

**PHYSIOLOGY AND MOLECULAR ECOLOGY OF CHEMOLITHOAUTOTROPHIC NITRATE
REDUCING BACTERIA AT DEEP SEA HYDROTHERMAL VENTS**

by

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A Dissertation submitted to the

Graduate School – New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Microbiology and Molecular Genetics

Written under the direction of

Professor Costantino Vetriani

and approved by

New Brunswick, New Jersey

October 2007

ABSTRACT OF THE DISSERTATION

Physiology and Molecular Ecology of Chemolithoautotrophic Nitrate Reducing Bacteria at Deep Sea Hydrothermal Vents

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Dr. Costantino Vetriani

At hydrothermal vent systems, the ability of microorganisms to use sulfur containing compounds for metabolic purposes has been long established while little is known regarding nitrogen metabolism. The objective of this thesis was to gain a better understanding of how microorganisms are involved in the cycling of nitrogen at deep sea hydrothermal vents through culture dependent and independent methods, isolation of novel nitrate reducing microorganisms, and phylogenetic surveys (16S rRNA gene, citrate lyase (*aclA* and *aclB*), and periplasmic nitrate reductase (*napA*)) of isolates and environmental samples. Sulfide, fluid, and bacterial filament samples from three separate hydrothermal vent sites (Rainbow, Logatchev, and Broken Spur) along the Mid Atlantic Ridge (MAR) and from 9°N on the East Pacific Rise (EPR) were used for isolation and phylogenetic surveys. Several novel autotrophic nitrate ammonifying bacterial strains belonging to the *Epsilonproteobacteria* were isolated with strain TB2 described as a new

species, *Caminibacter mediatlanticus*. Phylogenetic surveys of the 16S rRNA gene, *aclB*, and *napA* showed the dominance of *C. mediatlanticus* related organisms at the Rainbow hydrothermal vent system indicating that we had successfully isolated an environmentally relevant organism. The environmental survey for *napA* was the first to be completed for deep sea hydrothermal vents. The phylogenetic survey of *napA* in reference organisms and environmental samples indicated that there is a wide diversity of Epsilonproteobacterial related sequences present at hydrothermal vents, with the greatest diversity seen within the mesophilic temperature range. It also appears that the periplasmic nitrate reductase gene (*napA*) is possibly being laterally transferred between members among the *Epsilonproteobacteria*.

ACKNOWLEDGEMENTS

I would like to thank the members of my dissertation committee Dr. Constantino Vetriani, Dr. Max Haggblom, Dr. Lee Kerkhof, and Dr. Lily Young for their help and advice in completing this dissertation work.

I am indebted to a number of people who have helped with various aspects of this research project over the years. Cindy Van Dover for sulfide samples from Mid Atlantic Ridge hydrothermal vent sites. Valentin Starovoytov for electron micrographs of *Caminibacter mediatlanticus*. Ken Takai (Japan Agency for Marine-Earth Science and Technology) for kindly making genomic DNA of *Hydrogenimonas thermophila*, *Lebetimonas acidiphila*, *Sulfurimonas autotrophica*, and *Sulfurovum lithotrophicum* available to us. Melitza Crespo-Medina for work on the reverse transcription of napA in *C. mediatlanticus* and *Caminibacter profundus*. Michael Hugler for work on the enzyme measurements of the reverse TCA cycle in *C. mediatlanticus*. The crew of R/V *Atlantis*, and the crew and pilots of the deep-submergence vehicle *Alvin*, for their skilled operations at sea. Ruth Dorn, Ronald Lauck, and Nicole LeFur for technical assistance. The long line of undergraduate students who have performed research in Dr. Vetriani's laboratory over the years: Jasmine Ashraf, Susan Ellor, Kim Kendra, Vivian Ko, and My Do. I would also like to thank the members of the Vetriani, Haggblom, and Kerkhof laboratories and Dr. Stefan Sievert for support and useful discussions through the years.

I also thank the many sources that funded my research during the course of this dissertation: the H. Boyd Woodruff Fellowship in Microbiology, the Department of Biochemistry and Microbiology at Rutgers University, the Ph.D. Training Program in

Biotechnology at Rutgers University and The University of Medicine and Dentistry of
New Jersey-Robert Wood Johnson Medical School (NIH NIGMS 5 T32 GM08339).

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Chapter 1 – Introduction

BACKGROUND

Deep sea hydrothermal vents were discovered in 1977 on of the Galapagos Rift and instantly spawned intensive research into the macro- and microorganisms living at these geothermally active areas. Hydrothermal vents form at sites of geothermal activity such as mid-ocean spreading centers (e.g. Mid-Atlantic Ridge), subduction zones, and midplate hotspots (e.g. Hawaii) (Seyfried Jr. and Mottl 1995). At spreading centers, new oceanic seafloor is formed by the upwelling of fresh lava through extensive cracks in the Earth's crust. Subduction zones form when one crustal plate sinks beneath another such as off the coast of Japan. Hawaii is a perfect example of a hotspot, which form in the middle of crustal plates. The location of various hydrothermal vents can be seen in Figure 1.1.



Figure 1.1 World map of known hydrothermal vent sites.

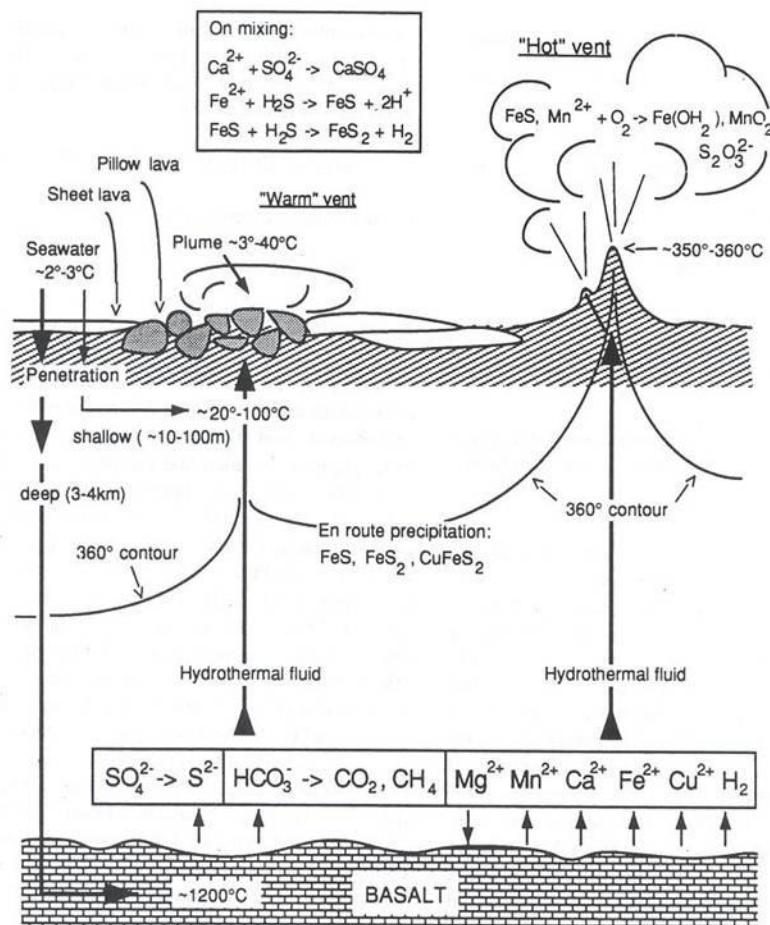


Fig. 2: Major processes during the hydrothermal circulation of seawater through the oceanic crust at mid-ocean ridges [redrawn from Jannasch and Mottl, 1985].

Figure 1.2 Water flow at hydrothermal vents. Adapted from Jannasch 1985.

At these geothermal areas seawater percolates down through cracks in the seafloor into areas of bedrock near magma chambers where it is heated and chemically altered due to high temperature interactions with basaltic rock (Figure 1.2). The seawater becomes highly reduced and depleted of many oxidized compounds (oxygen, sulfate, nitrate, etc.) while becoming enriched in carbon dioxide, methane, helium, hydrogen, and various metals (Al, Se, Fe...). Table 1.1 shows representative changes in chemical species and metal ions at hydrothermal vents as compared to seawater (Von Damm et al. 1985a; Von Damm et al. 1985b; Tunnicliffe et al. 1986; Von Damm 1990; Lilley et al.

1993; Seyfried Jr. and Mottl 1995; McCollom and Shock 1997). The chemistry of hydrothermal vents is variable depending upon geographic location, whether there is phase separation in the fluid, and sedimentation.

Table 1.1 Chemical composition of hydrothermal fluids. Modified from Jannasch 1985

Chemical Species	Guaymas Basin (sedimented) ^a	21°N East Pacific Rise (unsedimented) ^a	Seawater ^a
H ₂ S	5800	7300	0
H ₂	8000	50000	1
CH ₄	1000	1600	1
NH ₄	1500	0	0
SO ₄ ⁻²	0	0	27900
NO ₃ ⁻	nd ^b	nd	40
Fe	56	1664	0.001
Mn	139	960	0.001
pH	5.9	3.4	8.1

^a concentrations given in μM

^b not measured

The buoyant superheated hydrothermal seawater makes its way back up to the seafloor surface and vents into the bottom seawater producing either black smokers, where the exiting temperature can be as high as $\sim 350^\circ\text{C}$, white smokers ($100 - 300^\circ$), or diffuse flows, with temperatures ranging from ~ 2 to 50°C due to mixing with cold seawater ($2-4^\circ\text{C}$) in the shallow seafloor subsurface. The chemistry and temperature of the vent fluids depends on the amount of subsurface mixing with ambient seawater. Black smokers, which are characterized by higher temperature, are formed from the precipitation of sulfides, like pyrite (iron sulfide), when the hydrothermal fluids begin to mix with cold bottom seawater. White smokers get their appearance from the precipitation of silica, anhydrite, and barite. Diffuse flows generally do not contain any metal ions in concentrations high enough to form precipitates, since most have already

precipitated in the subsurface due to mixing with ambient seawater. Individual vent sites range from a few meters to over 100 meters across (Seyfried Jr. and Mottl 1995).

When these areas were first discovered, the high concentration of biological activity in the form of clams, tubeworms, and other animals amazed the scientists, as the deep-sea was supposed to be a relatively barren desert. However, biomass was so abundant at hydrothermal vents that they resembled oases of life. Since the vents were far from the surface with the “life giving” sun and phototrophic based food chain, and since that detritus arriving from the photic zone was not enough to support all of the organisms present (otherwise the entire abyssal plain would be lush with life), scientists began to look at the vents themselves for clues as to where these systems were gaining their energy from. What they found was that these ecosystems are based upon chemolithoautotrophy and the energy driving these systems came from the chemical redox reactions made possible by the hydrothermal fluid chemistry (Karl et al. 1980).

MICROORGANISMS

The microorganisms at the vents live within the microniches located in the vent structures, subsurface seafloor, water plumes, and on biological (invertebrates) and non-biological surfaces, at the steep gradients of chemistry, temperature, and pH that are formed by the transition from hydrothermal fluid (anaerobic, reduced, and hot) to ambient seawater (oxidized, oxygenated, and cold) (Karl 1995; McCollom and Shock 1997; Huber et al. 2003). The temperature range that these organisms live at extends from ambient seawater (2°C) to 121°C (Kashefi and Lovley 2003), and their metabolism spans from aerobic and anaerobic chemolithoautotrophy to heterotrophy.

Chemolithoautotrophic microorganisms at the vents use the reduced chemical species and metal ions found within the vent fluid (e.g. H_2 , H_2S , iron, manganese, and sulfur) in combination with more oxidized compounds (e.g. nitrate, sulfate, and oxygen) from the entrained seawater to form the redox reactions that provide energy through aerobic and/or anaerobic respiration for the cells to grow. Free oxygen is often limited in the vent structures due to the highly reduced condition of the hydrothermal fluids. The microorganisms that are able to fix carbon dioxide into biomass through chemolithoautotrophic processes form the base of the hydrothermal vent's food chain and provide food for organisms of higher trophic levels (Van Dover and Fry 1994). Invertebrates, like the *Riftia* tube worms or *Alvinella*, may either form symbiotic relationships with microorganisms in order to gain the nutrients they need (Cavanaugh et al. 1981; Rau 1981; Tunnicliffe et al. 1985; Wood and Kelly 1989), or they may consume the free living microorganisms by filter feeding or grazing.

Over the years a number of different techniques have been used to investigate what types of microorganisms are present at hydrothermal vents. These techniques have included both culture dependent and culture independent approaches. Early microbial investigations were aimed at cultivation of free-living organisms from the sulfide structures and water samples. This early investigation led to discovery of several new species of mesophilic chemolithoautotrophic bacteria (Karl et al. 1980; Ruby et al. 1981) and of thermo- and hyperthermophilic bacteria and archaea (Belkin et al. 1986; Stetter et al. 1987; Jannasch et al. 1988). A great deal of work has also been focused on the epi- and endosymbiotic microorganisms that have formed relationships with invertebrates at the vents, however many of these organisms still remain uncultured. Once molecular tools

(e.g. PCR) became widely available, the diversity and composition of the vent microbial communities became to be investigated with techniques that bypassed the cultivation steps. Phylogenetic analysis of cloned 16S rRNA genes from samples of vent structures, vent fluids, and microorganisms retrieved from *in situ* growth chambers show a wide distribution of bacteria and archaea including groups which have no known cultivated representative (Takai and Horikoshi 1999; Takai and Sako 1999; Reysenbach et al. 2000; Corre et al. 2001; Longnecker and Reysenbach 2001; Hugler et al. 2003; Takai et al. 2003). From these studies it has emerged that some groups of microorganisms are common at deep-sea hydrothermal vents. Among the archaea representatives belonging to the Euryarchaeota and Crenarchaeota are often found (Takai and Horikoshi 1999; Takai and Sako 1999; Nercessian et al. 2003). Most of the main phylogenetic groups of the bacteria have been found at the vent systems, including the *Cytophaga/Flavobacterium/Bacteroides* group, *Aquificae*, and the *Proteobacteria*. One bacterial group, the *Epsilonproteobacteria*, has been found to be extremely widespread and prevalent in the hydrothermal systems. *Epsilonproteobacteria* have been found in association with invertebrates, microbial mats, and chimney structures at the vents (Moyer et al. 1995; Polz and Cavanaugh 1995; Reysenbach et al. 2000; Lopez-Garcia et al. 2003; Campbell et al. 2006). They are also some of the first colonizers on *in situ* colonization devices (Lopez-Garcia et al. 2003; Takai et al. 2003). While these culture-independent approaches have greatly expanded our knowledge of the diversity and community composition of vent microorganisms, they do not provide information about the metabolic capabilities of the organisms from which the clones were derived (Takai et al. 2003). Currently molecular tools are being used to provide hints as to the metabolic

capabilities of the microbial communities (Campbell et al. 2003; Mehta et al. 2003; Campbell and Cary 2004). These molecular studies help complement cultivation-dependent approaches.

Within the last decade attempts at culturing representatives of the groups commonly seen at the vents have been very successful. A prime example of this is the isolation of several members of the *Epsilonproteobacteria* from subgroups previously only known through 16S rRNA gene surveys (Alain et al. 2002; Miroshnichenko et al. 2002; Takai et al. 2003). Other groups include the *Archaeoglobus* in the archaea, members of the *Aquificae* (*Persephonella* and *Desulfurobacterium*). (Stetter 1988; Burggraf et al. 1990; Huber et al. 1997; Gotz et al. 2002; L'Haridon et al. 2006) Now that organisms with a wide variety of metabolic abilities are being cultivated questions can be asked about how they bring the biogeochemical cycles (carbon, nitrogen, sulfur...) together at the deep-sea hydrothermal vents. One of the key biogeochemical cycles that has been little investigated is the nitrogen cycle (Figure 1.3).

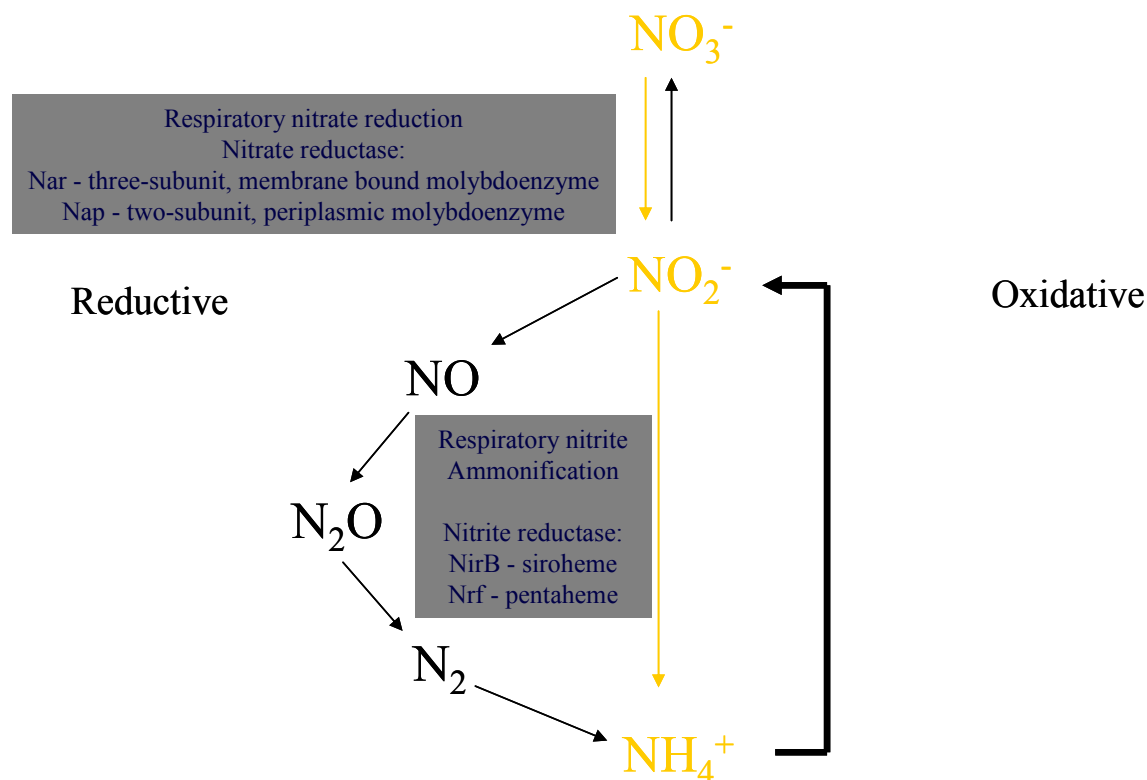


Figure 1.3 Abbreviated Nitrogen cycle

NITROGEN CYCLE

The extent of the role that microorganisms play in nitrogen cycling at the vents is much less understood than their role in terrestrial systems. In the nitrogen cycle microorganisms play several critical roles. The fixation of dinitrogen gas, the largest nitrogen reservoir, into ammonia is exclusively carried out by microorganisms under both aerobic and anaerobic conditions and appears to be possible at temperatures up to 92°C (Mehta and Baross 2006). This ammonia is then either incorporated into biomass or oxidized to nitrite and then to nitrate by nitrifying organisms. The *nifH* gene which encodes a subunit of the dinitrogenase protein involved in nitrogen fixation has been amplified from samples taken at the Juan de Fuca ridge (Mehta et al. 2003).

Another pool of nitrogen available at hydrothermal vents is nitrate which is reduced through various steps to either ammonia or dinitrogen gas, completing the cycle. While nitrate is depleted in reduced hydrothermal fluids, it is available in seawater at concentrations in the micromolar range (Butterfield et al. 1997). Microorganisms will reduce nitrate for one of three purposes: 1) Assimilation, used for cell growth, 2) Dissimilation, to dissipate off extra reducing power generated by cellular processes, and 3) Respiration, as a terminal electron acceptor in the generation of an electron motive force for ATP synthesis, usually under anaerobic conditions. Nitrate respiration can be accomplished through one of two enzymatic systems: Nar or Nap. Both systems, Nar and Nap, can be active within a single organism (Sears et al. 1997) and both use a molybdenum containing cofactor, a molybdopterin guanine dinucleotide (MGD), that is believed to be involved in the transfer of oxygen atoms from nitrate (Berks et al. 1995). These two systems can be distinguished based on the location of the molybdenum binding site by their location within the cell: the Nap system resides in the periplasmic space and Nar is attached to the cytoplasmic side of the cell membrane (Richardson et al. 2001).

The question of what advantage is conferred to organisms having both systems has been studied in *Escherichia coli*. Under nitrate limiting/carbon plentiful conditions, *E. coli* strains that can only express the Nap enzyme (mutants defective for Nar), have been shown to have a selective advantage over those expressing only Nar (mutants defective for Nap). However, when nitrate is plentiful and carbon is limiting *E. coli* strains expressing Nar have the advantage (Potter et al. 1999). Nap was originally studied due to questions over what role nitrate reduction under aerobic conditions plays in the

environment (Carter et al. 1995). This system has been found throughout the *Proteobacteria* and is encoded chromosomally or on megaplasms (Romermann and Friedrich 1985; Warnecke-Eberz and Friedrich 1992; Siddiqui et al. 1993). The exact physiological role of Nap in these bacteria appears to vary from group to group and includes both aerobic and anaerobic nitrate reduction respectively for dissipation of extra reducing power and respiration.

Nitrate is not present in pristine hydrothermal fluids, but is available in the seawater that is entrained by the rising fluid in the sea floor or by diffusion through the vent structure. When used as a terminal electron acceptor under anaerobic conditions, nitrate has a high redox potential when it is combined with hydrogen. As late as 1995 the amount of nitrate was considered to be too low for nitrate respiration to occur (Jannasch 1995). However, since then, numerous organisms, many of which belonging to the *Epsilonproteobacteria*, have been shown to use nitrate (concentrations greater than 10 mM) as a terminal electron acceptor in batch cultures, in combination with hydrogen or other reduced compounds including organic substrates (Alain et al. 2002; Gotz et al. 2002; Alain et al. 2003; Nakagawa et al. 2005; Voordeckers et al. 2005). The ecological significance of nitrate respiration in these organisms is still debated since many are also capable of using sulfur compounds as alternate electron acceptors. Ammonia has been detected at hydrothermal vents, but is attributed to the thermogenic decomposition of sediments that either overlie the site, e.g. Guaymas Basin (Von Damm et al. 1985b) or are buried at the site, e.g. Juan de Fuca Ridge (Von Damm et al. 1985a). The conversion of nitrate to ammonia through a dissimilatory nitrate reduction pathway could create a pool of bioavailable nitrogen for use by other organisms in the vent environment.

In the past several years, organisms capable of respiratory nitrate reduction have been isolated from hydrothermal vent samples (Alain et al. 2002; Huber et al. 2002; Alain et al. 2003; Miroshnichenko et al. 2003; Takai et al. 2003; Miroshnichenko et al. 2004; Vetriani et al. 2004). Other organisms, like *Ferroglobus placidus*, have been found to use nitrate as an electron acceptor but do not reduce it completely to nitrogen gas or ammonia by denitrification or ammonification, respectively (Hafenbradl et al. 1996; Vorholt et al. 1997). Organisms known to reduce nitrate to ammonia (see Figure 1.4) are spread across a wide range of genera in the archaea and bacteria. Most are obligate chemolithotrophs, but heterotrophs are also known, e.g. *Caldithrix abyssi*. (Miroshnichenko et al. 2003). Many of these nitrate reducing organisms can also use other alternate electron acceptors, such as elemental sulfur, or even oxygen allowing them to adapt to changing conditions within the vents (Alain et al. 2002; Miroshnichenko et al. 2004). Another common metabolic thread among nitrate respiring microorganisms at the vents is that many are autotrophic.

