MICROBIAL ECOLOGY OF DEEP-SEA HYDROTHERMAL VENTS: PHYSIOLOGY AND CELL-TO-CELL COMMUNICATION IN ANAEROBIC CHEMOSYNTHETIC *BACTERIA*.

By

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ABSTRACT OF DISSERTATION

Microbial ecology of deep-sea hydrothermal vents: physiology and cell-to-cell communication in anaerobic chemosynthetic *Bacteria*.

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The global influence of mid-oceanic ridges (MOR) first became apparent through continental drifting– its immanent force easily appreciated in today's resulting continents. The role of MORs as a source of global-ocean chemistry is less apparent but equally immense. Key to these processes is fluid-rock reactions between circulating seawater and hot new basalt. With the discovery of hydrothermal vent ecosystems in the 1970's, yet another important consequence of rock-fluid interaction was established in chemosynthesis. Early photographic descriptions of "frosted white and yellow precipitates" covering basalt rocks close to discharged hydrothermal fluids, with benthic communities emerging from them, referred to the now known chemosynthetic biofilms that interact with hydrothermal fluids. These microorganisms have a pivotal role in transforming the geochemistry of Earth's oceans.

The main objectives of this dissertation are to study anaerobic chemosynthetic vent microorganisms, and to explore the molecular ecology of these biofilm communities. Initial approaches included isolation of anaerobic chemosynthetic microorganisms resulting in the description of two novel bacterial species: the epsilonproteobacterium *Nautilia nitratireducens* strain MB-1^T, and *Phorcys thermohydrogeniphilus* strain HB-8^T, a new genus in the *Aquificales*. Both bacteria are obligate thermophilic anaerobes, capable of hydrogen oxidation coupled to sulfur- and nitrate-reduction.

Further investigation focused on mechanisms regulating vent biofilms, the dominant growth strategy in vent microbial communities. Quorum-sensing (QS), a mechanism relying on cell density and the production of extracellular signals for cell-cell communication, is used by many microbial species to regulate biofilm formation.

One QS signal is Autoinducer-2, whose precursor is synthesized by the LuxS enzyme. To study QS in vent environments, *Caminibacter mediatlanticus* and *Sulfurovum lithotrophicum*, cultured members of the well-represented *Epsilonproteobacteria*, were used as model systems. The *luxS* gene and transcripts were detected in their genomes and during growth, respectively; these *luxS*-expressing cultures induced bioluminescence, a QS response, in a *Vibrio harveyi* reporter strain. Detection of *luxS* transcripts *in-situ*, also indicated that QS is likely occurring in natural vent biofilms. This data demonstrates that vent *Epsilonproteobacteria* posses the *luxS*/AI-2 system for cell-cell communication. This work is relevant to our overall understanding of microbial phenotypic plasticity in response to environmental factors.

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Dedication

I want to dedicate this thesis to my parents, César Pérez-Robles and Milagros Rodríguez-Rivera in Puerto Rico, for all the time that we have not been together physically, although our hearts have always been next to each other. For their unconditional love and support all troughout my life; I am extremely grateful for being daughter to such wonderful people.

Table of Contents

List of Tables

Page

List of Figures

Page

Chapter 1

Introduction

Mid Ocean Ridges and deep-sea hydrothermal vent systems

The ocean crust covers 60% of Earth's surface at an average depth of 3800 m (Edwards *et al.*, 2005; Miroshnichenko, 2004). A significant fraction of ocean crust is exposed at Mid Ocean Ridge (MOR) systems, the most prominent mountain range system on the surface of Earth (Figure 1.1). The global MOR system consists of a 60,000 km-long volcanic mountain chain along the ocean floor (Edwards *et al.*, 2005; Macdonald, 1982). The ridge is located between the tectonic plates of the Earth's rigid outer shell that are separating at speeds of ~ 10 to 170 mm y^{-1}. Eruption of seafloor lava at MOR systems continually introduces new magma along the plate boundary generating new seafloor and a volcanically active ridge (Macdonald, 1982; Macdonald *et al.*, 1991).

The circulation of seawater through the oceanic crust along mid ocean ridges passes through deep areas of high temperatures and pressures where mineral leaching and chemical reductions occur (Jannasch, 1995), actively advecting hydrological systems (Schrenk *et al.*, 2010). As a result of this interaction, the water changes its chemical composition and gets overheated, but still remains liquid because of the enormous hydrostatic pressure (Jannasch & Mottl, 1985; Karl, 1995; Miroshnichenko, 2004; Santelli *et al.*, 2008). These generated hydrothermal fluids are hot, metal-enriched, acidic and strongly reducing (i.e. H_2S, H_2, and CH_4) compared to seawater (Delaney *et al.*, 1998; Jannasch & Mottl, 1985; Karl, 1995). When the buoyant hydrothermal fluids are vented at the seafloor, they mix rapidly with the surrounding cold, oxidized seawater (Lupton *et al.*, 1985; McCollom, 2000) precipitating the minerals contained in the fluids in the form polymetallic sulfides. Two general types of venting, focused and diffused flows, have been defined based on temperature and oxygen concentration associated with the fluids (Delaney *et al.*, 1998; Jannasch & Mottl, 1985; Karl, 1995). Hydrotherml fluids released from focused flow vents reach temperatures of up to 350°C and are highly reduced and, in contact with cold, oxygenated seawater at the seafloor, forms sulfide structures known as "black smokers" (Figure 1.1b). In contrast, diffuse flow fluids are generated when hot fluids mix with cold, oxygenated seawater in the shallow subseafloor, resulting in warm, mildly oxidized

fluids (up to 60°C). Typically, deep-sea hydrothermal vents occur at depths of 800 to 5,000 m, which corresponds to hydrostatic pressures of 80 to 500 bars (8-50 MPa) (Miroshnichenko, 2004).

Overall, the magmatic system at ocean ridges supplies the energy to drive seafloor hydrothermal systems, as well as the geothermal sources controling ocean chemistry (Macdonald *et al.*, 1991).

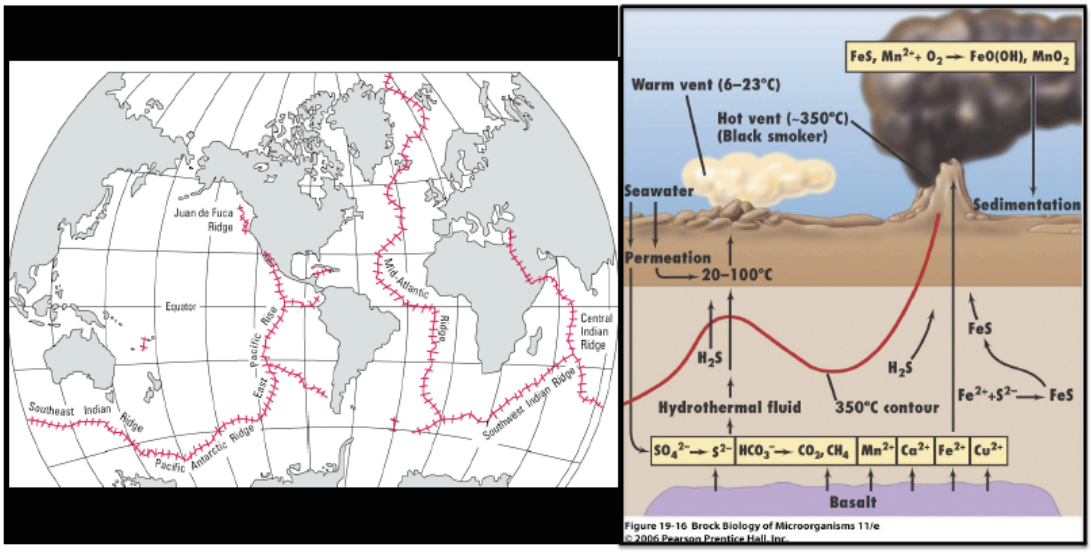

Figure 1.1 (a) Global Mid Oceanic Ridges (USGS). (b) Hydrothermal fluids circulation (Madigan *et al.*, 2006).

Chemosynthetic primary production at deep-sea hydrothermal vents

The discovery of deep-sea hydrothermal vents has significantly modified the established views on the deep sea that was long known as a cold, dark, high pressure and nutrient-poor environment (Corliss *et al.*, 1979; Jeanthon, 2000). For a long time it was thought that the input of energy for the heterotrophic production of biomass in the absence of light was limited to the sedimentation of particulate organic matter from the photosynthetically productive surface waters to the bottom of the ocean. The decomposition and mineralization of this organic carbon occurs largely in the upper 200-300 m layer of the world oceans averaging at about 95% of their total primary productivity. Of the remaining 5%, only about one fifth reaches the sea floor at greater depths in particulate form (Honjo & Manganini, 1993; Jannasch, 1995). With the discovery of metabolic activities in the permanently dark deep-sea, based on geothermal rather than solar energy, a new dimension was added to this general notion (Jannasch, 1995). In geothermal systems, water-

rock interactions at high temperature generate hydrothermal fluids enriched in reduced inorganic chemical species that provide a source of energy, or "geofuels" for microbial oxidations. Microorganisms transform chemical energy into biochemical energy (ATP), which is then used to fix carbon dioxide (Jannasch, 1995; Lonsdale, 1977). This process, called chemosynthesis or chemolithoautotrophy, emphasizes that not only the carbon source but also the energy source is inorganic, providing a basis for primary production of organic carbon in the deep-sea hydrothermal vents (Delaney *et al.*, 1998; Jannasch & Mottl, 1985; Karl, 1995). These ecosystems often referred to as "chemosynthetic communities", have proved to be unsurpassed when compared to any other biological system on Earth in terms of biomass production rate (Delaney *et al.*, 1998; Jannasch & Mottl, 1985; Karl, 1995).

General characteristics of this habitat include the following extreme environmental conditions: high temperature and hydrostatic pressure, complete absence of light, elevated concentrations of heavy metals, sharp gradients of all physico-chemical parameters and concentrations of substances required for metabolism (Miroshnichenko, 2004).

Aerobic vs. anaerobic chemosynthesis: Ecological and evolutionary considerations

Hydrothermal fluid mixing with water along MOR systems allow microbial habitats to exist over almost infinite ranges of temperatures based on seawater/hydrothermal fluid ratios ranging from very small within the vents to high at the surface (McCollom, 2000; McCollom & Shock, 1997). Hydrothermal fluids and seafloor rocks are rich in reduced chemical species, while ambient seawater provide microorganisms with a source of oxidized chemical species (Karl, 1995; McCollom, 2000). Chemolithoautotrophs can use the resulting redox disequilibria by catalyzing the reactions and converting the chemical energy released into biochemical energy for the production of cellular biomasss (McCollom, 2000). A consequence of the rapid mixing in hydrothermal plumes of two fluids with disparate oxidation states is that typically aerobic and anaerobic reactions can both provide energy in the same environment.

Under aerobic or micro-aerobic conditions, bacteria conserve energy for CO_2 fixation by coupling the oxidation of reduced inorganic chemical species (e.g., hydrogen, sulfide, elemental sulfur, thiosulfate,

iron, manganese, etc.) to the reduction of O_2 (Table 1.1.) (Jannasch & Mottl, 1985; Von Damm, 1990). Under anaerobic conditions, microorganisms use terminal electron acceptors other than O_2 (e.g. S^0, SO_4^{2-}, NO_3^-, CO_2, etc.) (Jannasch & Mottl, 1985).

Microbial process	Chemosynthetic reaction
	Anaerobic
Nitrate reduction	$CO_2 + 6H_2 + H^+ + NO_3^- \rightarrow [CH_2O] + NH_4^+ + OH^- + 3H_2O$
Sulfate reduction	$CO_2 + 6H_2 + 2H^+ + SO_4^{2-} \rightarrow [CH_2O] + H_2S + 5H_2O$
Methanogenesis	$2\ CO_2 + 6H_2 \rightarrow [CH_2O] + CH_4 + 3H_2O$
Sulfur reduction	$CO_2 + 3H_2 + S^0 \rightarrow [CH_2O] + H_2S + H_2O$
Iron reduction	$Fe^{3+} + 4H_2 + CO_2 \rightarrow [CH_2O] + Fe^{2+} + H_2O$
	Aerobic
Sulfide oxidation	$CO_2 + H_2O + H_2S + O_2 \rightarrow [CH_2O] + H_2SO_4$
Methanotrophy	$CO_2 + CH_4 + O_2 \rightarrow [CH_2O] + CO_2 + H_2O$
Thiosulfate oxidation	$CO_2 + 2H_2O + Na_2S_2O_3 + O_2 \rightarrow [CH_2O] + Na_2SO_4 + H_2SO_4$

Table 1.1 Examples of anaerobic and aerobic chemosynthesis based on redox couples available to chemolithoautotrophs at deep-sea hydrothermal vents.

From an ecological perspective, anaerobic chemosynthetic microorganisms are "strict" primary producers in vent ecosystems since they do not depend on photosynthetically derived oxygen. In contrast, aerobic chemosynthesis depends on free O_2 which is a byproduct of oxygenic photosynthesis (Jannasch, 1995). Hence, aerobic chemosynthetic microorganisms are not, strictly speaking, primary producers. Studying seafloor microbial chemosynthetic processes confounds standard ecological descriptions in part because of the difficulty of elucidating and describing the scale of relevant processes (Schrenk *et al.*, 2010). Metabolically, seawater represents a very large reservoir of potential oxidants, and the hydrothermal fluid is a researvoir of potential reductants, but the degree to which they are available concomitantly to chemosynthetic microbial communities is not well known (Edwards *et al.*, 2005). At the present time, microbially-mediated oxidation of reduced chemical species relative to the abiotic oxidation is largely unknown for vent environments, so it is difficult to quantify the fraction of the available energy that is effectively utilized by microbes (McCollom, 2000). Given that chemical and microbial oxidations represent

a competitive process for the microbial communities (Jannasch, 1995), it becomes relevant to understand bacterial adaptations to overcome this competition.

From an evolutionary standpoint, anaerobic chemosynthetic microorganisms are quite interesting. First, the majority of anaerobic chemosynthetic microorganisms are also thermophiles, and phylogenetic studies indicate that they occupy the deepest branches of the tree of life (Figure 1.2) (Jannasch, 1995). Second, many anaerobic thermophiles use elemental sulfur or carbon dioxide, two products of geothermal processes, as terminal electron acceptors. Third, anaerobic respiration is thought to have predated aerobic respiration, which evolved as a consequence of the release of oxygen in the atmosphere by oxygenic phototrophs. All together, these three independent lines of evidence, based on phylogeny, physiology and the reconstruction of the distribution of oxygen in the atmosphere throughout the Earth's history, suggest an early ancestry for anaerobic thermophiles and the possibility that deep-sea hydrothermal vents might have played a role in the origin of life on Earth (Jannasch, 1995).

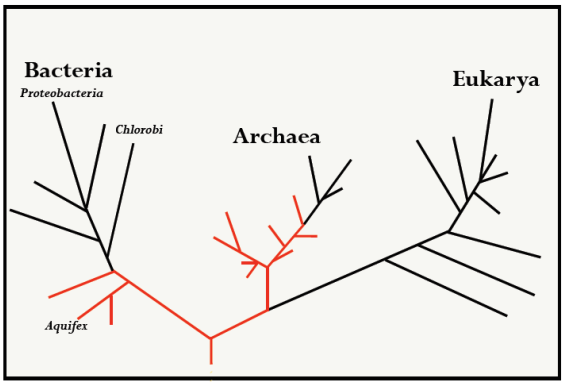

Figure 1.2 Positioning of hyperthermophilic microorganisms (shown in red) in the 16SrRNA based tree of life (Stetter, 1996).

Anaerobic chemosynthetic microbial communitites

At deep-sea vents, chemosynthetic microorganisms link geochemical and biological processes by transferring energy and carbon from the geothermal source to the higher trophic levels. Identifying the mechanisms of energy and carbon at the biochemical, physiological and ecological levels is critical to understanding the system. In general, there is a correlation between temperature and redox state in

hydrothermal fluids from deep-sea vents, in that high temperature fluids tend to be more reduced, while moderate temperature fluids are more oxidized (Luther *et al.*, 2001). In line with this observation, vent thermophiles and hyperthermophiles (e.g., *Aquificales* and *Archaea*) tend to be anaerobic and inhabit high temperature, highly reduced niches, while moderately thermophiles and mesophiles tend to be facultative anaerobes and/or microaerobes (e.g., *Epsilonproteobacteria*) and colonize habitats characterized by moderate temperature and less reducing regimes (Campbell *et al.*, 2006; Stetter, 1995). Furthermore, lab-based physiological studies of chemosynthetic microbes have revealed that the ability to use certain redox couples (e.g., H_2 oxidation, coupled to nitrate and/or sulfur reduction) is conserved across different taxonomic groups (Miroshnichenko & Bonch-Osmolovskaya, 2006). Within individual species, however, a certain level of metabolic versatility (e.g., the ability to use multiple oxidants) is evident (Table 1.1) (Campbell *et al.*, 2006). At deep-sea vents, chemosynthetic anaerobes become relevant not only as primary producers in their immediate environment, but also as important players in linking the carbon cycle to the ocean's sulfur and nitrogen cycle (Figure 1.3). **Therefore, this work has been aimed to elucidate the taxonomy and physiology of anaerobic chemosynthetic bacteria from deep-sea hydrothermal vents.**

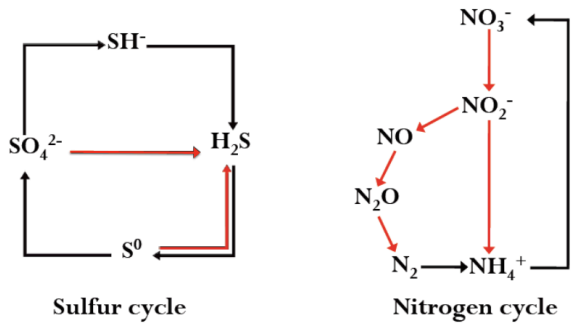

Figure 1.3 Anaerobic pathways performed by chemosynthetic bacteria (shown in red) within the sulfur and nitrogen cycles (simplified versions).

Epsilonproteobacteria and their relevance at deep-sea hydrothermal vents

It has become evident that *Epsilonproteobacteria* are one of the most revalent bacterial groups at deep-sea hydrothermal vents (Nakagawa *et al.*, 2005; Nakagawa *et al.*, 2007). *Epsilonproteobacteria* include two valid described orders: the *Nautiliales* and the *Campylobacterales* (Figure 1.4) (Garrity *et al.*, 2005;

Miroshnichenko *et al.*, 2004). The order *Nautiliales* comprises the genera *Nautilia*, *Caminibacter*, and *Lebetimonas* (Alain *et al.*, 2002; Miroshnichenko *et al.*, 2002; Takai *et al.*, 2005), which include hydrogen-oxidizing bacteria isolated from deep-sea hydrothermal vents. The order *Campylobacterales* includes the families *Campylobacteraceae*, *Helicobacteraceae* and *Hydrogenimonaceae* (Meinersmann *et al.*, 2002; Takai *et al.*, 2004), represented, among others, by the pathogenic *Campylobacter* and *Helicobacter* spp. Based on phylogenetic analyses, *Epsilonproteobacteria* fall into four robust phylogenetic clusters, classified as *Nautiliales*, *Arcobacter*, *Sulfurospirillum* and an environmental sequence cluster that consists of sequences retrieved from various marine systems (for example, deep-sea hydrothermal vents, vent fauna and deep-sea marine subsurfaces) and terrestrial systems (for example, groundwater, caves and springs) (Campbell *et al.*, 2006).

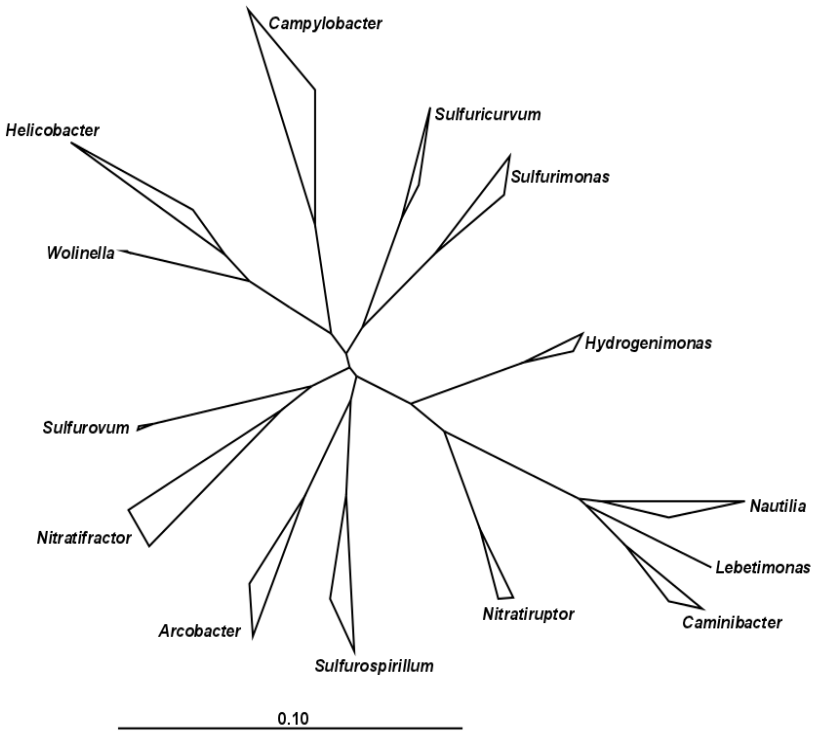

Figure 1.4 Phylogeny of cultured *Epsilonproteobacteria*. The tree was constructed using the ARB neighbor joining method from 16S ribosomal RNA genes of 129 cultured epsilonproteobacterial species.

Several environmental surveys have shown that *Epsilonproteobacteria* have been found at, and sometimes dominate, four main deep-sea hydrothermal vent-specific habitats: 1) mats on the surfaces of

rocks, chimneys and animal surfaces, 2) discharged vent fluids and subseafloor, 3) hydrothermal vent plume and 4) symbiont associations with vent animals such as *Alvinella pompejana*, *Alviniconcha aff. Hessleri*, and *Rimicaris* spp. (Campbell *et al.*, 2006; Longnecker & Reysenbach, 2001; Moussard *et al.*, 2006). The dominance of this group usually is associated to environments characterized by high temperature (40-60°C), anoxic and sulfide-rich vent fluids and mesophilic (25-35°C), moderately oxidized vents (Campbell *et al.*, 2006; Longnecker & Reysenbach, 2001; Moussard *et al.*, 2006). *Epsilonproteobacteria* may maximize overall ecosystem function, because of their high biomass and growth rates, rapid adaptations to changing geochemical conditions (Campbell *et al.*, 2003), the possibility of special modes for attachment to surfaces (Alain *et al.*, 2004) and metabolic versatility and phylotypic diversification (Campbell *et al.*, 2006; Lopez-Garcia *et al.*, 2003). Potentially, this allows *Epsilonproteobacteria* to establish themselves as the primary (and possibly the first) colonizers in the dynamic hydrothermal flow vent environment (Campbell *et al.*, 2006). Ecological principles indicate that there is a tendency for the most productive species in an ecosystem to be the most dominant in a habitat, thereby pushing other species to comparatively lower densitites (Campbell *et al.*, 2006). However, despite the dominance and widespread distribution of *Epsilonproteobacteria*, their ecological significance in hydrothermal systems remains to be elucidated (Nakagawa *et al.*, 2005).

