25.815	' S	177°	6.933	' W	550	0.032
34	Lau Havre gap	25°	07.472	' S	177°	27.053	' W	2000	0.017
35	Monowai - Hinepuia	25°	05.305	' S	177°	12.663	' W	1099	0.010
36	Hinepuia	26°	23.804	' S	177°	15.064	' W	345	0.330
37	Hinepuia - Rakahore	26°	38.181	' S	177°	13.676	' W	1002	0.009
38	Gamble	27°	12.528	' S	177°	25.999	' W	102	0.056
39	Gamble	27°	12.491	' S	177°	26.349	' W	303	0.045
40	Putoto - Gamble	27°	36.406	' S	177°	53.388	' W	2201	0.010
41	Putoto	27°	52.675	' S	177°	36.004	' W	608	0.022
42	North of Hinetapeka	28°	20.866	' S	177°	39.869	' W	749	0.009
43	Hinetapeka	28°	39.193	' S	177°	47.373	' W	182	0.031
44	Macauley	30°	12.417	' S	178°	26.750	' W	283	0.046
45	Wright	31°	51.001	' S	179°	11.258	' W	1094	0.045
46	Wrighy	31°	49.128	' S	179°	14.398	' W	795	0.009
47	Brothers	34°	53.069	' S	179°	4.431	' E	1288	0.100
48	Brothers	34°	53.069	' S	179°	4.431	' E	1360	0.132
49	Brothers	34°	53.069	' S	179°	4.431	' E	1176	0.170
50	Brothers	34°	50.196	' S	179°	3.359	' E	1483	0.038
51	Brothers	34°	53.089	' S	179°	4.077	' E	1271	0.249
52	Brothers	34°	51.817	' S	179°	3.746	' E	1205	0.110
53	Brothers	34°	52.004	' S	179°	3.370	' E	1301	0.260
54	Brothers	34°	52.004	' S	179°	3.370	' E	495	0.013
55	Brothers	34°	53.099	' S	179°	4.263	' E	1135	0.275
56	Brothers	34°	54.143	' S	179°	2.965	' E	1498	0.011
57	Brothers	34°	52.613	' S	179°	3.972	' E	1191	0.400
58	Brothers	34°	53.835	' S	179°	2.355	' E	1319	0.050
59	Brothers	34°	47.790	' S	179°	6.184	' E	1090	0.009

Appendix H: Publications

Journal articles and manuscripts

Hetzer, A., C. J. Daughney, and H. W. Morgan. 2006. Cadmium ion biosorption by the thermophilic bacteria *Geobacillus stearothermophilus* and *G. thermocatenulatus*. *Applied and Environment Microbiology*, 72:4020-4027.

Hetzer, A., H. W. Morgan, I. R. McDonald, and C. J. Daughney. 2007. Microbial life in Champagne Pool, a geothermal spring in Waiotapu, New Zealand. *Extremophiles*, 11:605-614.

Hetzer, A., I. R. McDonald, and H. W. Morgan. In press. *Venenivibrio stagnispumantis* gen. nov., sp. nov., a thermophilic hydrogen-oxidizing bacterium isolated from Champagne Pool, Waiotapu, New Zealand. *International Journal of Systematic and Evolutionary Microbiology*.

Conference proceedings

Hetzer, A., H. W. Morgan, R. Ronimus. 2004. Cadmium toxicity experiments using aerobic thermophilic bacteria. The 49th annual conference of the New Zealand Microbiological Society (NZMS) in Palmerston North, New Zealand.

Abstract text:

Cadmium is considered to be an important environmental pollutant that is toxic to many organisms. So far, only a few studies about heavy metal tolerance of thermophilic microorganisms have been described. The aim of our investigation is to isolate heavy metal tolerant microorganisms and gain a deeper insight into their detoxification strategies.
Thermophilic bacteria of the genera *Bacillus, Brevibacillus, Geobacillus,*

Anoxybacillus and *Thermus* are aerobic and have overlapping ranges of optimal pH values and temperatures for growth but differ in the composition of their cell walls. In cadmium toxicity experiments more than 50 species of the genus *Bacillus, Brevibacillus, Geobacillus, Anoxybacillus and Thermus* were tested in the presence of up to 3.2 mM cadmium.

Interestingly, two different patterns of response in the growth curves occurred: an increasing concentration of cadmium led either to a decreasing optical density at a given point of time or to a delay of the onset of positive growth phases. The bacteria that displayed the highest cadmium tolerance, 9 species of the tested genera, were selected for further cultivation experiments using two different media. The ability to tolerate cadmium depended on the selected medium. The results of this study will be presented.

Hetzer, A., R. Ronimus, C. J. Daughney, and H. W. Morgan. 2005. Cadmium biosorption onto two thermophilic bacteria. The 50th annual conference of the New Zealand Microbiological Society (NZMS) in Dunedin, New Zealand.