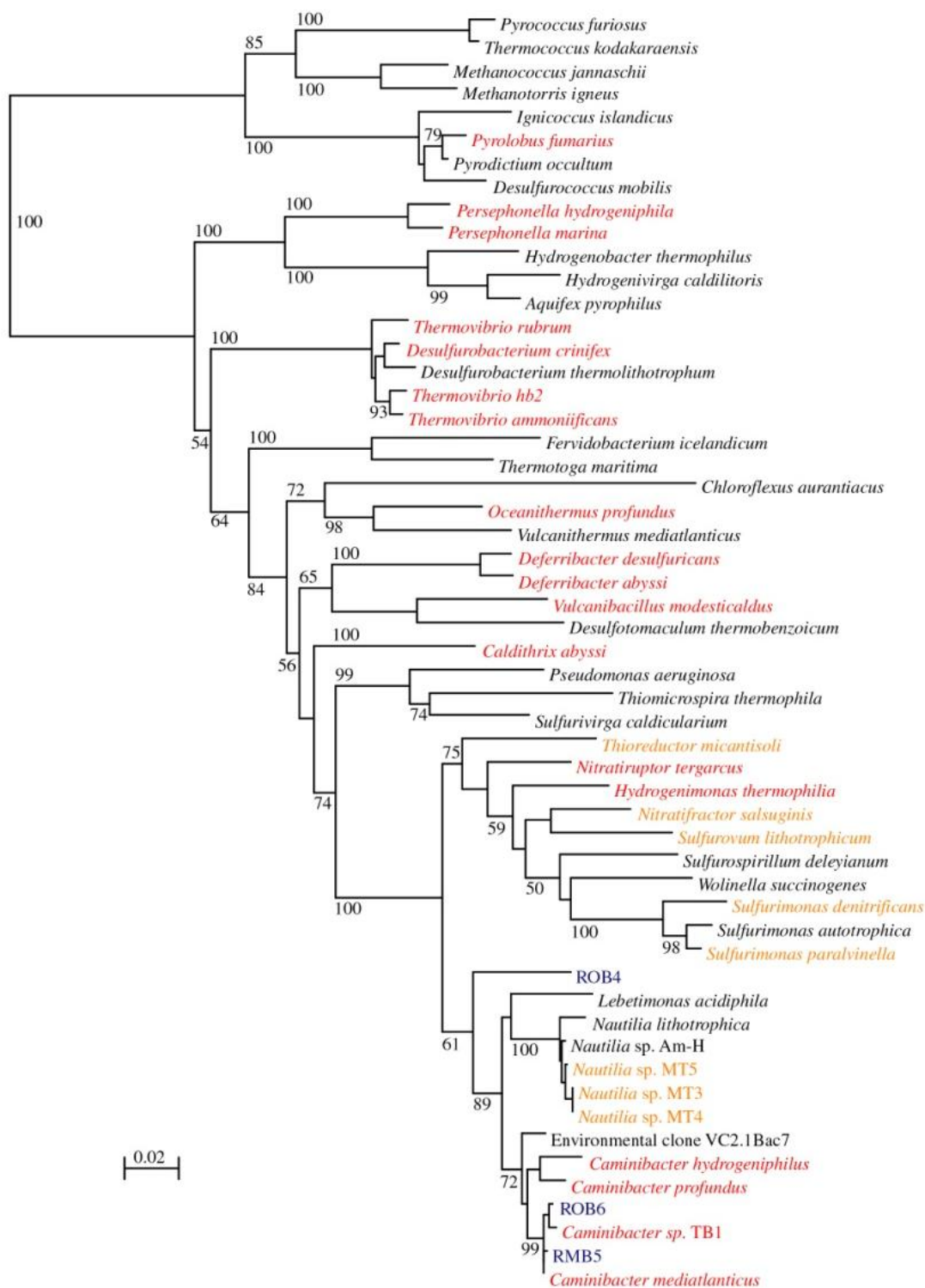


Figure 1.4 16S rRNA gene phylogenetic tree of nitrate ammonifying bacteria and archaea from hydrothermal vents. Thermophilic nitrate reducing organisms are in red; mesophilic nitrate reducing organisms are in orange 16S rRNA clones are in blue.

CARBON DIOXIDE FIXATION

Carbon dioxide fixation by autotrophic organisms is considered a critical component of any food web. Autotrophic organisms are capable of using carbon dioxide as the sole source of carbon for growth and are spread across a number of genera and can be either free living or in symbiotic association with other animals. In most ecosystems, autotrophic fixation of carbon dioxide is primarily provided through phototrophic fixation by green plants, algae, or cyanobacteria that use light as an energy source. However, a number of microorganisms are capable of carbon fixation through chemosynthesis. Chemolithoautotrophic organisms use energy gained from chemical reactions to fix carbon dioxide and are often found in lightless areas such as cave systems, deep-sea habitats, sub surface areas.

Among microorganisms, several different pathways of autotrophic carbon fixation have been described, including the Calvin cycle, the reductive citric acid cycle (reductive TCA cycle, rTCA), the reductive acetyl CoA pathway, and the 3-hydroxypropionate cycle (Fuchs 1989). Several lines of evidence indicate that all of these carbon fixation systems are present at both terrestrial and marine hydrothermal systems. The Calvin cycle is the most familiar cycle, it is used by green plants as well as a number of bacteria, e.g. cyanobacteria (Martin and Schnarrenberger 1997). The key diagnostic enzyme for this cycle is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). The diversity and distribution of the two main forms of RuBisCO, Form I and Form II, has been studied to see if there are differences in their distribution due to conditions such as geography, type of organism, or environmental conditions (Elsaied and Naganuma 2001). These studies have shown that the two forms are preferentially used under high or low

carbon dioxide concentrations, respectively. Form I, which appears to be better adapted to the presence of oxygen, is generally found in aerobic carbon dioxide fixers while Form II, which is better adapted to higher concentrations of carbon dioxide, is generally found in anaerobic organisms. Form II is believed to be more similar to the ancestral RuBisCO protein (Elsaied and Naganuma 2001). Some bacteria are known to possess both forms of the enzyme (Gibson and Tabita 1977). Among the endosymbionts of invertebrates present at hydrothermal vents there has been evidence, both enzymatic and genetic, that the Calvin cycle is used. Both types of RuBisCO have been found in use by endosymbionts and are usually divided by invertebrate type and depend upon the type of environment that they are exposed to (since the different forms have different advantages) (Haygood 1996; Elsaied and Naganuma 2001). Radiotracer experiments have been carried out to follow the metabolic path of carbon dioxide after uptake within tissue samples and whole organisms (Felbeck 1983). Genetic studies have found that multiple versions of RuBisCO can be present in the endosymbionts of *Riftia pachyptila* genome but with only one type, Form II, being expressed (Robinson and Cavanaugh 1995; Robinson et al. 1998).

The rTCA cycle (Figure 1.5) is essentially the TCA cycle operating in reverse, and requires the activity of two enzymes, ATP citrate lyase and 2-oxoglutarate:ferredoxin oxireductase. This cycle has been found in both strict anaerobes and microaerophilic organisms, bacteria and archaea, even though several of the enzymes are oxygen sensitive (Beh et al. 1993; Hugler et al. 2003). It has been hypothesized that the rTCA cycle could have possibly been the first autotrophic system to evolve, due to the fact that it can be autocatalytic (Wachtershauser 1990). However there are questions as to whether key

chemical components necessary for this cycle were present on the early earth.

Experiments have since shown that citric acid, a key component of the rTCA cycle, could be formed by abiotic reactions using minerals and conditions present in hydrothermal environments (Cody et al. 2001).

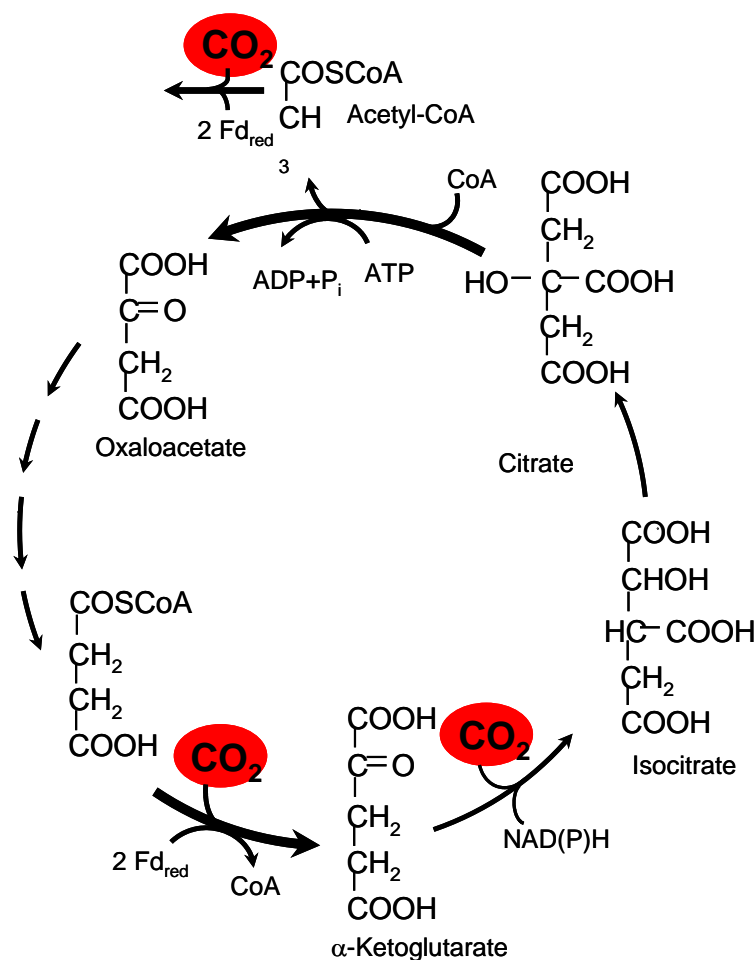


Figure 1.5 Schematic of the reductive TCA (rTCA) cycle; Courtesy of M. Hügler

The reductive acetyl CoA pathway is identified by acetyl-CoA synthase also known as CO dehydrogenase due to its ability to oxidize carbon monoxide to carbon dioxide. Organisms that are able to use this pathway include *Desulfobacterium autotrophicum* and *Clostridium thermoaceticum*. (Pezacka and Wood 1984; Schaunder et

al. 1989) The 3-hydroxypropionate cycle is unidirectional with two key enzymes, malonyl-CoA reductase and propionyl-CoA synthase (Menendez et al. 1999; Alber and Fuchs 2002). Some studies have found that the genes for multiple carbon fixation pathways can be found within the same organism while only one of those pathways is actually active (Hugler et al. 2003).

STUDY SCOPE AND OBJECTIVES

The goal of this study is to gain a better understanding of nitrate reduction at deep-sea hydrothermal vents through the isolation and characterization of novel nitrate ammonifying organisms and through the phylogeny of the 16S rRNA gene and relevant functional genes of the isolated organisms and environmental DNA samples. To accomplish the goal of isolation of nitrate reducing organisms we began with a 16S rRNA survey of selected sites on the Mid Atlantic Ridge to assess the likelihood of these organisms being present. Samples that contained 16S rRNA gene clones related to known nitrate reducing organisms were inoculated into nitrate reducing medium, under autotrophic conditions, and watched for growth. After growth was detected cultures were transferred several times, and isolation procedures used to obtain pure cultures for further study. PCR and/or enzyme tests were performed on the pure isolates to determine the pathway of carbon fixation. PCR was also performed to establish the presence of a gene for a nitrate reductase used in respiration, in this case *napA*. Environmental surveys to assess the presence of the rTCA carbon fixation pathway and for *napA* were performed on samples from the Mid Atlantic ridge and East Pacific Rise. Results from these

surveys were used to assess if the organisms cultured and isolated were of an environmentally important group.

OBJECTIVES:

- To isolate and characterize novel autotrophic nitrate reducing organisms using samples from hydrothermal vents.
- Assess their phylogenetic distribution by comparing isolates to known organisms and to 16S rRNA gene clones derived from hydrothermal vent sites.
- Expand knowledge of how these organisms connect biogeochemical cycles (nitrogen, carbon, sulfur, etc.) at hydrothermal vent systems.
- Address what nitrate reduction pathway(s) are utilized.
- Address which of the known carbon fixation cycles is used by the autotrophic nitrate reducing isolates.

Chapter 2 - Culture dependent and independent analyses of 16S rRNA and ATP citrate lyase genes: a comparison of microbial communities from different black smoker chimneys on the Mid-Atlantic Ridge

Submitted to the journal Extremophiles

INTRODUCTION

The steep chemical (redox, pH) and temperature gradients present at deep-sea hydrothermal vents provide numerous unique niches that microorganisms can colonize. In particular, the walls of black smoker chimneys are characterized by the rapid transition from reduced, high temperature conditions (in the interior section of the chimneys) to more oxidized, low temperature ones (at the interface between the chimneys and seawater). This transition occurs as a continuum when the hot, reduced hydrothermal fluids percolate from the interior conduits towards the outside walls of the chimneys, transporting hydrogen, reduced sulfur species and carbon dioxide, among other compounds. The flux rate and the chemical composition of the fluids (which in turn affect the mineral composition of the chimney) are likely to influence the formation of the chemical and temperature gradients. When the reduced fluids mix with oxidized chemical species (e.g., oxygen, nitrate and sulfate) diffusing inward from ambient seawater, microorganisms take advantage of the available redox potentials and convert chemical energy into ATP that can be used for carbon dioxide fixation and other anabolic

processes. Whole-cell hybridization studies of microbial communities associated with black smokers revealed that the highest density of microorganisms were found in the upper and outer parts of the chimneys, although the inner parts contained detectable amounts of cells (Harmsen et al. 1997). More recent studies that integrated whole cell hybridization with 16S rRNA gene surveys revealed that there was a transition from higher density, mixed bacterial and archaeal communities near the exterior of the chimney to lower density, archaea dominated communities in the interior of the structure (Schrenk et al. 2003). Surveys of the diversity of archaea associated with active black smokers showed that the communities in the chimney structure consisted, for the most part, of hyperthermophilic archaea and of several new archaeal groups, and that the various phylotypes were differently distributed within the chimney structure, possibly as a function of the thermal and redox gradients (Takai and Horikoshi 1999; Takai et al. 2001; Nercessian et al. 2003; Schrenk et al. 2003). In one of these studies, the majority of 16S rRNA gene sequences obtained from the exterior of the chimney were related to archaeal taxa previously recovered from benthic and pelagic environments, including the crenarchaeotal marine group I and uncultured benthic Euryarchaeota. In contrast, the interior regions of the chimney were colonized by methanogens, *Thermococcales*, and *Archaeoglobales*, in addition to uncultured crenarchaeal phylotypes related to sequences previously isolated from deep subsurface habitats (Schrenk et al. 2003). Culture independent studies of functional genes in black smokers further confirmed the occurrence of thermophilic and mesophilic methanogens, and revealed the presence of sulfate reducing bacteria and archaea and of methanotrophic bacteria (Nakagawa et al. 2004; Nercessian et al. 2005). Furthermore, 16S rRNA-based studies of vent microbial

communities indicated that *Epsilonproteobacteria* were associated with sulfide structures (Longnecker and Reysenbach 2001; Hoek et al. 2003), and showed that between 66 and 98% of the microorganisms associated with various types of colonization substrates that were deployed in the vicinity of chimney orifices belonged to this class of the Proteobacteria (Lopez-Garcia et al. 2003; Alain et al. 2004).

Recent work on the isolation of pure cultures from black smokers is complementing the culture-independent approaches by providing physiological information, for example suggesting that specific groups of organisms occupy discrete temperature niches within the chimney structures. For instance, under autotrophic conditions, temperatures between 30-65°C appear to best support growth of anaerobic or microaerobic *Epsilonproteobacteria* (e.g., *Nautilia*, *Caminibacter*, *Hydrogenimonas*, etc.), while temperatures between 50-80°C generally support growth of thermophiles of the phylum *Aquificae* (e.g., *Desulfurobacterium*, *Thermovibrio*, *Persephonella*), and growth temperatures >75°C for the most part select for hyperthermophilic archaea (Miroshnichenko and Bonch-Osmolovskaya 2006). In particular, several new species of *Epsilonproteobacteria* have been isolated from deep-sea hydrothermal vents during the past few years (reviewed in (Campbell et al. 2006; Miroshnichenko and Bonch-Osmolovskaya 2006). Along with the culture-independent studies, physiological information derived from this pure cultures is helping to establish the relevance of *Epsilonproteobacteria* as primary producers, early colonizers, and as metazoan episymbionts at deep-sea vents (Campbell et al. 2006).

One of the pathways for autotrophic CO₂ fixation in some anaerobic and microaerobic bacteria is based on a tricarboxylic acid cycle which operates in reverse

(Buchanan and Arnon 1990). This reductive tricarboxylic acid cycle (rTCA) leads to the fixation of CO₂ and to the synthesis of acetyl coenzyme A, which is carboxylated to pyruvate and then used in further anabolic processes. The three key enzymes that are essential to run the rTCA cycle are ATP citrate lyase, 2-oxoglutarate:ferredoxin oxidoreductase and fumarate reductase. The first evidence for the occurrence of the rTCA cycle in deep-sea hydrothermal vent microbial communities was obtained from metagenome studies of the episymbiotic community associated with the vent polychaete *Alvinella pompejana* and from rTCA-related gene surveys (Campbell et al. 2003; Campbell and Cary 2004). Recent reports demonstrated that pure cultures of *Epsilonproteobacteria* and *Aquificae*, which include representatives of hydrothermal vent bacteria, fix carbon dioxide via the rTCA cycle (Hügler et al. 2005; Takai et al. 2005; Ferrera et al. 2007; Hügler et al. 2007).

Here we present a study of the microbiology of chimney structures from three hydrothermal vent sites along the Mid-Atlantic Ridge (MAR): two ultramafic-hosted systems, Rainbow and Logatchev, and one basalt-hosted system, Broken Spur. We carried out a comparative analysis between 16S rRNA genes and the alpha and beta subunits of ATP citrate lyase, *aclA* and *aclB*, retrieved from natural microbial communities and from reference strains isolated from several different vent sites (including the MAR, the East Pacific Rise, the Central Indian Ridge, the Okinawa Trough and the Mariana Arc). Our results revealed significant differences in the composition of the microbial community of the three MAR sites and further defined the phylogeny of the ATP citrate lyase genes.

MATERIALS AND METHODS

Sample collection. Fragments of active, high temperature black smoker chimneys were collected from the “Rainbow” (36° 14’N, 33° 54’W; depth 2305 m), “Logatchev” (14° 45’N, 44° 58’W; depth 3000 m), and “Broken Spur” (29° 10’N, 43° 10’W; depth 3060 m) vent fields on the Mid-Atlantic Ridge, during a cruise aboard R/V *Atlantis* (cruise AT 05-03, July 2001), and from the East Pacific Rise (EPR; 9° 10’N, 104° 17’W; depth 2500 m) during cruise AT 11-10 (April 2004). The samples were collected using the manipulator of the DSV *Alvin* and stored in boxes on the submersible’s working platform for the rest of the dive. On the surface, samples were transferred to the ship’s laboratory and subsamples were either frozen at -80°C for nucleic acid extraction, or stored at 4°C under a dinitrogen atmosphere for enrichments and isolations. Sample locations are summarized in Table 2.1.

Enrichments, isolations and reference strains. Primary enrichment cultures were initiated by adding about 1 ml of inoculum (prepared by resuspending approximately 1 g of chimney sample in 1 ml of anaerobic artificial seawater) to 10 ml of modified SME media that had been prepared as previously described (Stetter et al. 1983). SME media mimics the chemical composition of hydrothermal vent fluids. A head space of H₂/CO₂ (80%/20%) was added. Carbon dioxide would act as the carbon source for the enrichments, while the hydrogen would act as the electron donor. Nitrate was added as previously described as the electron donor (Vetriani et al. 2004). Incubation temperatures were 55°C for the isolation of *Nautilia* spp. from EPR samples, and 65°C for the Broken Spur enrichment culture. Long-term stocks were prepared by supplementing 50 µL of DSMO (Fisher Scientific) to 1 mL of culture, and stored at -80°C. The reference strains

Table 2.1 Locations of sampling stations and clonal libraries constructed in this study.

Vent	Cruise/Dive #	Latitude	Longitude	Depth (m)	Temperature (°C) ^b	Library ^a 16S rRNA gene Citrate lyase
Rambow, MAR	R/V <i>Atlantic</i> Cruise AT 05-03	36°N14'	33°W54'	2305	158	ROB RMB ROA
	DSV <i>Alvin</i> Dive 3678					RacIB
Logatchev, MAR	R/V <i>Atlantic</i> Cruise AT 05-03	14°N45'	48°W58'	3000	348	L7B L7A L8B
	DSV <i>Alvin</i> Dive 3667-3668 (1run=2 vent)					ND ^d
Broken Spur, MAR	R/V <i>Atlantic</i> Cruise AT 05-03	29°N10'	43°W10'	3060	ND	BSE
	DSV <i>Alvin</i> Dive 3675					BSEacIA BSEacIB BSacIA BSacIB
9°N, EPR	R/V <i>Atlantic</i> Cruise AT 11-10	9°N50'	104°W17'	2500	339°	-
	DSV <i>Alvin</i> Dives 3999, 4002, 4008					-

^a See Material and Methods for library and clone naming^b Fluid temperature measured with DSV *Alvin* HiHT probe at chimney orifice^c Fluid temperature at P vent on Dive 4008^d *acIB* gene fragment not detected by PCR

used in this study included: *Caminibacter mediatlanticus* DSM 16658, *Caminibacter* sp. strain TB-1 (Voordeckers et al. 2005), *Caminibacter hydrogeniphilus* DSM 14510 (Alain et al. 2002), *Caminibacter profundus* DSM 15016 (Miroshnichenko et al. 2004), *Hydrogenimonas thermophila* JCM 11971 (Takai et al. 2004), *Lebetimonas acidiphila* DSM 16356 (Takai et al. 2005), *Sulfurimonas autotrophica* DSM 16294 (Inagaki et al. 2003), and *Sulfurovum lithotrophicum* JCM 12117 (Inagaki et al. 2004).

Preparation of cell extracts and enzyme assays. *Caminibacter mediatlanticus* DSM 16658 (Chapter 3) was used as a reference strain for activity assays of enzymes involved in the reductive TCA cycle. Cell extracts of *C. mediatlanticus* were prepared using a mixer mill (type MM 301, Retsch, Haare, Germany) according to (Hügler et al. 2005). Protein concentrations in cell extracts were determined by the method of (Bradford 1976) using bovine serum albumin as standard. Enzyme assays (0.5 ml assay mixture) were performed in stoppered 0.5 ml glass cuvettes at 55°C. Reactions involving pyridine nucleotides were followed spectrophotometrically at 365 nm ($\epsilon_{365 \text{ nm}} \text{ NAD(P)H} = 3.4 \bullet 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). Reactions involving benzyl viologen (BV) were followed spectrophotometrically at 578 nm ($\epsilon_{578 \text{ nm}} \text{ BV} = 8.6 \bullet 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). ATP citrate lyase activity was determined according to (Hügler et al. 2007). 2-Oxoglutarate:BV oxidoreductase, pyruvate:BV oxidoreductase, fumarate reductase, malate dehydrogenase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, and pyruvate dehydrogenase activities were measured according to references (Hügler et al. 2003; Hügler et al. 2005).

DNA extraction. Total genomic DNA was extracted from 1.7-2.1 g of four chimney subsamples (Rainbow 3678 out, Logatchev 3667, Logatchev 3668, and Broken Spur

3675) using the UltraClean Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA, USA) with the following protocol modifications: Bead beating was extended for 20 seconds and it was followed by heating at 70°C for 5 min. For subsample Rainbow 3678C-mid, 14.59 g was extracted using the Mega Prep UltraClean Soil DNA according to the manufacturer's specifications (Mo Bio Laboratories, Solana Beach, CA, USA). Cells obtained from 10 ml of pure cultures and from an enrichment culture inoculated with a chimney sample from the Broken Spur site were extracted using the UltraClean Microbial DNA Isolation Kit according to the protocol supplied with the kit (Mo Bio Laboratories, Solana Beach, CA, USA).