Pure cultures of deep-sea vent *Epsilonproteobacteria* have provided evidence that show that the majority of these cultured microbes are mesophilic to thermophilic, and grow under suboxic or anaerobic conditions (Campbell *et al.*, 2006; Nakagawa *et al.*, 2007; Perez-Rodriguez *et al.*, 2010; Voordeckers *et al.*, 2005). Cultured representatives utilize a wide spectrum of electron donors and acceptors (i.e. hydrogen, sulfur compounds, nitrate and oxygen), and many of them share the ability to use the same electron donors and acceptors (Table 1.2).

Cultured chemosynthetic *Epsilonproteobacteria* are autotrophic and have been shown to assimilate CO_2 via the reductive TCA cycle—a putatively ancient metabolic pathway of autotrophic carbon fixation that occurs under anaerobic conditions and appears to be significant at deep-sea vents (Hugler & Sievert, 2011; Preuss *et al.*, 1989). Until recent studies on chemolithoautotrophic *Epsilonproteobacteria*, the reverse Tricarboxylic Acid Cycle (reverse TCA) cycle had been described in only a few microorganisms including

the green sulfur bacterium *Chlorobium limicola*, a few members of the *Deltaproteobacteria* and some members of the thermophilic *Aquificales* and *Thermoproteaceae* (Fuchs, 1989; Hügler *et al.*, 2003).

Caminibacter mediatlanticus and *Sulfurovum lithotrophicum*, two cultured chemosynthetic *Epsilonproteobacteria* whose genomes have been sequenced (references), represent two categories of vent *Epsilonproteobacteria*: the first is a strictly anaerobic, hydrogen-oxidazing thermophile, while the second is a microaerobic, facultative anaerobic, thiosulfate-oxidizing mesophile. Given their physiological and metabolic characteristics, *C. mediatlanticus* and *S. lithotrophicum* were used as laboratory model systems to investigate their ecology.

					H_2		$S_2O_3^{2-}$	S^0	
Organism	**Isolation Site[a]**	**Temperature (°C)**	**End Product of Nitrate Respiration**	NO_3^-	S^0	O_2	NO_3^-	NO_3^-	O_2
Sulfurovum lithotrophicum[b]	MOT, Iheya, sediments	28-30	N_2	-	-	-	+	+	+
Sulfurimonas paralvinellae[c]	MOT, Iheya, *Paralvinella*	30	N_2	+	-	+	+	+	+
Thioreductor micantisoli[d]	MOT, Iheya, sediments	32	NH_4^+	+	+	-	-	-	-
Nitratifractor salsuginis[e]	MOT, Iheya, chimney	37	N_2	+	+	+	-	-	-
Nautilia nitratireducens[f]	EPR, 9°N, chimney	55	NH_4^+	+	+	-	-	-	-
Hydrogenimonas thermophila[g]	CIR, Kairei Field, colonizer	55	NH_4^+	+	+	+	-	-	-
Nitratiruptor tergarcus[e]	MOT, Iheya, chimney	55	N_2	+	+	+	-	-	-
Caminibacter profundus[h]	MAR, Rainbow, vent cap	55	NH_4^+	+	+	+	-	-	-
Caminibacter mediatlanticus[i]	MAR, Rainbow, chimney	55	NH_4^+	+	+	-	-	-	-
Caminibacter hydrogeniphilus[l]	EPR, 13°N, *Alvinella*	60	NH_4^+	+	+	-	-	-	-

[a] MOT: Mid-Okinawa Trough; EPR: East Pacific Rise; CIR: Central Indian Ridge; MAR: Mid-Atlantic Ridge.
[b] Inagaki, F. *et. al.*, 2004. *Int. J. Syst. Evol. Microbiol.* 54:1477.
[c] Takai, K. *et. al.*, 2006. *Int. J. Syst. Evol. Microbiol.* 56:1725.
[d] Nakagawa, S. *et. al.*, 2005. *Int. J. Syst. Evol. Microbiol.* 55:599.
[e] Nakagawa, S. *et. al.*, 2005. *Int. J. Syst. Evol. Microbiol.* 55:925.
[f] Pérez-Rodríguez, I. *et. al.*, 2010. *Int. J. Syst. Evol. Microbiol.* 60:1182.
[g] Takai, K. *et. al.*, 2004. *Int. J. Syst. Evol. Microbiol.* 54:25
[h] Miroshnichenko, M. L. *et. al.*, 2004. *Int. J. Syst. Evol. Microbiol.* 54:41.
[i] Voordeckers, J.W. *et. al.*, 2005. *Int. J. Syst. Evol. Microbiol.* 55:773.
[l] Alain, K. *et. al.*, *Int. J. Syst. Evol. Microbiol.* 52:1317.

Table 1.2 Metabolic capabilities and optimal temperatures for growth of several cultured *Epsilonproteobacteria* from deep-sea vent environments (Vetriani, unpublished).

Because of the metabolic characteristics and ecotype preferences of the modern vent *Epsilonproteobacteria*, together with the putative ancestral nature of marine deep-sea vent environments, it is

not far-reaching to hypothesize that, as the *Epsilonproteobacteria* are dominant and important organisms in modern vents, this group could have been significant to ecological and biogeochemical processes throughout much of Earth's history (Campbell *et al.*, 2006).

Interestingly, chemolithoautotrophic *Epsilonproteobacteria* are phylogenetically related to important pathogens; such relationships might provide insights into the origins of virulence in pathogenic relatives of the *Helicobacter* and *Campyobacter* genera (Nakagawa *et al.*, 2007). These deep-sea vent *Epsilonproteobacteria* diverged before their pathogenic relatives based on small-subunit rRNA gene trees, and comparative genomics have shown multiple systems for respiration, sensing and environmental response common to all members (Nakagawa *et al.*, 2007). These previously unrecognized evolutionary links between important human/animal pathogens and the nonpathogenic, symbiotic, chemolithoautotrophic deep-sea relatives provide evidence on the role that genomic plasticity, a characteristic of this group, has played in the diversification of microbial life (Nakagawa *et al.*, 2007).

Chemosynthetic microbial biofilms at deep-sea hydrothermal vents

The first photographs of fissures in basalt rocks close to hydrothermal vents were described to be "frosted with white and bright yellow chemical precipitates", with remarkable communities of benthic invertebrates emerging from them (Lonsdale, 1977). It is now known that these "frosted precipitates" are mostly composed of chemosynthetic bacterial communities, or biofilms, that mediate the transfer of carbon and energy from the geothermal source to the higher trophic levels (Figure 1.5). Direct observations of dense cell suspensions released from warm vents also provide striking evidence that microbial biofilms are extended to subseafloor environments (Jannasch, 1995).

The observed locations of abundant hydrothermal vent associated microbial biofilms include: (1) free-living bacterial populations associated with the discharged vent fluids and presumably growing and reproducing within the sub-seabed system (Figure 1.5a), (2) free-living microbial mats growing on the surface strata that are exposed to flowing vent waters (Figure 1.5b,c), and (3) endo- and episymbiotic associations of microorganisms within the deep-sea hydrothermal vent plumes (Jeanthon, 2000; Karl, 1995).

The high rock surface to fluid ratio at the seafloor provides very high surface area for microbial attachment; furthermore biofilms may represent a critical step in the primary colonization of deep-sea hydrothermal vents, as they allow microorganisms to maximize access to nutrients and energy sources in an environment typically characterized by high turbulence. Finally, the matrix that encases biofilms may provide protection from predation and/or exposure to toxic substances, hence improving the community environment in ways that are simply impossible for individual cells (Nadell *et al.*, 2009).

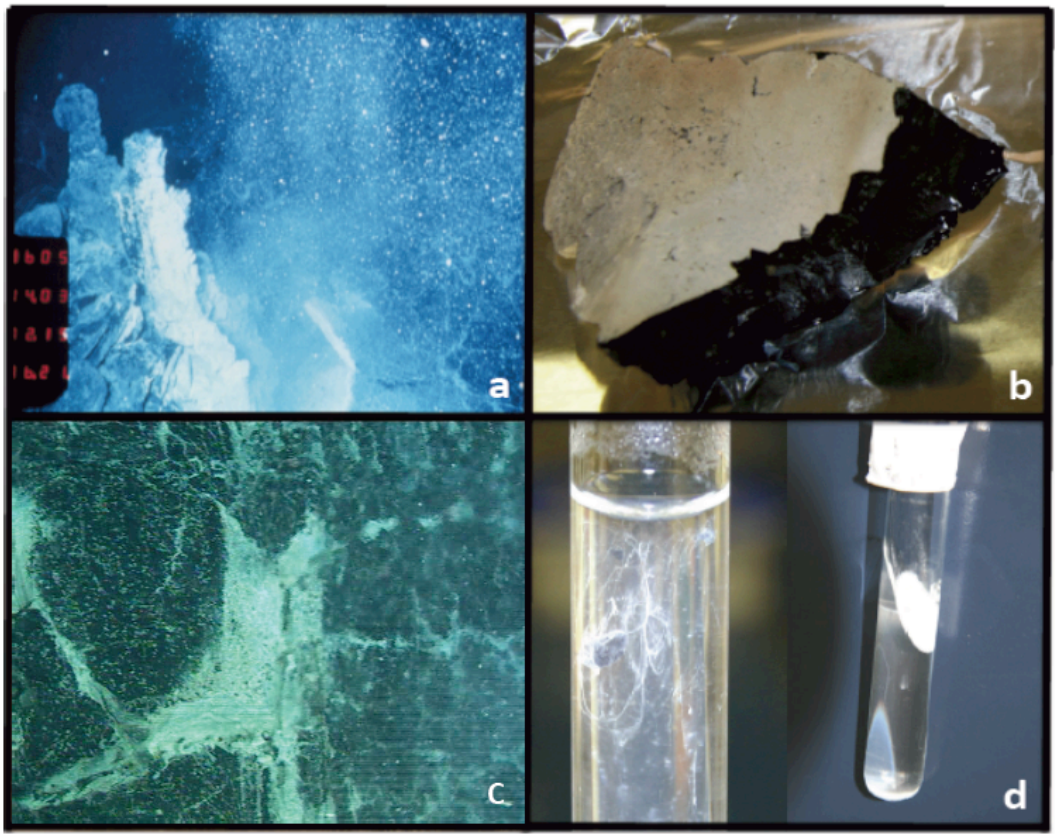

Figure 1.5 Release of chemosynthetic bacteria from the subsurface via hydrothermal venting (a). Vent microorganisms attached to basalt (b) and associated with the fluid-venting on the seafloor (c). Biofilm-forming pure cultures isolated from deep-sea vents.laboratory (d).

The role of cell-to-cell communication (quorum sensing) in biofilm formation

Biofilm formation occurs when communities of cells attach to a surface and embed themselves in secreted polymers (Davies *et al.*, 1998; Hammer & Bassler, 2003). The structure of biofilms is clearly

influenced by a number of biological factors such as twitching motility (Dalton *et al.*, 1996), growth rate (Heydorn *et al.*, 2000), cell signaling (Davies *et al.*, 1998), and exopolysaccharide (Gates *et al.,* 2003) production (Flemming *et al.*, 2000); the physical growth environment also plays a significant role in the determination of biofilm structure (Stoodley *et al.*, 2002). Accordingly, biofilms confer on their members considerable advantages, including the ability to resist challenges from predators, antibiotics, and other external threats (Nadell *et al.*, 2009). At the same time, surface association is an efficient means of lingering in a favorable microenvironment with accessible nutrients rather than being swept away by the current (Watnick & Kolter, 2000). In addition to spatial localization, surfaces may provide the protective niche in which attached cells create a localized homeostatic environment (Stoodley *et al.*, 2002).

In most bacteria, biofilms are made possible by the expression of sets of genes that result in phenotypes that differ profoundly from free-living cells of the same species (Stoodley *et al.*, 2002). Even within single species biofilms, genetically identical cells in the biofilm can diverge in a wide variety of traits, including basic metabolic activity (Xu *et al.*, 1998), antibiotic tolerance (Balaban *et al.*, 2004), spore formation (Branda *et al.*, 2001), and the secretion of extracellular polymers (Nadell *et al.*, 2009; Vlamakis *et al.*, 2008). Although no single mechanism is responsible for the expression of these functions, many species use quorum sensing (QS), to modulate surface attachment (Dunne, 2002), motility (Schuster & Greenberg, 2006), EPS production (Davies *et al.*, 1998), and dispersal (Hammer & Bassler, 2003; Nadell *et al.*, 2009). Quorum sensing, or cell-cell communication, involves the accumulation of signal molecules that enable bacteria to sense when the minimal number of bacteria, or "quorum", has been achieved for a concerted response to be initiated (Bassler *et al.*, 1994). The idea is that QS turns on the cooperative expression of specific genes when it is most useful to do so: at high cell density (Keller & Surette, 2006; Rumbaugh *et al.*, 2009). Biofilm formation and quorum sensing are central and often interconnected features of bacterial social life (Nadell *et al.*, 2009).

Because biofilms are ubiquitous in the bacterial realm, it has been proposed that biofilm formation within prokaryotic communitites is an ancient life strategy, the development of which was facilitated by the localization of cells on solid substrates (Hall-Stoodley *et al.*, 2004; Stoodley *et al.*, 2002). The basis for this

speculation is the fact that the earliest fossil records of life are surface-associated microbial mats (Rasmussen, 2000; Reysenbach & Cady, 2001), and that this trait is found in the most ancient linages of archaeal and bacterial lines, the *Korarchaeota* and *Aquificales* (Reysenbach & Cady, 2001; Stoodley *et al.*, 2002). Because biofilm communities use the phenotypic plasticity of bacterial genomes in varying patterns of expression, the study of quorum sensing, which controls the cooperative expression of biofilm-specific genes, is critical to understanding the mechanisms underlying this microbial life strategy (Stoodley *et al.*, 2002).

Autoinducer-2 quorum sensing system

Quorum sensing was first characterized in the marine bacteria *Vibrio harveyi* and *Vibrio fisheri* as the mechanism that regulates bioluminescence (Bassler *et al.*, 1994). One of the quorum sensing systems in these microorganisms is mediated by the autoinducer-2 (AI-2) signaling molecule, which is predicted to be a furanosyl borate diester and is synthesized by the product of the enzyme LuxS (Figure 1.6) (Kaper *et al.*, 1999). The LuxS enzyme converts *S*-ribosylhomocysteine to 4,5-dihydroxyl-2,3-pentanedione (DPD), catalyzing AI-2 formation. (Chen *et al.*, 2002). It is believed that AI-2 is used by these bacteria as an interspecies communication system (Bassler *et al.*, 1997; Bassler *et al.*, 2002).

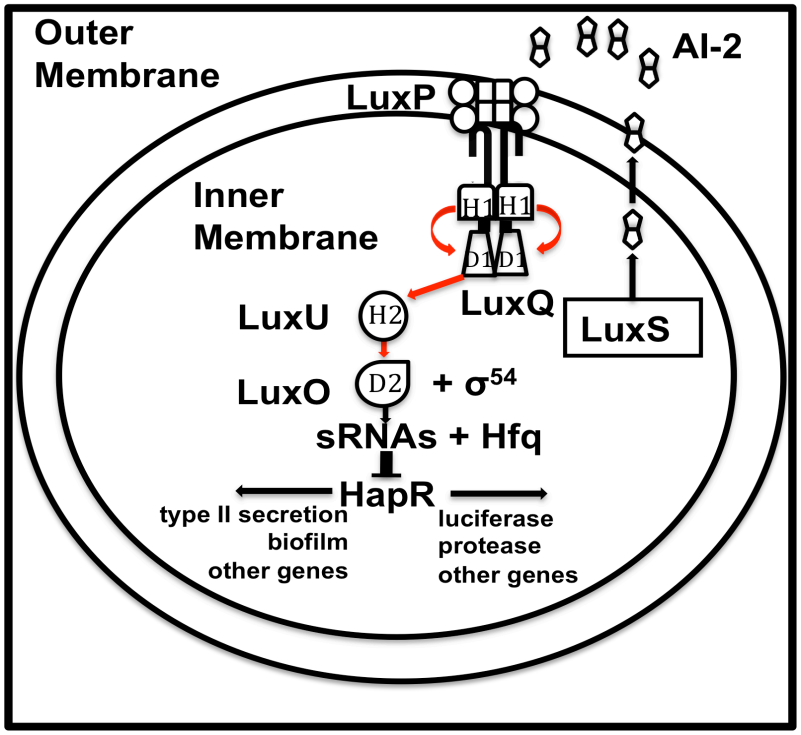

Figure 1.6 LuxS quorum sensing in *Vibrio harveyi*. This system includes the AI-2 and the sensor LuxPQ. The AI-2 synthase is LuxS. (Bassler & Henke, 2004).

Special attention has been given to the Autoinducer-2 (AI-2) type QS system since it has been recognized to be widespread across the bacterial kingdom and has been proposed by many to function as a "Bacterial Esperanto" (Figure 1.7a) (Guillemin *et al.*, 2007). We have found that the genome of chemosynthetic *Epsilonproteobacteria* from deep-sea vent hydrothermal vents encode for *luxS* (Figure 1.7b). In particular, we have identified the *luxS* gene in representative cultures of two chemosynthetic *Epsilonproteobacteria, Caminibacter mediatlanticus* and *Sulfurovum lithotrophicum*. Because pathogenic *Epsilonproteobacteria* from the *Helicobacter* and *Campylobacter* genera have been demonstrated to utilize the *luxS* dependent AI-2 quorum sensing system to regulate biofilm formation and pathogenesis (Elvers & Park, 2002; Forsyth & Cover, 2000; Itoh *et al.*, 2003; Loh *et al.*, 2004; Wright *et al.*, 2000), we have hypothesized that *luxS* plays a role in QS also in *C. mediatlanticus* and *S. lithotrophicum*.

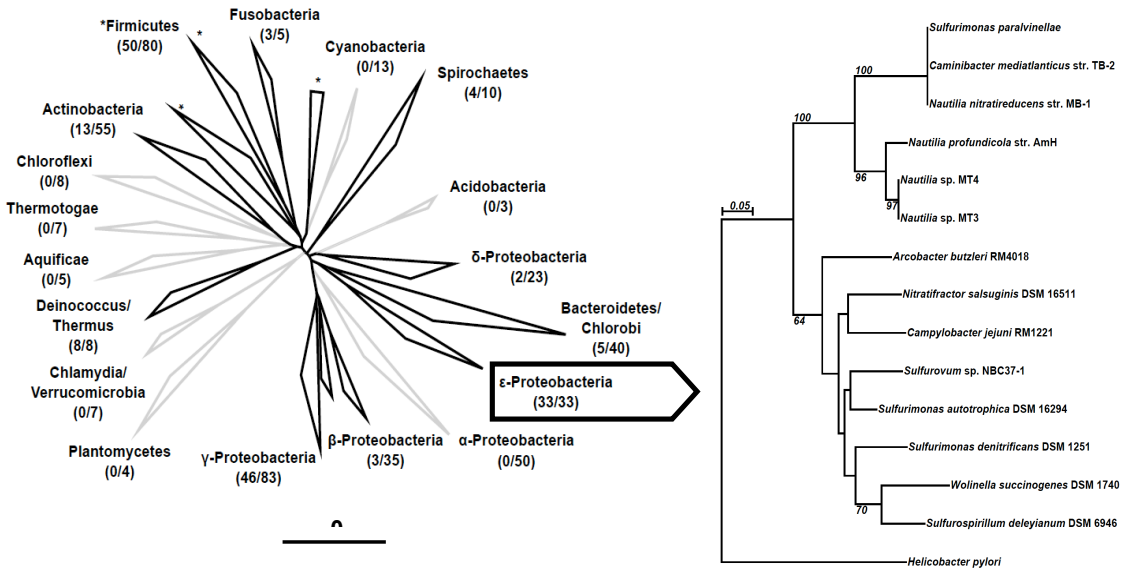

Figure 1.7 (a) Distribution of LuxS among phyla of the domain Bacteria. An initial Blast search for *Vibrio harveyi* and *Salmonella enterica serovar Typhimurium* LuxS was performed in complete bacterial genomes, followed by a Blast search using representative LuxS obtained from each major group shown. LuxS homologs were indentified in 167 *Beta-*, *Delta-*, *Gamma-* and *Epsilonproteobacteria, Firmicutes, Fusobacteria, Actinobacteria, Deinococcus/Thermus, Spirochaetes* and *Bacteroidetes/Chlorobi* (shown in black). The number of hits within respective phyla are indicated, followed by the total number of genomes surveyed. The tree was constructed using the ARB neighbor joining method from 16S ribosomal RNAs of the total 469 bacterial species surveyed. The bar represents 0.10 base substitutions per nucleotide. (b) Phylogenetic tree of *luxS* homologous amino acid sequence found in the genome of several *Epsilonproteobacteria*.

However, several studies have questioned the validity of the AI-2 QS system, arguing that the pivotal role of LuxS in the Activated Methyl Cycle (AMC; the major source of methyl groups in the cell), explains its wide distribution (Figure 1.8) (Turovskiy *et al.*, 2007; Vendeville *et al.*, 2005). Nevertheless, the importance of the LuxS/AI-2 QS system for the coordination of virulence induction, biofilm formation and bioluminescence has been well established in many microorganisms (Bassler *et al.*, 1994; Chung *et al.*, 2001;

Forsyth & Cover, 2000; McNab *et al.*, 2003; Rhee *et al.*, 2003; Shimizu *et al.*, 2002; Stevenson & Babb, 2002; Winzer *et al.*, 2002; Wright*, et al.*, 2000)**.**

Because LuxS bears the unique feature of contributing both to cellular metabolism through the activated methyl cycle and to the regulation of gene expression via QS, AI-2 synthesis could theoretically gauge not only the density of a population but also its metabolic state (Bassler, 2002). In deep-sea vent environments chemical oxidation and reduction represent competitive processes for the microbial communities; QS could provide important insights into bacterial adaptations to overcome competition.