Abstract text:

Biosorption of metals is the action of forming compounds with metal ions by cell surfaces thereby lowering the effective concentration of the metal ions from a solution. The chemical composition of microbial cell walls and exopolysaccharides can vary considerably within a species displaying qualitative and quantitative varieties of proton-active functional groups that may act as ligands for metal ions.

Microbial biosorption influences the distribution and availability of metal ions in some natural systems and it is utilized in waste treatment of metal-polluted water and soil. Although it having been subject of many studies, as yet, there is still not a single report describing the metal adsorption by thermophilic bacteria that has been published. The aim of our investigation is to generate surface complexation models for two thermophilic bacteria that describe the interaction between the binding sites exposed on the cell surfaces and cadmium ions.

Thermophilic bacteria of the genera *Aneurinibacillus*, *Anoxybacillus*, *Bacillus*, *Brevibacillus* and *Geobacillus* were tested in initial cadmium toxicity experiments. The highest tolerance to cadmium, 400 – 3200 µM, was observed for the species belonging to the genus *Geobacillus*. *Geobacillus thermocatenulatus* and *Geobacillus stearothermophilus* were selected for further investigations. Acid-base titrations were conducted to determine the concentrations and deprotonation constants of binding sites onto the bacterial surfaces. Cadmium adsorption experiments were performed to determine the thermodynamic stability constants for the metal complexes. Based on the experimental data surface complexation models were developed.

The binding capacities for protons and cadmium ions were higher for *G. thermocatenulatus* than those for *G. stearothermophilus*. Cadmium adsorption increased with pH and biomass for both bacteria. The parameters for the surface complexation models will be presented in comparison to published data for mesophilic microorganisms.

Hetzer, A., C. J. Daughney, and H. W. Morgan. 2006. Cadmium ion biosorption onto the thermophilic bacteria *Geobacillus stearothermophilus* and *G. thermocatenulatus*. The 11th International Symposium on Microbial Ecology (ISME) in Vienna, Austria.

Abstract text:

Microbial biosorption influences the distribution and availability of metal ions in some natural and anthropogenic environments and it is utilized in waste management of metal-polluted water and soil. Although several previous investigations have applied surface complexation models (SCMs) to describe the extent of cation adsorption by mesophilic bacteria, not a single study was ever published about thermophilic microorganisms. We assume that mesophilic and thermophilic bacteria differ in cell wall composition and therefore may display different biosorption behaviors.

The aim of our study is to develop SCMs for the thermophilic Gram-positive bacteria *Geobacillus stearothermophilus* and *G. thermocatenulatus* characterizing the complexation between cadmium ions and chemically reactive functional groups within and onto the cell wall as a function of pH and biomass. Experimental data from electrophoretic mobility, potentiometric titration, and cadmium ion adsorption experiments were used quantifying the concentrations and deprotonation constants of binding sites and to determine the thermodynamic stability constants for the metal-bacteria complexes. In comparison to recent reported model parameters obtained for mesophilic bacteria, our results indicate the functional group, with a deprotonation constants p*K* value of approximately 3.8, to be more dominant in cation complexation reactions accounting for 66 and 80% of all titrable groups for *G. thermocatenulatus* and *G. stearothermophilus*, respectively. The experimental results show that the two thermophilic species studied in this investigation display binding capacities for protons and cadmium ions that are different from the mesophilic species that have been studied to date.

Hetzer, A., H. W. Morgan, I. R. McDonald, and C. J. Daughney. 2007. Survey of microbial life in a hydrothermal spring rich in arsenic compounds. The 9th International Conference on the Biogeochemistry of Trace Elements (ICOBTE) in Beijing, China.

Abstract text:

Champagne Pool, one of New Zealand's largest terrestrial hot springs and rich in metalloids such as arsenic and antimony compounds, has been subject to extensive geological and geochemistry descriptions. Only few microbiological studies recently described microbial activity based on culture-independent methods. In the current investigation, a combined approach of culture and culture-independent studies was applied to investigate microbial life in Champagne Pool. Culture-independent methods such as microscopy, DNA yield, and ATP content confirmed relatively low biomass in Champagne Pool compared to other terrestrial hot springs within New Zealand and low cell numbers of 5.6 ± 0.5 x10^6 cells per ml were determined. Denaturing Gradient Gel Electrophoresis (DGGE) and 16S rRNA (small-subunit

ribosomal nucleic acid) gene clone libraries analyses indicated low microbial diversity and the presence of *Sulfurihydrogenibium*, *Nevskia*, *Paracoccus, Sulfolobus* and *Thermofilum*-like populations in Champagne Pool. Three novel species were isolated and phylogenetic analysis of 16S rRNA gene sequences revealed relationships with members of the genera *Thermoanaerobacter*, *Sulfurihydrogenibium*, and *Thermococcus*. One of the isolate represents a novel genus and was able to grow in the presence of relatively high concentrations of arsenic and antimony compounds.