DNA amplification by PCR. Archaeal and bacterial 16S rRNA genes were amplified using the archaeal domain specific forward primer 16F (5'-CTGGTTGATCCTGCCAG-3') and the bacterial domain specific forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), respectively, in combination with universal primer 1517R (5'-ACGGCTACCTTG-TTACGACTT-3'). PCR conditions for amplification reactions were as follows: 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s, with a final extension time of 7 min during the last cycle. The *acIB* gene was amplified from pure cultures (*Nautilia* spp. and *Caminibacter* spp.), from the Rainbow and Broken Spur natural communities, and from the Broken Spur enrichment culture (Table 2.1) using primers 892F and 1204R and PCR conditions as described by Campbell et al. (2003), while primers F2 and R5 and PCR conditions as described by Hügler et al. (2005) were used to amplify the *acIA* gene from natural communities, from the Broken Spur enrichment culture (Table 2.1) and from the following strains: *Caminibacter mediatlanticus*, *Caminibacter hydrogeniphilus*,

Hydrogenimonas thermophila, *Lebetimonas acidiphila*, *Sulfurimonas autotrophica*, and *Sulfurovum lithotrophicum*.

Library construction, Restriction Fragment Length Polymorphism screening and sequence. The amplified 16S rRNA and *acl* gene fragments were gel-purified using the QIAGEN Gel Spin purification kit (Qiagen, Santa Clarita, CA, USA), cloned into either pCR II or pCR4-TOPO plasmid vectors, and the ligation products were transformed into competent *E. coli* Oneshot cells (Invitrogen, Inc., Carlsbad, CA, USA). Nine environmental libraries (six 16S rRNA and three ATP citrate lyase gene libraries) were constructed from different chimney samples, and three libraries were constructed from an enrichment culture (one 16S rRNA and two ATP citrate lyase gene libraries) (Table 2.1). A total of 275 randomly chosen clones (132 16S rRNA gene clones and 143 ATP citrate lyase clones) were analyzed for insert-containing plasmids by direct PCR followed by gel electrophoresis of the amplified products. Forty-one archaeal and seventy-eight bacterial 16S rRNA gene clones from the environmental libraries, and thirteen bacterial clones from the Broken Spur enrichment culture were screened by Restriction Fragment Length Polymorphism (RFLP) as previously described (Reed et al. 2006). Representative clones for each library showing unique RFLP patterns were selected and their sequences (about 1,400 nucleotides) was determined for both strands on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). A total of sixty *aclA* and eighty-three *aclB* clones were screened by PCR, and representative inserts were sequenced (about 970 nucleotides for *aclA* fragments and about 290 nucleotides for *aclB* fragments).

Phylogenetic analyses. Sequences were assembled using the AutoAssembler Program (Applied Biosystems, Foster City, CA). For the detection of putative chimeric sequences,

both Pintail (Ashelford et al. 2005) and the Check_Chimera 2.7 program of the Ribosomal Database Project II were used (<http://rdp.cme.msu.edu/html/index.html>, (Cole et al. 2003)). Two bacterial phylotypes from Logatchev, represented by a single clone each, appeared to be chimeras and were eliminated from the phylogenetic analysis. The remaining 16S rRNA gene sequences were aligned using ClustalX v 1.8 (Thompson et al. 1997) and manually adjusted using Seaview (Galtier et al. 1996). Phylogenetic distances were calculated using the Jukes-Cantor model and the neighbor joining method was used to evaluate tree topologies. Phylo_win was utilized to plot tree topologies (Galtier et al. 1996) and their robustness was tested by bootstrap analysis with 1,000 resamplings. ATP citrate lyase gene fragments were translated using the online tool EMBOSS Transeq (<http://www.ebi.ac.uk/emboss/transeq/>) and the amino acid sequences were aligned with ClustalX v 1.8. Phylogenetic distances were calculated using the Observed Divergence matrix and the neighbor joining method was used to evaluate tree topologies.

Clone naming. 16S rRNA gene clones retrieved from the Rainbow site were named using the following format: Rxy#, where R stood for Rainbow, x for outside (O) or middle (M) layer, y for either archaeal (A) or bacterial (B), and # indicated the clone number. Logatchev 16S rRNA gene clones were labeled Lxy# where L stood for Logatchev, x for dive number 3667 (7) or 3668 (8), y for either archaeal (A) or bacterial (B), and # for the number of the clone in the library. One representative 16S rRNA gene clone for the Broken Spur enrichment culture was named Broken Spur Enrichment 8. *aclB* clones retrieved from the Rainbow and Broken Spur sites were named RaclB# and BSaclB#, respectively, *aclA* clones from Broken Spur were named BSaclA#, and *aclA* and *aclB* clones retrieved from the Broken Spur enrichment culture were named

BSEaclA# and BSEaclB#, respectively.

Nucleotide sequence accession numbers. The sequences from this study are available through GenBank under the following accession numbers: EF644656 to EF644685, EF644759 to 644814, and EF644827 to EF644847.

RESULTS

Pure cultures. Enrichment cultures for thermophilic, chemolithotrophic, hydrogen-oxidizing organisms were obtained by inoculating 10 ml of anaerobic SME medium (Stetter et al. 1983), supplemented with 0.1% nitrate, with approximately 1 ml of slurries obtained from black smoker chimneys. The isolation (from a MAR black smoker) and characterization of *Caminibacter mediatlanticus* DSM 16658 and of *Caminibacter* sp. strain TB-1, two anaerobic, chemolithoautotrophic *Epsilonproteobacteria*, is reported in Chapter 3. Three moderately thermophilic, anaerobic, hydrogen-oxidizing and nitrate-reducing, chemolithoautotrophic bacteria, designated as strains MT3, MT4, and MT5, were isolated, using the dilution to extinction technique, from the walls of active deep-sea hydrothermal vents chimneys collected at the 9°N site on the EPR. All three strains were grown at 55°C. Phylogenetic analysis of the 16S rRNA gene indicated that all three organisms belonged to the *Epsilonproteobacteria*, and that they were closely related to *Nautilia lithotrophica* (Miroshnichenko et al. 2002), with 96 – 98% sequence identity (Figure 2.1).

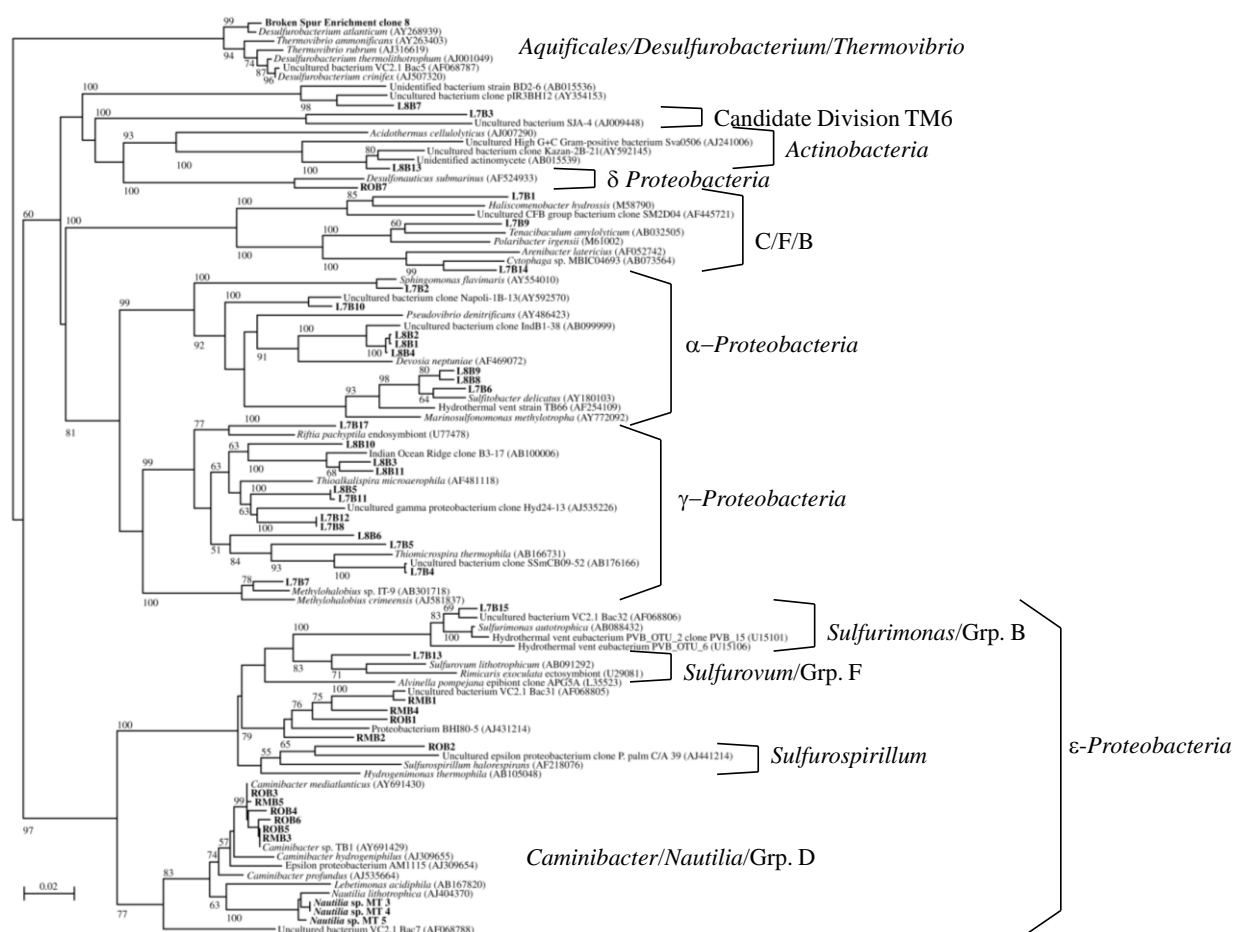
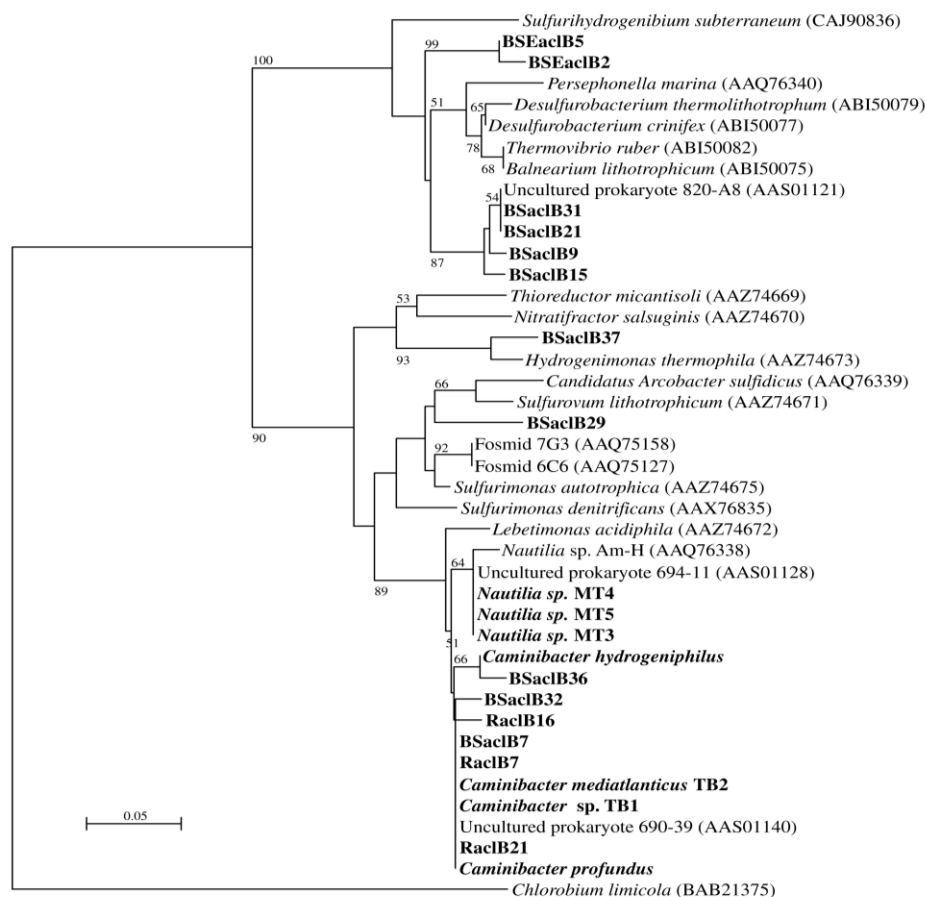


Figure 2.1 Phylogenetic analysis of bacterial 16S rRNA gene sequences from the Rainbow (ROB and ROM) and Logatchev (L7B and L8B) black smokers, and from the Broken Spur enrichment culture. The tree was constructed using the neighbor-joining method from a similarity matrix based on the Jukes-Cantor distance model. Percentages greater than 50% of bootstrap resampling (of 1000 resamplings) that support each topological element are indicated near the nodes. Representative sequences of rRNA genes obtained in this study are in bold. The scale bar is 2 substitutions per 100 sequence positions.

The genes encoding for either the large and/or the small subunit of ATP citrate lyase, *aclA* and *aclB*, were amplified from the three newly isolated *Nautilia* strains (*aclB*), *Caminibacter* spp. (*aclA* and *aclB*), *Hydrogenimonas thermophila* (*aclA*), *Lebetimonas acidiphila* (*aclA*), *Sulfurimonas autotrophica* (*aclA*), and *Sulfurovum lithotrophicum* (*aclA*). Phylogenetic analyses of the amino acid sequence deduced from

the *aclB* gene placed *Nautilia* sp. strains MT3, MT4, and MT5, *C. mediatlanticus*, *Caminibacter* sp. strain TB1, *C. profundus* and *C. hydrogeniphilus* in two closely related clusters distinct from the *aclB* sequences from other *Epsilonproteobacteria* (Figure 2.2a). In line with the phylogenetic analysis, the AclB amino acid sequence of the *Caminibacter* strains had more identity to the sequences of the *Nautilia* strains (95-97% identity) than to the sequences of other vent *Epsilonproteobacteria* (Table 2.2).

a)



b)

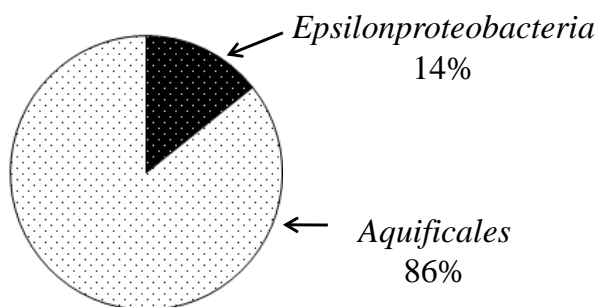
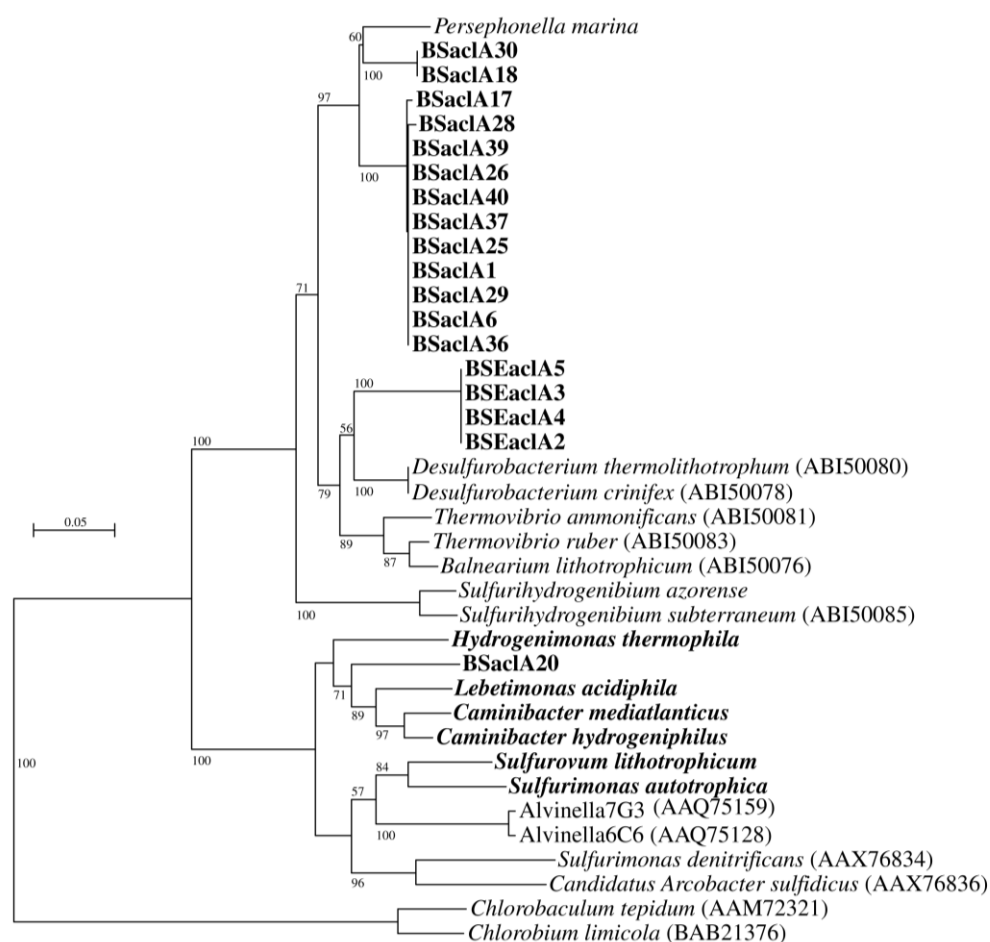


Figure 2.2 Neighbor-joining phylogenetic analysis of the amino acid sequence deduced from a fragment of the gene encoding for the beta subunit of ATP citrate lyase (*aclB*) from the Rainbow (RaclB) and Broken Spur (BSaclB) black smokers, and from the Broken Spur enrichment culture (BSEaclB). Percentages greater than 50% of bootstrap resampling (of 1000 resamplings) that support each topological element are indicated near the nodes. Representative sequences obtained in this study are in bold. The scale bar is 5 substitutions per 100 sequence positions (a). Frequency of *aclB* clones from the Broken Spur black smoker (b).

Phylogenetic analysis of the amino acid sequence deduced from the *aclA* fragment showed that the sequences of *C. mediatlanticus* and *C. hydrogeniphilus* were closely related to each other (95% identity) and more distantly related to ATP citrate lyase from *Lebetimonas acidiphila* and *Hydrogenimonas thermophila*, while the AclA from *Sulfurimonas autotrophica* and *Sulfurovum lithotrophicum* formed a separate cluster related to uncultured episymbionts of the vent worm *Alvinella pompejana* (Figure 2.3a and Table 2.3)).

a)



b)

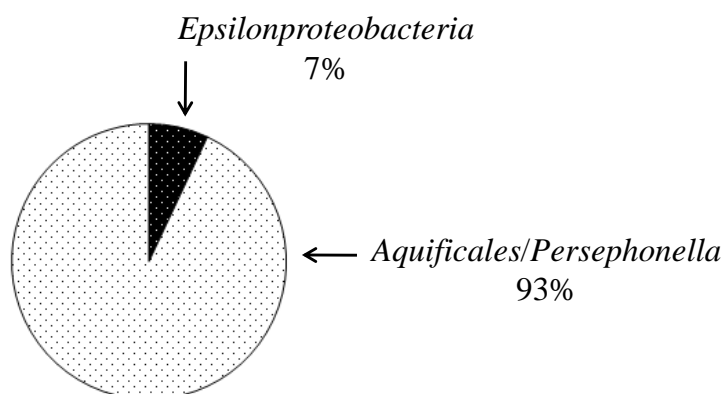
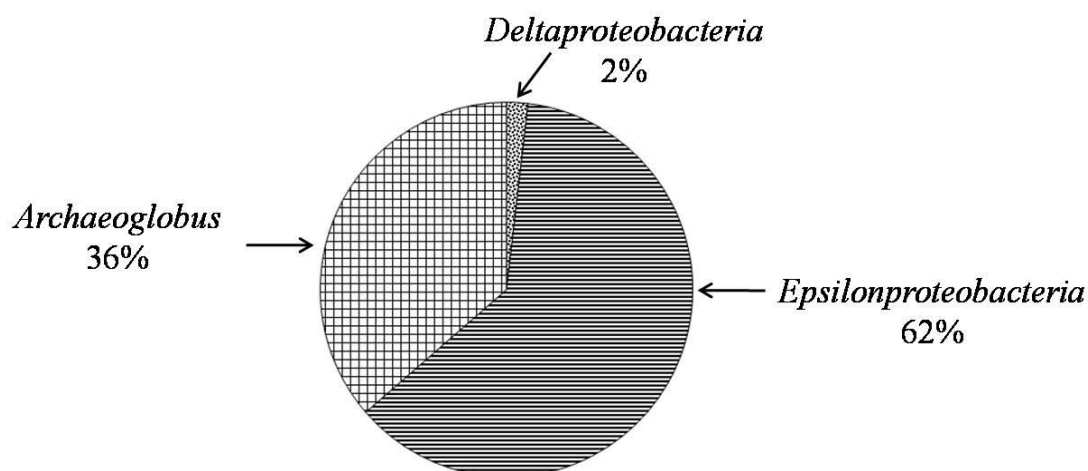


Figure 2.3 Neighbor-joining phylogenetic analysis of the amino acid sequence deduced from a fragment of the gene encoding for the alpha subunit of ATP citrate lyase (*aclA*) from the Broken Spur (BSaclA) black smoker and enrichment culture (BSEaclA). Percentages greater than 50% of bootstrap resampling (of 1000 resamplings) that support each topological element are indicated near the nodes. Representative sequences obtained in this study are in bold. The scale bar is 5 substitutions per 100 sequence positions (a). Frequency of *aclA* clones from the Broken Spur black smoker (b).

Phylogenetic analysis of the Rainbow microbial community. The majority (62%) of the clones retrieved from the Rainbow site were related to the *Epsilonproteobacteria*, and to the archaeal genus *Archaeoglobus* (36%), while only 2% of the Rainbow clones were related to the *Deltaproteobacteria* (Figure 2.4a). The bacterial 16S rRNA gene libraries from both the outside and middle sections of the Rainbow chimney were dominated by *Epsilonproteobacteria*, accounting for eleven of the twelve bacterial phylotypes sequenced from this site. Phylogenetic analysis placed six of these phylotypes (ROB3, ROB4, ROB5, ROB6, RMB3 and RMB5, representing about 40% of all the clones from the Rainbow chimney) in the *Caminibacter/Nautilia* group (also defined as Group D; Figure 2.1). The nearest cultivated relatives to these phylotypes are *C. mediatlanticus* and *Caminibacter* sp. strain TB-1 (99-100% sequence identity), both of which are hydrogen-oxidizing and nitrate-reducing thermophiles (optimum growth temperature 50-55°C), and were isolated from the Rainbow hydrothermal vent field (Voordeckers et al. 2005). Four phylotypes from the Rainbow site (RMB1, RMB2, RMB4, and ROB1) clustered into a group that was related (96-99% sequence identity) to epsilonproteobacterial sequences retrieved from an *in situ* growth chamber deployed at the Snake Pit hydrothermal vent site on the MAR (Reysenbach et al. 2000), and to organisms enriched from the tubes of *Alvinella pompejana* worms (Cambon–Bonavita, unpublished results). Phylotype ROB2 was related to clones obtained from the microbial

a)



b)

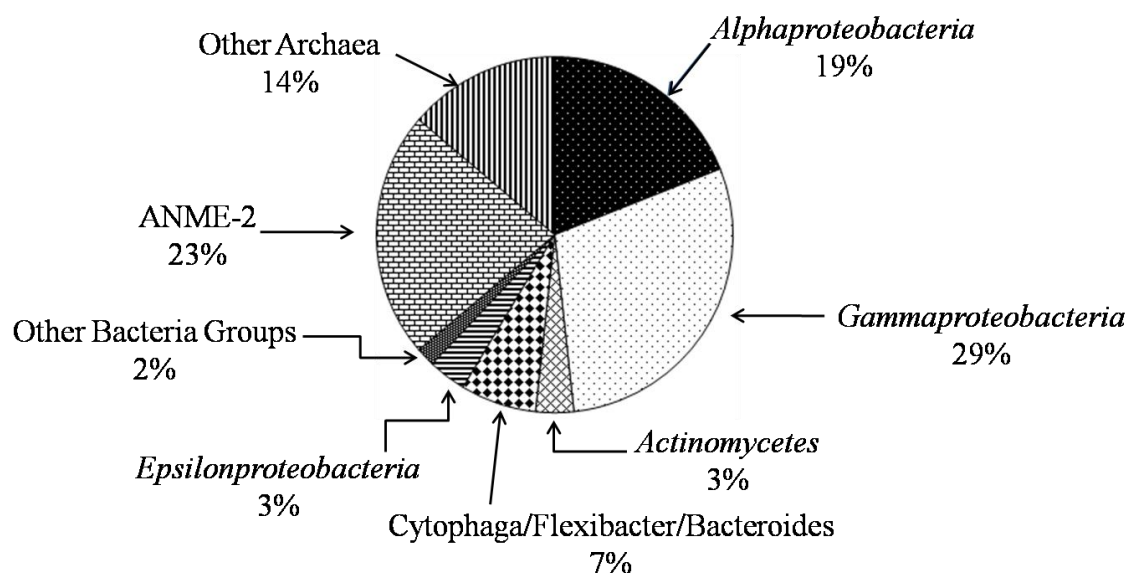


Figure 2.4 Frequency of bacterial and archaeal 16S rRNA gene clones from the Rainbow (a) and Logatchev (b) black smokers.

community associated with the invertebrate *Paralvinella palmiformis* (90% sequence identity) (Haddad et al. 1995; Alain et al. 2002), while the closest cultured relative to this phylotype was *Sulfurospirillum halorespirans* (89% sequence identity) (Luijten et al.