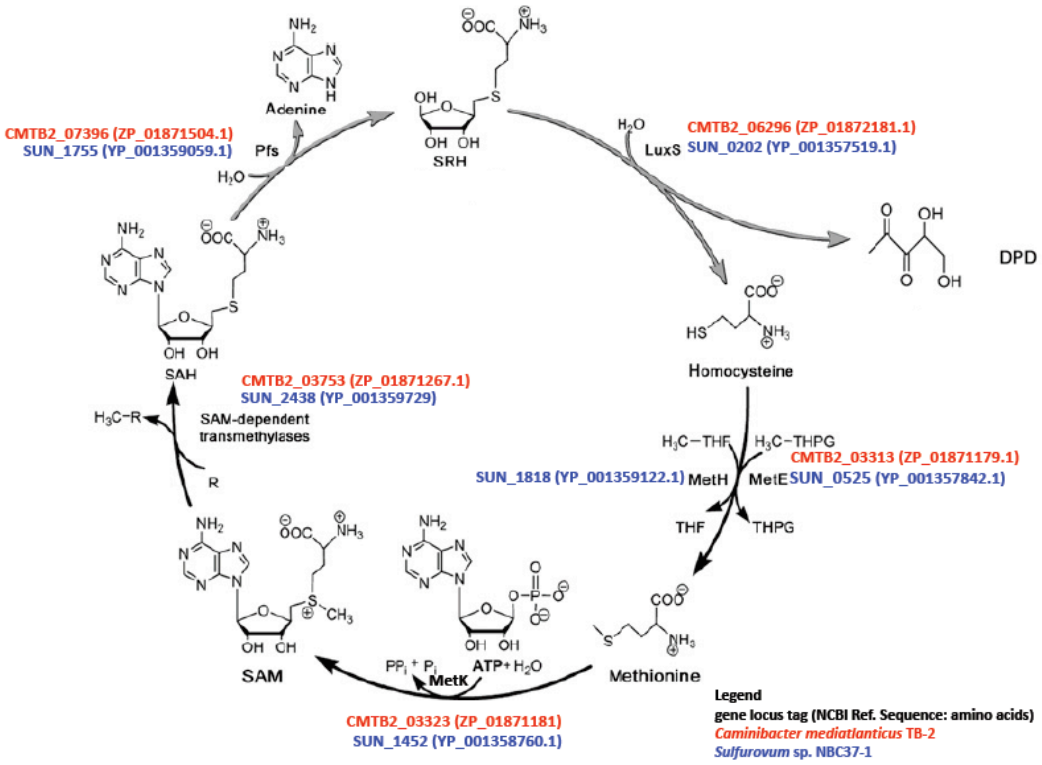

Figure 1.8 Genes involved in the Activated Methyl Cycle in *Caminibacter mediatlanticus* (red) and *Sulfurovum litthotrophicum* (blue). The activated methyl group (CH_3) of SAM is used for methylation of RNA, DNA, certain metabolites and proteins, leading to the formation of the toxic metabolite S-adenosylhomocysteine (SAH). SAH is then removed and the cycle completed by one of two routes, depending on the organism. One route involves the one-step conversion of SAH to HCY by SAH hydrolase (SahH), the other requires the production of S-ribosylhomocysteine (SRH) by Pfs and then the generation of HCY from this by LuxS, which simultaneously generates 4,5-dihydroxy-2,3-pentanedione (DPD). Cyclic derivatives of DPD are generated spontaneously, some of which include the Autoinducer-2 type molecules (Chen *et al.*, 2002; Miller *et al.*, 2004; Nedvidek *et al.*, 1992; Vendeville *et al.*, 2005; Winzer *et al.*, 2003). Image modified from (Turovskiy *et al.*, 2007)

Study scope and objectives

The work presented in this dissertation has been motivated by the desire of understanding the large but often underappreciated role that chemosynthetic microorganisms play in the transfer of energy and carbon from the ocean crust to the ocean basins, making them central players to global ocean biogeochemical cycles. More specifically, the objectives of this study are: 1. to expand our understanding of the phylogenetic and functional diversity of anaerobic chemosynthetic bacteria associated with hydrothermal vent systems; 2. to explore the role of these bacteria in chemosynthetic biofilms. To this end, we have carried out the isolation and characterization of novel bacterial strains, and we have used *Caminibacter mediatlanticus* and *Sulfurovum lithotrophicum* (two cultured chemosynthetic *Epsilonproteobacteria* whose genomes have been sequenced) as model systems to investigate the *luxS*-based QS system.

Gaining information on the physiology and metabolic properties of described chemosynthetic bacteria from deep-sea hydrothermal vent environments is essential to our understanding of the characteristcs of these natural microbial communities. Culture independent analyses are only informative when the phylogenetic position of environmental sequences are in the vincity of known isolates with known metabolic properties. Only then, we can obtain hints toward the physiological potential of environmental "phylotypes" (Jannasch, 1994). Moreover, cultured chemosynthetic representatives are important tools in the laboratory that can be used as model systems to explore ecological and evolutionary questions, which would be impossible otherwise in such an inaccessible environment.

Chapter 2

Nautilia nitratireducens sp. nov, a thermophilic, anaerobic, chemosynthetic, nitrate ammonifying bacterium isolated from a deep-sea hydrothermal vent on the East Pacific Rise.

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Introduction

In deep-sea hydrothermal vents, primary productivity relies on microorganisms that use different metabolic strategies to convert chemical energy into biochemical energy and fix carbon dioxide. It has been found that *Epsilonproteobacteria* are abundant in deep-sea hydrothermal vent systems and contribute substantially to the primary productivity of these ecosystems (reviewed in Campbell *et al.*, 2006). Two orders are currently described within the class *Epsilonproteobacteria*: *Nautiliales* and *Campylobacterales* (Garrity *et al.*, 2005; Miroshnichenko *et al.*, 2004). The order *Nautiliales* comprises the genera *Caminibacter*, *Nautlilia*, and *Lebetimonas* (Alain *et al.*, 2002; Miroshnichenko *et al.*, 2002; Takai *et al.*, 2005), which include hydrogen-oxidizing bacteria isolated from deep-sea hydrothermal vents. These bacteria have been found in association with invertebrates, chimney edifices or *in-situ* colonization devices. At present, the genus *Nautilia* is composed of three anaerobic, thermophilic chemolithotrophic species: *Nautilia lithotrophica* (Miroshnichenko *et al.*, 2002), *Nautilia profundicola* (Smith *et al.*, 2008) and *Nautilia abyssi* (Alain *et al.*, 2009). In this study, we describe a novel thermophilic, chemosynthetic, strictly anaerobic, nitrate ammonifying *Epsilonproteobacterium* that was isolated from a deep-sea hydrothermal vent on the East Pacific Rise (EPR) at 9° North.

Material and methods

Fragments of active, high-temperature, black smoker chimneys were collected from the L-vent field (fluid temperature: 346°C) on the EPR at depth of 2,523 m during R/V Atlantis cruise AT 15-6 (July, 2006). 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. Enrichment culture for thermophilic, chemolithoautotrophic organisms were obtained by inoculating 10 ml of modified SME medium (Stetter *et al.*, 1983; Vetriani *et al.*, 2004) supplemented with 10 % w/v nitrate and under H_2/CO_2

(80:20; 200 kPa), with 1 g of the black smoker chimney sample resuspended in 1 ml of anaerobic artificial seawater. The primary enrichments were incubated shipboard at 28 °C. Aliquots (0.1 ml) of the original cultures were subsequently transferred to fresh medium back in the laboratory, and pure cultures were isolated by three consecutive series of dilutions to extinction followed by isolation of single colonies on plates containing SME medium solidified with 1 g l^{-1} Phytagel (Sigma). Plates were incubated in an anaerobic jar (Oxoid) pressurized with H_2/CO_2 (80:20; 200 kPa). During the isolation procedures the cultures were incubated at 35 °C.

Long term stocks of the new isolate were prepared by adding 50 μl DSMO (Fisher Scientific) to 1 ml culture and were stored at –80 °C. Cells were routinely stained in 0.1 % acridine orange and visualized with an Olympus BX 60 microscope with an oil immersion objective (UPlanFl 100/1.3). Transmission electron micrographs were obtained as previously described (Vetriani, *et al.*, 2004). Growth rates (μ ; h^{-1}) were estimated as μ = (ln N2-ln N1)/(t2-t1), where N2 and N1 are numbers of cell ml^{-1} at times (in h) t2 and t1. Generation times (tg; h) were calculated as tg= (ln2)/ μ . All growth experiments were carried out in duplicate in modified SME medium supplemented with 10 % w/v nitrate under H_2/CO_2 gas phase (80:20; 200 kPa), unless stated otherwise. Quantitative determinations of nitrate, nitrite and ammonium were carried out spectrophotometrically using a Lachat QuickChem automated ion analyzer according to the manufacturer's specifications (Diamond, 1993; Diamond, 1993). Qualitative determination of hydrogen sulfide was carried out as previously described (Vetriani *et al.*, 2004).

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. Catalase activity was detected by the formation of gas bubbles after concentrated cells were resuspended in 70 μl of a 3 % solution of H_2O_2 at room temperature.

The effect of organic substrates on the growth of strain MB-1^T was investigated by adding the following substrates to the medium under an H_2/CO_2 (80:20; 200 kPa) gas phase: lactate, peptone, tryptone, acetate, formate, Casamino acids, D(+)-glucose, sucrose, fructose, galactose (each at 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: N_2/CO_2 (80:20; 200 kPa), N_2 (100 %; 200 kPa) or H_2 (100 %; 200 kPa). The ability of MB-1^T to use alternative electron acceptors was tested by adding thiosulfate (4 mM), sulfate (7 mM), sulfite (4.1 mM), arsenate (5 mM), selenate (5 mM), sulfur (3 % w/v) and oxygen (0.5 %, v/v) to nitrate depleted medium.

Genomic DNA was extracted from cells of strain MB-1^T by using the UltraClean microbial DNA isolation kit (MoBio). The 16S rRNA gene was selectively amplified from the genomic DNA by PCR and sequenced as described previously (Vetriani *et al.*, 1999; Vetriani *et al.*, 2004). Sequences were aligned automatically using CLUSTAL X and the aligment was manually refined using SEAVIEW (Galtier *et al.*, 1996; Thompson *et al.*, 1997). Neighbor-joining trees were constructed by using the least-squares algorithm of De Soete from a normal evolutionary distance matrix, using Phylo_Win (DeSoete, 1983; Perriere & Gouy, 1996). Approximately 1227 homologous nucleotides were included in the analysis, and 500 bootstrap replicates were carried out to provide confidence estimates for phylogenetic tree topologies. The DNA G + C content of MB-1^T was determined by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH, Braunschweig, Germany) by HPLC analysis of deoxyribonucleosides as described by Mesbah, *et al.* 1989. The genomic DNA G + C content of strain MB-1^T was determined by HPLC analysis of the deoxyribonucleosides.

Results

The pure culture obtained was designated as strain MB-1^T. The optimal growth temperature for strain MB-1^T was determined by incubating cultures between 25 and 75 °C (at 5 °C intervals). Strain MB-1^T grew at temperatures between 25 and 65 °C, with optimal growth at 55 °C. No growth was observed at 20 or 75 °C (Figure 2.1a). All subsequent experiments were carried out at 55 °C. The optimal salt requirement was determined by varying the concentration of NaCl between 5 and 45 g l^{-1}, at 5 g l^{-1} intervals. Strain MB-1^T grew at NaCl concentrations between 10 and 35 g l^{-1} with optimal growth at 20 g l^{-1} (no growth was observed at 5 and at 40 g l^{-1}; Figure 2.1b). The optimal pH for growth was determined as previously described (Voordeckers *et al.*, 2005). Growth of strain MB-1^T occurred between pH 4.5 and pH 8.5, with

an optimum at pH 7.0 (Figure 2.1c). Under optimal conditions, the generation time of isolate MB-1^T was 45.6 min. MB-1^T was a strictly anaerobic, chemolithoautotrophic bacterium that used nitrate, hydrogen and carbon dioxide as the terminal electron acceptor, electron donor and carbon source, respectively. Under these conditions, nitrate was reduced to ammonium in stochiometric amounts, and nitrite did not accumulate in the culture medium (Figure 2.2).

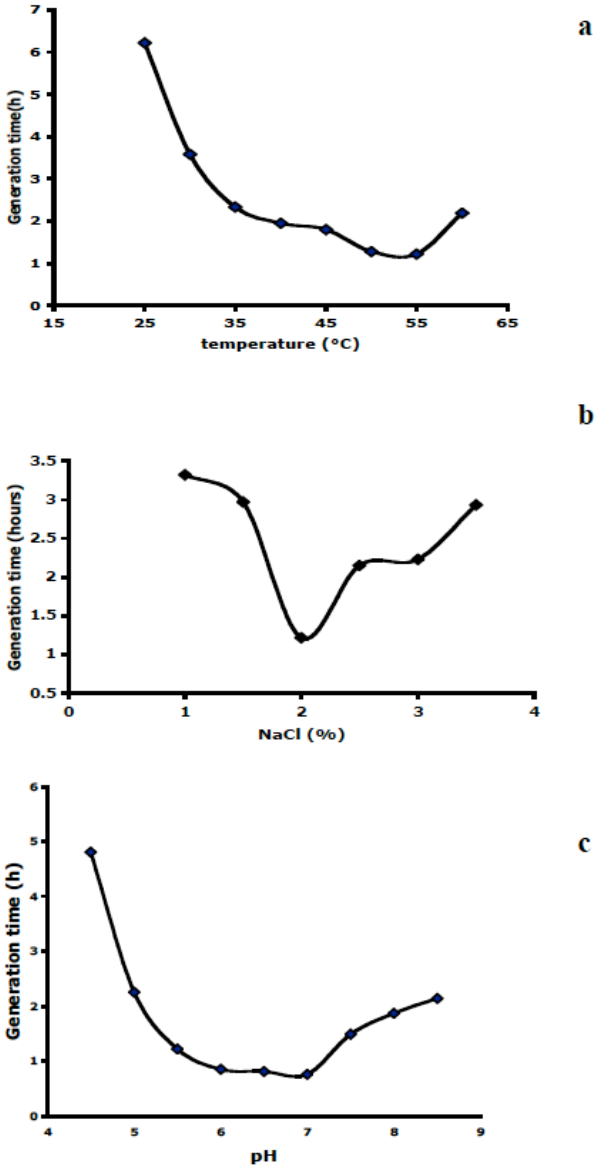

Figure 2.1 Generation time (in h) of *Nautilia nitratireducens* sp. nov. MB-1^T under different growth conditions. (a) Temperature; (b) NaCl concentration; (c) pH.

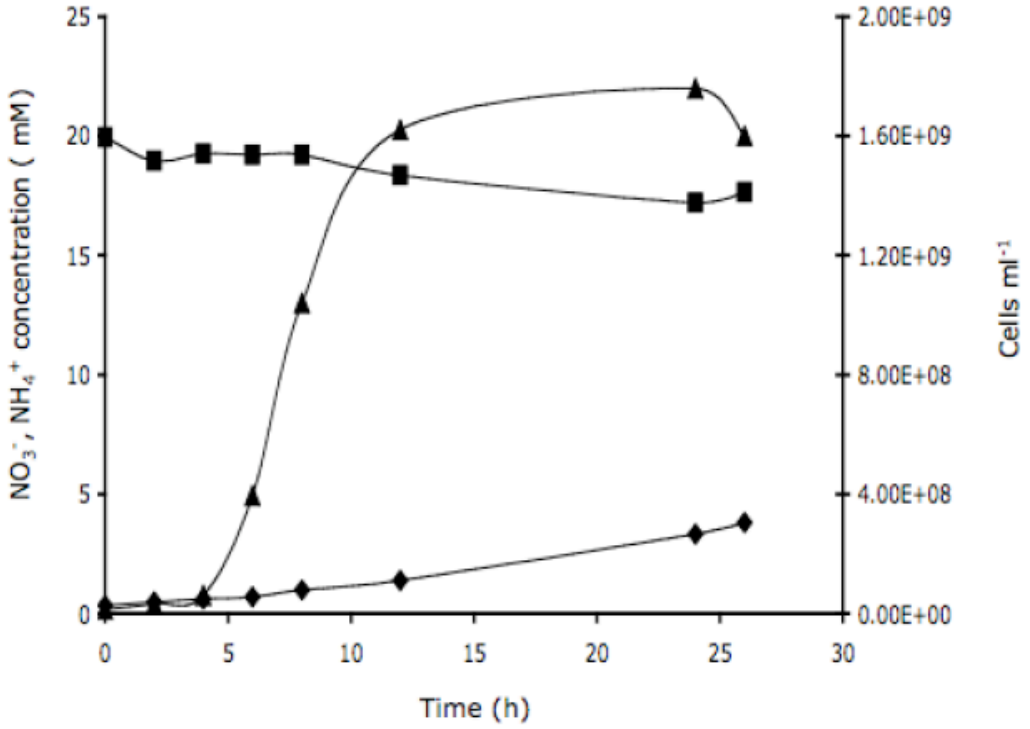

Figure 2.2 Nitrate consumption and ammonium formation during growth of *Nautilia nitratireducens* sp. nov. MB-1^T. ▲, growth curve; ■, nitrate utilization; ◆, ammonium production

Strain MB-1^T did not grow when oxygen, arsenate, sulfate or sulfite were used as electron acceptors. However, MB-1^T was able to grow when selenate, elemental sulfur or thiosulfate were used as electron acceptors. Generation times under these conditions were 3.8 h, 6.0 h and 99 min, respectively. Under an H_2/CO_2 gas phase, no growth of MB-1^T occurred in the presence of lactate, peptone and tryptone, while sub-optimal growth occurred in the presence of acetate and formate (in the latter cases the generation time was several hours). No inhibition of growth was observed under a H_2/CO_2 (80: 20; 200Pka) gas phase in the presence of casamino acids, glucose, sucrose, fructose, galactose and yeast extract (0.1 and 1.0g l^{-1}). MB-1^T grew in the presence of acetate, formate, casamino acids, sucrose, galactose and yeast extract (0.1 g l^-

[1]) under a N_2/CO_2 gas phase, indicating that the strain could use these substrates as electron donors besides H_2. However, under a N_2 gas phase, strain MB-1^T grew only in the presence of formate, indicating that the strain could only use this substrate and CO_2 as carbon sources. Strain MB-1^T was inhibited by chloramphenicol, ampicillin, streptomycin and kanamycin, and exhibited catalase activity.

MB-1^T cells were short rods, approximately 1-1.5 μ m in length and 0.3-0.5 μ m in width, and divided by constriction (Figure 2.3). Cells stained Gram-negative. The organism was motile and possessed one or more polar flagella, which were observed in electron micrographs of platinum-shadowed cells (Fig. 1b). The presence of endospores was not observed.

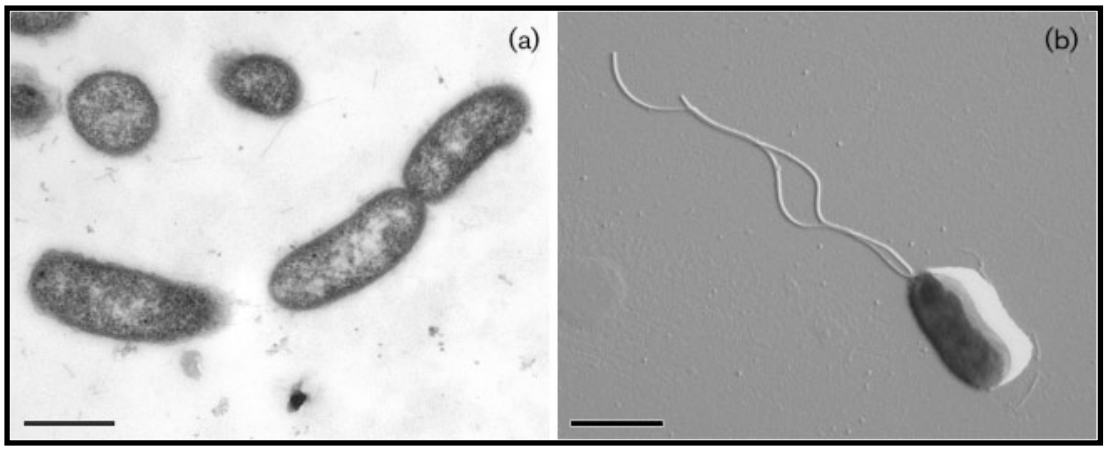

Figure 2.3 Electron micrographs of strain MB-1^T; thin section of cells showing cell morphology and division by constriction (a) and platinum-shadowed cell showing multiple polar flagella (b). Bars, 0.5 μm (a) and 1.0 μm (b)

Phylogenetic analysis of the 16S rRNA gene sequence placed strain MB-1^T within the class '*Epsilonproteobacteria*' (Figure 2.4). Strain MB-1^T was placed in a discrete cluster in the genus *Nautilia*, and its next closest cultured relatives were *Nautilia profundicola*, *Nautilia abyssi* and *Nautilia lithotrophica* (95%, 94% and 93 % sequence identity, respectively). The genomic DNA G + C content of strain MB-1^T was 36.0 mol%. Strain MB-1^T was assigned to the genus *Nautilia*, although this organism could be differentiated from the previously described *Nautilia* species by means of several physiological characteristics (Table 2.1). Strain

MB-1^T could be distinguished from all other *Nautilia* species by a lower optimum salinity and its inability to growth in concentrations greater than 35 g l^{-1} NaCl. Furthermore, strain MB-1^T had the widest temperature range among the *Nautilia* spp., and it is the only *Nautilia* sp. able to grow at a pH of 4.5. Strain MB-1^T also differs from the other *Nautilia* spp. for its ability to use nitrate or thiosulfate as terminal electron acceptors. The generation time of strain MB-1^T using sulfur as terminal electron acceptor was the same as *N. profundicola* (6 h), and longer than *N. lithotrophica* (140 min) and *N. abyssi* (120 min), respectively. However, under optimal conditions (with nitrate as the terminal electron acceptor) strain MB-1^T had a generation time shorter than all other *Nautilia* species (45.6 min). Physiological, phylogenetic and genetic analyses indicated that strain MB-1^T is not related to *N. lithotrophica*, *N. profundicola* or *N. abyssi* at the species level, and therefore strain MB-1^T represents a novel species within the genus *Nautilia*, for which we propose the name *Nautilia nitratireducens*. Recently, *Epsilonproteobacteria* have been recognized to play a significant role in the ecology of deep-sea hydrothermal vents and other sulfidic environments (reviewed inCampbell *et al.*, 2006). The physiological and metabolic versatility of *N. nitratireducens* strain MB-1^T, including the wide temperature, pH and salinity growth ranges, and its ability to utilize various terminal electron acceptors (nitrate, sulfur, thiosulfate and selenate) may contribute to its adaptability to the steep physico-chemical gradients found at deep-sea vents.

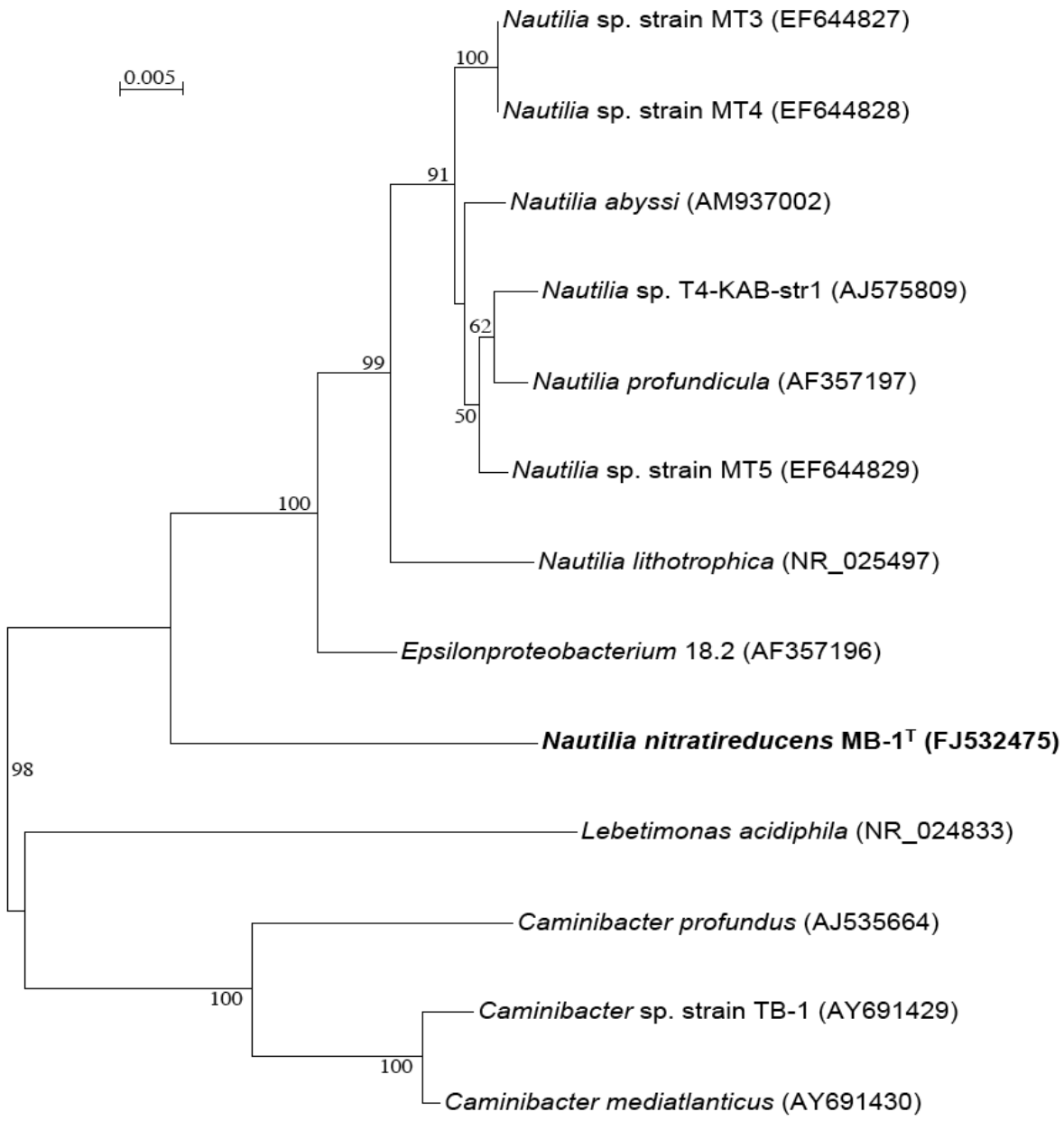

Figure 2.4 Phylogenetic position of strain MB-1^T based on 16S rRNA gene sequences in a neighbour-joining tree. Bootstrap values (>50%) based on 500 replications are shown as percentages at branch nodes. Bar, 0.5 substitutions per nucleotide position.