Hetzer, A., H. W. Morgan, I. R. McDonald, and C. J. Daughney. 2007. Microbial life in a hydrothermal spring rich in arsenic compounds. The 17th V. M. Goldschmidt conference in Cologne, Germany.

Abstract text:

The geothermal spring Champagne Pool in Waiotapu, New Zealand has an estimated volume of 50,000 m^3 and discharges fluid at 75° C, which is oversaturated with arsenic and antimony compounds such as orpiment (As_2S_3) and stibnite (Sb_2S_3) that precipitate and form orange deposits. Although Champagne Pool is geochemically well characterized only few studies addressed its role as a potential habitat for microbial life. In the current investigation, a combined approach of first culture-independent studies followed by culturing experiments was applied to describe microbial density and diversity within Champagne Pool. ATP measurements and epifluorescence microscopy showed relatively low biomass in Champagne Pool compare to other terrestrial hot springs within New Zealand and relatively low cell numbers of 5.6 ± 0.5 x10^6 cells per ml. Denaturing Gradient Gel Electrophoresis and 16S rRNA gene clone libraries analyses indicated low microbial diversity and the abundance of hydrogen-oxidizing and sulfur-dependent populations, which were dominated by members belonging to the order *Aquificales*. On account of the results culture media were designed and two novel bacteria and a novel archaeon were successfully isolated. Experiments suggested that the observed relatively low biomass and biodiversity might be due to the presence of volatile components (H_2S,

methyl and hydride derivatives of arsenic and antimony) within the spring which are inhibiting microbial growth.

Hetzer, A., H. W. Morgan, I. R. McDonald, and C. J. Daughney. 2007. Champagne Pool: Cocktail of geothermal energy, arsenic compounds and microbial life. The 18th International Symposium on Environmental Biogeochemistry (ISEB) in Taupo, New Zealand.

Abstract text:

The terrestrial hot spring Champagne Pool in Waiotapu, New Zealand is approximately 65 m in diameter and discharges fluid at 75° C and pH 5.5, which is oversaturated with arsenic and antimony compounds such as orpiment (As_2S_3) and stibnite (Sb_2S_3) that precipitate and form orange deposits. Although Champagne Pool is geologically and geochemically well characterized only few studies investigated its role as potential habitat for microbial life forms using culture-independent methods. However, a successful isolation of microorganisms obtained from Champagne Pool has not been previously reported.

In the current investigation, a combined approach of first culture-independent studies following culturing experiments was applied to describe microbial density and diversity within Champagne Pool. Culture-independent methods consisting of adenosine 5'-triphosphate (ATP) measurements and epifluorescence microscopy indicated relatively low biomass in Champagne Pool compare to other terrestrial hot springs within New Zealand and relatively low cell numbers of $5.6 \pm 0.5 \times 10^6$ cells per ml. Denaturing Gradient Gel Electrophoresis and 16S rRNA gene clone libraries analyses indicated low microbial diversity and the abundance of *Sulfurihydrogenibium*, *Nevskia*, *Paracoccus, Sulfolobus* and *Thermofilum*-like populations which were dominated by members belonging to the order *Aquificales*, with a distant phylogenetic relationship to *Sulfurihydrogenibium*.

On account of the results culture media were selected and designed to target the enrichment of hydrogen-oxidizing and sulfur-dependent microorganisms. Two novel bacteria and a novel archaeon were successfully obtained having 16S rRNA gene sequences with similarities of around 98% between isolate CP.B1

Thermoanaerobacter tengcongensis, 94% between isolate CP.B2 and *Sulfurihydrogenibium azorense*, and 99% between CP.B3 and *Thermococcus waiotapuensis*, respectively. Isolate CP.B2 represents a novel genus within the *Aquificales* order, for which the name *Venenivibrio stagnispumantis* gen. nov., sp. nov. is proposed. *V. stagnispumantis* cells grew chemolithotrophically under microaerophilic conditions with H_2 as electron donor and O_2 as electron acceptor and displayed tolerance to relatively high concentrations of arsenic and antimony compounds (8 mM As^{3+}, 15 mM Sb^{3+}, and >20 mM As^{5+}), but growth was not observed when As^{3+} and As^{5+} were provided as the sole electron donor and acceptor pair. Arsenic resistance was mediated by a two-component energy-dependent efflux system (ArsA and ArsB).

Cruise Report

C. J. Daughney and A. Hetzer. 2006. Microbial studies. In *Cruise report: NZAPLUME III New Zealand American Plume mapping Expedition. September 23 – October 17, 2004*, pp. 49-52. Edited by C. E. J. de Ronde. Institute of Geological & Nuclear Sciences, Lower Hutt, New Zealand.