2003). Clone ROB7, the only Rainbow phylotype found outside of the *Epsilonproteobacteria*, was related (94% sequence identity) to *Desulfonauticus submarinus*, a moderately thermophilic (optimum growth temperature 45°C), sulfate-reducing *Deltaproteobacterium* isolated from 13°N on the EPR (Audiffren et al. 2003).

All archaeal sequences retrieved from the outside wall of the Rainbow chimney belonged to the order *Archaeoglobales*. Clones ROA1, ROA2, and ROA3 were related to *Archaeoglobus veneficus* (Huber et al. 1997) (95-97% sequence identity), and clone ROA3 was the dominant phylotype, accounting for 85.7% of the Rainbow archaeal library (Figure 2.5).

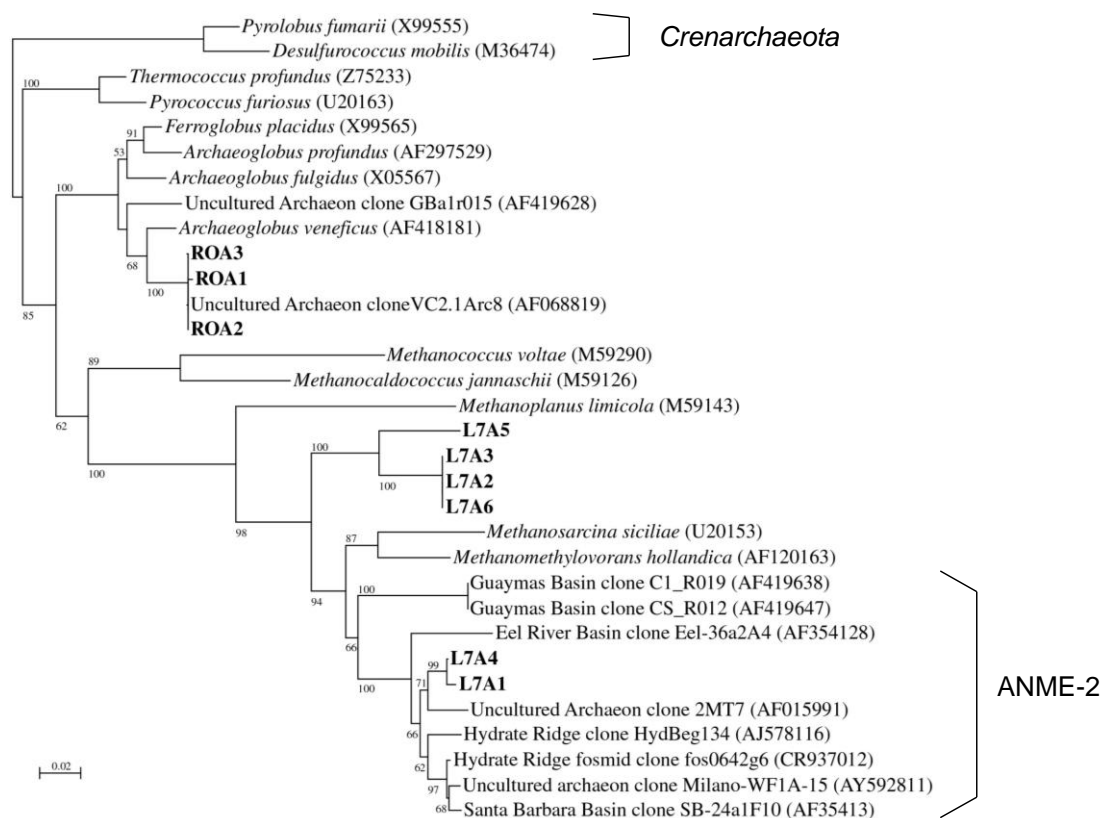


Figure 2.5 Phylogenetic analysis of archaeal 16S rRNA gene sequences from the Rainbow (ROA) and Logatchev (L7A) black smokers. The tree was constructed using the neighbor-joining method from a similarity matrix based on the Jukes-Cantor distance model. Percentages greater than 50% of bootstrap resampling (of 1000 resamplings) that support each topological element are indicated near the nodes. Representative sequences of rRNA genes obtained in this study are in bold. The scale bar is 2 substitutions per 100 sequence positions.

The gene encoding for the small subunit of ATP citrate lyase, *acIB*, was amplified from DNA extracted from the microbial community of the Rainbow black smoker. All the Rainbow *acIB* sequences, represented by clones RaclB16, 7 and 21, were placed within the *Caminibacter* cluster, and their amino acid sequence had 98-99% identity to the ATP citrate lyase of *C. mediatlanticus* (Figure 2.2a and Table 2.2).

Phylogenetic analysis of the Logatchev microbial community. The Logatchev microbial community was much more diverse than the Rainbow community (Figures 2.4a and b). The epsilonproteobacterial phylotypes retrieved from the Logatchev black

smokers were phylogenetically distinct from the Rainbow sequences (Figure 2.1). The closest cultured relatives to phylotypes L7B13 and L7B15 were *Sulfurovum lithotrophicum* (Group F; 92% sequence identity) and *Sulfurimonas autotrophica* (Group B; 94% sequence identity), respectively, two mesophilic (optimum growth temperature 25-30°C) sulfur and thiosulfate-oxidizing bacteria isolated from the Okinawa Trough (Inagaki et al. 2003; Inagaki et al. 2004).

The alphaproteobacterial related phylotypes retrieved from the Logatchev black smokers were clustered into four main groups (Figure 2.1). The first group consisted of three phylotypes, L8B1, L8B2, and L8B4, which were closely related (96% sequence identity) to a 16S rRNA gene sequence (IndB1-38) retrieved from an inactive chimney of the Kairei hydrothermal vent field on the Indian Ocean Ridge (Suzuki et al. 2004), and more distantly related (92-93% sequence identity) to *Devosia neptuniae*, a symbiotic, dinitrogen fixing *Alphaproteobacterium* found in the root-nodule of the aquatic legume *Neptunia natans* (Rivas et al. 2003). The second group consisted of three phylotypes, L7B6, L8B8, and L8B9, which accounted for approximately 10% of the clones from Logatchev and grouped closely with the genus *Sulfitobacter* (97% sequence identity) and more distantly (93-96% sequence identity) with *Methylosulfonomonas methylovora* (Holmes et al. 1997). The third alphaproteobacterial group included one phylotype, L7B10, which was closely related to 16S rRNA sequences retrieved from a Mediterranean mud volcano (represented in Figure 2.1 by clone Napoli 1B-13; 98% sequence identity) (Heijs, unpublished results). The closest cultured relative of L7B10 was *Pseudovibrio denitrificans* (93% sequence identity) (Shieh et al. 2004). The fourth alphaproteobacterial group from the Logatchev black smokers also consisted of one

phylotype, L7B2, which was related to *Sphingopyxis flavimaris* (98% sequence identity), a mesophilic bacterium (optimum growth temperature 30°C) isolated from the Yellow Sea in Korea (Yoon and Oh 2005).

Phylogenetic analysis placed the gammaproteobacterial phylotypes retrieved from the Logatchev black smoker samples into five groups (Figure 2.1). The first group consisted of four phylotypes, L7B8, L7B11, L7B12 and L8B5, which accounted for about 7% of the total bacterial clones from that site. These phylotypes were related (92-93% identity) to clones retrieved from a sediment sample located above a gas hydrate deposit on the Cascadia Margin, Oregon (Knittel et al. 2003). The nearest cultivated relative to these phylotypes (91-92% sequence identity) was *Thioalkalispira microaerophila*, a sulfur oxidizing lithoautotroph isolated from a soda lake in Egypt (Sorokin et al. 2002). The second group of gammaproteobacterial phylotypes included three clones, L8B3, L8B10, and L8B11, whose closest relative (96%, 90%, and 96% sequence identity, respectively) was a clone retrieved from a deep-sea vent located on the Indian Ridge (clone B3-17 in Figure 2.1) (Suzuki et al., unpublished results). The third group of gammaproteobacterial phylotypes, represented by L7B4, L7B5, and L8B6, was related to *Thiomicrospira thermophila* (94%, 90% and 92% sequence identity, respectively) (Takai et al. 2004). L7B4 was most closely related to a bacterial clone, SSmCB09-52, from a hydrothermal system on Suiyo Seamount (98% sequence identity) (Hara, unpublished results). The fourth group included one clone, L7B17, which was related to an endosymbiotic bacterium of the vent tubeworm, *Riftia pachyptila* (91% sequence identity) (Feldman et al. 1997). The fifth gammaproteobacterial group, represented by L7B7, was related (94% sequence identity) to *Methylohalobius*

crimeensis, a mesophilic (optimum growth temperature 30°C) methanotroph from a hypersaline lake in Ukraine (Heyer et al. 2005), and to an isolate, *Methylohalobius* sp. strain IT-9 (93% sequence identity), from a shallow hydrothermal vent off Japan (Hirayama, unpublished results).

Three phylotypes from the Logatchev site, L7B9, L7B14 and L7B1, were related to the C/F/B group (Figure 2.1). The closest cultured relatives to L7B9 and L7B14 were *Tenacibaculum amylolyticum* (90% sequence identity) (Suzuki et al. 2001) and *Cytophaga* sp. MBIC04693 (94% sequence identity) (Matsuo et al. 2003), which are both algae-associated bacteria. Phylotype L7B1 was distantly related to *Haliscomenobacter hydrossis* (89% sequence identity), which was isolated from activated sludge (Gherna and Woese 1992).

Finally, three Logatchev phylotypes were placed in unique clades (Figure 2.1). Clone L8B13 was related to the *Actinobacteria*, clone L8B7 was related (90% sequence identity) to a bacterium isolated from deep-sea sediments (strain BD2-6 in Figure 2.1) (Li et al. 1999), and clone L7B3 was related to an uncultivated bacterium from a trichlorobenzene degrading consortium that belongs to the candidate division TM6 (von Wintzingerode et al. 1999).

The Logatchev archaeal community was more diverse than the Rainbow one, and it was dominated by members of the *Euryarchaeota*. Four of the Logatchev phylotypes, L7A2, L7A3, L7A5, and L7A6, formed a distinct cluster related to both the *Methanosarcinales* (88% and 90% sequence identity to *Methanosarcina siciliae* and *Methanomethylovorans hollandica*, respectively), and the *Methanomicrobiales* (83% sequence identity to *Methanoplanus limicola*), and accounted for about 38% of the

archaeal clones from this site (Figure 2.5). Phylogenetic analysis showed that a second cluster of sequences, represented by L7A1 and L7A4, accounted for about 62% of the archaeal clones retrieved from Logatchev. These phylotypes were related to a lineage whose organisms are involved in the anaerobic oxidation of methane (ANME-2) (Orphan et al. 2001), and which were retrieved from both Eel River and Hydrate Ridge sediments (the sequences of L7A1 and L7A4 had 94-97% identity to the 16S rRNA gene from representatives of the ANME-2 group).

No *aclB* gene fragment could be amplified from the Logatchev black smoker microbial community, although PCR was repeated several times using DNA templates obtained from three independent extractions (Table 2.1).

Phylogenetic analysis of the ATP citrate lyase from the Broken Spur bacterial community and enrichment culture. An enrichment culture was obtained by inoculating, in anaerobic SME medium, a black smoker sample collected from the Broken Spur site on the MAR. The incubation temperature for this enrichment was 65°C. The 16S rRNA gene was amplified from the enrichment culture, cloned into *E. coli*, and several clones were screened by RFLP analysis. A single hydrogen-oxidizing, autotrophic bacterium dominated the Broken Spur enrichment culture. Its 16S rRNA gene clustered within the phylum *Aquificae*, with 98% sequence identity to *Desulfurobacterium atlanticum* (L'Haridon et al. 2006) (Figure 2.1).

The genes encoding for the large and small subunits of the ATP citrate lyase, *aclA* and *aclB*, were amplified from DNA extracted from the microbial community and from the enrichment culture of the Broken Spur black smoker. In contrast to the Rainbow community, both the *aclA* and *aclB* libraries constructed from DNA extracted from the

Broken Spur black smoker community were dominated by clones related to the *Aquificales*, while a smaller number of clones were related to the *Epsilonproteobacteria*. Phylogenetic analyses placed 86% of the amino acid sequences deduced from the *aclB* clones from Broken Spur (Figure 2.2b), represented by clones BSaclB9, 15, 21 and 31, in a cluster related to the ATP citrate lyase from the genera *Thermovibrio*, *Desulfurobacterium* and *Persephonella*, within the *Aquificales* (Figure 2.2a). The AclB amino acid sequences deduced from clones BSaclB15 and 31 were most similar to the ATP citrate lyase of *Persephonella marina* (89% and 91% identity; Table 2.2). Only 14% of the Broken Spur *aclB* clones were related to the *Epsilonproteobacteria* (Figure 2.2b): three of these clones, BSaclB7, 32 and 36, were related to *Caminibacter* spp., clone BSaclB29 was related to ATP citrate lyase from *Candidatus Arcobacter sulfidicus* and *Sulfurimonas autotrophica*, and clone BAaclB37 was related to the sequence of *Hydrogenimonas thermophila* (Figure 2.2a and Table 2.2).

Similarly, 93% of the *aclA* clones from Broken Spur were related to the *Aquificales* (Figure 2.3b), and more specifically to the genus *Persephonella* (clusters represented by clones BSaclA30 and BSaclA17; Figure 2.3a and Table 2.3). Only 7% of the Broken Spur *aclA* clones, represented by clone BSaclA20, were related to the *Epsilonproteobacteria* (Figures 2.3a and b). Phylogenetic analysis placed clone BSaclA20 in a cluster with the AclA from *C. mediatlanticus*, *C. hydrogeniphilus*, *Hydrogenimonas thermophila*, and *Lebetimonas acidiphila* (Figure 2.3a and Table 2.3).

In line with the 16S rRNA gene results, phylogenetic analysis placed the AclB sequences retrieved from the Broken Spur enrichment culture, represented by clones BSEaclB2 and 5, in a cluster related to the *Aquificales* (Figure 2.2a). The deduced amino

acid sequences of the BSEaclB clones were most similar to the AclB of *Persephonella marina* (87-88% identity), to that of the Broken Spur chimney clones (86-89% identity to clones BSaclB9, 15, 21 and 31) and to the AclB of *Desulfurobacterium thermolithotrophum* (Figure 2.2a and Table 2.2). The aclA sequences retrieved from the Broken Spur enrichment culture, represented by clones BSEaclA2, 3, 4, and 5, were related to *Desulfurobacterium* spp. (Figure 2.3a and Table 2.3).

Activities of Enzymes of the Reductive TCA Cycle in *Caminibacter mediatlanticus*. In order to establish whether the rTCA cycle operates in *C. mediatlanticus*, the activity of enzymes of the rTCA cycle were tested in cell extracts of this strain. The activities of all enzymes of the rTCA cycle, including that of the ATP-dependent citrate lyase (encoded by the *aclBA* genes), could be detected in *C. mediatlanticus*, indicating that a functional rTCA cycle is present in this organism (Table 2.2). In contrast, the activities of enzymes specific to the oxidative TCA cycle, such as the 2-oxoglutarate dehydrogenase and the pyruvate dehydrogenase, which are not shared with the rTCA, could not be detected (Table 2.4).

Table 2.4 Specific activities (nmol min⁻¹ (mg cell protein)⁻¹) of enzymes of the reductive TCA cycle in *C. mediatlanticus*^a

Enzyme activity tested	<i>Caminibacter mediatlanticus</i>
Assay temperature (°C)	55
ATP citrate lyase	275
2-Oxoglutarate : BV oxidoreductase	330
Pyruvate:BV oxidoreductase	160
Fumarate reductase (BV)	710
Isocitrate dehydrogenase (Miroshnichenko et al.)	45
Isocitrate dehydrogenase (NADP)	7800
Malate dehydrogenase (NADH)	4080
2-Oxoglutarate dehydrogenase (Miroshnichenko et al.)	n.d.
2-Oxoglutarate dehydrogenase (NADP)	n.d.
Pyruvate dehydrogenase (Miroshnichenko et al.)	n.d.
Pyruvate dehydrogenase (NADP)	n.d.

DISCUSSION

Comparative analysis of the microbial diversity in black smokers from the MAR.

Phylogenetic analysis of the middle and outside sections of the Rainbow chimney did not show significant differences: *Epsilonproteobacteria* dominated both sections of the chimney, and they were distributed in two main clusters of sequences (Figure 2.1).

Because of the high concentration of hydrogen measured in Rainbow hydrothermal emissions (Charlou et al. 2002), it was not unexpected that about 40% of all the clones retrieved from the Rainbow chimney were closely related to hydrogen-oxidizing *Caminibacter* spp. (up to 100% sequence identity). Moreover, three thermophilic, chemolithoautotrophic, hydrogen-oxidizing *Epsilonproteobacteria*, *C. profundus*, *C.*

^a Specific activities (nmol min⁻¹ (mg cell protein)⁻¹) of enzymes of the reductive TCA cycle. Mean values were obtained from at least five measurements. Standard errors were less than ± 20 %. n.d., no activity detected, detection limit < 1 nmol min⁻¹ (mg cell protein)⁻¹.

mediatlanticus (Chapter 3), and *Caminibacter* sp. strain TB-1 (Chapter 3), were previously isolated from this site (Miroshnichenko et al. 2004; Voordeckers et al. 2005). Overall, the Rainbow black smoker communities showed a very limited diversity (Figure 2.4a), and all the cultured relatives to the Rainbow clones were strictly anaerobic thermophiles (e.g., *Caminibacter* spp., *Desulfonauticus submarinus*) or hyperthermophiles (e.g., *Archaeoglobus* spp.) (Figures 2.1 and 2.5). The thermophilic and anaerobic nature of the Rainbow community implies a relatively low dilution of the reduced hydrothermal fluids with cold, oxygenated seawater within the chimney wall. In contrast, two independent studies (Lopez-Garcia et al. 2003; Nercessian et al. 2005) showed that the microbial communities of hydrothermally influenced sediments collected within the limits of the Rainbow vent field were more phylogenetically diverse, and had a higher representation of pelagic microbial taxa than the Rainbow chimney. The sediment communities investigated in both these studies, which are likely to be less impacted by hydrothermal fluids than chimneys, included only a few epsilonproteobacterial clones, which were related to group B (which comprises members of the genus *Sulfurimonas*) and to group F (which comprises *Sulfurovum lithotrophicum*), and none of the sediment clones was related to thermophilic microorganisms.

In contrast to the Rainbow black smoker community, only 3% of all the clones retrieved from the two Logatchev samples were related to the *Epsilonproteobacteria* (Figure 2.4b), and none of these sequences were related to the *Caminibacter/Nautilia* group (Figure 2.1). The few epsilonproteobacterial clones retrieved from the Logatchev chimney were related to two microaerobic, mesophilic, sulfur and thiosulfate oxidizing bacteria, *Sulfurovum lithotrophicum* (group F) and *Sulfurimonas autotrophica* (group B)

(Inagaki et al. 2003; Inagaki et al. 2004), which do not use hydrogen as an electron donor. The remaining sequences from the Logatchev samples were phylogenetically diverse, included a relatively large fraction of *Gamma*- and *Alphaproteobacteria* (29% and 19%, respectively; Figure 2.4b), and none of the bacterial and archaeal clones retrieved from the Logatchev samples were related to thermophilic microorganisms (Figures 2.1 and 2.5). Furthermore, the gene encoding for the beta subunit of ATP citrate lyase could not be amplified from this chimney, suggesting that the use of the rTCA cycle for carbon dioxide fixation was not widespread throughout the autotrophic fraction of the Logatchev community. The mesophilic, aerobic and microaerobic nature of the Logatchev bacterial community implies that extensive mixing (dilution) of the high temperature, reduced hydrothermal fluid with cold, oxygen-rich seawater may be occurring within the chimney wall. For comparison, in a recent study Perner et al. (2007) investigated high temperature fluids and chimney samples collected at the Logatchev vent site and, differently from our study, found a dominance of clones related to the *Epsilonproteobacteria* (up to 49% of the bacterial clones) and a smaller fraction of sequences related to other thermophilic bacteria. The differences in the composition of the Rainbow and Logatchev black smoker communities described in our study, and between the Logatchev chimneys investigated by Perner et al. (Perner et al. 2007) and in our study, imply that the microhabitats within the chimney walls, which are in part defined by redox and temperature gradients, may be highly variable from site to site and from chimney to chimney within the same site. Although both Rainbow and Logatchev are ultramafic-hosted vent systems, there are substantial differences in the fluid chemistry at these sites. For instance, rare earth elements and transition metals (in particular iron and manganese) are much more

abundant in the hydrothermal fluids at Rainbow than at Logatchev (Douville et al. 2002; Marques et al. 2006). Moreover, geochemical differences have been reported between black smokers within the Logatchev field, mostly related to subsurface mixing with seawater, which results in cooling and chemical alteration of the fluids emanating from some of the chimneys (Schmidt et al. 2006). While the thermophilic and mesophilic nature of the Rainbow and Logatchev communities, respectively, may not be directly related to the temperatures of the fluids emitted by the two chimneys (the temperature of the Logatchev fluid was higher than that of the Rainbow fluid; Table 2.1), it is possible that specific differences in the fluid chemistry (and therefore in the mineral composition) of the two sulfide structures, may affect the temperature and redox gradients within the chimney walls. In particular, differences in the permeability of the mineral structures may influence the flux of hydrothermal fluids through the Rainbow and Logatchev chimney walls, by increasing or decreasing the amount of fluid/seawater mixing, and in turn affect the temperature and redox gradients (i.e., the availability of the geothermal sources of energy, such as molecular hydrogen). These differences could then be reflected in the composition of the microbial communities at the two sites.

Potential for anaerobic methane oxidation in the Logatchev archaeal community.

About 23% of the total clones (62% of the archaeal clones) retrieved from the Logatchev black smokers were affiliated with the ANME-2 lineage of the *Methanosarcinales*, which is involved in the anaerobic oxidation of methane (Orphan et al. 2001). While ANME-related sequences have been previously recovered from marine hydrothermal sediments (Teske et al. 2002) and, more recently, one sequence was recovered from hydrothermal emissions at Logatchev (Perner et al. 2007), this is the first report of abundant ANME

sequences associated with black smokers. The possibility that anaerobic methane oxidation occurs within the chimney walls at Logatchev is consistent with the very high concentrations of methane (up to 3.5 mM) (Charlou et al. 1998; Schmidt et al. 2006) that have been measured in hydrothermal fluids at this site, and with the detection of methyl coenzyme M reductase encoding genes (*mcrA*) related to the ANME lineage from the Logatchev community (Reed and Vetriani, unpublished results). However, further work to assess methane oxidation rates and/or to detect molecular signatures of the ANME lineages will be necessary to test this hypothesis.