Characteristic	1	2	3	4
Catalase	+	+	ND	ND
Temperature for growth (°C)				
Range	25–65	30–55	33–65	37–68
Optimum	55	40	60	53
NaCl concentration for growth (g l^{-1})				
Range	10–35	20–50	20–40	8–50
Optimum	20	30	30	30
pH for growth				
Range	4.5–8.5	6.0–9.0	5.0–8.0	6.4–7.4
Optimum	7.0	7.0	6.0–6.5	6.8–7.0
Terminal electron acceptors				
Nitrate	+	−	−	−
Thiosulfate	+	−	−	−
Selenate	+	ND	ND	ND
Electron donors				
Acetate	+	ND	−	−
Formate	+	+	−	+
Yeast extract	+ (0.1 g l^{-1})	ND	−	−
Heterotrophic growth	−	−	+	−
Growth with formate as carbon source	+	+	−	+
Shortest generation time (h)	0.75	6	2	1.2
DNA G+C content (mol%)	36.0	33.5	35	34.7

Table 2.1 Differentiating features of strain MB-1^T and the type strains of *N.profundicola*, *N. abyssi* and *N. lithotrophica*.

Strains: 1, *Nautilia nitratireducens* sp. nov. MB-1^T (data from this study); *N. profundicola* AmH^T (Smith *et al.*, 2008; Stetter, 1995); *N. abyssi* PH1209^T (Alain *et al.*, 2009); *N. lithotrophica* 525^T (Miroshnichenko *et al.*, 2002). +, growth; -, no growth; ND, no data available.

Description of *Nautilia nitratireducens* sp. nov. *Nautilia nitratireducens* (ni.tra.ti.re.du.cens. N.L. n. *nitras –atis*, nitrate; N.L. part. adj. *reducens*, converting to a different condition, reducing; N.L. adj. *nitratireducens*, reducing nitrate). Cells are rod-shaped (1–1.5 μm long, 0.3–0.5 μm wide). Motile by means of one or more flagella. Obligate anaerobe. Gram-negative. Catalase positive. Growth occurs between 25 and 65°C, 10 and 35 g l-1 NaCl-1 and pH 4.5 and 8.5. Optimal growth conditions are 55°C, 20 g l-1 NaCl and pH 7.0 (shortest generation time 45.6 min). Growth occurs under strictly anaerobic, chemolithoautotrophic conditions in the presence of H_2 and CO_2 with nitrate, sulfur, selenate and thiosulfate as terminal electron acceptors. The following are not utilized as electron acceptors: oxygen, arsenate, sulfate and sulfite. No growth occurs in the presence of lactate, peptone and tryptone. Acetate, casamino acids, formate, sucrose, galactose (each at 2 g l^{-1}) and yeast extract (0.1 g l^{-1}) are used as energy source under a N_2/CO_2 gas phase. Formate is utilized as a carbon source. Sensitive to chloramphenicol, ampicillin, streptomycin and kanamycin (each at 100 mg ml^{-1}). Genomic DNA G + C content is 36.0 mol%. The type strain is MB-1^T (= DSM 22087^T = JCM 15746^T), which was isolated from the walls of an active deep-sea hydrothermal vent on the East Pacific Rise at 9° 50' N, 104°17' W.

Chapter 3

Phorcys thermohydrogeniphilus gen. nov., sp. nov., a thermophilic, chemolithoautotrophic, nitrate-ammonifying bacterium isolated from a microbial biofilm from a deep-sea hydrothermal vent .

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Introduction

The phylum *Aquificae* is generally considered, based on phylogenetic analyses of rRNA genes and concatenated monophyletic protein genes, to be one of the deepest and earliest branching groups within the *Bacteria* (Battistuzzi *et al.*, 2004; Reysenbach, 2001). Cultivated representatives of the phylum *Aquificae* are thermophilic or hyperthermophilic bacteria isolated from marine and continental geothermal environments (Reysenbach, 2001). This phylum includes three families (*Aquificaceae*, *Desulfurobacteriaceae* and *Hydrogenothermaceae*) within the single order *Aquificales* (Burggraf *et al.*, 1992; L'Haridon *et al.*, 2006; Reysenbach, 2001; Reysenbach, 2001). The family *Aquificaceae* is composed of five genera, *Hydrogenobacter*, *Aquifex*, *Thermocrinis*, *Hydrogenobaculum* and *Hydrogenivirga* (Huber *et al.*, 1998; Huber *et al.*, 1992; Kawasumi *et al.*, 1984; Nakagawa, 2004; Stohr *et al.*, 2001). The family *Hydrogenothermaceae* (Eder & Huber, 2002) is formed by the genera *Hydrogenothermus*, *Persephonella* and *Sulfurihydrogenibium* (Gotz *et al.*, 2002; Stohr, *et al.*, 2001; Takai *et al.*, 2003). The family *Desulfurobacteriaceae* (L'Haridon *et al.*, 2006) includes the genera *Desulfurobacterium*, *Thermovibrio* and *Balnearium* (Huber *et al.*, 2002; L'Haridon *et al.*, 1998; L'Haridon *et al.*, 2006). Here, we report the isolation and characterization of a strain that represents a novel lineage within the *Desulfurobacteriaceae*. This strain is a thermophilic, chemolithoautotrophic, strictly anaerobic, hydrogen-oxidizing bacterium that was isolated from an active deep-sea hydrothermal vent on the East Pacific Rise.

Materials and Methods

Samples of microbial biofilms were collected during R/V *Atlantis* cruise AT 15-28 from an experimental microbial colonization device that was deployed on the wall of a diffuse flow venting sulfide structure colonized by the tubeworm *Alvinella pompejana* ("Jumeaux" site, depth 2621 m, East Pacific Rise, 12° 48 N, 103° 56 W). The experimental microbial colonization device was deployed using the manipulator

of the DSV *Alvin*, collected three days later, and stored in a box on the submersible's working platform for the rest of the dive. At the time of recovery, white microbial biofilms covered the colonization device and a few tubes of the tubeworm *Alvinella pompejana* that were associated with the device. Subsamples were transferred to the ship's laboratory and stored at 4 °C under a dinitrogen atmosphere. Primary enrichment cultures were initiated shipboard by inoculating a fragment of biofilm-containing *A. pompejana* tube in 10 ml of modified SME medium (Stetter *et al.*, 1983). The primary enrichments were incubated shipboard at 75 °C. Back in the laboratory, aliquots (0.1 ml) of the original cultures were subsequently transferred to fresh medium and pure cultures were isolated by several consecutive series of dilutions to extinction. During the isolation procedures the cultures were incubated at 75 °C. Long-term stocks were prepared by adding 50 µl DSMO (Fisher Scientific) to 1 ml culture and were stored at –80 °C.

Cells were routinely stained in 0.1 % acridine orange and visualized with an Olympus BX 60 microscope with an oil immersion objective (UPlanFl 100/1.3). Transmission electron micrographs were obtained as previously described (Vetriani *et al.*, 2004). Biofilms samples were collected from the gas/liquid interphase of batch cultures, fixed in Trump's EM fixative, washed in 0.2 M cacodylate buffer (EMS, Hatfield, PA), dehydrated in ethanol, subjected to critical point drying and sputter coating with Au/Pd, and visualized on a AMRAY -1830I Scanning Electron Microscope (AMRAY Inc., Bedford MA).

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 numbers of cell ml^{-1} at times (in h) t_2 and t_1, respectively. Generation times (t_g; h) were calculated as $t_g = (\ln 2)/\mu$. All growth experiments were carried out in duplicate in modified SME medium supplemented with 10% (w/v) nitrate under H_2/CO_2 unless stated otherwise. Quantitative determinations of nitrate, nitrite and ammonium were carried out spectrophotometrically using a Lachat QuickChem automated ion analyzer according to the manufacturer's specifications (Diamond, 1993; Diamond, 1993)

The optimal growth temperature for strain HB-8^T was determined by incubating cultures at temperatures between 60 and 85 °C (at 5 °C intervals). All other experiments were carried out at 75 °C. The optimal salt requirement was determined by varying the concentration of NaCl between 5 and 45 g l^{-1}, at 5 g l^{-1} intervals. The optimal pH for growth was determined as previously described (Perez-Rodriguez *et*

al., 2010). 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 75 °C and an ethanol control was performed for chloramphenicol. Catalase activity was detected by the formation of gas bubbles after concentrated cells were resuspended in 70 μl of a 3 % solution of H_2O_2 at room temperature.

The effect of organic substrates on the growth of strain HB-8^T was investigated by adding the following substrates to the medium under an H_2/CO_2 gas phase (80:20; 200 kPa): acetate, formate, lactate, peptone, tryptone, Casamino acids, D(+)-glucose, sucrose (all at 2 g l^{-1}) and yeast extract (0.1 and 1 g l^{-1}). These same compounds were tested under N_2/CO_2 (80:20; 200 kPa), H_2 (100 %; 200 kPa) or N_2 (100 %; 200 kPa) gas phase, for their possible use as electron and/or carbon sources. The ability of strain HB-8^T to use alternative electron acceptors was tested by adding 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.

Genomic DNA was extracted from cells of strain HB-8^T, and both the 16S rRNA and a 909 bp fragment of the *aclA* (encoding the alpha subunit of the ATP citrate lyase) genes were selectively amplified from the genomic DNA by PCR and sequenced as described previously (Vetriani *et al.*, 1999; Vetriani *et al.*, 2004). Sequences were aligned automatically using CLUSTAL X, the aligment was manually refined using SEAVIEW (Galtier *et al.*, 1996; Thompson *et al.*, 1997). Neighbor-joining trees were constructed as previously described (Perez-Rodriguez *et al.*, 2010). Maximum likelihood trees were constructed using PhylML (Gouy *et al.*, 2010) and the Jukes and Cantor nucleotide substitution model for the 16S rRNA (Jukes & Cantor, 1969), or the LG amino acid substitution model for the ATP citrate lyase (Le & Gascuel, 2008). Pairwise nucleotide similarity values were calculated using the EzTaxon web-based tool (www.eztaxon.org). The DNA G + C content of HB-8^T was determined by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH, Braunschweig, Germany) by HPLC analysis of deoxyribonucleosides as described by (Mesbah *et al.*, 1989).

Chemotaxonomic analyses of strain HB-8^T, which included cellular fatty acid composition, polar lipids and respiratory quinones, were carried out by the Identification Service and Dr. Brian Tindall (DSMZ,

Braunschweig, Germany) on 200 mg of freeze-dried cells grown under optimal culture conditions by the authors.

Results

The pure culture obtained using this procedure was designated strain HB-8^T. HB-8^T cells were rod-shaped, approximately 1-1.5 μm in length and 0.5 μm in width, and divided by constriction (Figure 3.1a). Cells stained Gram-negative. The presence of polar flagella was observed in electron micrographs of platinum-shadowed cells (Figure 3.1b). Batch cultures of strain HB-8^T formed a 5 μm thick biofilm at the gas/liquid interphase (Figure 3.1c), and the presence of endospores was not observed.

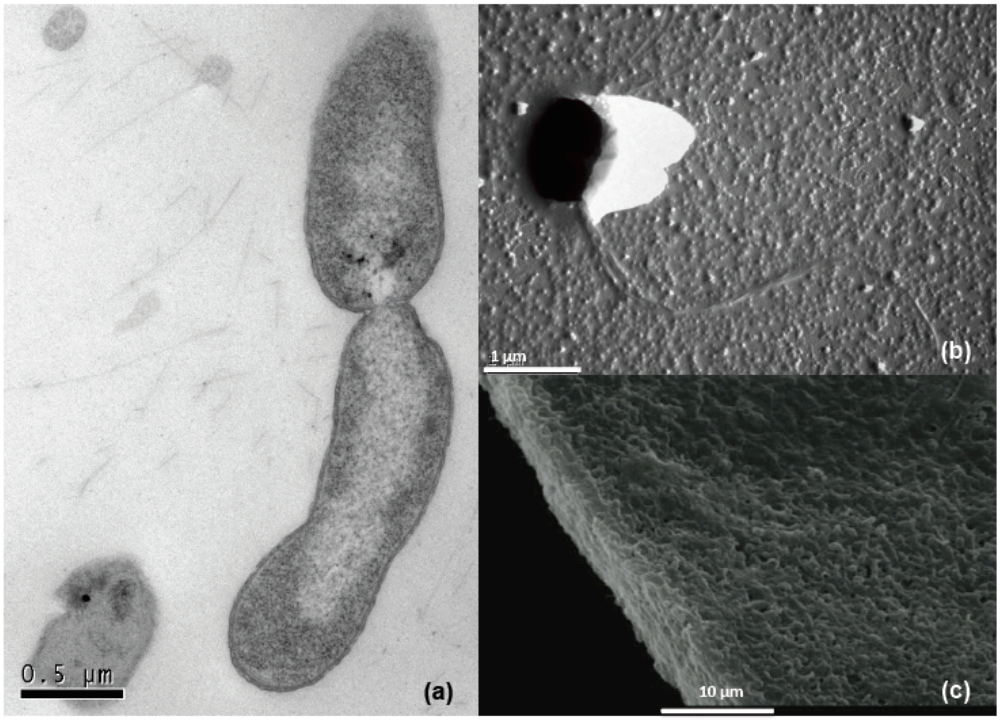


Figure 3.1 (a) Electron micrograph of strain HB-8^T; thin section of cells showing cell morphology and division by constriction (a), platinum-shadowed cell showing multiple polar flagella (b), and scanning electron micrograph of biofilm forming at the gas-liquid interphase (c). Bars, 0.5 μm.

(b) Electron micrograph of platinum-shadowed HB-8^{T}cell, showing multiple polar flagella. Bars 0.5 μm (a), 1.0 μm (b) and 10 μm (c).

Strain HB-8^T grew at temperatures between 65 and 80 °C, with optimal growth at 75 °C. No growth was detected at 60 or 85 °C (Figure 3.2a). HB-8^T grew at NaCl concentrations between 15 and 35 g l^{-1} with optimal growth at 30 g l^{-1} (no growth was detected at 10 and at 40 g l^{-1}; Figure 3.2b). Growth of strain HB-8^T occurred between pH 4.5 and pH 8.5, with an optimum at pH 6.0 (Figure 3.2c). Under optimal conditions, the generation time of strain HB-8^T was 26 min. HB-8^T was a strictly anaerobic, chemolithoautotrophic bacterium that used nitrate, hydrogen and carbon dioxide as the primary electron acceptor, electron donor and carbon source, respectively. Time course measurements of nitrate, nitrite, ammonium and concomitant growth under these conditions showed that nitrate was reduced to ammonium, and nitrite did not accumulate in the culture medium (Figure 3.3). Strain HB-8^T was resistant to kanamycin and streptomycin while it was inhibited by chloramphenicol and ampicillin. When exposed to H_2O_2, strain HB-8^T exhibited catalase activity detected by the formation of gas bubbles.

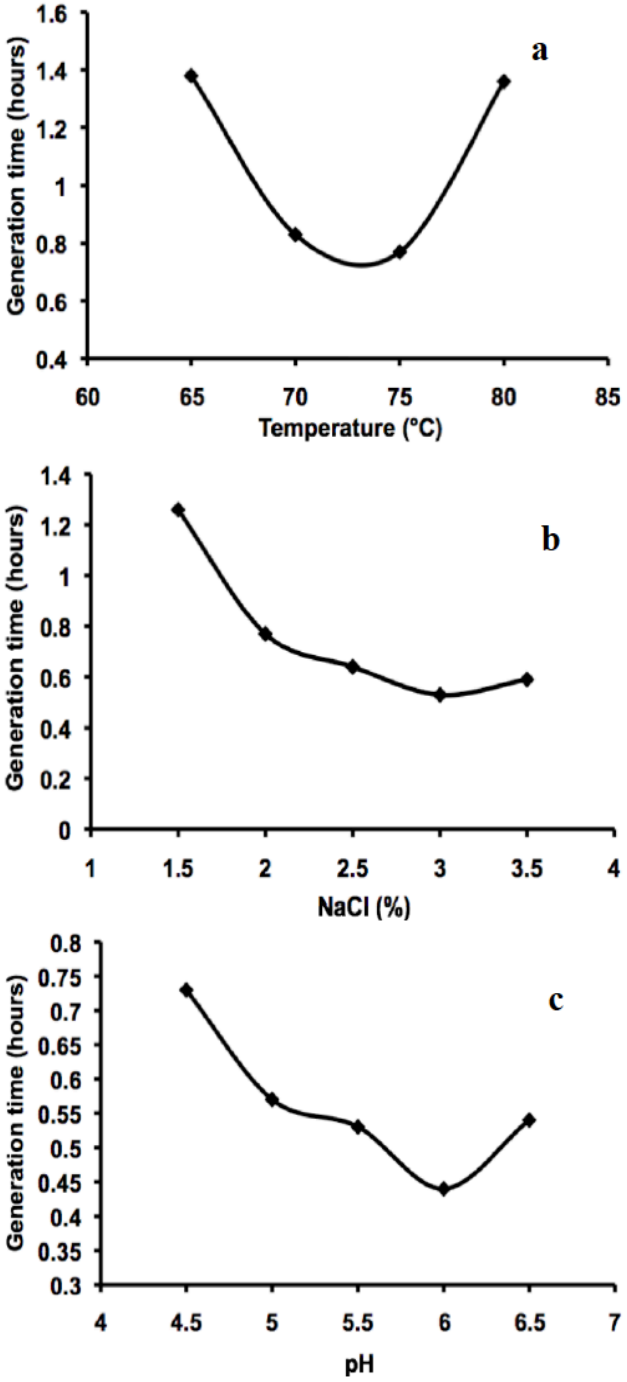


Figure 3.2 Generation time (in h) of *Phorcys thermohydrogenophilus* sp. nov. HB-8^{T} under different growth conditions. (a) Temperature; (b) NaCl concentration; (c) pH.

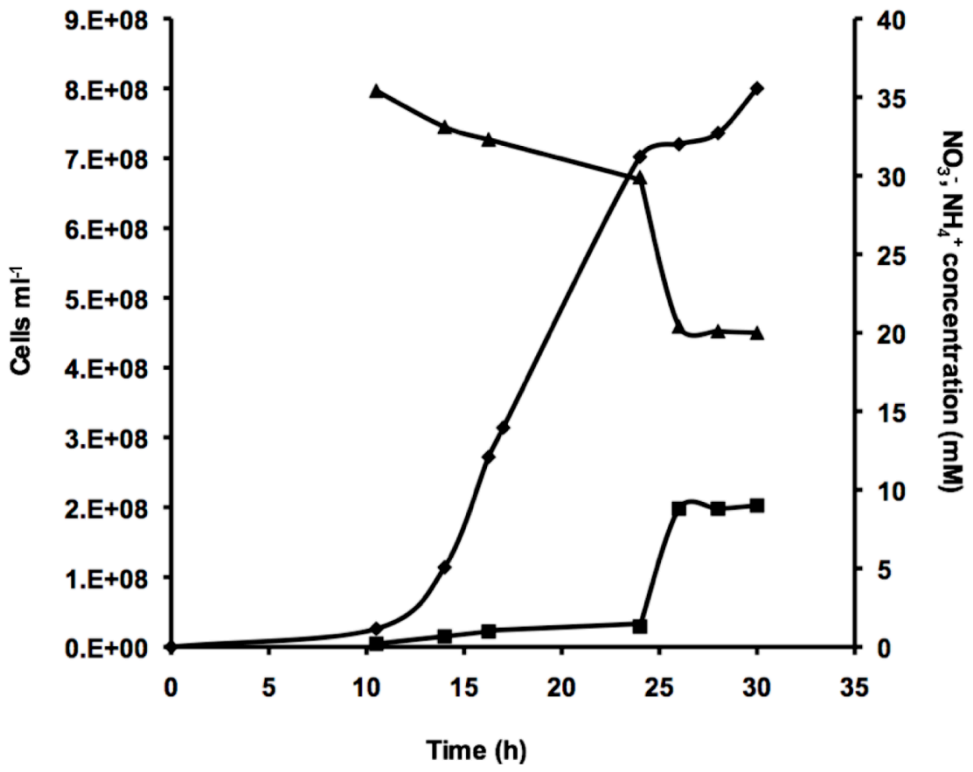


Figure 3.3 Nitrate consumption and ammonium formation during grwoth of *Phorcys thermohydrogenophilus* sp. nov. HB-8^T ◆, growth curve; ▲, nitrate utilization; ■, ammonium production

No Growth of HB-8^T was observed in the presence of lactate (2 g l^{-1}), acetate (2 g l^{-1}), formate (2 g l^{-1}) and tryptone (2 g l^{-1}). Casamino acids (2 g l^{-1}), glucose (2 g l^{-1}), sucrose (2 g l^{-1}) and yeast extract (1.0 and 0.1 g l^{-1}) did not affect growth of HB-8^T under a H_2/CO_2 (80: 20; 200 Pka) gas phase, but no growth was observed in the presence of the same compounds under N_2/CO_2 (80:20; 200 kPa), H_2 (100 %; 200

kPa) or N_2 (100 %; 200 kPa) gas phase, indicating that the organic substrates were not used as electron and/or carbon sources.

Strain HB-8^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. However, HB-8^T was able to grow with elemental sulfur (3 % w/v) as electron acceptor. Under these conditions, the generation time of strain HB- was 8.6 hours, and about 4-10 mM of hydrogen sulfide (measured as previously described by Vetriani, *et al.*, 2004) was produced from the reduction of elemental sulfur.

Phylogenetic analysis of the 16S rRNA gene placed strain HB-8^T within the class '*Aquificae*' (Fig. 3.4). Both maximum-likelihood and neighbor-joining phylogenetic analyses showed that strain HB-8^T represented a novel phylogenetic lineage related to members of the *Desulfurobacteriaceae* family (Figs. 3.4 and 3.5). However, the two methods of phylogenetic inference provided slightly different results: while the maximum-likelihood method of tree reconstruction consistently placed strain HB-8^T within the *Desulfurobacteriaceae* family (Fig. 3.4), the neighbor-joining tree suggested that the novel lineage diverged prior to the differentiation of its closest cultured relatives (Fig. 3.5). In line with the phylogenetic analyses, pairwise nucleotide similarity values indicated that the 16S rRNA gene from strain HB-8^T had a similar level of homology to those from representatives of the three genera of the *Desulfurobacteriaceae*. The similarity values were: 95.5 % to *Thermovibrio ruber* strain ED11/3LLK8, 95.4 % to *Balnearium lithotrophicum* strain 17S and 94.9 % to *Desulfurobacterium thermolithotrophum* strain BSA, all within the range (90-96 %) accepted as an indication of genus-level differentiation (Gillis *et al.*, 2001).

Since members of the *Desulfurobacteriaceae* family are known to fix CO_2 via the reverse citric acid cycle (L'Haridon *et al.*, 2006), we further explored the phylogeny of the ATP citrate lyase of strain HB-8^T, a key enzyme in this cycle. Both maximum-likelihood and neighbor-joining phylogenetic analyses of the amino acid sequence derived from the *aclA* gene consistently placed the ATP citrate lyase of strain HB-8^T on a discrete lineage (Figs. 3.6 and 3.7). When we compared the intra-genus and inter-genera identities of the amino acid sequences of the ATP citrate lyase of members of the *Desulfurobacteriaceae* family, we found that the enzymes of strains within the *Desulfurobacterium* genus were 98 – 99% identical, while the enzymes of *T.*

ruber and *B. lithotrophicum* had a 96 % match. In comparison, the ATP citrate lyase of strain HB-8^T was 89 %, 87 % and 91 % identical to that of *T. ruber*, *B. lithotrophicum* and *D. thermolithotrophum*, respectively, which is well below the observed intra-genus range (98 – 99 %).

The genomic DNA G + C content of strain HB-8^T, determined by HPLC analysis of the deoxyribonucleosides, was 47.8 mol%, which was similar to that of *T. ruber* but higher than that of *B. lithotrophicum and D. thermolithotrophum* (Table 3.1).

Chemotaxonomic analyses of strain HB-8^T, which included cellular fatty acid composition, polar lipids and respiratory quinones, were carried out by the Identification Service and Dr. Brian Tindall (DSMZ, Braunschweig, Germany) on 200 mg of freeze-dried cells grown under optimal culture conditions by the authors. The major cellular fatty acids of strain HB-8^T, analyzed as the methyl ester derivatives (Labrenz *et al.*, 1998), were $C_{18:1w7c}$ (59.1 %) and $C_{18:0}$ (23.7 %), while $C_{16:0}$ and $C_{20:1w7c}$ were present in lower amounts (4.9 % and 5.7 %, respectively). These results show that the overall composition of the fatty acids of strain HB-8^T was comparable to that of other members of the *Desulfurobacteriaceae* family (L'Haridon *et al.*, 2006).

The two predominant polar lipids of strain HB-8^T, identified by their staining behavior on thin layer chromatography plates (Tindall, 1990a; Tindall, 1990b), were phosphatidylethanolamine and phosphatidylglycerol, while minor phospholipids included phosphatidylinositol and phosphatidylaminopentanetetrol. The polar lipid profile of strain HB-8^T differentiated this bacterium from other members of the *Desulfurobacteriaceae* family, whose major polar lipids were shown to be phosphatidylinositol and phosphatidylaminopentanetetrol (L'Haridon *et al.*, 2006).

Analysis of the respiratory lipoquinones of strain HB-8^T by two dimensional thin layer chromatography followed by HPLC of the eluted products (Tindall, 1990a; Tindall, 1990b), revealed that strain HB-8^T synthesized novel quinones, unlike any of known references. The retention times of the quinones of strain HB-8^T were significantly longer than any of the quinones analyzed from members of the *Aquificales* to date (B. J. Tindall, personal communication). This indicates that the isoprenoid side chains of the novel quinones are likely to be longer than those of the quinones previously identified in other members of the *Aquificales*.

When compared to its closest relatives *T. ruber, B. lithotrophicum and D. thermolithotrophum*, strain HB-8^T showed the shortest generation time, the broadest pH range, and the highest mol% DNA G + C content (Table 3.1). Furthermore, strain HB-8^T differed from *B. lithotrophicum* and *D. thermolithotrophum* in its ability to use nitrate and from *D. thermolithotrophum* in its inability to use sulfite and thiosulfate as alternative electron acceptors (Table 3.1). Finally, phylogenetic analyses of both the 16S rRNA gene and of the ATP citrate lyase of strain HB-8^T, along with its unique polar lipids and lipoquinones profiles, reinforce the differences of this strain from the other members of the *Desulfurobacteriaceae* family. Overall, the phylogenetic, physiological and chemotaxonomic characteristics of strain HB-8^T suggest that this bacterium can be classified as a novel genus, for which we propose the name *Phorcys* gen. nov. The type species is *Phorcys thermohydrogeniphilus* sp. nov., with the type strain HB-8^T (=DSM 24425^T =JCM 17384^T).

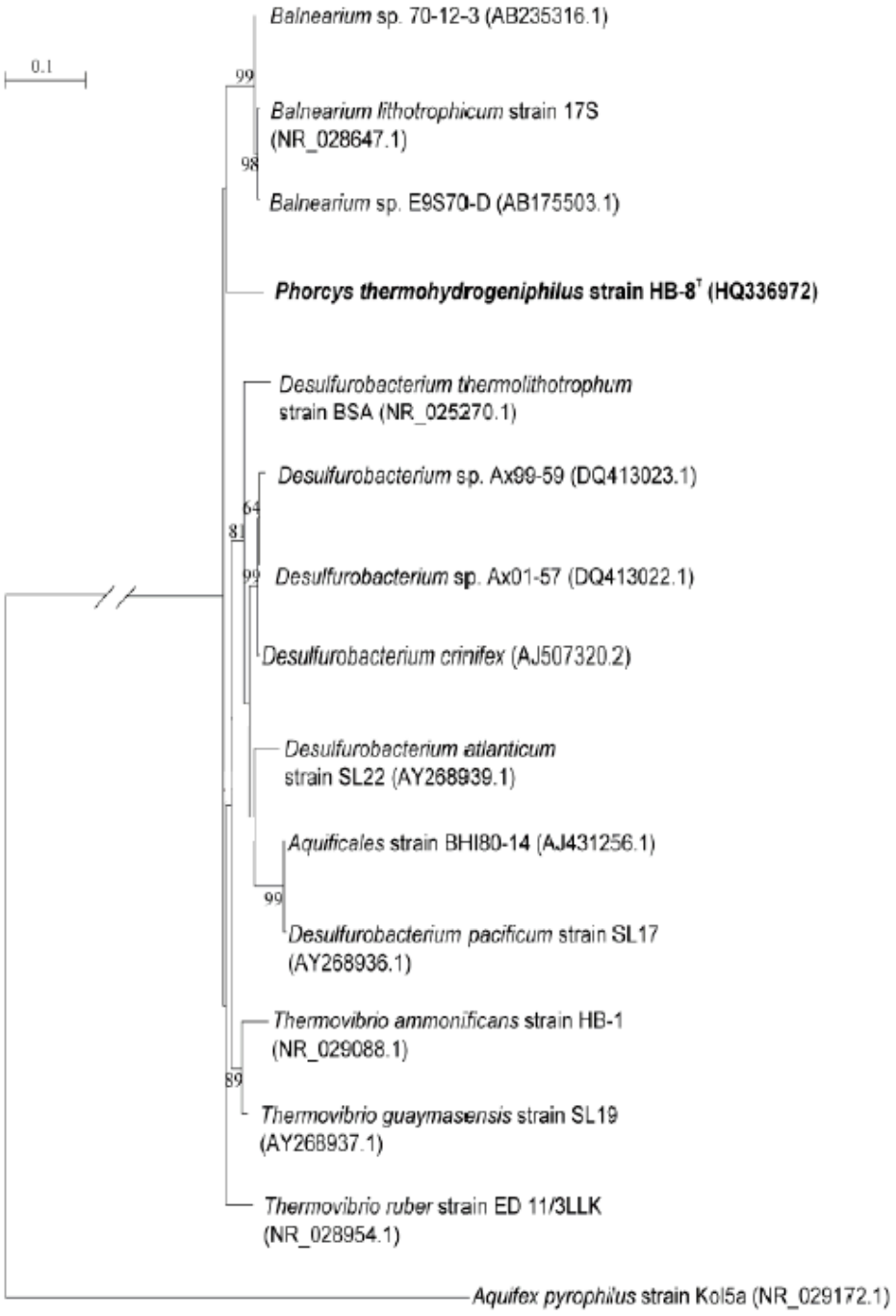


Figure 3.4 Maximum-likelihood phylogenetic tree inferred from 16S rRNA gene sequences showing the position of *Phorcys thermohydrogeniphilus* gen. nov, sp. nov. HB-8^T relative to representative strains of the *Desulfurobacteriaceae* family. Bootstrap values based on 100 replications are shown as percentages at branch nodes. Bar, 0.1 % substitutions per nucleotide position.

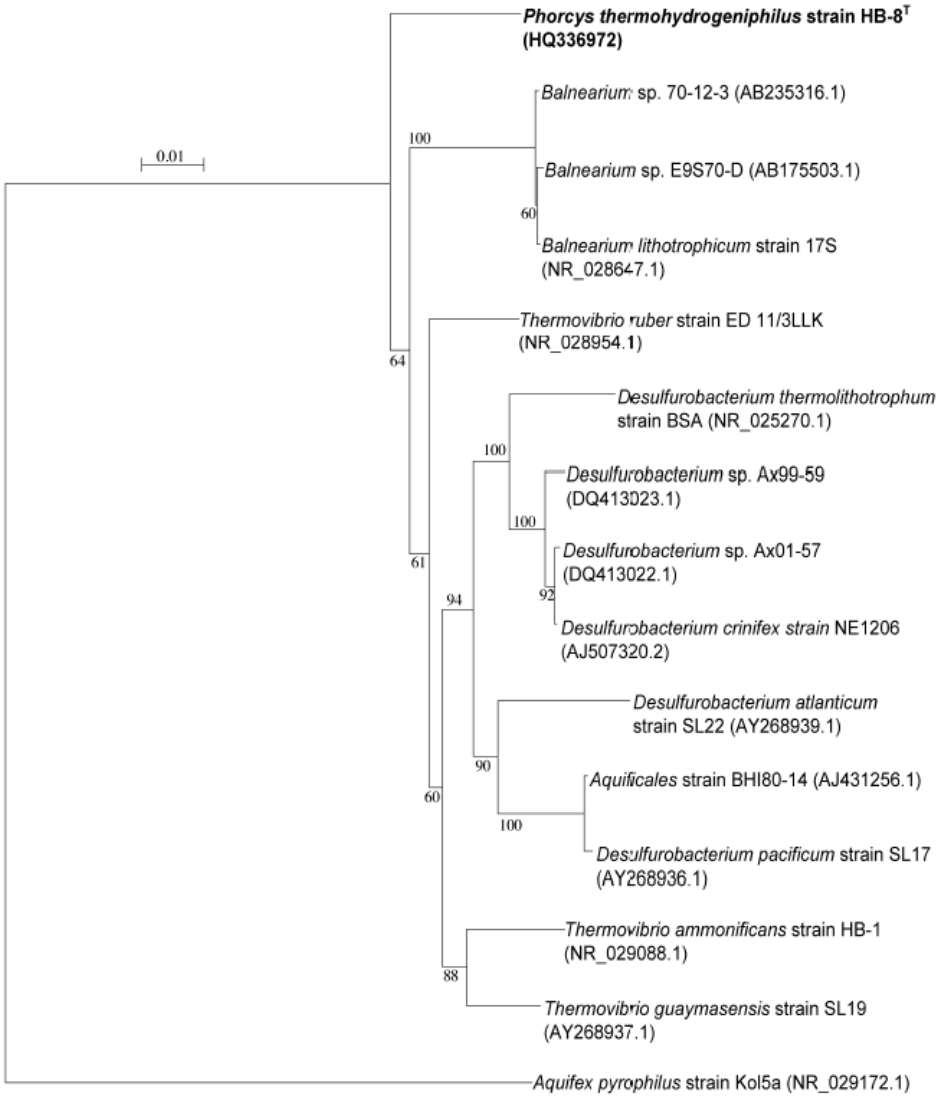


Figure 3.5 Neighbor-joining phylogenetic tree inferred from 16S rRNA gene sequences showing the position of *Phorcys thermohydrogeniphilus* gen. nov, sp. nov. HB-8^T relative to representative strains of the *Desulfurobacteriaceae* family. Bootstrap values based on 100 replications are shown as percentages at branch nodes. Bar, 0.01 % substitutions per nucleotide position.

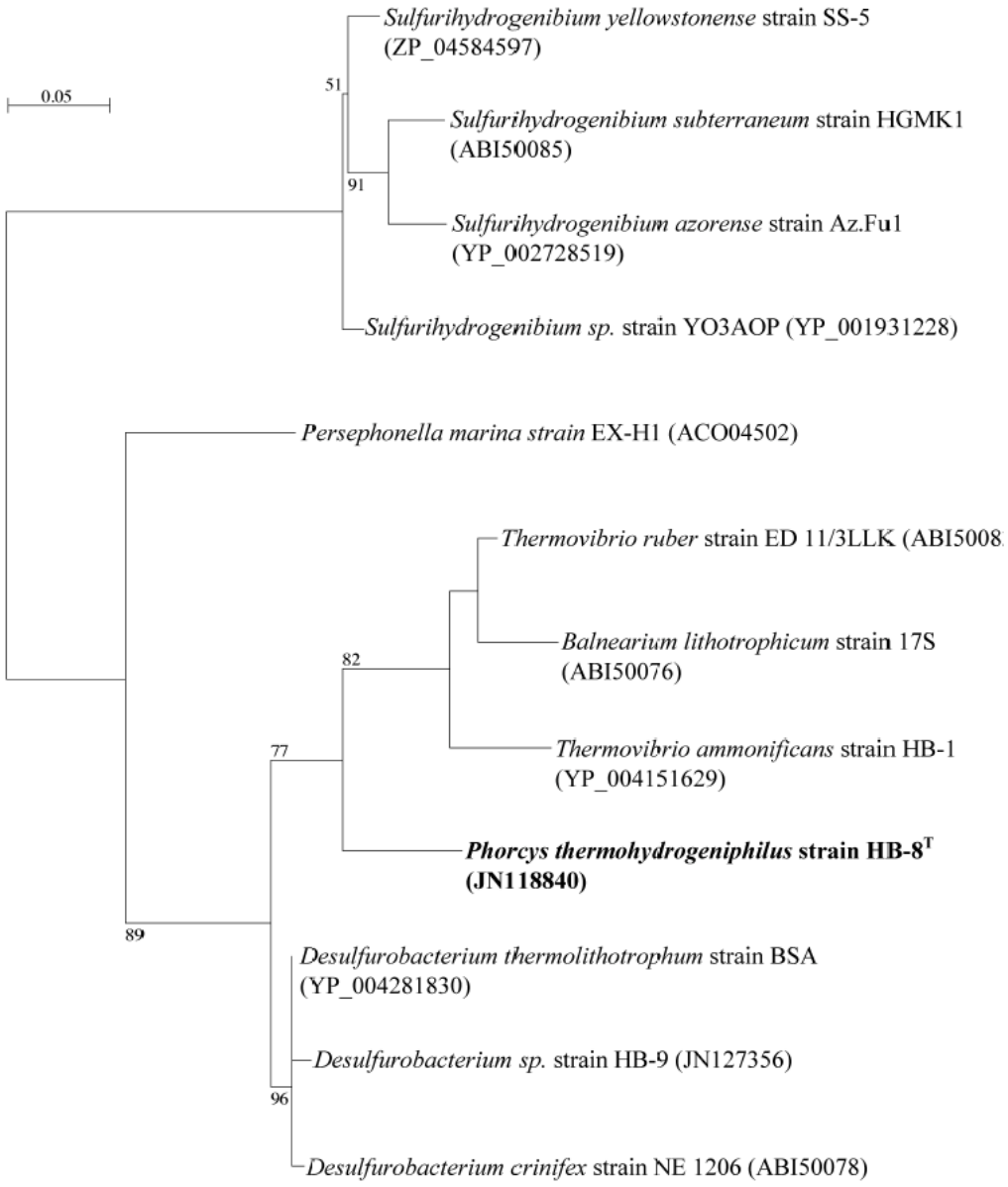


Figure 3.6 Maximum-likelihood phylogenetic tree inferred from the amino acid sequences deduced from a fragment of the *aclA* gene (encoding for the alpha subunit of the ATP citrate lyase), showing the position of *Phorcys thermohydrogeniphilus* gen. nov, sp. nov. HB-8^T relative to representative strains of the *Desulfurobacteriaceae* and *Hydrogenothermaceae* families. Bootstrap values based on 100 replications are shown as percentages at branch nodes. Bar, 5 % estimated substitutions.

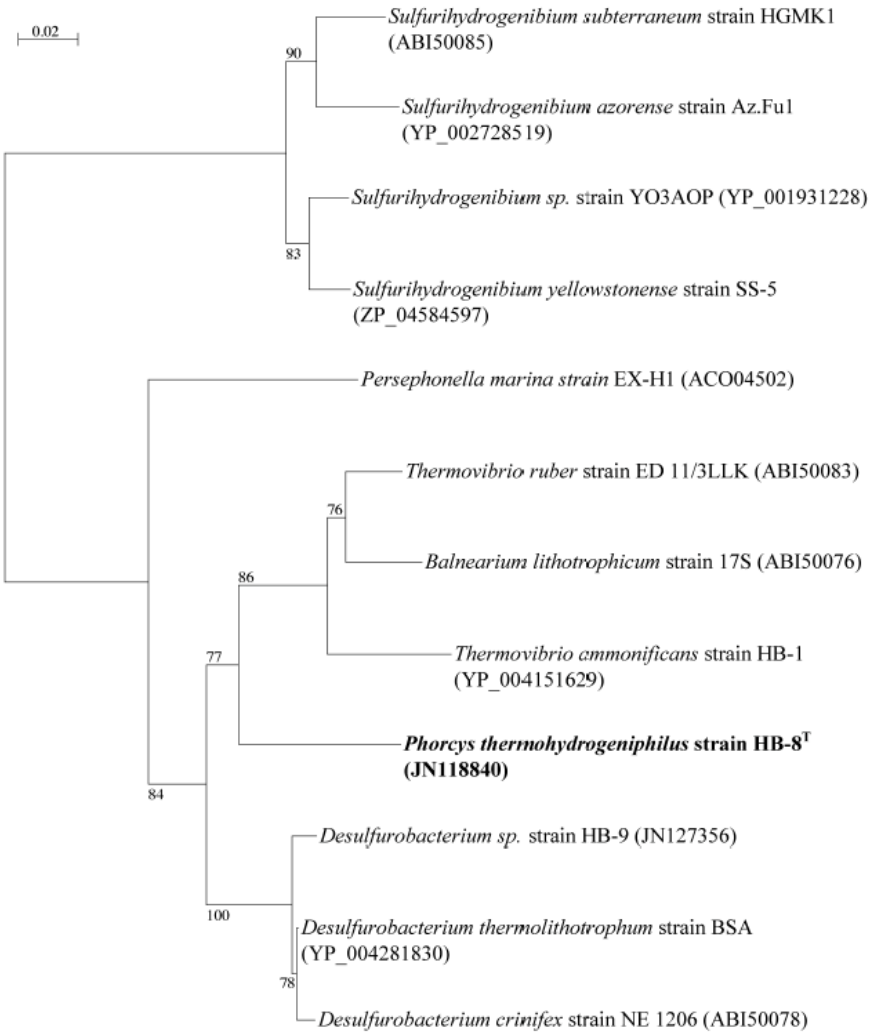


Figure 3.7 Neighbor-joining phylogenetic tree inferred from the amino acid sequences deduced from a fragment of the *aclA* gene (encoding for the alpha subunit of the ATP citrate lyase), showing the position of *Phorcys thermohydrogeniphilus* gen. nov, sp. nov. HB-8^T relative to representative strains of the *Desulfurobacteriaceae* and *Hydrogenothermaceae* families. Bootstrap values based on 100 replications are shown as percentages at branch nodes. Bar, 2 % estimated substitutions.

Characteristic	**1**	**2**	**3**	**4**
DNA G+C Content (mol%)	47.8	46	34.6	36
Temperature for growth (°C)				
Range	65-80	50-80	45-80	40-75
Optimum	75	75	70-75	70
NaCl concentration for growth (g l^{-1})				
Range	15-35	20-47	8-56	20-40
Optimum	30	30	32	30
pH for growth				
Range	4.5-8.5	5-6.5	5-7	4.4-8
Optimum	6	6	5.4	6
Terminal electron acceptors				
Nitrate	+	+	-	-
Sulfur	+	+	+	+
Sulfite	-	-	-	+
Thiosulfate	-	-	-	+
Shortest generation time (min)	26	60	80	135

Table 3.1 Differentiating features of *Phorcys thermohydrogeniphilus* gen nov., sp. nov. EPR-HB8^T and the type strains of *Thermovibrio ruber*, *Balnearium lithotrophicum* and *Desulfurobacterium thermolithotrophum*. Strains: 1, *Phorcys thermohydrogeniphilus* sp. nov. EPR-HB8^T (data from this study); 2, *Thermovibrio ruber* ED11/3LLK8 ^{T} (Huber *et al.*, 2002); 3, *Balnearium lithotrophicum* NE1206 ^{T} (Takai *et al.*, 2003); 4, *Desulfurobacterium thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998). +, growth; -, no growth.

Thermophilic, strictly anaerobic, hydrogen-oxidizing, chemolithoautotrophs such as *P. thermohydrogeniphilus* are likely to be important primary producers in high temperature and reducing habitats at deep-sea hydrothermal vents. The respiratory metabolism of *P. thermohydrogeniphilus*, along with that of other recently isolated chemolithoautotrophs (Miroshnichenko & Bonch-Osmolovskaya, 2006), suggests a widespread distribution of nitrate respiration in microorganisms from deep-sea hydrothermal vents. In particular, the very short generation time that we observed in *P. thermohydrogeniphilus,* along with its ability

to form biofilms and to grow chemolithoautotrophically by coupling hydrogen oxidation to nitrate or sulfur reduction, makes it extremely well adapted to the conditions found in active, high-temperature deep-sea vents.

Description of *Phorcys* gen. nov. *Phorcys* (Phor.cys N.L. from Gr. N. *Phorcys* ancient sea god who presided over the hidden dangers of the deep).