ATP citrate lyase and rTCA cycle in cultures and in vent natural communities. In this study we carried out, for the first time, a comparative survey of the *aclA* and *aclB* genes from natural vent microbial communities. In addition, we sequenced the *aclA* and *aclB* genes from representative *Epsilonproteobacteria* isolated from different deep-sea hydrothermal vents, further defining the phylogeny of this locus. A search of the *C. mediatlanticus* genome using TBLASTX revealed no evidence that this organism possessed the key enzymes for the Calvin cycle (RuBisCO and phosphoribulokinase), reductive acetyl CoA pathway (acetyl-CoA synthase), or 3-Hydroxypropionate cycle (malonyl-CoA reductase and propionyl-CoA synthase). Furthermore, we measured the activities of the enzymes of the rTCA cycle in *C. mediatlanticus* which, along with the detection of the *aclBA* genes by PCR and the presence of the genes necessary for all of the enzymes in the rTCA cycle, provided full evidence that CO₂ fixation occurs via the rTCA cycle in this strain (Table 2.4). This is consistent with previously published data that showed the activity of rTCA-related enzymes in other *Epsilonproteobacteria* (Hügler et al. 2005; Takai et al. 2005).

In line with the 16S rRNA gene analyses, all the Rainbow *aclB* sequences were closely related to *Caminibacter* spp. (Figure 2.2a). Combined with the enzymatic activities measured in *C. mediatlanticus* (Table 2.4), these results strongly suggest that members of the *Caminibacter* genus are the main primary producers in the Rainbow bacterial community. Our inability to detect *aclB* sequences in the Logatchev community may be explained by the low relative abundance of *Epsilonproteobacteria* in this sample (3%; Figure 2.4b).

In contrast to the Rainbow community, the Broken Spur *aclA* and *aclB* libraries were dominated by sequences related to the *Aquificales* (*Persephonella* and *Desulfurobacterium* genera), while *Epsilonproteobacteria* were much less represented. It is worth noting that the relative proportion of *Aquificales*- and *Epsilonproteobacteria*-related sequences was highly conserved in the *aclA* and *aclB* libraries from the Broken Spur black smoker community (Figures 2.2b and 2.3b). The data that we obtained from the Broken Spur natural community are consistent with our observation that the enrichment culture grown from the same sample (under anaerobic, autotrophic conditions at 65°C), was dominated by an organism closely related to *Desulfurobacterium* spp. (Figures 2.1, 2.2a and 2.3a). The combined 16S rRNA and ATP citrate lyase gene data for the Broken Spur natural community and enrichment culture suggest that thermophilic bacteria related to the *Aquificales* (*Desulfurobacterium* and *Persephonella*) are the dominant primary producers in the Broken Spur community.

CONCLUSIONS

In conclusion, comparative analyses of 16S rRNA and ATP citrate lyase genes indicated that the three MAR hydrothermal vent chimneys investigated in this study host very different microbial assemblages, probably as a consequence of differences in the fluid chemistry, mineral composition, redox and temperature gradients at the three sites. *Caminibacter*- and *Archaeoglobus*-related sequences dominated the Rainbow chimney, suggesting the thermophilic, autotrophic hydrogen oxidation and hyperthermophilic sulfate reduction were the main energy yielding pathways in this environment. The Logatchev bacterial community included several sequences related to sulfur-oxidizing bacteria and, in general, it appeared to be mesophilic and microaerobic. The archaeal component of the Logatchev community was dominated by sequences related to the ANME-2 lineage, suggesting that anaerobic oxidation of methane may also be occurring in this environment. Finally, comparative analyses the 16S rRNA and ATP citrate lyase genes from the Broken Spur chimney and enrichment culture suggested that *Aquificales* of the genera *Desulfurobacterium* and *Persephonella* were the main bacterial primary producers in this environment.

Chapter 3 - *Caminibacter mediatlanticus* sp. nov., a thermophilic, chemolithoautotrophic, nitrate-ammonifying bacterium isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge

Published in IJSEM

Voordeckers, J. W., V. Starovoytov, and Costantino Vetriani. (2005). "*Caminibacter mediatlanticus* sp. nov., a thermophilic, chemolithoautotrophic, nitrate-ammonifying bacterium isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge." International Journal of Systematic and Evolutionary Microbiology **55**: 773-9.

ABSTRACT

A thermophilic, anaerobic, chemolithoautotrophic bacterium, designated as strain TB-2^T, was isolated from the walls of an active deep-sea hydrothermal vent chimney on the Mid-Atlantic Ridge at 36° 14'N, 33° 54'W. Cells were Gram-negative rods approximately 1.5 µm in length and 0.75 µm in width. Strain TB-2^T grew between 45 and 70°C (optimum 55°C), 10 and 40 g l⁻¹ NaCl (optimum 30 g l⁻¹), and pH 4.5 and 7.5 (optimum 5.5). Generation time under optimal conditions was 50 min. Growth occurred

under chemolithoautotrophic conditions with H₂ as the energy source and CO₂ as the carbon source. Nitrate or sulfur was used as the electron acceptor, with resulting production of ammonium and hydrogen sulfide, respectively. Oxygen, thiosulfate, sulfite, selenate, and arsenate were not used as electron acceptors. Growth was inhibited by the presence of acetate, lactate, formate, and peptone. The G+C content of the genomic DNA was 25.6%. Phylogenetic analysis of the 16S rRNA gene indicated that this organism is closely related to *Caminibacter hydrogeniphilus* and *Caminibacter profundus* (95.9% and 96.3% similarity, respectively). On the basis of phylogenetic, physiological, and genetic considerations it is proposed that the organism represents a novel species within the genus *Caminibacter*. The type strain is *Caminibacter mediatlanticus* TB-2^T (= DSM 16658^T = JCM 12641^T).

INTRODUCTION

Until very recently, the class *Epsilonproteobacteria* was thought to comprise a single order, the *Campylobacterales*, and two families, the *Campylobacteraceae* and the *Helicobacteraceae* (Kerstens et al. 2003). These two families include the genera *Arcobacter*, *Campylobacter*, *Sulfurospirillum*, *Thiomicrospira*, *Thiovolum*, *Helicobacter* and *Wolinella*. The organisms that belong to these genera are mesophilic, microaerobic or anaerobic bacteria, which are found in aquatic environments or in association with animals. However, the recent characterization of a number of novel *Epsilonproteobacteria* revealed that the taxonomy of this class is more complex than previously recognized, (Campbell et al. 2001; Alain et al. 2002; Miroshnichenko et al.

2002; Takai et al. 2003; Miroshnichenko et al. 2004). A new order, the *Nautiliales*, was recently proposed to include two genera, *Caminibacter* and *Nautilia*, both of which comprise thermophilic bacteria isolated from deep-sea hydrothermal vents (Miroshnichenko et al. 2004). *Nautilia lithotrophica*, a thermophilic, anaerobic, sulfur-reducing bacterium was isolated from tubes of the vent polychaete *Alvinella pompejana*, and it is the only representative of this genus (Miroshnichenko et al. 2002). *Caminibacter hydrogeniphilus* and *Caminibacter profundus* are both thermophilic, sulfur and nitrate-reducing bacteria that were isolated from deep-sea hydrothermal vents (Alain et al. 2002; Miroshnichenko et al. 2004). In addition to anaerobic growth, *Caminibacter profundus* is also able to grow microaerobically. *Caminibacter hydrogeniphilus* was isolated from *Alvinella pompejana* tubes, while *Caminibacter profundus* was isolated from biomass collected using an environmental growth chamber. Furthermore, four new genera within the *Epsilonproteobacteria* were recently described: *Sulfurimonas*, *Sulfurovum*, *Sulfuricurvum* and *Hydrogenimonas*. *Sulfurimonas autotrophica*, *Sulfurovum lithotrophicum* and *Sulfuricurvum kujiense* are mesophilic, facultatively microaerobic, sulfur and thiosulfate-oxidizing bacteria (Inagaki et al. 2003; Inagaki et al. 2004; Kodama and Watanabe 2004). While *Sulfurimonas autotrophica* and *Sulfurovum lithotrophicum* were isolated from deep-sea hydrothermal sediments, *Sulfuricurvum kujiense* was isolated from an underwater crude oil storage cavity. *Hydrogenimonas thermophila* is a thermophilic, facultatively microaerobic, hydrogen-oxidizing bacterium isolated from a deep-sea hydrothermal vent on the Central Indian Ridge (Takai et al. 2004). Overall, the discovery of these novel organisms revealed a broad taxonomic diversity within the *Epsilonproteobacteria*, and indicates that a revision of the classification of these

organisms is timely.

Culture-independent analyses of microbial communities associated with sulfide structures and vent invertebrates indicated that *Epsilonproteobacteria* are widely distributed at deep-sea hydrothermal vents throughout the world's oceans (Haddad et al. 1995; Polz and Cavanaugh 1995; Cary et al. 1997; Reysenbach et al. 2000; Campbell et al. 2001; Corre et al. 2001; Longnecker and Reysenbach 2001; Alain et al. 2002; Hoek et al. 2003; Huber et al. 2003). Furthermore, experiments where various types of colonization substrates were deployed in the vicinity of active deep-sea vents revealed that between 66 to 98% of the microorganisms associated with these substrates belonged to the *Epsilonproteobacteria* (Lopez-Garcia et al. 2003; Takai et al. 2003; Alain et al. 2004). Overall these observations suggest that *Epsilonproteobacteria* represent a dominant fraction of the microbial communities at deep-sea hydrothermal vents. Here we describe the isolation and characterization of a novel thermophilic, chemolithoautotrophic, strictly anaerobic, nitrate-ammonifying *epsilonproteobacterium* that was isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge.

MATERIALS AND METHODS

Enrichment Cultures and Isolation. Fragments of active, high temperature black smoker chimneys were collected from the “Rainbow” vent field on the Mid-Atlantic Ridge (36° 14'N, 33° 54'W) at a depth of 2,305 m, during a cruise aboard R/V *Atlantis* (cruise AT 05-03, July 2001). The samples were collected using the manipulator of the DSV *Alvin* and stored in boxes on the submersible's working platform for the rest of the

dive. On the surface, samples were transferred to the ship's laboratory and subsamples were stored at 4°C under a dinitrogen atmosphere until use in the laboratory. Primary enrichment cultures were initiated by adding about 1 ml of inoculum (prepared by resuspending approximately 1 g of chimney sample in 1 ml of anaerobic artificial seawater) to 10 ml of modified SME media that had been prepared as previously described (Stetter et al. 1983; Vetriani et al. 2004). For the isolation of single colonies, plates containing modified SME medium solidified with 1 g l⁻¹ phytagel were used. Plates were incubated in an anaerobic jar (Oxoid) pressurized with H₂/CO₂ (80:20, 70 kPa). Long-term stocks were prepared by supplementing 50 µL of DMSO (Fisher Scientific) to 1 mL of culture, and stored at -80°C.

Metabolic Tests. Growth rates (μ ; h⁻¹) were estimated as: $\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1)$, where N_2 and N_1 are no. cells ml⁻¹ at time (in h) t_2 and t_1 . Generation times (t_g ; h) were calculated as: $t_g = (\ln 2) / \mu$. All growth experiments were carried out in duplicate. Optimal growth temperature for strain TB-2^T was determined by incubating cultures between 40 and 80°C (at 5°C intervals). All other experiments were carried out at 55°C. Optimal salt requirement was determined by varying the concentration of NaCl between 10 and 45 g l⁻¹, at 5g l⁻¹ intervals. Optimal pH for growth was determined by varying the pH in the culture medium between 4.0 and 8.5, using the following buffers at a concentration of 10 mM: acetate at pH 4.0, 4.5, and 5.0, MES at pH 5.5 and 6.0, PIPES at pH 6.5 and 7.0, HEPES at pH 7.5, and TRIS at pH 8.0 and 8.5. Antibiotic resistance was tested in the presence of ampicillin, chloramphenicol, kanamycin, and streptomycin (all 100 µg ml⁻¹). All antibiotics were added aseptically before incubation at 55°C and an ethanol control was performed for chloramphenicol. The effect of organic substrates upon the growth of

strain TB-2^T was investigated by the addition of the following substrates to the medium under an H₂/CO₂ gas phase (80:20; 200 kPa): acetate (2 g l⁻¹), formate (2 g l⁻¹), lactate (2 g l⁻¹), peptone (2 g l⁻¹), tryptone (2 g l⁻¹), Casamino acids (2 g l⁻¹), D-(+)-glucose (2 g l⁻¹), sucrose (2 g l⁻¹), and yeast extract (0.1 and 1 g l⁻¹). These substrates were also tested as possible energy and/or carbon sources by using the following gas phases: N₂/CO₂ (80:20; 200 kPa; 200 kPa), N₂ (100%; 200 kPa) or H₂ (100%; 200 kPa). The ability of TB-2^T to use alternate electron acceptors was tested by the addition of thiosulfate (0.1%, w/v), sulfite (0.1% w/v), arsenate (5 mM), selenate (5 mM), sulfur (3% w/v), and oxygen (0.5% v/v) to nitrate depleted media.

Analytical Techniques. Quantitative determination of nitrate, nitrite and ammonium were carried out spectrophotometrically using a Lachat QuickChem automated ion analyzer according to manufacturer specifications (Diamond 1993; Diamond 1993). Qualitative hydrogen sulfide determination was carried out as previously described (Vetriani et al. 2004). For the determination of catalase, cells were collected by centrifugation from 1.5 ml of an overnight culture, resuspended in 70 µl of a 3% solution of H₂O₂, and incubated both at 55°C and room temperature. A cell free 3% solution of H₂O₂ was used as a negative control. The presence of catalase was detected by the formation of gas bubbles.

Microscopy. Cells were routinely stained in 0.1% acridine orange and visualized with an Olympus BX 60 microscope with an oil immersion objective UPlanF1 100/1.3. For ultrathin sections, cells were fixed for 3 h in Karnovsky's fixative (formaldehyde, 4% v/v and glutaraldehyde, 1% v/v, in 0.1 M Millonig's phosphate buffer, pH 7.3), followed by incubation in 1% osmium tetroxide for 1 h and dehydration in a graded ethanol series.

Cells were then embedded in Epon-Araldite and sectioned with a diamond knife by using an LKB 2088 ultramicrotome (LKB Produkter). Thin sections were stained with a 5% uranyl acetate (w/v) solution in 50% ethanol for 15 min and then with a 0.5% lead citrate (w/v) solution in CO₂-free, double distilled water for 2 min. For direct visualization, cells were fixed and applied onto a copper Formvar/carbon-coated grid. The grids were air-dried and shadowed with 2 nm Pt/C (angle, 15°) by using a High Vacuum Freeze-Etch unit BAF 300 (Balzers). Electron micrographs were taken on a model JEM 100 CX transmission electron microscope (JEOL LTD).

Phylogenetic analysis. Genomic DNA was extracted from cells of strain TB-2^T using the UltraClean Microbial DNA Isolation Kit (MoBio). The 16S rRNA gene was selectively amplified from the genomic DNA by PCR as described previously (Vetriani et al. 1999; Vetriani et al. 2004), and its sequence was determined for both strands on an ABI 3100-Avant Genetic Analyzer (Applied Biosystems). Sequences were aligned automatically using ClustalX and the alignment was manually refined using SEAVIEW (Galtier et al. 1996; Thompson et al. 1997). Neighbor-joining trees were constructed by the least-squares algorithm of DeSoete from a normal evolutionary distance matrix, using Phylo_Win (DeSoete 1983; Perriere and Gouy 1996). Approximately 1204 homologous nucleotides were included in the analysis, and 500 bootstrap replicates were carried out to provide confidence estimates for phylogenetic tree topologies. The determination of DNA base composition for TB-2^T, and DNA-DNA hybridization between *Caminibacter profundus* and TB-2^T, were carried out as previously described (Vetriani et al. 2004).

RESULTS AND DISCUSSION

Enrichment culture for thermophilic, chemolithotrophic organisms were obtained by inoculating 10 ml of modified SME media, which had been supplemented with 0.1% nitrate or 3% w/v elemental sulfur, with 1 ml of slurries from a high temperature vent (158°C) located on the Mid-Atlantic Ridge. Headspace atmosphere was composed of 80% hydrogen and 20% carbon dioxide. Cultures were incubated at 50°C, 65°C, and 80°C. Turbidity was observed within two days and 0.1 ml of the original cultures were subsequently transferred to fresh medium. Two independent cultures, supplemented with nitrate as the terminal electron acceptor, showed consistent growth after repeated transfers at 50°C and 65°C, respectively. Pure cultures were obtained by isolation of single colonies on solidified medium. Both cultures were short rods and were designated strains TB-1 (50°C) and TB-2^T (65°C). Preliminary phylogenetic analysis of the 16S rRNA gene indicated that strains TB-1 and TB-2^T were closely related (sequence identity: 99%) and TB-2^T was chosen for further characterization. TB-2^T cells were short rods approximately 1.5 to 2.0 µm in length and 0.75 µm in width that stained gram negative (Figure 3.1a). The cell envelope of TB-2^T included a cytoplasmic membrane surrounded by the periplasmic space and an outer membrane (Figure 3.1b). Ultrathin sections revealed the presence of stacked membranes (Figure 3.1b). TB-2^T possessed one or more polar flagella as observed in platinum shadowed electron micrographs (Figure 3.1c). The presence of spores was never observed and the cells divided by constriction.

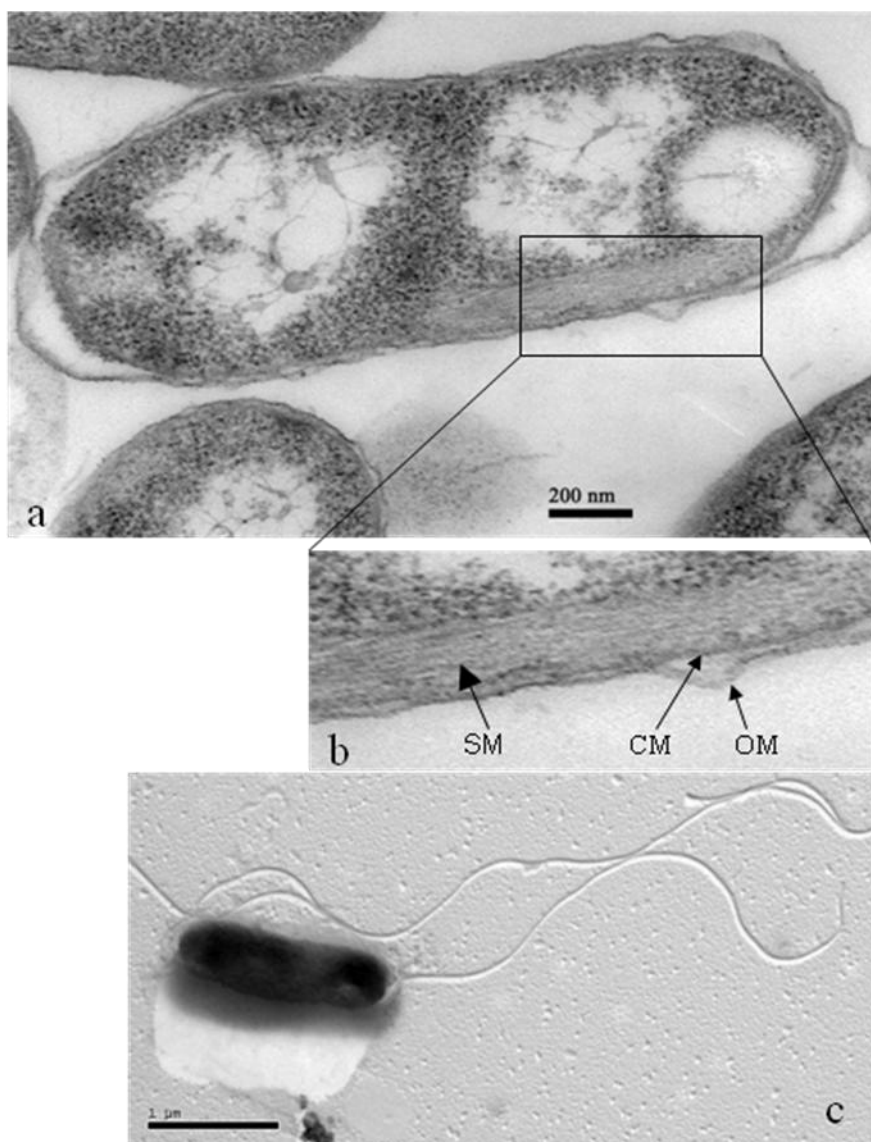


Figure 3.1 Electron micrographs of strain TB-2^T (a) Electron micrograph of a thin section of cells of strain TB-2^T showing cell morphology and stacked membrane feature. (b) Ultrastructural details of stacked membranes and cell envelope of strain TB-2^T. SM, stacked membrane; CM, cytoplasmic membrane; OM, outer membrane. (c) Electron micrograph of platinum-shadowed TB2^T cell showing multiple polar flagella.

Strain TB-2^T grew at temperatures between 45°C and 70°C with optimal growth at 55°C (optimal growth for TB-1 was 50°C). No growth was detected at 40 and 75°C. TB-

2^T grew at NaCl concentration between 10 and 40 g l⁻¹ with optimal growth at 30 g l⁻¹ (no growth was detected at 45 g l⁻¹). Growth of strain TB-2^T occurred between pH 4.5 and pH 7.5, with an optimum at pH 5.5. Under optimal conditions, the generational time of isolate TB-2^T was 50 min. TB-2^T is a strictly anaerobic, chemolithoautotrophic bacterium that used nitrate, H₂, and carbon dioxide as the primary electron acceptor, electron donor and carbon source, respectively. Under these conditions nitrate was reduced to ammonium in stoichiometric amounts, and nitrite did not accumulate in the culture medium (Figure 3.2). Growth of TB-2^T was also supported by elemental sulfur as the terminal electron acceptor, with concomitant production of H₂S. Under these conditions, TB-2^T underwent a lag phase of about 12 hrs, and the generation time was 7.0 hours. Strain TB-2^T did not grow when oxygen (0.5% v/v), arsenate (5 mM), selenate (5 mM), thiosulfate (0.1% w/v), or sulfite (0.1% w/v) were used as electron acceptors. In nitrate-containing medium, the presence of oxygen (0.5 v/v) inhibited growth. In contrast, *Caminibacter profundus* grew in medium with H₂/CO₂/O₂ (79.75:19.75:0.5; 200 kPa) as the gas phase.

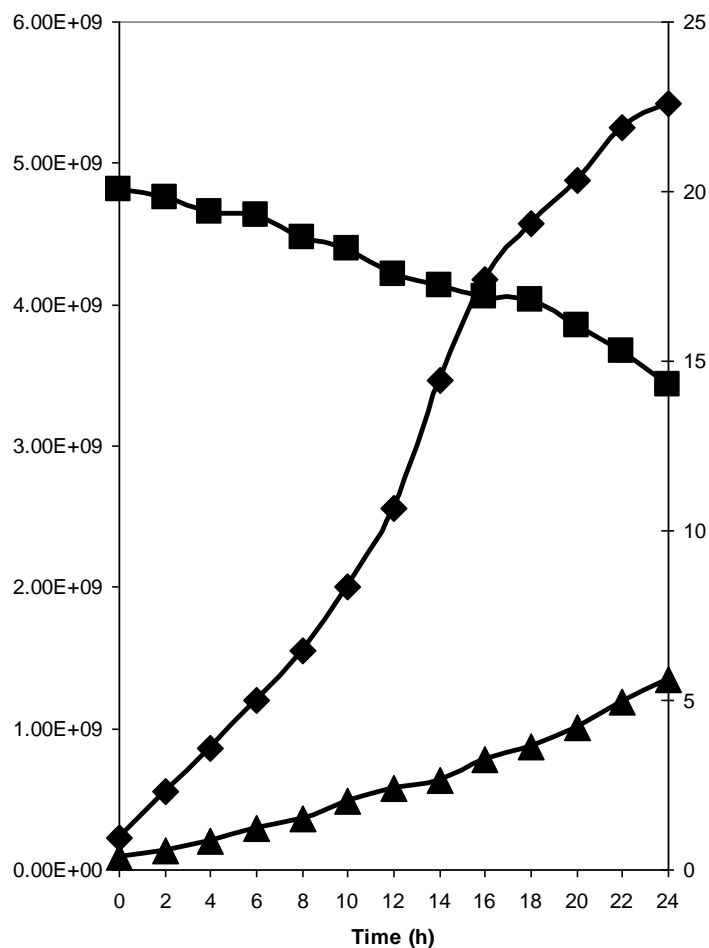


Figure 3.2 Nitrate consumption and ammonium formation during growth of strain TB-2^T. (♦) Growth curve, (■) nitrate utilization, and (▲) ammonium production.