Cells are rod-shaped with polar flagella. Cells stain Gram-negative and do not form spores. Thermophilic, strictly anaerobic and chemolithoautotrophic. Growth by reduction of nitrate and formation of ammonium. Genomic DNA G + C content is 47.8 mol%. Major cellular fatty acids are $C_{18:1w7c}$ and $C_{18:0}$, and the predominant polar lipids are phosphatidylethanolamine and phosphatidylglycerol. Synthesize novel quinones. On the basis of phylogenetic analyses of both the 16S rRNA gene and the ATP citrate lyase, the genus *Phorcys* forms a separate lineage within the phylum *Aquificae*, and it is related to the genera *Thermovibrio, Desulfurobacterium* and *Balnearium*. Members of the genus *Phorcys* inhabit marine geothermal environments. The type species of the genus is *Phorcys thermohydrogeniphilus*.

Description of *Phorcys thermohydrogeniphilus* sp. nov. *Phorcys thermohydrogeniphilus* (*therm.o.hy.dro.ge.ni'phil.us;* Gr. fem. n. *therme* heat; L.N. *hydrogenus* hydrogen; Gr. N. *phylus* friend; N.L. adj. *thermohydrogeniphilum* heat- and hydrogen-liking, referring to its ability to grow lithotrophically on H_2 at elevated temperature.

Cells are rod-shaped (1-1.5 μm in length and 0.5 μm in width), with several polar flagella. Gram-negative. Catalase positive. Growth occurs between 65 and 80 °C, 15 and 35 g $NaCl^{-1}$ and pH 4.5 and 8.5. Optimal growth conditions are 75°C, 30 g $NaCl^{-1}$ and pH 6.0 (shortest generation time 26 min). Growth occurs under strictly anaerobic, chemolithotrophic conditions in the presence of H_2 and CO_2 with nitrate or elemental sulfur as electron acceptors, and the formation of ammonium and hydrogen sulfide, respectively. The following are not utilized as electron acceptors: oxygen, arsenate, selenate, thiosulfate and sulfite. Acetate, lactate and formate inhibit growth under a H_2/CO_2 gas phase. No chemoorganotrophic growth occurs in the presence of Casamino acids, glucose, sucrose and yeast extract. Cellular fatty acids are $C_{18:1w7c}$ (59.1 %), $C_{18:0}$ (23.7 %), $C_{16:0}$ (4.9 %) and $C_{20:1w7c}$ (5.7 %). Resistant to kanamycin and streptomycin; sensitive to chloramphenicol and ampicillin (each at 100 mg ml^{-1}). Genomic DNA G + C content is 47.8 mol%. The type strain is HB-8^T, (=DSM 24425^T =JCM 17384^T), which was isolated from an active deep-sea hydrothermal vent on the East Pacific Rise at 12° 48 N, 103° 56 W.

Chapter 4

Quorum sensing in chemosynthetic *Epsilonprotebacteria* and natural biofilms from deep-sea hydrothermal vents

Introduction

Bacteria release intercellular signaling molecules for cell-cell communication in the form of small peptides, acyl homoserine lactones (AHLs) and autoinducer molecules (reviewed in Turovskiy *et al.*, 2007). This phenomenon, called quorum-sensing (QS), relies on cell density and the production of such signalling molecules (Bassler *et al.*, 1994; Keller & Surette, 2006) to turn on the cooperative production of extracellular products at high cell density (Rumbaugh *et al.*, 2009). One quorum sensing system that is widespread across the bacterial domain (Figure 1.7a) and has been proposed to function as universal language for interspecies communication is based on the product of the LuxS enzyme which is involved in the production of autoinducer-2 (AI-2) molecules (Guillemin *et al.*, 2007). However, LuxS has an alternative role in the cell, in which it functions as an integral component of the Activated methyl cycle (AMC)—the major methyl donor of the cell (Turovskiy *et al.*, 2007; Vendeville *et al.*, 2005). This link could also provide an explanation for the widespread conservation of the *luxS* gene, although further investigation is required to confirm this possibility (Guillemin *et al.*, 2007).

We have found in the genome of chemosynthetic *Epsilonproteobacteria* from deep-sea hydrothermal vents a putative *luxS* gene (Figure 1.7b). Chemosynthetic *Epsilonptroteobacteria*, which represent one of the most cosmopolitan taxa at deep-sea vents, exist primarily as integrated communities or biofilms. Our hypothesis is that coordinated gene expression is central to this growth strategy, integrating individual microbial activities comparable to those of multicellular organisms (Stoodley *et al.*, 2002). We have hypothesized that *luxS,* which appears to have a role in AMC in chemosynthetic *Epsilonproteobacteria* (Figure 1.8), has an alternative role in QS. Because pathogenic *Epsilonproteobacteria*, such as *Helicobacter* and *Campylobacter* spp., have been demonstrated to utilize the *luxS* dependent AI-2 quorum sensing system to regulate biofilm formation and pathogenesis (Elvers & Park, 2002; Forsyth & Cover, 2000; Itoh *et al.*, 2003; Loh *et al.*, 2004; Wright *et al.*, 2000), we argue that it is possible that this trait is also shared among their

deep-sea chemosynthetic relatives. We selected *Caminibacter mediatlanticus* and *Sulfurovum lithotrophicum*, two cultured and fully sequenced chemosynthetic *Epsilonproteobacteria,* as model systems to investigate the *luxS*-based QS system. *These* two cultured chemosynthetic *Epsilonproteobacteria,* are representatives of the epsilonproteobacterial members that have been shown to occur along thermophilic and mesophilic temperature gradients in vent environments (Table 1.2). We believe that the metabolic characteristics (microaerophilic to anaerobic metabolisms) and ecotype preferences (mesophilic to thermophilic and moderately to fully reduced environments) of these cultured microorganisms are representative of the natural *Epsilonproteobacteria* that occur at deep-sea vents. In this study we demonstrate that *C. mediatlanticus* and *S. lithotrophicum* express the *luxS* gene during growth, and simultaneously induce a QS response in a reporter strain, *Vibrio harveyi*. Furthermore, we show that the *luxS* gene is actively transcribed *in-situ* in two chemosynthetic biofilm communities collected from a high and a low temperature vent. All together, our data indicate that chemosynthetic *Epsilonproteobacteria* may use an AI-2 based QS system for cell-to-cell communication.

Methods

Bacterial strains, growth conditions and culture media.

Caminibacter mediatlanticus strain TB-2 (DSM 16658^T) was isolated and described previously in our laboratory (Voordeckers*, et al.*, 2005). *Sulfurovum lithotrophicum* (DSM 23290^T; (Inagaki *et al.*, 2004), was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and *Vibrio harveyi* strains BB170 and BB120 were kindly provided to us by Dr. Chikindas (Rutgers University) and Dr. Bassler (Princeton University). *Caminibacter mediatlanticus* was grown in modified SME medium that had been prepared as previously described (Stetter*, et al.*, 1983) under optimal conditions (Voordeckers*, et al.*, 2005). These include a gas phase of H_2/CO_2 (20%: 80%), nitrate as terminal electron acceptor, 55°C, pH 5.5 and 3% NaCl. *Sulfurovum lithotrophicum* was grown using 356 MJ basal medium as described previously (Inagaki*, et al.*, 2004) under nitrate-reducing conditions. These include a gas phase of N_2/CO_2 (80%:20%), nitrate as terminal electron acceptor, 30°C, pH 5.5-6.0 and 3% NaCl. Strains of *Vibrio harveyi* were grown in modified Autoinducer Bioassay (AB) medium according (Bassler *et al.*, 1994). Time course experiments for

Caminibacter mediatlanticus and *Sulfurovum lithotrophicum* were performed in 1 liter of media in 2 liter bottles, in triplicate.

Sterile glass slides were added to previously reduced and sterilized media before time course experiments to monitor bacterial surface colonization during growth. All the experiments that involved *Sulfurovum lithotrophicum* were performed by Marie Bolognini, an MS graduate student in the Vetriani laboratory who worked under my supervision.

Scanning electron microscopy.

Scanning electron micrographs were obtained from cells growing as biofilms on glass slides added to the growth media. To this end, we removed glass slides at different times during growth and prepared them for microscopy as previously described by Vetriani *et al.*, 2004.

Autoinducer-2 bioassay.

The currently accepted and widely used AI-2 detection assay utilizes the *luxN*-null mutant, *Vibrio harveyi* BB170, as a reporter strain that produces bioluminescence in response to QS signals (Bassler *et al.*, 1994). This strain synthesizes two types of autoinducer molecules, the acyl homoserine lactone AI-1, and the product of the LuxS enzyme, AI-2. However, due to the lack of LuxN, the functional receptor for AI-1 (Turovskiy *et al.*, 2007), *V. harveyi* BB170 cannot respond to the AI-1-mediated signal. Hence, this strain responds exclusively to the AI-2 autoinducer.

The assay was initiated by growing *V. harveyi* BB170 cultures for 16 hours in modified Autoinducer-2 Bioassay (AB) medium (Turovskiy & Chikindas, 2006) with aeration at 30°C. The cultures were diluted 1:5,000 in fresh AB medium to obtain 10^5 CFU/mL. The rationale for this assay is that at lower cell densities (10^4–10^7 CFU/ml) the reporter cells respond to externally added AI-2 because the level of endogenously produced autoinducers has not reached the critical point for the bioluminescent response to take place (Bassler *et al.*, 1994). Cell-Free Supernatants (CFS) preparations of *C. mediatlanticus* and *S. lithotrophicum* were added to the diluted *V. harveyi* to a 10% v/v final concentration, and these cultures were shaken (140 RPM) at 30°C for 3-7 h (Guillemin *et al.*, 2007). Bioluminescence measurements were taken every 30 min with Luminoscan TL *plus* (ThermoLabsystems). Non-inoculated *C. mediatlaticus* and *S.*

lithotrophicum media were used as a negative controls, while the CFS from wild-type *V. harveyi* strain BB120 (OD600 of 0.5-0.6) were used as a positive control. The activity of AI-2 is expressed in relative bioluminescence units (light intensity was normalized to the negative controls) of the reporter strain. Relative bioluminescence unit values were expressed as fractions of bioluminescence induction at each time point measured, divided by the maximal bioluminescence induction value obtained from each strain.

CFS were prepared from collected cell suspensions from time course growth experiments. These samples were filtered through a 0.2 µm-pore-size filter and subsequently centrifuged at 6,000 × g for 30 min (Nalgene Nunc International). CFS for both strains were assayed for AI-2 activity. AI-2 bioassays were performed in duplicates for each individual time course experiments.

Samples for direct cell-counts were taken at different time points during the AI-2 bioassay to determine the number of *V. harveyi* BB170 cells per milliliter. 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).

RNA extraction.

Total RNA was isolated from *C. mediatlanticus* and *S. lithotrophicum* cultures at different phases during growth. Culture samples of 50 ml for *C. mediatlanticus* and 75 ml for *S. lithotrophicum* were preserved using RNA later and stored at -80°C until further processing. Before RNA extraction, centrifugation was performed at 6,000rpm at 4°C. RNA was extracted from the cell pellets using the RNAeasy Mini kit (Qiagen, USA) and treated with TURBO DNase (Ambion, USA), as recommended by the manufacturer. The quantity and quality of the RNA obtained was evaluated both spectrophotometrically on a NanoDrop ND-1000 spectrophotometer (NanoDrop, USA) and by agarose gel electrophoresis.

Total RNA was also extracted from environmental biofilm samples previously preserved in RNAEasy (Qiagen). The total RNA was treated with DNase I (Invitrogen) to remove traces of DNA. DNA contamination was excluded following the same methodology used for pure culture RNA samples.

Real-time quantitative RT-PCR analysis for pure cultures.

Specific transcripts were quantified by qRT-PCR using iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, USA) and the real-time detection system iCycler iQ (Bio-Rad, USA). Reactions in triplicate for each individual sample, were assembled according to manufacturer's protocol, and using 0.3 μg of total RNA for *C. mediatlanticus* and 0.15μg of total RNA for *S. lithotrophicum* in a 15-μl reaction. Primers were manually designed and analyzed using the Oligo Analyzer 3.1 from IDT (Integrated DNA Technologies), available through the following website: http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx/oligocalc.asp, to have a composition suitable for use in qRT-PCR (Table 4.1). Specificity of each pair of primers was confirmed by PCR amplification using the genomic DNA of each strain followed by sequencing. The efficiency of the PCR amplifications was determined from the slopes of the dilution curves of the target RNA. The cycle threshold (C_t) values obtained were used to calculate the absolute changes in gene expression. For the development of standard curves for targeted genes, RT-PCR was performed on a dilution series of amplified gene fragments using the iQ SYBR Green Supermix (Bio-Rad, USA).

Table 4.1 16S rRNA and *luxS* designed primers for One Step RT-PCR and qRT-PCR

Primers	Sequence	Primer size (bp)	Fragment Size (bp)
Primers for PCR dilutions for qRT-PCR standard curves			
C. mediatlanticus qPCR 16S rRNA F	5'-GGAGCATGTGGTTTAATTCG-3'	20	680
Univ p1517R	5'-ACGGCTACCTTGTTACGACTT-3'	21	680
C. mediatlanticus luxS 1F	5'-TTGCTCCATCTAACGAATGC-3'	20	367
C. mediatlanticus luxS 1R	5'-AAAATGCCAGCACCTGCTAT-3'	20	367
Univ p8F	5'-GCTGGCGGCGTGCTTAACAC-3'	20	727
S. lithototrophicum qPCR16S rRNA R	5'-CTGTTTGCTCCCCACGCTTT-3'	20	727
S. lithototrophicum luxS F	5'-CTAGAATGATCGCTCCCGCA-3'	20	421
S. lithototrophicum luxS R	5'-CCAATACCTCTGTCTAACAC-3'	20	421
Primers for RNA samples qRT-PCR			
S. lithototrophicum qPCR 16S rRNA F	5'-GCGGCCTTTTAAGTTGGATGTG-3'	22	201
S. lithototrophicum qPCR 16S rRNA R	5'-CTGTTTGCTCCCCACGCTTT-3'	20	201
C. mediatlanticus qPCR 16S rRNA F	5'-GGAGCATGTGGTTTAATTCG-3'	20	179
C. mediatlanticus qPCR 16S rRNA R	5'-AGGGTTGCGCTCGTT-3'	15	179
S. lithototrophicum qPCR luxS F	5'-CTCACCTATGGGGTGCAGAA-3'	20	198
S. lithototrophicum qPCR luxS R	5'-GTGCGATCTCCTTAGCCTCT-3'	20	198
C. mediatlanticus qPCR luxS F	5'-TTGCTCCATCTAACGAATGC-3'	20	134
C. mediatlanticus qPCR luxS R	5'-TGGAGAGCCCAAAAATGAAG-3'	20	134
Primers for One-Step RT-PCR			
SL and SD luxS 1F	5'-ATGCCACTATTAGAYAGTT-3'	19	456
SL and SD luxS 1R	5'-ACCAATRCSTCTSTCTAACAC-3'	21	456
Cm and Np luxS 1F	5'-TKGCKCCRTCKARCGAATGC-3'	20	295
Cm and Np luxS Ralt	5'-GAYKTAAGATTTGCAAAACC-3'	20	295

Sampling of vent natural chemosynthetic biofilms.

Samples of microbial biofilms were collected during R/V *Atlantis* cruises AT 15-15 (January – February 2007) and AT 15-28 (December 2007 – January 2008). Two experimental microbial colonization devices (Figure 4.1) that were deployed and recovered in the course of these cruises using the Deep-Submergence Vehicle *Alvin,* were used for this study: the first experimental colonizer, designated CV9, was deployed for seven days on a fissure with no associated megafauna venting 10 - 15°C fluids ("Mk 33", depth 2506 m, East Pacific Rise, 9° 509 N, 104° 189 W). The second colonizer, designated CV41, was deployed for three days on the wall of a sulfide structure colonized by *Alvinella pompejana* tubeworm and venting 25 – 40°C fluids ("Jumeaux" site, depth 2621 m, East Pacific Rise, 12° 48 N, 103° 56 W). At the time of recovery, white microbial biofilms covered the colonization devices and a few tubes of the tubeworm

Alvinella pompejana were associated with CV41. Subsamples were transferred to the ship's laboratory, preserved in RNA-preserving solution (RNAlater®; Ambion) (Rosenow *et al.*, 2003) and stored at 4 °C under a dinitrogen atmosphere. Parameters of the microbial colonization devices used in this work are summarized in Table 4.2. The fluid temperatures were measured in-situ at the top of each device. Temperatures at the bottom of the colonizers are expected to be 10 - 25°C hotter because of the steep temperature gradients at the vents.

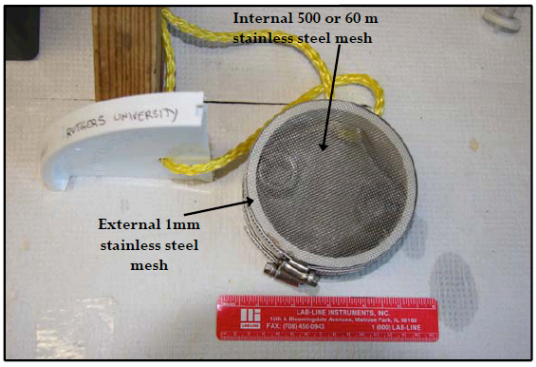


Figure 4.1 Stainless steel mesh experimental microbial colonizers. Hydrothermal fluids flow through the device allowing colonizing microorganisms to attach to the mesh.

Table 4.2 Data relative to the deployment of the two experimental microbial colonization devices, CV9 and CV41.

	CV9	**CV41**
Deployment Site	9°N EPR, Mk33	13°N EPR, Jumeaux
Depth	2506m	2621m
Temperature	10 - 15°C	25 - 40°C
Vent Type	Diffuse flow	Side wall of a diffusing sulfide
Associated Megafauna	None	*Alvinella pompejana*
Deployment Length	7 day	3 day
Deployment/Recovery (Alvin Dives)	4305/4312	4389/4392

Detection and phylogenetic analyses of *luxS* transcripts in vent natural biofilms

(This part of the work was carried out by Jessica Ricci, an undergraduate student in the Vetriani laboratory).

Total DNase-treaded RNA (0.5 µg) was reversed transcribed in 25-µl reactions using SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen). Two sets of primers, the first based on the *luxS* genes of *Sulfurovum lithotrophicum* and *Sulfurimons denitrificans* (SL and SD luxS primers; see Table 4.1) and the second based on the *luxS* genes of *Caminibacter mediatlanticus* and

Nautilia profundicola (CM and NP luxS primers: see Table 4.1) were designed and used in the One-Step RT-PCR reactions to amplify the *luxS* gene transcripts from the biofilms collected from the two microbial colonizers, CV9 and CV41. Both primer sets were used to probe *luxS* trascripts in both microbial colonizers. However, *luxS* amplification on CV9 was only obtained with SL and SD primers, while *luxS* amplification was only obtained in CV41 with CM and NP primers.

The amplified *luxS* transcripts were gel-purified using the MinElute™ Gel Extraction Kit (Qiagen), cloned into the pCR4-TOPO plasmid vector, and the ligation products were transformed into *Escherichia coli* One Shot® TOP10 Competent Cell using the TOPO TA Cloning® Kit for Sequencing (Invitrogen).

Fifty-two and forty-six randomly chosen clones from CV9 and CV41 *luxS* environmental libraries were analyzed respectively, from insert-containing plasmids by direct PCR followed by gel electrophoresis of the amplified products. The resulting PCR fragments were screened by restriction fragment length polymorphism (RFLP) analysis using the RsaI restriction endonuclease (Boehringer Mannheim). The RFLP reactions were then analyzed on a 3% MetaPhor® Agarose (Cambrex Bio Science) gel. Representative *luxS*-containing plasmid vectors showing unique RFLP patterns were purified using the MiniPrep Plasmid Purification Kit (Qiagen) and sequenced using the T3 primer. Sequences were determined for both strands on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA).

The *luxS* sequences were translated into the amino acid sequence using EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq/) and assembled using the AutoAssembler Program (Applied Biosystems, Foster City, CA). Sequences were then 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 and the neighbor joining method 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 100 re-samplings.

Results

Biofilm formation in *C. mediatlanticus* and *S. lithotrophicum* under laboratory conditions.

Throughout this study, we consistently observed the ability of chemosynthetic bacteria to form biofilms both *in situ* (Figure 1.3 a-c) and as pure cultures in our laboratory (Figure 1.3d; Figure 3.1c). In this work, by using *C. mediatlanticus* and *S. lithotrophicum*, our model systems of choice, we have been able to further explore the ability of chemosynthetic vent *Epsilonproteobacteria* to grow as single species biofilms by scanning electron microscopy (SEM; Figure 4.2)

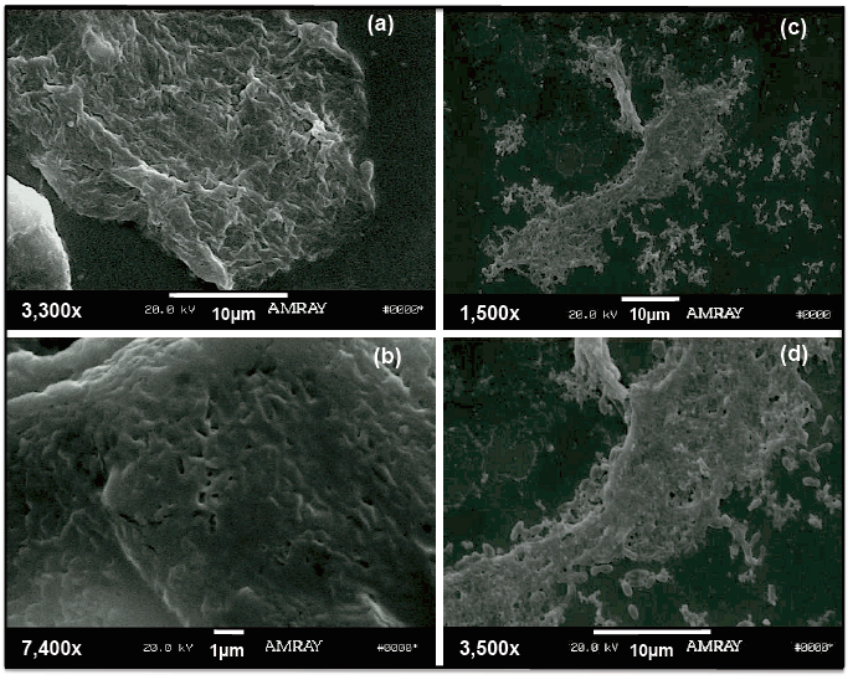


Figure 4.2 SEM of (a,b) *Sulfurovum lithotrophicum* and (c,d) *Caminibacter mediatlanticus* biofilms under nitrate-reducing conditions at 200 hours and 28 hours, respectively. Individual bacterial cells are difficult to appreciate because they appear to be encased in an exopolysaccharide matrix.

The supernatant of *C. mediatlanticus* and *S. lithotrophicum* cultures induces bioluminescence in a *V. harveyi* reporter strain: Autoinducer-2 bioassay.

AI-2 assays were performed in triplicate with spent media of both *C. mediatlanticus* and *S. lithotrophicum* grown under nitrate-reducing conditions, and an induction of bioluminescence in a *V. harveyi*

report strain was observed in both cases (Figure 4.3). The bioluminescence induction for both organisms appears to be maximal during the late exponential/early stationary phase of growth followed by a decrease in bioluminescence induction during the death phase.

Bioluminescence induction is expressed as the value of bioluminescence induction over the maximum bioluminescence induction by each strain. Positive controls are 2.2 ± 0.7 and 2.9 ± 0.8, respectively. For all AI-2 bioassays performed, cell-counts of the *V. harveyi* reporter strains were monitored at the beginning and at the end of each assay. This procedure was performed to verify that variations in bioluminescence induction were not a result of different *V. harveyi* cell densities in individual assays. Cell counts for each AI-2 assays performed for *C. mediatlanticus* and *S. lithotrophicum* were in the same range and are shown in table 4.3.

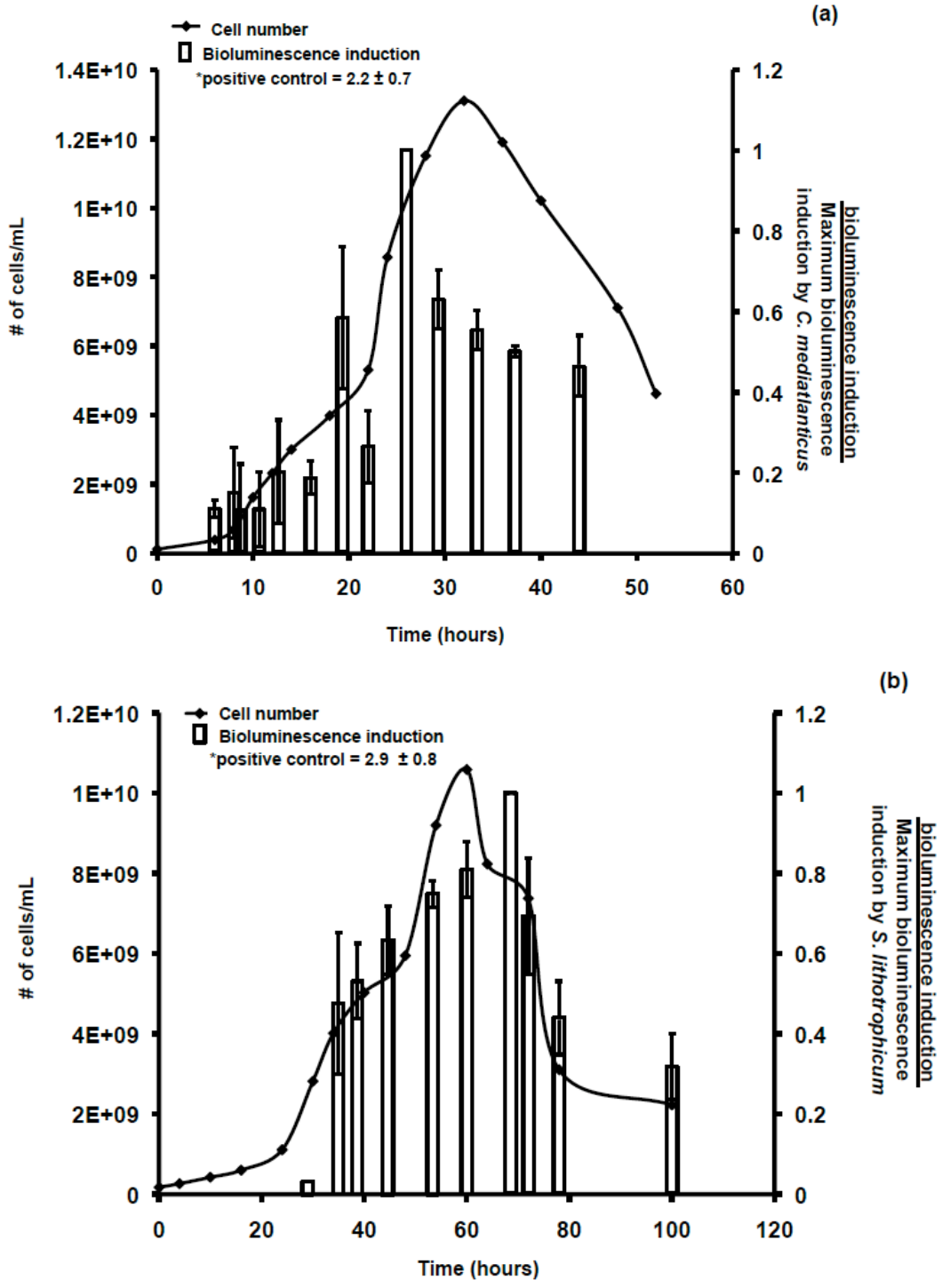


Figure 4.3 AI-2 detection assay for *C. mediatlanticus* (a) and *S. lithotrophicum* (b) grown under nitrate-reducing conditions. Growth curves and bioluminescence values are based on triplicate assays.

Table 4.3 *Vibrio harveyi* BB170 direct cell-counts during the AI-2 bioassay for *Caminibacter mediatlanticus* and *Sulfurovum lithotrophicum*. Duplicates (α and β) were performed for each individual growth assay (A, B and C). Samples for each assay (A,B,C) are labeled with the number representing a time point (in hours) within the time course experiment. Positive control (positive) and negative controls (α- and β-) values are also shown.

Caminibacter mediatlanticus			***Sulfurovum lithotrophicum***		
Bottle A					
Sample	**t=0 h (cells/ml)**	**T=5 h (cells/ml)**		**t=0 h (cells/ml)**	**t=4 h (cells/ml)**
positive	3×10^7	8.8×10^8	positive	6.2×10^7	1.2×10^8
α-	3.2×10^7	9.2×10^8	α-	6.2×10^7	1.4×10^8
α10	3.6×10^7	5.8×10^8	α24	5.3×10^7	1.2×10^8
α24	4×10^7	5.6×10^8	α60	6.8×10^7	1.5×10^8
α48	3.6×10^7	8.4×10^8	α100	6.3×10^7	1.5×10^8
β-	2.4×10^7	4.5×10^8	β-	6.2×10^7	1.2×10^8
β10	3.4×10^7	4.7×10^8	β24	5.2×10^7	1.4×10^8
β24	3.6×10^7	6.3×10^8	β60	6×10^7	1.4×10^8
β48	3×10^7	1.1×10^9	β100	6.2×10^7	1.4×10^8
Bottle B					
Sample					
positive	2.8×10^7	2.4×10^8	positive	6.5×10^7	1.5×10^8
α-	2.8×10^7	2×10^8	α-	6.5×10^7	1.4×10^8
α10	3×10^7	2.1×10^8	α24	6.2×10^7	1.5×10^8
α24	3.4×10^7	2.2×10^8	α60	7.2×10^7	1.5×10^8
α48	3×10^7	2×10^8	α100	7.1×10^7	1.6×10^8
β-	2.8×10^7	2.4×10^8	β-	7.1×10^7	1.5×10^8
β10	3.2×10^7	2×10^8	β24	6.9×10^7	1.4×10^8
β24	3.2×10^7	2.3×10^8	β60	7.3×10^7	1.7×10^8
β48	3.2×10^7	1.9×10^8	β100	7.1×10^7	1.6×10^8
Bottle C					
Sample					
positive	3.8×10^7	2.8×10^8	positive	6×10^7	1.1×10^8
α-	4.2×10^7	2.4×10^8	α-	6×10^7	1.1×10^8
α10	5×10^7	3.1×10^8	α24	5.1×10^7	1.42×10^8
α24	3.4×10^7	3.6×10^8	α60	5.3×10^7	1.6×10^8
α48	6.2×10^7	3.1×10^8	α100	5.4×10^7	2.1×10^8
β-	4.2×10^7	2.2×10^8	β-	5.8×10^7	1.2×10^8
β10	4.6×10^7	3.5×10^8	β24	5.3×10^7	1.2×10^8
β24	5.2×10^7	3.5×10^8	β60	6.3×10^7	1.7×10^8
β48	5.2×10^7	2.8×10^8	β100	6.2×10^7	1.6×10^8

Quantification of *luxS* transcripts in *C. mediatlanticus* and *S. lithotrophicum*.

Quantification of the expression of *luxS* during bacterial growth was performed in *C. mediatlanticus* and *S. lithotrophicum* in the same time-course experiment used for the AI-2 bioassay (Figure 4.4 and 4.5). By monitoring the changes in gene expression, a relation can be established between *luxS* and the QS-induced bioluminescence response. Data from both *C. mediatlanticus* and *S. lithotrophicum* show a similar trend where *luxS* is differentially expressed during growth, with maximal expression occurring in the early exponential phase, and declining to minimal expression in the late exponential, stationary and death phase (Figures 4.4 and 4.5). Expression of the *luxS* gene in both *C. mediatlanticus* and *S. lithotrophicum* is negatively correlated to bioluminescence induction in *V. harveyi*, as shown in Figure 4.4b and 4.5b.

16S rRNA controls were prepared with the qRT-PCR procedure, as we monitored the changes in expression in the *luxS* gene. The 16S rRNA gene, being a housekeeping gene, is consistently expressed during growth with no significant differences (Figure 4.6 and 4.7). Expression of the 16S rRNA gene in both *C. mediatlanticus* and *S. lithotrophicum* is not correlated to bioluminescence induction in *V. harveyi*, as shown in Figure 4.6b and 4.7b. Thus, changes in *luxS* expression are reliable in this procedure.

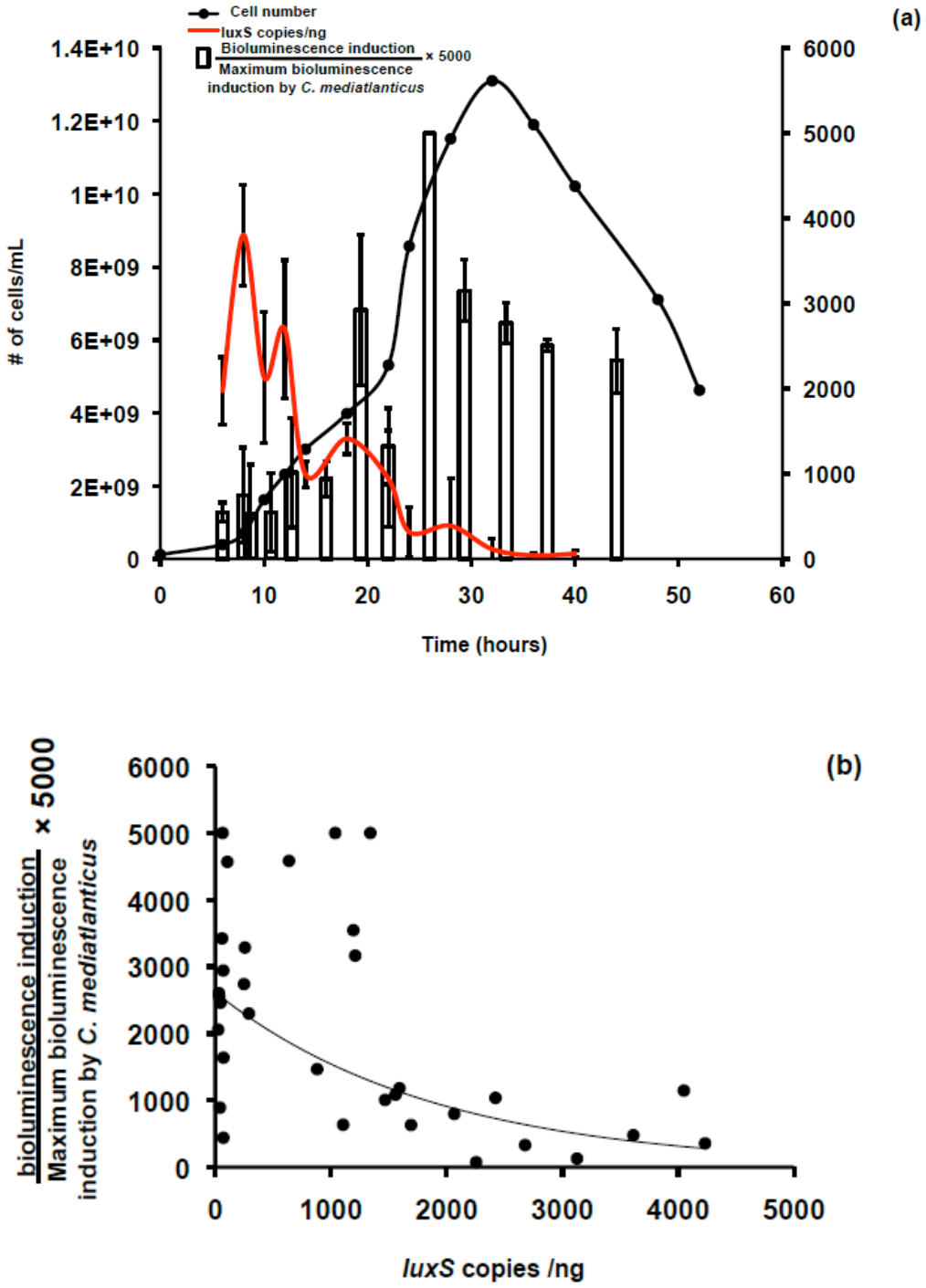


Figure 4.4 Expression of the *luxS* gene in *C. mediatlanticus* during growth under nitrate reducing conditions (a). Correlation between *luxS* expression and bioluminescence induction in *V. harveyi* as *C. mediatlanticus* grows is significant (Pearson correlation =-0.532; $p<0.01$; n =30) (b). Values are the averages of triplicates samples.

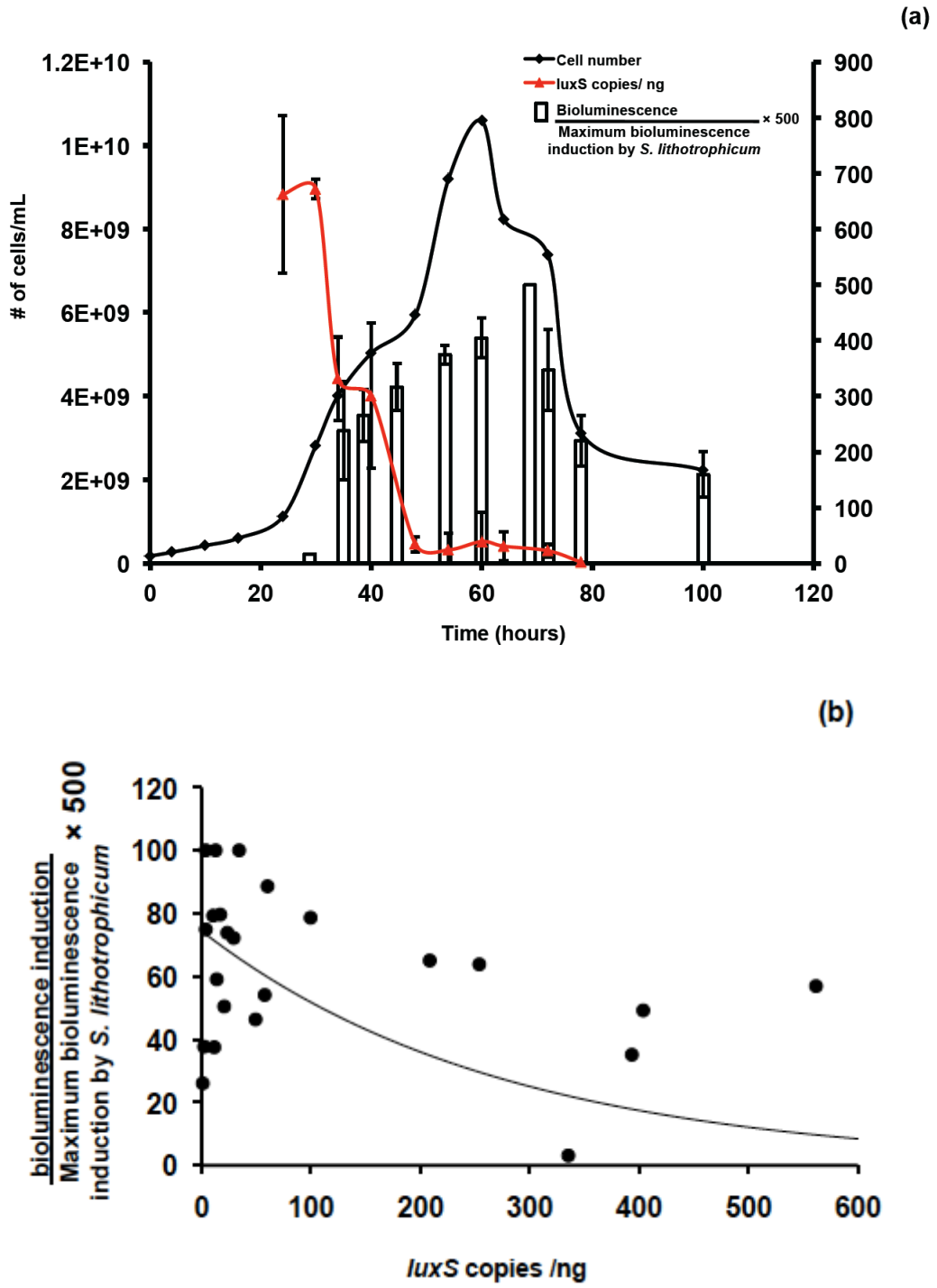


Figure 4.5 Expression of the *luxS* gene in *S. lithotrophicum* during growth under nitrate reducing conditions (a). Correlation between *luxS* expression and bioluminescence induction in *V. harveyi* as *S. lithotrophicum* grows is significant (Pearson correlation =-0.716; $p<0.01$; n =26) (b). Values are the averages of triplicates samples.

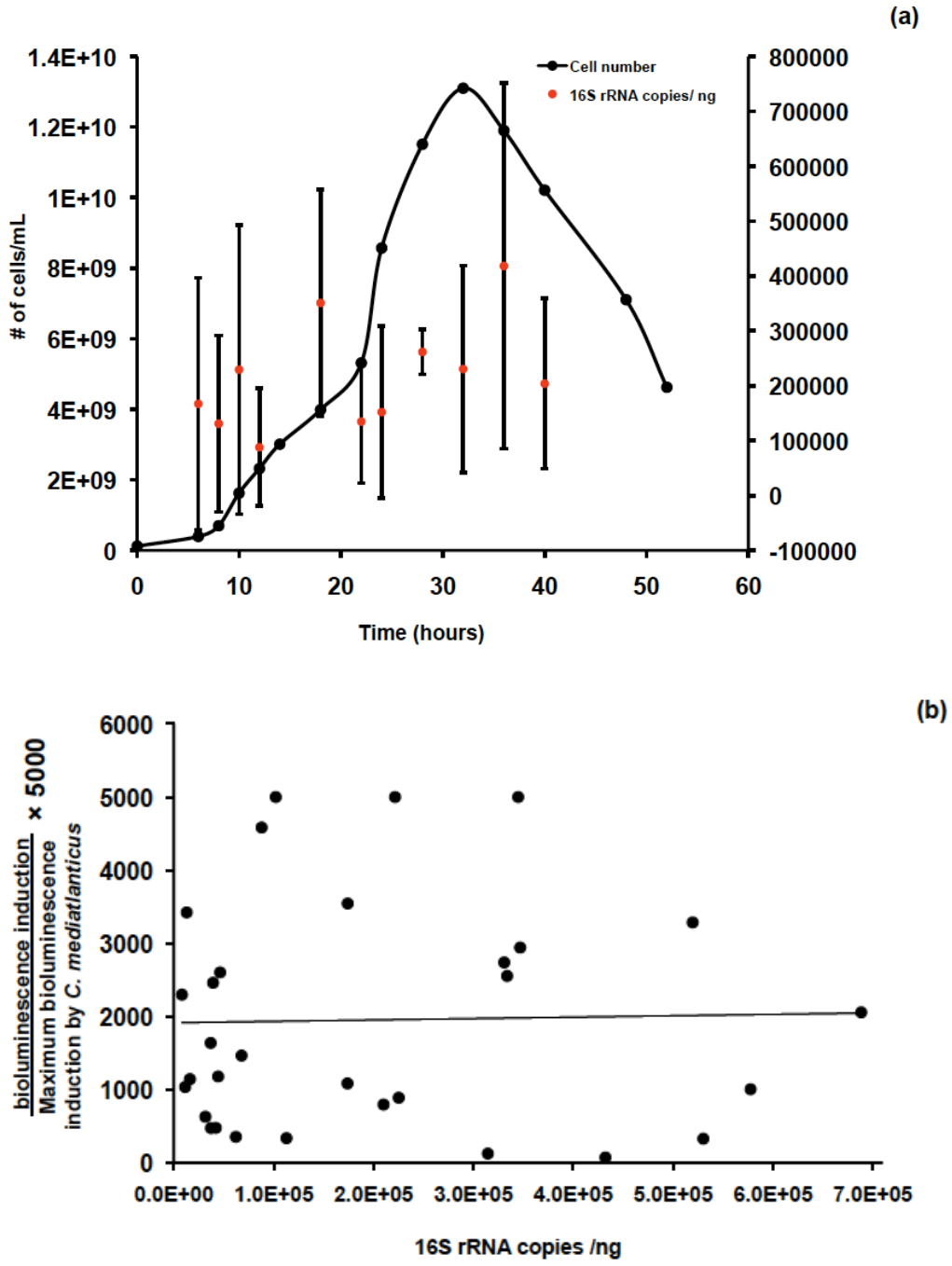


Figure 4.6 Expression of the 16S rRNA gene in *C. mediatlanticus* during growth under nitrate reducing conditions (a). Correlation between 16S rRNA gene expression and bioluminescence induction in *V. harveyi* as *C. mediatlanticus* grows is not significant (Pearson correlation =0.0240; p> 0.10; n =31) (b). Values are the averages of triplicates samples.

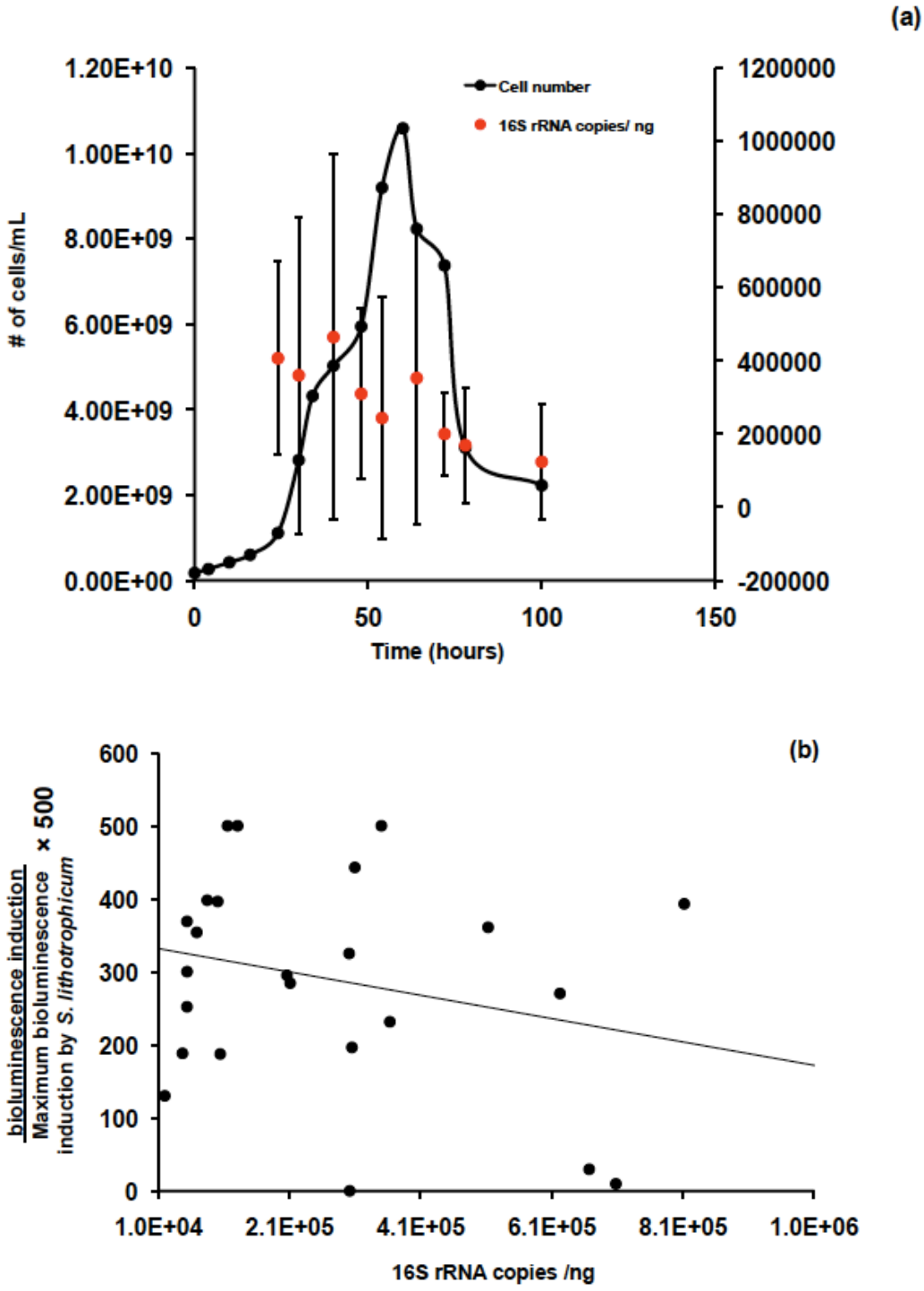


Figure 4.7 Expression of the 16S rRNA gene in *S. lithotrophicum* during growth under nitrate reducing conditions (a). Correlation between 16S rRNA gene expression and bioluminescence induction in *V. harveyi* as *S. lithotrophicum* grows is not significant (Pearson correlation =-0.3071; p> 0.10; n =26) (b). Values are the averages of triplicates samples.