Growth of TB-2^T was inhibited by the presence of acetate (2 g l⁻¹), formate (2 g l⁻¹), lactate (2 g l⁻¹), and peptone (2 g l⁻¹) under an H₂/CO₂ gas phase. No inhibition was observed under a H₂/CO₂ (80; 20; 200 kPa) gas phase in the presence of tryptone (2 g l⁻¹), casamino acids (2 g l⁻¹), yeast extract (0.1 and 1 g l⁻¹), sucrose (2 g l⁻¹), and glucose (2 g l⁻¹). However, no growth was observed with these substrates under N₂/CO₂ or H₂ gas phase. Strain TB-2^T was inhibited by chloramphenicol, ampicillin and streptomycin, but

not by kanamycin. Strain TB-2^T exhibited weak catalase activity after concentrated cells were incubated in the presence of H₂O₂, both at 55°C and at room temperature.

The genomic DNA G+C content of strain TB-2^T, determined by HPLC analysis of the deoxyribonucleosides, was 25.6 mol%. DNA-DNA hybridization experiments with *Caminibacter profundus* revealed a similarity of 35.7% between the organisms.

Phylogenetic analysis of the 16S rRNA, carried out using the neighbor-joining method, placed both strains TB-2^T and TB-1 within the class *Epsilonproteobacteria* (Figure 3.3). Both these strains, whose sequences were 99% similar, were placed in a discrete cluster in the genus *Caminibacter* (Figure 3.3). The next closest relatives to both TB-1 and TB-2^T were *Caminibacter hydrogenophilus* and *Caminibacter profundus* (95.9 and 96.3% sequence similarity, respectively), which branched in separate clusters (Figure 3.3). High bootstrap values supported the branching topology of the four *Caminibacter* strains (Figure 3.3).

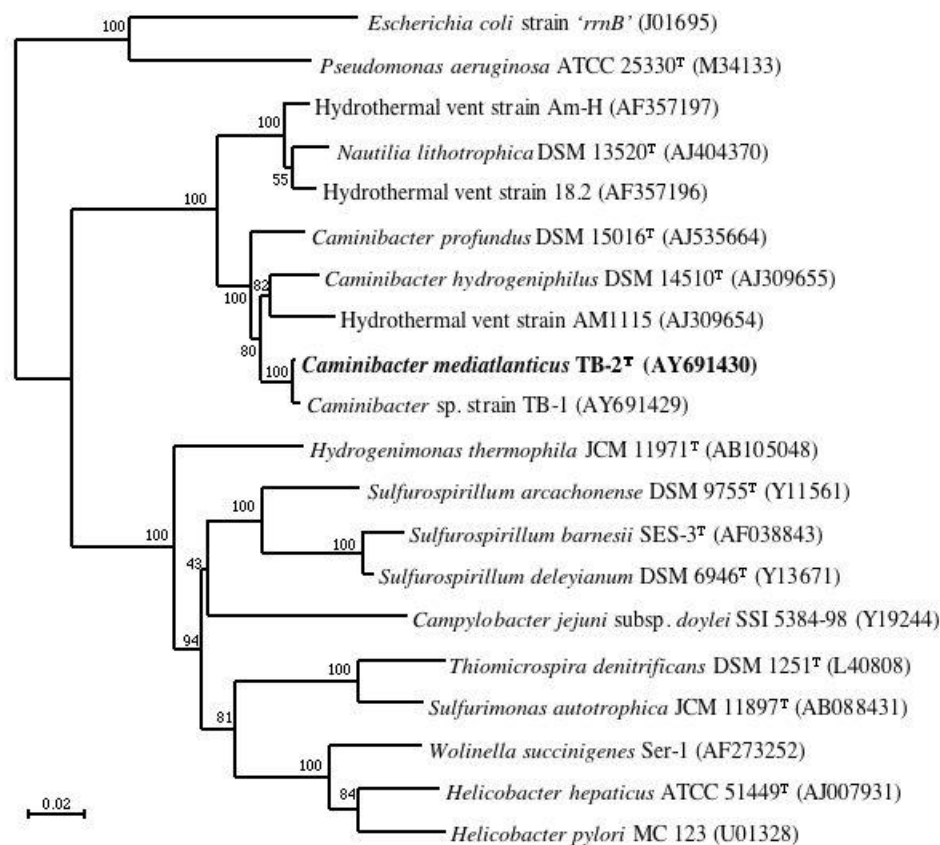


Figure 3.3 Phylogenetic position of *Caminibacter mediatlanticus* (strain TB-2^T). The neighbor-joining tree was constructed using Phylo_Win. Bar, 2% estimated base substitutions.

Strain TB-2^T could be assigned to the genus *Caminibacter*, although this organism could be differentiated from the previously described *Caminibacter* species by several physiological characteristics (Table 3.1). The G+C content of TB-2^T (25.6 mol%) was lower than that of either *Caminibacter hydrogenophilus* (29±1 mol%) or *Caminibacter profundus* (32.1 mol%) (Alain et al. 2002; Miroshnichenko et al. 2004). TB-2^T could be distinguished from *Caminibacter hydrogenophilus* by a lower optimum temperature, higher optimum salinity and shorter generation time; it could be distinguished from *Caminibacter profundus* by a lower optimum pH, the inability to use

oxygen as an electron acceptor, a slightly longer generation time, and susceptibility to the antibiotic chloramphenicol (Table 3.1). Furthermore, DNA-DNA hybridization of strain TB-2^T and *Caminibacter profundus*, which were both isolated from a vent site on the Mid-Atlantic Ridge, showed a similarity of 35.7%, indicating that the two organisms were not related at the species level (Wayne et al. 1987). Both physiological and genetic analyses indicated that TB-2^T was a new species within the *Caminibacter* genus, for which we propose the name *Caminibacter mediatlanticus* (type strain, TB-2^T).

Table 3.1 Differentiating features of *C. hydrogenophilus*, *C. profundus*, and *C. mediatlanticus* TB-2^T.

Taxa: 1, *C. hydrogenophilus*; 2, *C. profundus*; 3, *C. mediatlanticus* TB-2^T

Feature	1	2	3
Optimal growth parameters			
Temperature	60°C	55°C	55°C
pH	5.5-6	6.9-7.1	5.5
Salinity	20-25 g l ⁻¹	30 g l ⁻¹	30 g l ⁻¹
Microaerophilic growth	-	Max. 2% (v/v) Opt. 0.5% (v/v)	-
Chloramphenicol resistance	-	+	-
DNA G+C content (mol%)	29±1 mol%	32.1 mol%	25.6 mol%
Generation time	1.5 hr	40 min	50 min

Respiratory nitrate ammonification is an energy-conserving pathway, widespread in mesophilic prokaryotes (e.g., *Escherichia coli*, *Wolinella succinogenes*, *Sulfurospirillum deleyianum*, *Desulfovibrio desulfuricans*; reviewed in (Potter et al. 2001; Simon 2002), in which nitrate is reduced to nitrite, which is subsequently reduced to

ammonium. Therefore, compared to denitrification, nitrate ammonification represents a “shortcut” in the biological nitrogen cycle. Because of the primary importance of the geothermally produced sulfur species at deep-sea hydrothermal vents, historically S^0 has been used as the primary electron acceptor in experimental strategies used for the isolation of thermophilic organisms (Baross and Deming 1995). In contrast, nitrate is depleted in hydrothermal fluids (but available in seawater), and only recently its role as a terminal electron acceptor in anaerobic respiration of thermophilic organisms has been established in more detail only recently (Huber et al. 1996; Blochl et al. 1997; Alain et al. 2002; Huber et al. 2002; Alain et al. 2003; Miroshnichenko et al. 2003; Miroshnichenko et al. 2004; Vetriani et al. 2004). These studies revealed that, along with sulfur reduction, the lithotrophic reduction of nitrate to ammonium is a bioenergetic pathway found in several thermophiles, including the hyperthermophilic archaeon *Pyrolobus fumarii* (Blochl et al. 1997) and several, phylogenetically diverse, thermophilic bacteria. These bacteria include *Thermovibrio ruber*, *Thermovibrio ammonificans*, *Desulfurobacterium crinifex* (class Aquificae) (Huber et al. 2002; Alain et al. 2003; Vetriani et al. 2004), *Caminibacter hydrogeniphilus*, *Caminibacter profundus*, and *Caminibacter mediatlanticus* (class Epsilonproteobacteria) (Alain et al. 2002; Miroshnichenko et al. 2004), *Cadithrix abyssi* (novel bacterial lineage) (Miroshnichenko et al. 2003), and *Ammonifex degensii* (class Clostridia) (Huber et al. 1996). Most of these thermophilic, nitrate-ammonifying organisms are also capable of autotrophic carbon dioxide fixation. In light of the widespread distribution, importance, and physiological characteristic of thermophilic Epsilonproteobacteria in deep-sea geothermal environments, it is likely that

these organisms provide a relevant contribution to both primary productivity and the biogeochemical cycling of carbon, nitrogen and sulfur at hydrothermal vents.

Description of *Caminibacter mediatlanticus* sp. nov.

Caminibacter mediatlanticus (me.di.at.lan'ti.cus. L. adj. *medius* middle; L. adj. *atlanticus* Atlantic; N.L. masc. Adj. *mediatlanticus* middle Atlantic).

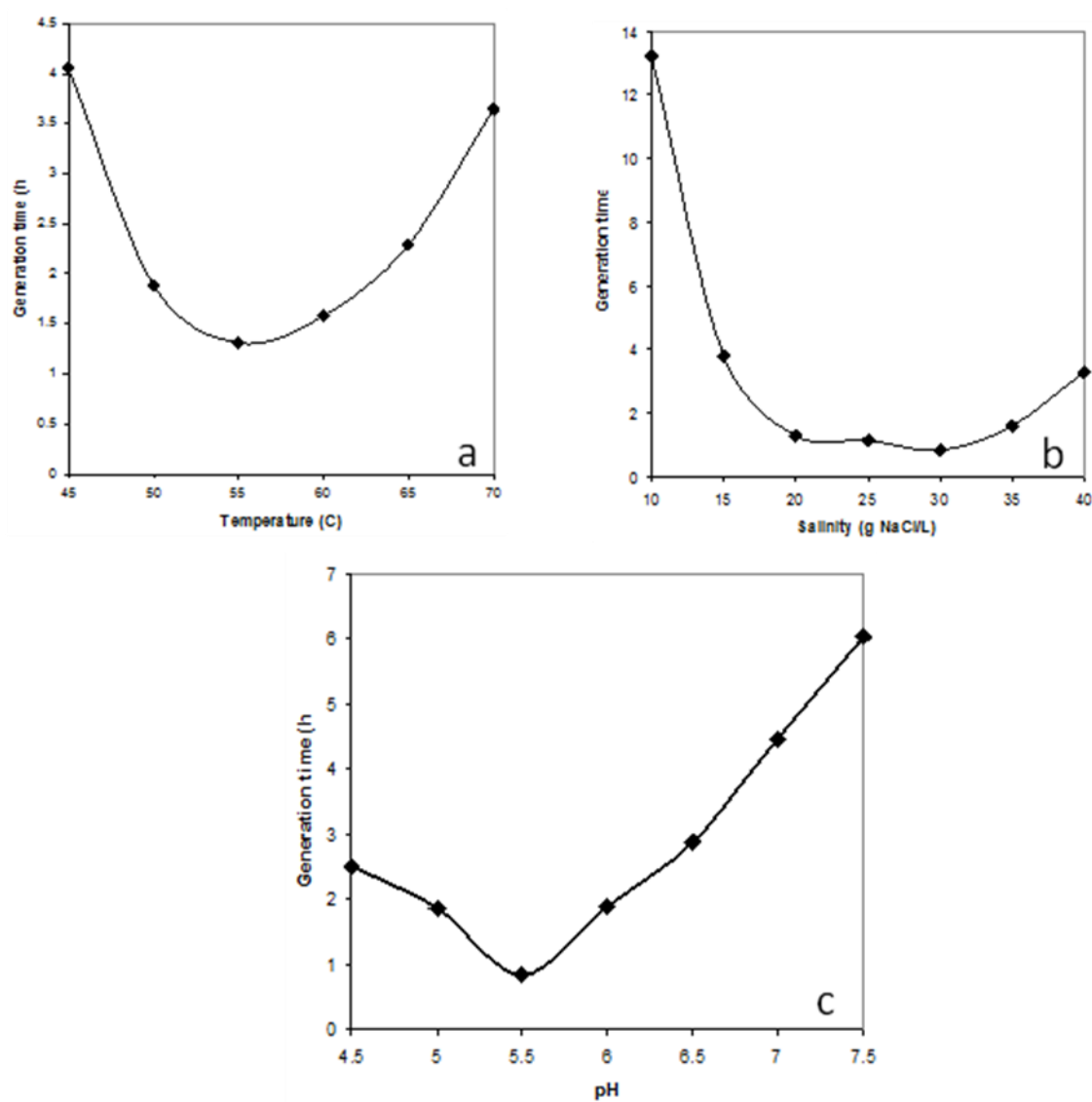
Cells are Gram-negative rods approximately 1.5 μm in length and 0.75 μm in width. Growth occurs between 45 and 70°C, 10 and 40 g l⁻¹ NaCl, and pH 4.5 and 7.5. Optimal growth conditions are 55°C, 30 g l⁻¹ NaCl, and pH 5.5 (generation time 50 minutes). Growth occurs under strictly anaerobic, chemolithotrophic conditions in the presence of H₂ and CO₂ with nitrate or sulfur as electron acceptors and the formation of ammonia or hydrogen sulfide respectively. Oxygen, selenate, arsenate, thiosulfate, or sulfite were not utilized as electron acceptors. Acetate, lactate, formate, and peptone inhibit growth. No chemoorganoheterotrophic growth occurs on tryptone, Casamino acids, yeast extract (0.1 g l⁻¹), sucrose, or glucose. Sensitive to chloramphenicol, ampicillin and streptomycin, but resistant to kanamycin (each at 100 mg ml⁻¹). Genomic DNA G+C content is 25.6 mol%.

The type strain is TB-2^T (= DSM 16658^T = JCM 12641^T), which was isolated from the walls of an active deep-sea hydrothermal vent on the Mid-Atlantic Ridge at 36° 14'N, 33° 54'W.

ADDENDUM

Utilization of nitrite and sulfate as an electron acceptor with hydrogen as the electron donor was not determined.

Supplementary Material 3.1 Generation time (in h) of strain TB-2^T under different growth conditions. (a) Temperature; (b) NaCl concentration; (c) pH.



Chapter 4 - Detection of the periplasmic nitrate reductase (NapA) in chemolithoautotrophic *Epsilonproteobacteria* and in deep-sea hydrothermal vent microbial communities

Submitted to the journal Applied and Environmental Microbiology

Introduction

Because of the primary importance of the geothermally produced sulfur species at deep-sea hydrothermal vents, the contribution of vent microorganisms to the sulfur cycle has been extensively investigated (reviewed in (Jannasch 1995; Karl 1995; Reysenbach et al. 2002)). The vast majority of hyperthermophiles, excluding the methanogens, take advantage of electron transfer among sulfur-bearing compounds. For example, the reduction of elemental sulfur to sulfide is prevalent among the thermo- and hyperthermophiles and, until recently, this was believed to be the sole energy-yielding process in numerous thermophilic, autotrophic organisms. However, the fact that sulfur appears as the most frequently required terminal electron acceptor used by anaerobic thermophiles might reflect a bias in the most common experimental strategies used in past for the isolation of these organisms, which usually included sulfur as the main tested electron acceptor (Baross and Deming 1995). Recently, the isolation and characterization of a number of novel microorganisms from deep-sea hydrothermal vents, for the most part chemolithoautotrophs, revealed that most of them can use nitrate in addition to sulfur as their terminal electron acceptors (the end product of nitrate reduction being either

nitrite, dinitrogen or ammonium) (Nakagawa et al. 2005; Campbell et al. 2006; Miroshnichenko and Bonch-Osmolovskaya 2006). In most cases these bacteria reduce nitrate at the expense of molecular hydrogen (and less frequently of thiosulfate), and some of these organisms have a shorter doubling time when nitrate, rather than sulfur, is supplemented as a terminal electron acceptor (Vetriani et al. 2004; Voordeckers et al. 2005). Many (but not all) of these recently isolated chemolithotrophic, nitrate-reducing organisms belong in the *Epsilonproteobacteria*, and their wide distribution throughout the world's oceans has been confirmed by culture-independent analyses, which revealed the occurrence of *Epsilonproteobacteria* in geographically dispersed deep-sea vent sites (reviewed in (Campbell et al. 2006). Furthermore, experiments that were carried out using various types of colonization substrates that were deployed in the vicinity of active deep-sea vents, revealed that between 66 to 98% of the microorganisms associated with these substrates belonged to the *Epsilonproteobacteria* (Lopez-Garcia et al. 2003; Takai et al. 2003; Alain et al. 2004). Overall these observations indicate that *Epsilonproteobacteria* represent a large fraction of the primary producers at deep-sea hydrothermal vents, and imply that the use of nitrate as a terminal electron acceptor in these environments may be more common than previously thought.

Data on the nitrogen chemistry of hydrothermal fluids indicate a depletion of both nitrate and nitrite in vent fluids, while ammonium has been found in appreciable quantities only in the end member fluids of vents from the Guaymas Basin and from both the Endeavour and the Explorer segments of the Juan de Fuca Ridge (Tunnicliffe et al. 1986; Karl et al. 1988; Lilley et al. 1993; Von Damm 1995). At these sites, the high concentration of ammonium has been attributed to the decomposition of sub-seafloor

organic matter associated with buried sediments, suggesting that ammonium is not a direct product of geothermal processes (i.e., seawater/rock interactions at elevated temperatures) (Edmond and Von Damm 1985; Lilley et al. 1993). Nitrate, however, is present in the bottom seawater (Millero 1996), and it is therefore available to be used by bacteria both as an electron acceptor and as a nitrogen source.

Prokaryotes are able to synthesize three distinct types of nitrate reductases. All three enzymes reduce nitrate to nitrite, but they are involved in different physiological processes (reviewed in Lin and Stewart 1998; Moreno-Vivian et al. 1999; Potter et al. 2001; Richardson et al. 2001). Figure 4.1 shows the enzymes involved in respiratory and assimilatory nitrate ammonification and denitrification. The NAS enzyme system is located in the cytoplasm and participates in nitrogen assimilation, while the other two nitrate reductases (NAR and NAP) are both linked to respiratory electron transport systems. The NAR enzyme is a three-subunit complex firmly attached to the cytoplasmic side of the membrane. The NAP enzyme is a two-subunit complex located in the periplasmic space of Gram-negative bacteria. All three types of nitrate reductases contain molybdenum bound to the bis-molybdopterin guanine dinucleotide cofactor at the active site. However, while the assimilatory enzyme (NAS) uses NAD(P)H or ferredoxin as reductant, the respiratory NAR and NAP enzymes ultimately take electrons from the membranous quinol pool. Several microorganisms, including *Escherichia coli*, encode for more than one type of nitrate reductase.

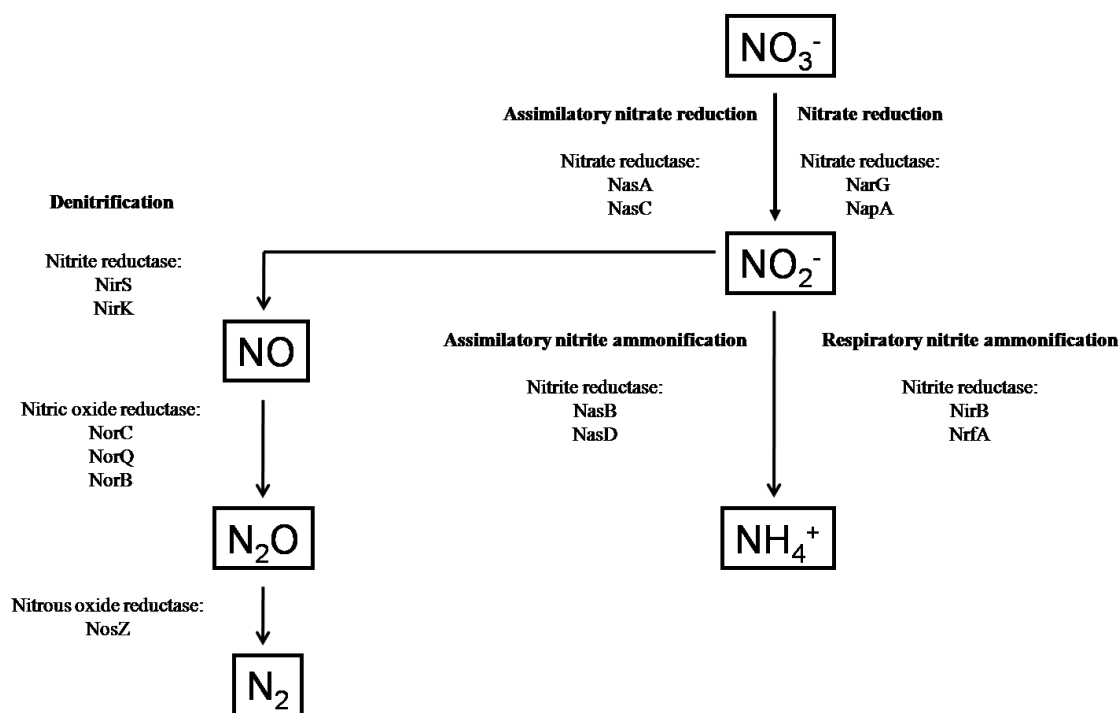


Figure 4.1 Genes involved in nitrate reduction.

The periplasmic NAP enzyme (encoded by the *napA* gene) has been described in several different organisms, and its physiological functions may vary in different bacteria (Moreno-Vivian et al. 1999; Potter et al. 2001; Richardson et al. 2001). For instance, in *Paracoccus* and *Rhodobacter* spp., NAP has a dissimilatory function to maintain redox balance during growth on highly reduced carbon sources (Richardson et al. 2001). In contrast, the NAP enzymes in some enteric and rumen bacteria, such as *E. coli* and *Wolinella succinogenes*, are expressed during anaerobic growth and catalyze the first step in the respiratory reduction of nitrate to ammonium (Grove et al. 1996; Simon et al. 2003). Within the *Epsilonproteobacteria*, the NAP enzyme has been detected in the genomes of the rumen bacterium *W. succinogenes* (Simon et al. 2003), the pathogens *Campylobacter jejuni* (Parkhill et al. 2000) and *Helicobacter hepaticus* (Suerbaum et al. 2003), and the nitrate *Sulfurimonas* (*Thiomicrospira*) *denitrificans* (GenBank accession

number: CP000153). The genes for NAP, but not NAR, are found within the genomes of these *Epsilonproteobacteria* indicating that the NAP system is utilized for respiratory nitrate reduction (Simon et al. 2000). However, to our knowledge the genes encoding for the nitrate reductase in *Epsilonproteobacteria* from deep-sea hydrothermal vents have not yet been identified.

In this study we have established for the first time that reference strains of *Epsilonproteobacteria* isolated from different deep-sea hydrothermal vents encode and express a periplasmic nitrate reductase (NAP enzyme), and we have investigated the phylogeny of this enzyme. Furthermore, we have assessed for the first time the diversity, distribution and phylogeny of the NAP enzyme in natural microbial communities from different vent microhabitats. Our results show an unprecedented diversity of the periplasmic nitrate reductase in deep-sea vent microbial communities, and provide insight on alternative energy metabolism in vent microorganisms.

Materials and Methods

Sample collection. Three types of samples were collected for this study using the DSV *Alvin*: 1) Fragments of active, high temperature black smoker chimneys were collected from the “Rainbow” (36° 14’N, 33° 54’W; depth 2305) and “Logatchev” (14° 45’N, 44° 58’W; depth 3000 m) vent fields on the Mid-Atlantic Ridge (MAR), during a cruise aboard R/V *Atlantis* (cruise AT 05-03, July 2001); 2) Hydrothermal fluids, which were collected in titanium samplers from two diffuse flow vents located at East Wall and at *Alvinella* Pillar, respectively, on the East Pacific Rise (EPR; 9° 10’N, 104° 17’W; depth 2500 m) during cruise AT 11-26 (May 2005), and which were filtered on 0.2 µm Supor

Gelman filters (Ann Arbor, MI); 3) Bacterial filaments, which were attached to a cage made of Vexar plastic mesh that had been deployed above a diffuse flow vent located at Marker 89 on the EPR for one year. All samples were collected using the manipulator of the DSV *Alvin* and stored on the submersible's working platform for the rest of the dive. On the surface, samples were transferred to the ship's laboratory, and subsamples were either frozen at -80°C for nucleic acid extraction, or stored at 4°C under a dinitrogen atmosphere for enrichments and isolations. Characteristics of samples and sampling locations are summarized in Table 4.1.

Reference strains. The *Epsilonproteobacteria* used as reference strains in this study were: *Caminibacter mediatlanticus* DSM 16658 (Voordeckers et al. 2005), *Caminibacter* sp. strain TB-1 (Voordeckers et al. 2005), *Nautilia* spp. strains MT-3, MT-4, and MT-5 (Chapter 1), *Caminibacter hydrogeniphilus* DSM 14510 (Alain et al. 2002), *Caminibacter profundus* DSM 15016 (Miroshnichenko et al. 2004), *Hydrogenimonas thermophila* JCM 11971 (Takai et al. 2004), *Thioreductor micantisoli* DSM 16661 (Nakagawa et al. 2005), *Sulfurimonas paralvinellae* DSM 17229 (Takai et al. 2006), and *Sulfurovum lithotrophicum* JCM 12117 (Inagaki et al. 2004). The reference strains were cultured according to the conditions described in the original references. The sequences for the 16S rRNA genes of the reference strains were downloaded from GenBank. The metabolic characteristics of the reference strains used in this study are summarized in Table 4.2.

Table 4.1 Locations of sampling stations and clonal libraries constructed in this study.

Vent	Cruise/Dive #	Latitude	Longitude	Depth (m)	Sample type; Temperature	<i>mgpA</i> library
Rainbow, MAR	R/V <i>Alionis</i> Cruise AT 05-03 DSV <i>Alvin</i> Dive 3678	36°N14'	33°W54'	2305	Seafloor; 158°C*	MAR seafloor
Logatchev, MAR	R/V <i>Alionis</i> Cruise AT 05-03 DSV <i>Alvin</i> Dive 3667- 3668 (Jrma2 vent)	14°N45'	48°W58'	3000	Seafloor; 348°C*	ND ^b
East Wall, EPR	R/V <i>Alionis</i> Cruise AT 11-26 DSV <i>Alvin</i> Dive 4109	9°N50'	104°W17'	2500	Fluids; 25°C	EPR EW fluid
<i>Abyssella</i> Pillar, EPR	R/V <i>Alionis</i> Cruise AT 11-26 DSV <i>Alvin</i> Dive 4112	9°N50'	104°W17'	2500	Fluids; 40°C	EPR AP fluid
MA 89, EPR	R/V <i>Alionis</i> Cruise AT 11-26 DSV <i>Alvin</i> Dive 4102	9°N50'	104°W17'	2500	Bacterial filaments on Ventur plastic mesh; 25°C	EPR white filament

* Temperature of the hydrothermal fluid measured in-situ at the chimney orifice

^b The *mgpA* gene was not detected in the Logatchev microbial community

Table 4.2. Metabolic characteristics of *Environirobacter* used as reference strains in this study.

Reference Organism	Isolation Site ^a	Temperature ^b (°C)	End Product of Nitrate Respiration	H ₂				S ₂ O ₃ ²⁻	
				NO ₃	S ⁰	O ₂	NO ₂	NO ₃	NO ₂
<i>Shigella sonnei</i>	MOT, Dapn, sediments	28-30	N ₂	-	-	-	-	+	+
<i>Shigella sonnei</i>	MOT, Dapn, <i>Paralimnula</i> polychaetes	30	N ₂	+	-	+	+	+	+
<i>Thiobacter autotrophicus</i>	MOT, Dapn, sediments	32	NH ₄ ⁺	+	+	-	-	-	-
<i>Nautella</i> spp. MT3, MT4, MT5	EPR, 9°N, chimney ^c	42-50	NH ₄ ⁺	+	ND ^d	ND	ND	ND	ND
<i>Hydrogenomonas thermophila</i>	CIR, Kuroi Field, microcolony	35	NH ₄ ⁺	+	+	+	+	-	-
<i>Camithobacter profundus</i>	NAR, Rainbow, vent cap	35	NH ₄ ⁺	+	+	+	+	-	-
<i>Camithobacter medianticus</i>	NAR, Rainbow, chimney	35	NH ₄ ⁺	+	+	-	-	ND	ND
<i>Camithobacter</i> sp. TB1	NAR, Rainbow, chimney	50	NH ₄ ⁺	+	+	-	-	ND	ND
<i>Camithobacter hydrogenophilus</i>	EPR, 13°N, <i>Alvinella</i> <i>pumeyana</i> tubes	60	NH ₄ ⁺	+	+	-	-	-	-

^a MOT: Mid-Atlantic Trough; EPR: East Pacific Rise; CIR: Central Indian Ridge; NAR: Mid-Atlantic Ridge.^b Optimum growth temperatures are indicated for all strains, with the exception of *Nautella* spp.^c Experiment not done.

DNA extraction. Total genomic DNA was extracted from: 1) About 2 g each of the Rainbow and Logatchev chimneys using the UltraClean Soil DNA extraction kit, according to the manufacturer's specifications (MoBio Laboratories, Solana Beach, CA, USA); 2) Biomass collected on filters from about 2.4 liters of hydrothermal fluids from East Wall and *Alvinella* Pillar, as described in Vetriani et al. (2003); 3) About 1 g of bacterial filaments, and cells recovered from 10 ml of a culture of each reference strain, using the UltraClean Microbial DNA Isolation Kit according to the protocol supplied with the kit (MoBio Laboratories, Solana Beach, CA, USA).

DNA amplification by Polymerase Chain Reaction (PCR). A fragment of about 1,150 base pairs of the *napA* gene, encoding for the periplasmic nitrate reductase, was amplified from the genomic DNA of each of the reference strains (Table 4.2), and from the total genomic DNA extracted from four of the five vent samples (Table 4.1). No PCR product for *napA* was obtained from the Logatchev chimney (Table 4.1). PCR was carried out using primers NapV16F (5'-GCNCCNTGYMGNTTYTGYGG-3') and NapV17R (5'-RTGYTGRTTRAANCCCATNGTCCA-3'), which were designed based on the *napA* sequence of *Paracoccus pantotrophus*, *Ralstonia eutropha* and *Escherichia coli* (Flanagan et al. 1999). PCR conditions for amplification reactions were as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, with a final extension time of 10 min during the last cycle.

RNA extraction and qualitative detection of *napA* transcripts. Total RNA was extracted from 20 ml of cultures of *C. mediatlanticus* and *C. profundus* grown in modified SME medium supplemented with either potassium nitrate or elemental sulfur

(Voordeckers et al. 2005), using the UltraClean Microbial RNA Isolation Kit according to the protocol supplied with the kit (MoBio Laboratories, Solana Beach, CA, USA). Six μ l of RNA were incubated for 15 min at room temperature with DNase I (Invitrogen, Inc., Carlsbad, CA, USA), followed by incubation for 10 min at 65°C with 25 mM EDTA. The total RNA was then used as a template for the qualitative detection of *napA* transcripts by Reverse Transcription PCR (RT-PCR), using the SuperScript III One-Step RT-PCR System with primers NapV16F and NapV17R according to the manufacturer's specifications (Invitrogen, Inc., Carlsbad, CA, USA).

Library construction, Restriction Fragment Length Polymorphism screening and sequence. The amplified *napA* gene fragments were gel-purified using the QIAGEN Gel Spin purification kit (Qiagen, Santa Clarita, CA, USA), cloned into the pCR4-TOPO plasmid vector, and the ligation products were transformed into competent *E. coli* Oneshot cells (Invitrogen, Inc., Carlsbad, CA, USA). Four *napA* environmental libraries were constructed: the MAR sulfide library from the Rainbow chimney, the EPR EW fluid and the EPR AP fluid libraries from the East Wall and *Alvinella* Pillar diffuse flow vents on the EPR, respectively, and the EPR white filament library from the bacterial filaments at Mk 89 on the EPR (Table 4.1). A total of 195 randomly chosen clones were analyzed for insert-containing plasmids by direct PCR followed by gel electrophoresis of the amplified products. One hundred and eighty nine *napA* clones were screened by Restriction Fragment Length Polymorphism (RFLP) analysis using the *MnII* restriction endonuclease. Representative clones for each library showing unique RFLP patterns were selected and their sequences (about 1,000 nucleotides) was determined for both strands on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA).

Phylogenetic Analyses. Sequences were assembled using the AutoAssembler Program (Applied Biosystems, Foster City, CA) and they were translated using the online tool EMBOSS Transeq (<http://www.ebi.ac.uk/emboss/transeq/>). The amino acid sequences were aligned with ClustalX v 1.8 (Thompson et al. 1997) and manually adjusted using Seaview (Galtier et al. 1996). Phylogenetic distances were calculated using the Observed Divergence matrix, and the neighbor joining method was used to evaluate tree topologies. Phylo_win was utilized to plot tree topologies (Galtier et al. 1996) and their robustness was tested by bootstrap analysis with 1,000 resamplings. The 16S rRNA gene sequences from the reference strains were aligned using ClustalX v 1.8 (Thompson et al. 1997) and manually adjusted using Seaview (Galtier et al. 1996). Phylogenetic distances were calculated using the Jukes-Cantor model and the neighbor joining method was used to evaluate tree topologies. Phylo_win was utilized to plot tree topologies (Galtier et al. 1996) and their robustness was tested by bootstrap analysis with 1,000 resamplings.

Nucleotide sequence accession numbers. The sequences from this study are available through GenBank under the following accession numbers: EF644686 to EF644758, EF644772 to 644780, EF644815 to EF644826, and EF683089 to EF683090.

Results

Detection and phylogenetic analysis of the NapA periplasmic nitrate reductase in vent *Epsilonproteobacteria*. A 1,150 base pair fragment of the *napA* gene, encoding for the periplasmic nitrate reductase, was successfully amplified from all the reference strains used in this study. Attempts to amplify different sized fragments of the *narG* gene (encoding for the cytoplasmic nitrate reductase) from *C. mediatlanticus* were not

successful (data not shown). More recently, the genome sequence of *C. mediatlanticus* was completed, and confirmed that this bacterium encodes only for the periplasmic nitrate reductase (data not shown). Phylogenetic analysis of the amino acid sequence deduced from the *napA* genes placed the enzymes from all the *Epsilonproteobacteria* in a group distinct from the gammaproteobacterial NapA (Figure 4.2). In particular, the four *Caminibacter* spp. were placed into a single group, within which the enzymes from *C. mediatlanticus* and strain TB-1 (98% sequence identity) formed a subgroup separated from those of *C. hydrogeniphilus* and *C. profundus* (93% sequence identity; Figure 4.2). The sequence identity between the enzymes from the two subgroups ranged between 84 and 87%. The NapA from the three *Nautilia* spp. was embedded in the *Caminibacter* group, and formed a subgroup closely related to the enzymes from *C. hydrogeniphilus* and *C. profundus* (87-88% sequence identity). In contrast with the NapA phylogeny, 16S rRNA gene based phylogenetic analysis placed the *Nautilia* spp. in a unique group, closely related but separated from the four *Caminibacter* spp. (Figure 4.2). The NapA from *Thioreductor micantisoli* was placed in an independent lineage related to the *Caminibacter/Nautilia* group (77% identity to the NapA from *C. mediatlanticus*), while the three sequences from *Helicobacter hepaticus*, *Wolinella succinogenes* and *Campylobacter jejuni* formed a group on their own (the NapA from *W. succinogenes* was 72% identical to that of *C. mediatlanticus*). The NapA of *Sulfurimonas paralvinellae* was more closely related to the enzyme of *Sulfurovum lithotrophicum* (84% identity) than to that of *Sulfurimonas denitrificans* (81% identity; Figure 4.2). Again, this is contrast with the 16S rRNA gene based phylogeny, which placed *Sulfurovum* on a separate lineage from *Sulfurimonas* spp. (Figure 4.2) (Takai et al. 2006). Finally, the NapA of

Hydrogenimonas thermophila formed an independent lineage, and its deduced amino acid sequence was 70% identical to that of *C. mediatlanticus* and 68% identical to those of *Sulfurospirillum barnesii* and *T. micantisoli* (Figure 4.2).



Figure 4.2 Comparison of Neighbour-joining phylogenetic analysis of the amino acid sequence deduced from *napA* (left) and 16S rRNA gene (right) of reference strains. The scale bar is 2 substitutions per 100 sequence positions.

Qualitative detection of *napA* transcripts in *Caminibacter* spp. Total RNA was extracted from cultures of *C. mediatlanticus* and *C. profundus*, which were grown anaerobically at their optimal growth temperatures (Table 4.2) under a CO₂/H₂ atmosphere (Figure 4.3a). When the RNA extracted from cultures of the two species grown in the presence of nitrate as their sole terminal electron acceptor was subjected to RT-PCR with *napA* specific primers, *napA* transcripts were detected in both cases (Figure 4.3b, lanes 3 and 4). A control where the PCR was carried out in the absence of a reverse transcription step did not generate a band (Figure 4.3b, lane 2). When the two cultures were repeatedly grown in the presence of elemental sulfur as their sole terminal electron acceptor, faint signals for the *napA* transcript were also detected, indicating that the periplasmic nitrate reductase may be constitutively expressed at a basal level (data not shown).

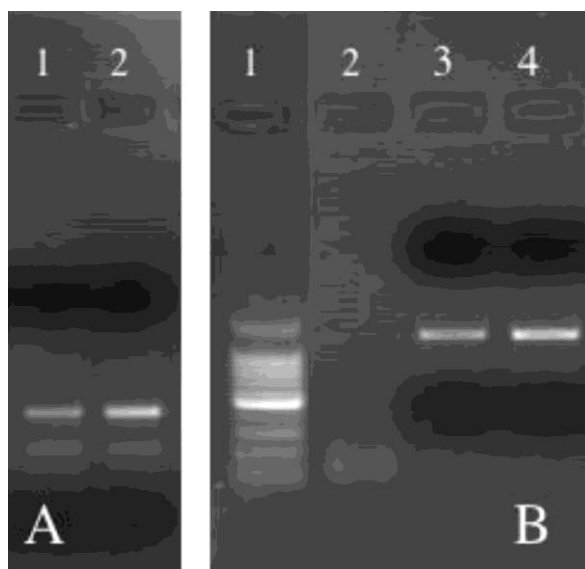


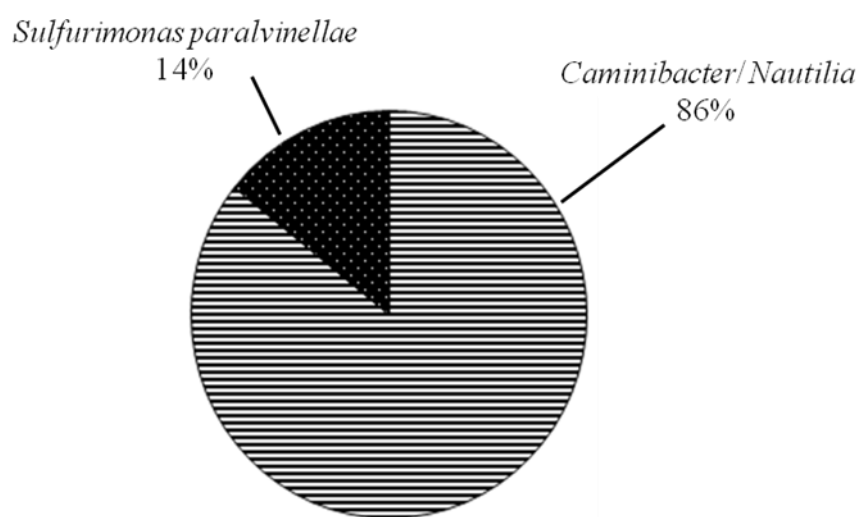
Figure 4.3 (a) Total RNA from nitrate-reducing *Caminibacter* cultures. Lane 1: *C. mediatlanticus*.; lane 2: *C. profundus*. (b) Detection of *napA* transcripts by RT-PCR. Lane 1: 100 bp ladder; lane 2: negative control (no RT); lane 3: *napA* reverse transcript from *C. mediatlanticus*; lane 4: *napA* reverse transcript from *C. profundus*.

Diversity and phylogenetic analysis of NapA nitrate reductase in vent microbial

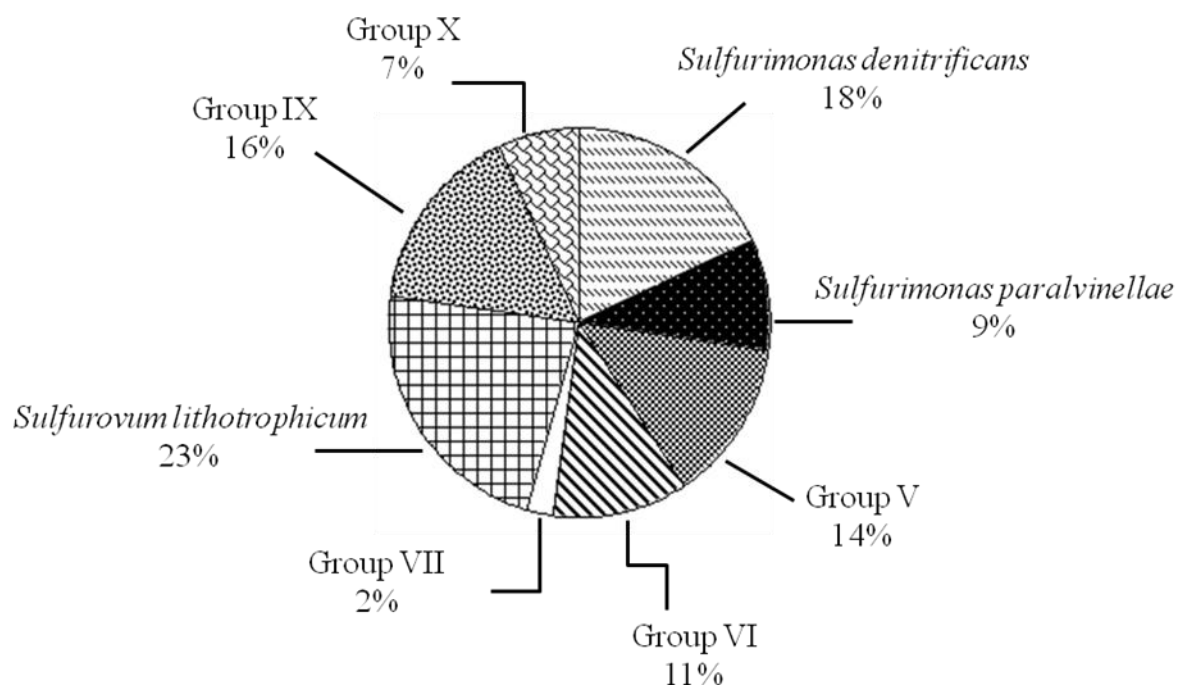
communities. Genomic DNA was successfully extracted from five samples, representing different vent microhabitats along a temperature gradient: two active, high temperature sulfides from the Rainbow (MAR sulfide) and Logatchev vent fields on the MAR, hydrothermal fluids from two sites on the EPR, East Wall (25°C; EPR EW fluid) and *Alvinella* Pillar (40°C; EPR AP fluid), and bacterial filaments attached to an artificial substrate (ambient temperature, about 2°C; EPR white filaments; Table 4.1). A 1,150 base pairs fragment of the *napA* gene was successfully amplified from four out of five samples, as no PCR product for *napA* was obtained from the Logatchev chimney (Table 4.1). All the *napA* gene fragments retrieved from the vent natural microbial communities investigated in this study were related to those of the *Epsilonproteobacteria*, and a total of fifteen NapA groups were identified based on phylogenetic clustering (Figures 4.4a-d and 5). Out of these fifteen groups, only four included environmental NapA sequences that were related to the enzymes from cultured organisms (Figure 4.5).

Eighty-six percent of the MAR sulfide NapA sequences were related to the enzymes from *Caminibacter* and *Nautilia* spp. (the amino acid sequence identity of these clones to the NapA of *C. mediatlanticus* ranged from 86 to 96%), while the remaining 14% of the sequences from this site was related to the enzyme from *S. paralvinellae* (84% average identity to the NapA of *S. paralvinellae*; Figures 4.4a and 4.5).

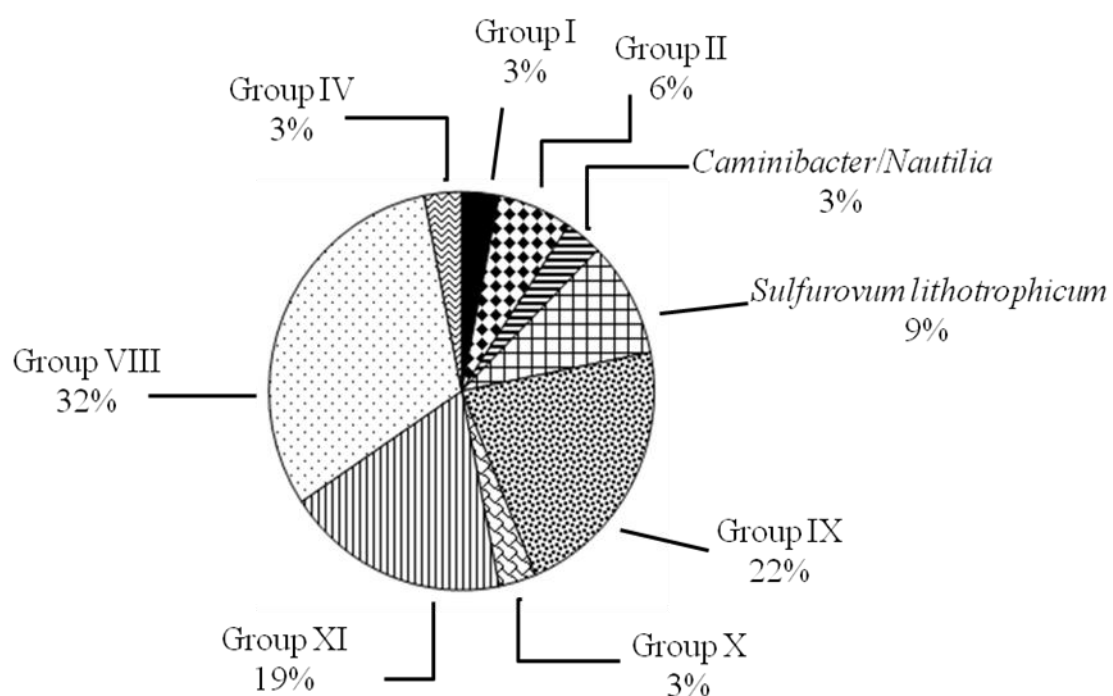
(4a)



(4b)



(4c)



(4d)

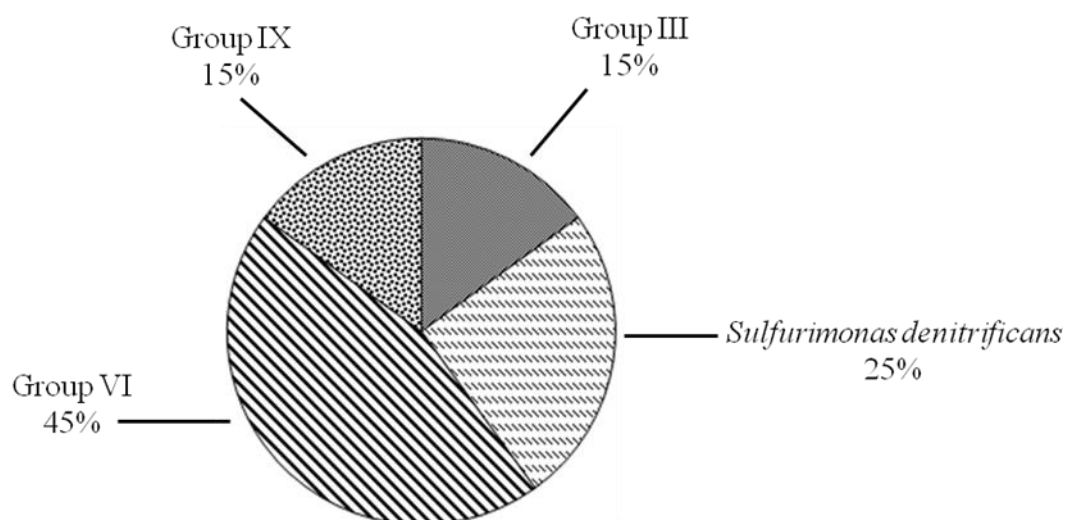


Figure 4.4 Frequency of NapA clones from the Rainbow black smoker (MAR) (a), East Wall fluids (25°C, EPR) (b), Alvinella Pillar (40 °C, EPR) (c), and white filaments (EPR) (d).

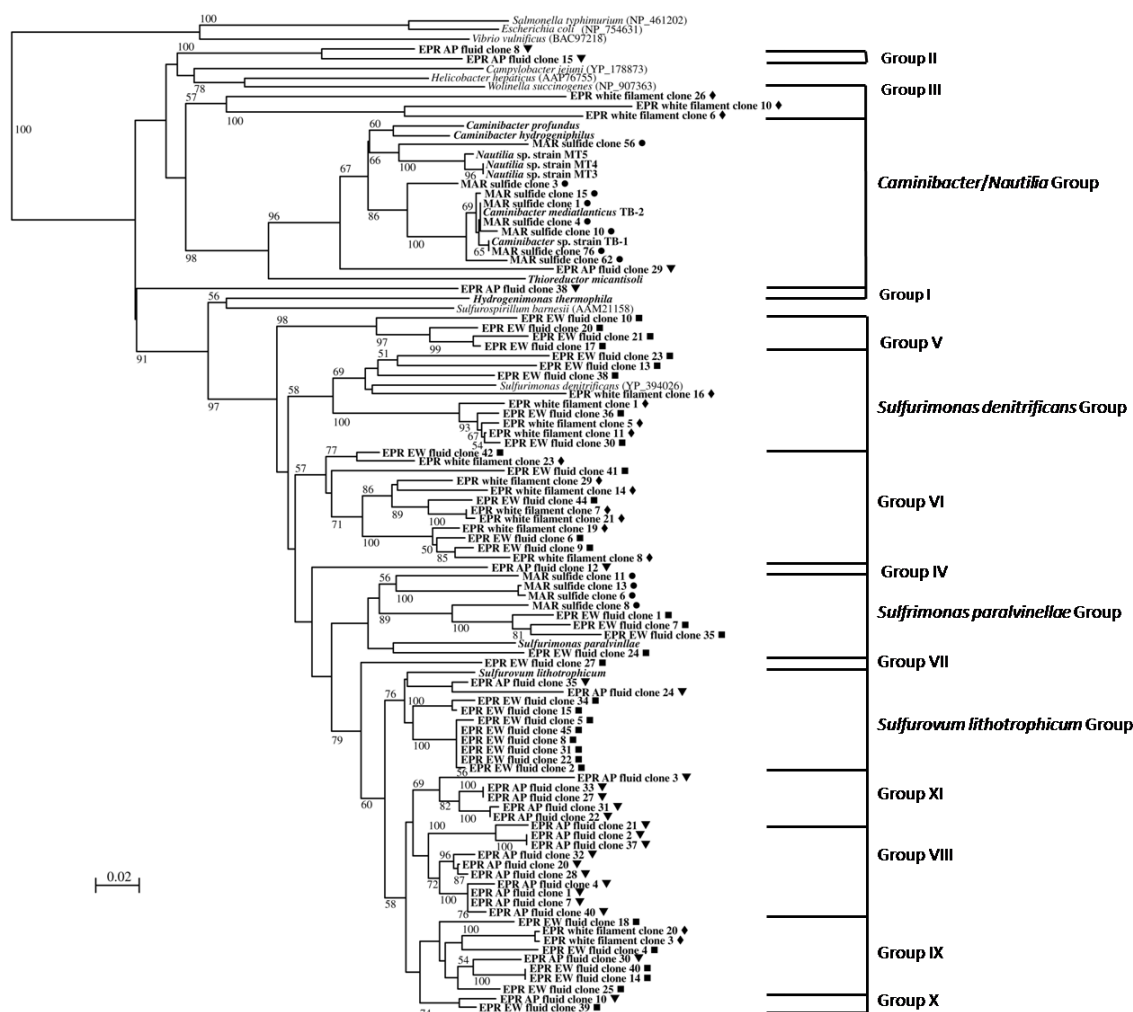


Figure 4.5 Phylogenetic tree of *napA* sequences from Rainbow black smoker (MAR sulfide), East Wall fluids (EPR EW fluid), Alvinella Pillar fluids (EPR AP fluid), and white filaments (EPR white filament). The tree was constructed using the neighbor-joining method from a similarity matrix based on the Observed Divergence model. Percentages greater than 50% of bootstrap resampling (of 1000 resamplings) that support each topological element are indicated near the nodes. Representative sequences of rRNA genes obtained in this study are in bold. The scale bar is 2 substitutions per 100 sequence positions.

The *napA* library from the EPR EW fluid was much more diverse than that from the MAR sulfide. A total of eight NapA groups were identified in the EPR EW fluids: the dominant groups included lineages related to the *Sulfurovum lithotrophicum* (23% of the clones; 90% average sequence identity to the *S. lithotrophicum* NapA) and to the *Sulfurimonas denitrificans* enzymes (18% of the clones; 82% average sequence identity

to the *S. denitrificans* NapA), while 9% of the clones were related to the *Sulfurimonas paralvinellae* enzyme (89% average sequence identity to the *S. paralvinellae* NapA; Figures 4.4b and 4.5). Phylogenetic analysis placed the remaining clones into five groups that did not have close cultured relatives. The average sequence identity of these groups (V, VI, VII, IX and X) to the *S. denitrificans*, *S. paralvinellae* and *S. lithotrophicum* enzymes ranged between 78 and 83%. Groups V and VII were unique to the EPR EW fluid (compare Figures 4. 4a-d).

The *napA* library from the EPR AP fluid included nine groups: the most frequently represented groups were VIII (32% of the clones; 78 and 86% sequence identity to the *S. paralvinellae* and *S. lithotrophicum* NapA, respectively), IX (22% of the clones) and XI (19% of the clones; 82 and 87% sequence identity to the *S. paralvinellae* and *S. lithotrophicum* NapA, respectively), while groups I, II, IV and X were less represented (Figure 4.4c). Groups I, II, IV and VIII were only detected in the EPR AP fluid. In particular, groups I and II, represented by EPR AP fluid clones 38 and 15, respectively (Figure 4.5), formed two unique lineages distantly related to the nitrate reductase from all the other cultured *Epsilonproteobacteria* (65 and 70% average sequence identity to the enzymes from cultured *Epsilonproteobacteria*, respectively). For comparison, the average amino acid sequence identity of these two clones to the NapA from the *Gammaproteobacteria* *E. coli*, *S. typhimurium* and *V. vulnificus* was 54%. Finally, 9% of the NapA clones from the EPR AP fluid were related to the *S. lithotrophicum* enzyme (90 to 94% sequence identity), and 3% of the clones were related to the *Caminibacter/Nautilia* enzymes (81% identity to *C. mediatlanticus*).

The *napA* library from the EPR white filament community included four groups: group VI was the most represented of all groups, accounting for 45% of the clones, while 25% of the clones were related to the NapA from *S. denitrificans* (83% average sequence identity to the *S. denitrificans* enzyme; Figures 4.4d and 4.5). Of the remaining two groups represented in this library, group III was only detected within the white filament community. EPR white filament clone 10, representing group III, had an average sequence identity of 61 and 54% to the NapA from cultured *Epsilon*- and *Gammaproteobacteria*, respectively.

Caminibacter and *Nautilia*-related periplasmic nitrate reductase were detected only in the MAR sulfide and in the EPR AP fluid libraries, while *S. lithotrophicum*-related enzymes were detected in the EPR EW and AP fluid libraries, and NapA related to the *S. denitrificans* and the *S. parvalvinellae* enzymes were detected in the EPR EW fluid and white filament libraries (Figures 4. 4a-d). Group IX enzymes were highly represented in all three EPR libraries, but were not detected in the MAR sulfide library (Figures 4. 4a-d).

Analysis of the primary structure of the NapA molybdenum-binding site.

Comparative analysis of the primary structure of the NapA region adjacent to the molybdenum-binding site (indicated by an asterisk in Figure 4.6) revealed signature amino acid residues characteristic of the different groups of enzymes. All the epsilonproteobacterial enzymes had a highly conserved tyrosine residue, which was substituted by a tryptophan in the gammaproteobacterial enzymes (solid circle in Figure 4.6). Furthermore, all the *Caminibacter* and *Nautilia*-related NapA, and the enzyme from *T. micantisoli*, had a conserved tryptophan residue replacing the phenylalanine found in

all the other sequences (solid diamond in Figure 4.6). All the *Sulfurimonas* and *Sulfurovum*-related NapA sequences, and the enzyme from *H. thermophila*, had a conserved alanine residue replaced by an asparagine in all other sequences, the *S. barnesii* enzyme being the only exception with a glycine in that position (solid triangle in Figure 4.6). The same group of *Sulfurimonas* and *Sulfurovum*-related NapA sequences, and the enzymes from *C jejuni* and EPR filament clone 10 had a conserved alanine residue, which was replaced by a serine in all the remaining epsilonproteobacterial enzymes (except for the EPR AP fluid clone 38, which had a proline) and by a methionine in the *Gammaproteobacteria* (solid square in Figure 4.6).

Discussion

Preliminary evidence for horizontal gene transfer of the *napA* gene within the *Epsilonproteobacteria*. Comparative phylogenetic analyses of the 16S rRNA gene and of the periplasmic nitrate reductase from *Gamma*- and *Epsilonproteobacteria* showed an overall good complementarity between the two trees (Figure 4.2). However, minor incongruencies were detected within the epsilonproteobacterial lineage, suggesting that horizontal gene transfer events may have occurred within discrete lineages in this group of organisms. For instance, the 16S rRNA genes from the *Nautilia* strains formed a lineage separated from the *Caminibacter* sequences, while their NapA sequences were placed in a branch closely related to *C. profundus* and *C. hydrogeniphilus*, suggesting that the *Nautilia napA* gene may have been acquired from *Caminibacter* spp. (Figure 4.2). The phylogenetic placement of the *Sulfurimonas paralvinellae* nitrate reductase further suggested the possibility that horizontal gene transfer of this locus occurred within the *Epsilonproteobacteria*, as this sequence was closer to a member of a different genus, *Sulfurovum lithotrophum*, than to *Sulfurimonas denitrificans* (Figure 4.2). Since both *Si. paralvinellae* and *So. lithotrophum* were isolated from the same site, and since they share the same temperature niche (Table 4.2), it is possible that an event of horizontal gene transfer occurred between the two organisms.

This preliminary evidence for horizontal gene transfer of *napA* is consistent with the observation that this locus is located on plasmids in several organisms, including *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Ralstonia eutropha* and *Paracoccus denitrificans* (Moreno-Vivian et al. 1999). Overall these observations suggest that the periplasmic nitrate reductase is a potential good candidate for horizontal gene transfer.

However, comparative genetic analyses of the organization of the *nap* operon in different species of *Epsilonproteobacteria* is necessary to provide further evidence for the occurrence of horizontal gene transfer within this group of organisms.

Community structure of nitrate-reducing *Epsilonproteobacteria* in deep-sea

hydrothermal vents. The community structure of nitrate-reducing microorganisms was investigated by surveying the distribution of the periplasmic nitrate reductase in four vent microhabitats representing different temperature and redox conditions. All the *napA* genes retrieved from the vent samples were related to epsilonproteobacterial enzymes, which indicated that at deep-sea hydrothermal vents, *napA*-catalyzed respiratory nitrate reduction to either dinitrogen gas or to ammonium (Table 4.2) is mediated by chemolithoautotrophic *Epsilonproteobacteria*. In contrast, a survey of the *narG* gene from the MAR and EPR vent samples examined in this study revealed that the cytoplasmic nitrate reductase was predominantly encoded by *Alpha* and *Gammaproteobacteria* (Bohnert, Perez and Vetriani, unpublished results). Furthermore, two previously published surveys of the periplasmic nitrate reductase in natural microbial communities showed that *napA* genes retrieved from freshwater and estuarine sediments were for the most part related to *Alpha*- and *Gammaproteobacteria* (Flanagan et al. 1999; Smith et al. 2007).

In general, the communities of *napA*-encoding nitrate-reducers in the EPR EW and AP fluids samples (in-situ temperatures: 25 and 40°C, respectively) were more diverse than those inhabiting the MAR sulfide (temperature of the fluid at the chimney orifice: 158°C) and the EPR white filaments (in-situ temperature: 2°C; Figures 4.4a-d). In particular, the observation that the NapA recovered from the MAR sulfide community

were predominantly related to enzymes from *Caminibacter* and *Nautila* spp. (Figures 4.4a and 4.5) is in line with data obtained from 16S rRNA gene and ATP citrate lyase (an enzyme involved in CO₂ fixation via the reductive tricarboxylic acid cycle) libraries constructed from the same chimney sample, which revealed a prevalence of *Epsilonproteobacteria* of the genus *Caminibacter* associated with this vent (Chapter 2). The isolation of *C. mediantlanticus*, *Caminibacter* sp. strain TB-1 (Voordeckers et al. 2005) and *C. profundus* (Miroshnichenko et al. 2004) from the Rainbow hydrothermal vent further confirmed the dominance of these organisms at this site. Overall, the dominance of thermophilic members of the genus *Caminibacter* in the MAR sulfide sample suggests in-situ temperatures in the 45-65°C range within the walls of this chimney. Our inability to amplify the *napA* gene from the Logatchev sulfide may be related to the very low abundance of *Epsilonproteobacteria* in this sample (Chapter 2).

Analyses of the periplasmic nitrate reductase from the EPR EW and AP fluids suggests that the moderate temperatures (25-40°C) and mildly reducing conditions usually associated with diffuse flow vents are conducive to the establishment of a diverse community of *Epsilonproteobacteria*. In general, a correlation exists between temperature and redox state of hydrothermal fluids, in that higher temperature fluids tend to be more reduced than lower temperature ones (Luther et al. 2001; Le Bris et al. 2006). To some extent, this may explain the presence of clones related to the mesophilic, microaerobic, thiosulfate-oxidizing *Sulfurimonas* spp. in both the 25°C EPR EW fluid and in the 2°C, substrate-associated white filament communities (Figures 4.4b and d) while, interestingly, they were not detected in the 40°C EPR AP fluid. In contrast, the occurrence in the AP fluid of sequences related to hydrogen-oxidizing

Caminibacter/Nautilia spp. may be related to the higher temperature (and more reducing conditions) of this vent (Figure 4.4c). Overall, the EPR EW fluid community shared three groups (*S. denitrificans*, VI and IX) with the white filament community and two groups (*S. lithotrophicum* and X) with that of the EPR AP fluid, while group IX was the only one shared between the EPR AP fluid and the filament communities (Figure 4.4b, c and d). Therefore, the placement of the EPR EW fluid community in an intermediate position between the AP fluid and the white filament community is consistent with the intermediate temperature and redox conditions of this vent.

Implications of NapA expression in nitrate depleted environments. In this study we have demonstrated, for the first time, that nitrate-reducing *Epsilonproteobacteria* from deep-sea hydrothermal vents encode for the NapA periplasmic nitrate reductase, and that this is the only nitrate reductase encoded by the *C. mediatlanticus* genome. TBLASTX searches of the *C. mediatlanticus* genome did not yield matches to enzymes involved in denitrification including: NirS (Cytochrome cd1 nitrite reductase), NirK (Cu-containing nitrite reductase), NorB (Cytochrome b subunit of NO reductase), NorC (Cytochrome c subunit of NO reductase), and NosZ (Nitrous oxide reductase). Furthermore, we have established that the *napA* gene is expressed in *C. mediatlanticus* and *C. profundus* cultures grown under nitrate reducing conditions, and we have gathered preliminary evidence that some level of basal expression of the *napA* gene occurs in *Caminibacter* spp. grown in the absence of nitrate in the culture medium. In *E. coli*, the expression of the *nap* operon is induced at low concentrations of nitrate (μM range), whereas the cytoplasmic nitrate reductase (Nar) becomes predominant at nitrate concentrations in the mM range (Cole 1996; Wang et al. 1999). These observations imply that, between the

two enzymes, NapA is better adapted to function in nitrate-depleted environments.

Epsilonproteobacteria include very different organisms in terms of ecology, such as deep-sea vent thermophiles (e.g., *Caminibacter*) and human pathogens (e.g., *Campylobacter*, *Helicobacter*). However, the NAP nitrate reductase system appears to be conserved within these very different bacteria. It is worth to note that nitrate concentrations in the μM range are common to both deep seawater and fluids in the human body (Lentner 1984; Millero 1996). Therefore, comparative analyses of the genes comprising the *nap* cluster in pathogenic and vent *Epsilonproteobacteria* may provide insights on the evolution of the periplasmic nitrate reductase complex, and possibly of nitrate scavenging strategies.

Chapter 5 – Conclusion

The objective of this thesis dissertation was to gain a better understanding of nitrogen cycling at deep-sea hydrothermal vents through the use of culture dependent and independent techniques to isolate and study the nitrate reducing microorganisms present in these environments. What has been found in this study and by other groups is that nitrate reducing microorganisms belong to several different genera in the bacteria and archaea and are capable of growth over a range of physiological conditions (temp, pH, and redox conditions) at deep-sea hydrothermal vents (Blochl et al. 1997; Gotz et al. 2002; Huber et al. 2002; Alain et al. 2003; Miroshnichenko et al. 2003; Miroshnichenko et al. 2003; Miroshnichenko et al. 2003; Nakagawa et al. 2004; Vetriani et al. 2004; L'Haridon et al. 2006) (*Epsilonproteobacteria* reviewed by Campbell (2006)). Since this project began in 2002 a number of *Epsilonproteobacteria* capable of nitrate reduction, including *C. mediatlanticus* (Voordeckers et al. 2005), have been isolated and described from deep-sea vents (Alain et al. 2002; Alain et al. 2003; Audiffren et al. 2003; Inagaki et al. 2004). The *Epsilonproteobacteria* have been found to be among the one of the most ubiquitous bacterial groups at the hydrothermal vent systems and among the earliest colonizers of new surfaces at these sites (Corre et al. 2001; Longnecker and Reysenbach 2001; Lopez-Garcia et al. 2003; Nakagawa et al. 2005; Perner et al. 2007). This raised the question of their importance to the cycling of not only nitrogen at the vents but other biogeochemical cycles as well. By colonizing fresh surfaces they are exposed to a wide range of conditions as hydrothermal fluids and ambient seawater mix over them making a number of electron donor/acceptor pairings available for possible use depending on the

amount of mixing and vent fluid composition. If hydrogen is found in high concentrations within the vent fluids nitrate reduction with hydrogen oxidation would become a possibility once the fluids become diluted with ambient seawater.

We performed environmental surveys for 16S rRNA genes at geographically distinct hydrothermal vent systems along the Mid Atlantic Ridge and found that the Rainbow hydrothermal vent system was dominated by a number of epsilonproteobacterial phylotypes related to the genus *Caminibacter*, members of which were known to be thermophilic nitrate reducers. Thermophilic archaeal sequences were also obtained from this site. The Logatchev system showed a diverse assemblage of bacteria, mostly related to mesophilic aerobes and microaerobes, but no phylotypes related to known nitrate reducing organisms.

Isolation efforts using samples from these MAR sites yielded autotrophic nitrate respiring isolates belonging to the *Epsilonproteobacteria* from the Rainbow vent site. One isolate, TB2, was eventually chosen for full characterization as a new species, *Caminibacter mediatlanticus*. Later isolation efforts involving fluids and sulfides from the 9°N site on the EPR resulted in the isolation of several strains of *Epsilonproteobacteria* belonging to the genus *Nautilia*, *Nautilia* sps. MT3, MT4, and MT5. All isolates belonged to the order *Nautiliales* and possessed similar metabolic characteristics: anaerobic growth, autotrophic, and the ability to respire nitrate to ammonia. 16S rRNA gene comparison showed that *C. mediatlanticus* was related to the dominant bacterial phylotypes present at Rainbow and that we had successfully brought into pure culture an environmentally relevant organism.

Because *C. mediatlanticus* and its sister isolate *Caminibacter* sp. TB1 were related to the dominant bacterial types at Rainbow we were provided with a chance to examine the metabolic capabilities of an environmentally relevant organism. Detailed characterization of *C. mediatlanticus* showed that it is able to use elemental sulfur as an alternate electron acceptor, however, unlike many of the other nitrate reducing *Epsilonproteobacteria* it was unable to utilize oxygen for respiration. *C. mediatlanticus* was also shown to have a functional rTCA cycle allowing it to fix carbon dioxide into biomass. A survey for the beta subunit (*aclB*) of ATP-citrate lyase, a key enzyme in the rTCA cycle, showed results similar to the 16S rRNA gene survey, with all *AclB* sequences related to *C. mediatlanticus*. Further work with the recently sequenced genome of *C. mediatlanticus* will yield more information about its metabolic capabilities.

Our survey of the periplasmic nitrate reductase *napA* gene at four hydrothermal vent microbial communities showed that all clones were related to epsilonproteobacterial derived sequences as opposed to the alpha and gammaproteobacterial sequences normally seen in freshwater and estuarine environments. The greatest diversity of *napA* sequences was seen in the mesophilic temperature range with lower amount of diversity seen at higher and lower temperatures. Overlap of specific NapA groups was seen between samples with similar temperatures. The NapA survey of the high temperature Rainbow hydrothermal vent site showed a predominance of NapA sequences related to *C. mediatlanticus*, as would be expected given the previous 16S rRNA gene data. The survey of cultured organisms yielded also evidence that the *napA* gene may have been horizontally transferred between different groups within the *Epsilonproteobacteria*. Further studies to assess the NapA phylogeny of hydrothermal vent nitrate reducing

organisms, including any future isolates, will be needed to resolve this issue. With only a few *napA* gene sequences available from reference organisms we can only speculate whether this diversity indicates that there are possibly a number of as yet uncultured nitrate, and most likely sulfur, reducing microorganisms within the chimney structures and fluids.

Taken together the data from this study provided insight into the cycling of nitrogen at deep sea hydrothermal vents. We performed the first survey for the Nap nitrate reduction system. The Nap system appears to be associated with one particular group of bacteria, the *Epsilonproteobacteria*, at the vent systems. The combined data provided a view of the chemolithoautotrophic, nitrate reducing bacterial community of the Rainbow hydrothermal vent system. We were able to cover the Rainbow site with surveys of the 16S rRNA, *acIB* (carbon fixation), and *napA* (nitrate respiration) genes and finally able to isolate and characterize a representative of the dominant organism phylotype whose metabolism can now be studied in detail.

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