In-situ detection and phylogenetic analysis of _luxS_ transcripts in vent natural biofilms.

The composition of the two biofilm communities collected on the experimental colonizers, CV9 and CV41, was analyzed using a metatranscriptomic approach by Jessica Ricci in the Vetriani laboratory, revealing that the two biofilm communities were dominated by *Epsilonproteobacteria*. The lower temperature biofilm (CV9; see Table 4.2) was dominated by mesophilic *Epsilonproteobacteria* related to the *Sulfurovum* and *Sulfurimonas* genera. In contrast, the higher temperature biofilm (CV41; see Table 4.2) was dominated by *Epsilonprotebacteria* belonging to the order *Nautiliales*, including the thermophilic *Caminibacter* and *Nautilia* spp. RT-PCR of *luxS* transcripts in the two biofilm communities revealed that the gene was expressed *in-situ*. Phylogenetic analyses revealed that the *luxS* transcripts amplified from the total RNA extracted from the two biofilms were related to *luxS* sequences from chemosynthetic *Epsilonproteobacteria* (Figure 4.8). In line with the composition of the two biofilms based on the retrieval of 16S rRNA transcripts, the *luxS* transcripts from the lower temperature biofilm (CV9; see Table 4.2) were related to homologous genes from *Sulfurovum* spp. and *Sulfurimonas* spp. (order *Campylobacterales*), while the transcripts detected in the biofilm associated with the higher temperature regime (CV41; see Table 4.2) was shown to be related to homologous genes found in *Caminibacter* spp. and *Nautilia* spp. (Figure 4.8).

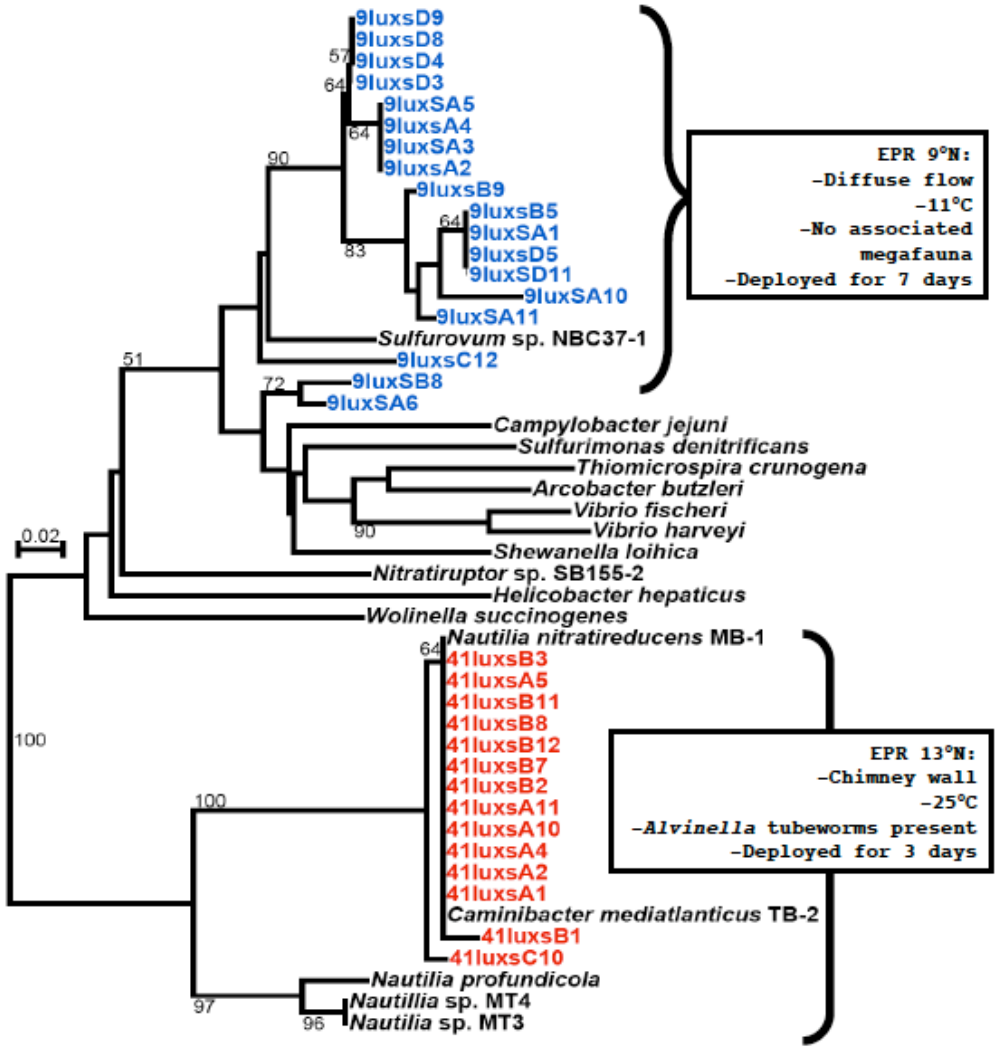


Figure 4.8 *In-situ* detection of epsilonproteobaterial *luxS* transcripts in two natural biofilm communities from a diffuse flow vent (in blue) and a sulfide structure (in red) on the East Pacific Rise.

Discussion

Deep-sea vent microbial communities must adapt rapidly to to changing environmental conditions, and biofilm formation appears to be a critical step during the colonization of deep-sea vents. In this study, we have observed that chemosynthetic *Epsilonproteobacteria*, similarly to their pathogenic counterparts, form biofilms on rock surfaces and associated invertebrates along the MOR system, as well as in laboratory grown

cultures. Pathogenic *Epsilonproteobacteria,* such as *Helicobacter* and *Campylobacter* spp., regulate the establishment of biofilms, which are crucial to their colonization in host tissues, via AI-2 dependent quorum sensing mechanisms (Forsyth & Cover, 2000; Loh *et al.*, 2004; Wright *et al.*, 2000). Like their pathogenic relatives, chemosynthetic *Epsilonproteobacteria* encode for the *luxS* gene, which specify for the LuxS enzyme responsible for AI-2 signal production in quorum sensing (Figure 1.7b). This work provides evidence that, in the chemosynthetic *Epsilonproteobacteria*, *Caminibacter mediatlanticus* and *Sulfurovum lithotrophicum,* the expression of the *luxS* gene is correlated to a QS response. The expression of *luxS* was monitored during growth of *C. mediatlanticus* and *S. lithotrophicum*, with consistent maximum expression occurring in the early exponential growth phase in both organisms. Maximal bioluminescence in the *V. harveyi* reporter strain was induced by supernatants from late-exponential/early-stationary phase cultures of both *C. mediatlanticus* and *S. lithotrophicum*. Differences in *luxS* expression are significantly correlated at the $p < 0.01$ level to the detection of an AI-2 molecule. Our data suggest that the LuxS/AI-2 system functions in *C. mediatlanticus* and *S. lithotrophicum* as a negative feedback in the regulation of *luxS* expression; at low levels of the AI-2 signaling molecule, *luxS* is expressed; however, when AI-2 accumulates in the culture medium, *luxS* expression is repressed. Overall, our data indicate that the LuxS/AI-2 system in *C. mediatlanticus* and *S. lithotrophicum* regulates the QS response, which appears to be conserved across taxonomic boundaries (Figure 1.7).

Part of this work involved targeting the LuxS/AI-2 system in natural chemosynthetic biofilms. Transcripts of the *luxS* gene were detected *in-situ* in natural biofilms that developed in vents characterized by different temperature regimes (Table 4.2), revealing that both termophilic (*e.g., Caminibacter* and *Nautilia* app.) and mesophilic (*e.g., Sulfurovum* spp.) *Epsilonproteobacteria* express *luxS* in their natural environment. Since we demonstrated that the expression of *luxS* in *C. mediatlanticus* and *S. lithotrophicum* is correlated to the induction of a QS response in laboratory experiments, it is possible that cell-to-cell communication signalling is occurring among the biofilm bacteria in their natural habitats via a LuxS/AI-2 based QS system.

Recent studies of the LuxS/AI-2 QS system in the pathogenic *Epsilonproteobacteria, Campylobacter* and *Helicobacter* spp., indicate that mutations in the *luxS* gene alters flagellar motility, EPS production, and virulence, all phenotypes related associated with the formation of biofilms (Elvers & Park, 2002; Forsyth &

Cover, 2000; Itoh *et al.*, 2003; Loh *et al.*, 2004; Wright *et al.*, 2000). These observations promped us to investigate QS in vent *Epsilonproteobacteria*.

Given the role of *luxS* in the Activated Methyl Cycle (Figure 1.8), it is possible that *luxS* can provide information to microbial communities not only about the cell density through QS, but also in terms of the metabolic state the community is experiencing (Bassler *et al.*, 1997). This is particularly important at deep-sea hydrothermal vents, where chemical oxidation and reduction represent a competitive process for the microorganisms. Quorum sensing based on the AI-2 signalling molecule could provide important insights into how bacteria adapt metabolically to overcome competition (Jannasch, 1995). Potential metabolic information received through the AI-2 molecule, could allow some individuals within a biofilm to perform genetic and behavioral shifts towards a different metabolic (and ecological) niche when being outcompeted by neighbouring bacteria. Perhaps, it is not always feasible for microorganisms within a biofilm to perform the most energetically favorable metabolism if being outcompeted by neighbours. This idea is based on the ecological concept of competitive exclusion principle, which states that complete competitors cannot coexist (Hardin, 1960). Given that the precursor of the AI-2 molecule, (S)-4,5-dihydroxy-2,3-pentanedione (DPD), can exist in several chemical conformations, it is likely that the outcome of the AI-2 signaling molecule depends on the immediate chemical milieu of the environment and on the microbial community metabolic state. Thus, the immediate chemistry of the environment could be dictating the type of signaling molecules being received, and consequently, gene regulation. We speculate that EPS production and secretion could be vital in providing a homeostatic chemical protection to the precursor molecule against external chemical conditions, which could alter the microbial signals. However, the way the chemistry of the vents affects the chemistry of the AI-2 signal and, in turn, gene regulation, is an area ripe for investigation. One important outcome of the study of the factors affecting the chemical structure of QS signals could provide informations on how to block bacterial communication in pathogenic bacteria.

The realization that deep-sea vent microbial biofilms could influence global biogeochemistry has been a major revelation in the geosciences (Schrenk *et al.*, 2010) given their strength in numbers and outstanding metabolic versatility (Nadell *et al.*, 2009). Thus, understanding the mechanisms that microorganisms evolved

to respond and interact with abiotic conditions at the molecular and microscopic scales has great implications to our understanding of their past and present influence on the global biogeochemical cycling of C, Fe, N, and other elements (Fredrickson & Zachara, 2008).

Chapter 5

Marinobacter-related species dominate nitrate-reducing communities encoding for the membrane-bound nitrate reductase (NarG) in different vent microbial habitats.

In dedicattion to the memory of Mariola Cuebas

Introduction

Dinitrogen gas is the most abundant component of the Earth atmosphere because, thermodynamically, nitrogen gas (N_2) is the most stable form of nitrogen (Falkowski, 1997, Madigan *et al.*, 2000). Due to the high amount of energy required to break the triple bond of molecular nitrogen, the reduction of N_2 is an energy demanding process. Therefore, despite its abundance in the atmosphere, most organisms can't assimilate it into the most important polymers of life: proteins and nucleic acids (Canfield *et al.*, 2010, Madigan *et al.*, 2000).

Since nitrogen fixation does not have significant effects in ocean ecosystems (Millero, 2006), its cycling in the ocean depends on other inorganic nitrogen forms, ammonia and nitrate (Falkowski *et al.*, 2008, Madigan *et al.*, 2000). Several of the key reduction-oxidation (redox) reactions of the biogeochemistry of nitrogen that are involved in cycling both nitrate and ammonia, are carried out in nature almost exclusively by microorganisms, and so microbial involvement in the ocean's nitrogen cycle is of great importance (Madigan, *et al.*, 2000).

One key microbiological redox reaction that is implicated in cycling many oxidation states of nitrogen is that of dissimilatory nitrate reduction. Nitrate-reducing microorganisms can thus be differentiated by: (a) bacteria which reduce nitrate to nitrite only, (b) bacteria which reduce nitrate and nitrite to ammonia through the dissimilatory nitrate reduction to ammonia (DNRA) process, and (c) bacteria which reduce nitrate and nitrite to atmospheric nitrogen and to gaseous oxides of nitrogen in the process of denitrification (Waksman *et al.*, 1933). Although these subsequent steps involve different enzymes and intermediates, the first step of nitrate reduction is identical and can be carried out by the same enzyme, the nitrate reductase (Argandona *et al.*, 2006).

Two types of dissimilatory nitrate reductases have been described in bacteria, which differ both in their location and structural and catalytic properties: one is membrane bound (Nar) and the other is located in the periplasm (Grove *et al.*, 1996; Philippot, 2005). Nar has been mainly studied in the non-denitrifying bacterium *Escherichia coli*, but also has been purified from a large variety of microorganisms, including *Archaea* (Afshar *et al.*, 2001). In contrast, the periplasmic nitrate reductase (Grove *et al.,* 1996) is present only in Gram negative bacteria (Philippot, 2005).

Several lines of investigation indicate that dissimilatory nitrate reduction plays an important role in higher temperature deep-sea vent ecosystems (Campbell *et al.*, 2006; Nakagawa *et al.*, 2005). When the reduced hydrothermal fluids mix within the walls of the vent chimneys 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 in carbon dioxide fixation and other anabolic processes (Voordeckers *et al.*, 2008). However, at deep-sea vents nitrate reduction is not limited to high temperature habitats. In effect, the physical and chemical characteristics of potential biomes in the subseafloor reflects the potential importance of nitrate as a terminal electron acceptor not only in mid-ocean ridges systems but also ridge flanks, island arc systems, abyssal plains, ocean island volcanoes, active continental margins and passive continental margins (Schrenk *et al.*, 2010). The increasing concentration of nitrate with the depth of the water in the open ocean (Millero, 2006; Waksman *et al.*, 1933) and the fact that nitrate is the most oxidized nitrogen form transformed by bacteria have directed a large body of research in marine microbiology towards global microbially-mediated transformation of nitrate in the sea.

This study focuses on the membrane-associated nitrate reductase and makes use of previously developed PCR primers that successfully amplify fragments of the *narG* gene that encodes the catalytic molybdenum-cofactor-containing α subunit of the enzyme (Gregory *et al.*, 2000). We carried out a comparative analysis between 16S rRNA genes and the α subunit of the *nar* nitrate reductase, *narG*, retrieved from natural vent microbial communities and from pure cultures isolated from several different hydrothermal vent sites, including the Mid Atlantic Ridge (MAR) and East Pacific Rise (EPR). The goal of

this work was to investigate the diversity and phylogeny of Nar-dependent nitrate-reducing microorganisms at deep-sea hydrothermal vents. Our findings indicate that, although there are significant differences in the composition of the microbial communities from different vent-associated microbial habitats, the distribution of the *narG* gene is similar among the natural microbial communities and the cultured strains, and suggest a relevant role of *Gammaproteobacteria* related to *Marinobacter* spp. in the Nar-dependent nitrate reduction in the world's deep oceans.

Materials and Methods

Sample collection

Three environmental samples from different deep-sea vent microhabitats were collected to investigate the diversity of nitrate-reducing bacteria: 1) Fragments of active, high temperature black smoker chimneys with in situ temperature of 158°C were collected from the "Rainbow" (36°14'N, 33°54'W; depth 2,305m) vent field on the Mid-Atlantic Ridge, during R/V Atlantis cruise AT 05-03 (Dive 3678, July 2001); 2) Diffuse flow fluids samples from "East Wall Mk EW1" (9°N, depth 2500m, 25°C) vent field on the East Pacific Rise (EPR) were collected using titanium samplers and filtered shipboard on 0.2μm Supor Gelman filters (Ann Arbor, MI) during R/V Atlantis cruise AT 15-26 (Dive 4109); 3) Samples of white bacterial filaments were collected from the acrylic mesh lid of an exclusion cage deployed for a year on "Mk 89" (9°N, depth 2500m, 2°C) diffuse flow vent field on the EPR during R/V Atlantis cruise AT 15-26 (Dive 4102). All 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 frozen at -80°C for nucleic acid extraction.

Pure cultures

Isolates screened in this study for their ability to reduce nitrate, as well as the presence of the *narG* gene, were obtained from Dr. Vetriani's laboratory culture collection of deep-sea vent microorganisms. Pure cultures were grown in anaerobic artificial seawater medium, in the presence and absence of 10% w/v potassium nitrate. Characteristics of isolates and their sampling location are summarized in Table 5.1.

DNA Extraction

Total genomic DNA was extracted from: 1) 2 g of chimney subsamples (Rainbow 3678) using the Ultra-Clean Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA, USA) with the following protocol modifications: Bead beating was extended for 20 s and it was followed by heating at 70°C for 5 min; 2) Biomass collected on filters from about 2.4 liters of hydrothermal fluids from East Wall, as described in Vetriani et al. 2004; 3) ~ 1g of bacterial filaments from Marker 89; and 4) cells recovered from 10 ml cultures for each pure cultures. The UltranClean Microbial DNA Isolation Kit was used for fluids, filaments and pure cultures according to the protocol supplied with the kit (Mo Bio Laboratories, Solana Beach, CA, USA).

DNA amplification by Polymerase Chain Reaction (PCR)

A fragment of approximately 440 bp of the *narG* gene, encoding for the catalytic molybdenum cofactor-containing α subunit of the membrane bound nitrate reductase, was amplified from the genomic DNA of each of the pure cultures (Table 5.1), and from that extracted from the three natural communities (chimney, diffuse flow fuids, and filaments). For this, an initial PCR reaction was performed with primers T37F and T39R (designed to amplify a fragment ~1600bp), followed with a nested PCR using primers T38R and W9F (previously designed primers by Gregory, *et al.*, 2000). A *narG* fragment of ~ 444 bp was successfully amplified from each of the clones from the environmental libraries through this procedure. PCR conditions for amplification reactions were as follow: 5 minutes at 94°C, one minute at 94°C, annealing at 55°C for 60s, and extension at 72°C for 1.5 minutes, with a final extension time of 10 minutes at 72°C during the last cycle. In order to reduce PCR bias, at least four independent PCR reactions per sample were carried out in parallel and pooled.

For the natural communities in this study, 16S rRNA gene amplification through PCR (primers: p8F and p1517R) and cloning were successfully accomplished by Mariola Cuebas.

Library construction, restriction fragment length polymorphism screening and sequence

The amplified *narG* 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). Three 16S rRNA gene and three *narG* environmental libraries were constructed: the EPR East Wall diffuse flow fluids library (Dive 4102), the EPR bacterial filaments at Marker 89 library (Dive 4109) and the MAR sulfide library from Rainbow chimney (Dive 3678).

Sixty randomly chosen clones from each 16S rRNA and *narG* environmental sample (total of 360 clones for both 16S rRNA and *narG*) were analyzed for insert-containing plasmids by direct PCR followed by gel electrophoresis of the amplified products. These were screened by restriction fragment length polymorphism (RFLP) analysis using *HaeIII* restriction endonuclease. Representative clones for each library showing unique RFLP patterns were selected and the sequences were determined for both strands on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). A summary showing the number of clones examined for each library is presented in the supplemental material (Table 5.2 and 5.3).

Phylogenetic analyses

16S rRNA nucleic acid and deduced NarG amino acid sequences (translated using the online tool EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq/) were assembled using the AutoAssembler Program (Applied Biosystems, Foster City, CA), 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 Observed Divergence matrix and the neighbor joining method was used to evaluate tree topologies. Phylo_win was used to plot tree topologies (Galtier *et al.*, 1996) and their robustness was tested by bootstrap analysis with 500 resamplings.

Statistical analyses

To compare the richness of *narG* and 16S rRNA profiles among the different libraries, rarefaction curves were constructed using the Analytic Rarefaction 1.3 program on restriction profiles obtained by the RFLP procedure. This data was complemented by calculating the diversity index of each *narG* and 16SrRNA library using the Shannon-Wiener Index.

Results

Pure cultures

Pure cultures from hydrothermal vent environments were obtained from the deep-sea microbiology laboratory culture collection. All cultured strains were where grown in anaerobic artificial seawater (supplemented with yeast extract 3g/L and peptone 2.5g/L) in the presence/ absence of NO_3^- to test for their ability to respire nitrate in anaerobic conditions. The isolates that could presumably utilize nitrate as terminal electron acceptor all belonged to the *Alcanivorax* and *Marinobacter* genera, as indicated by phylogenetic analyses of the 16S rRNA gene (Table 5.1).

The gene encoding the catalytic α-subunit of the membrane-bound nitrate reductase, *narG* was amplified from: (a) the *Alcanivorax* strains MAR12, EPR7 and EPR8, (b) *Marinobacter* strains EPR 80, 81, 108, 109 and 229 and (c) *Kribella* strain EPR178. A summary showing the number of isolates examined for growth in nitrate-reducing conditions and detection of *narG* gene is presented in Table 5.1

Table 5.1 Laboratory vent isolates tested for heterotrophic growth using nitrate as a terminal electron acceptor

	PCR product	ASW anaerobic + NO_3	ASW anaerobic without NO_3	Isolation Location	Temperature (°C)
Alcanivorax sp. MAR 12	+	+	-	Broken Spur Mussels	28
Alcanivorax sp. EPR 7	+	+	-	Mk 119 Vent	37-45
Alcanivorax sp. EPR 8	+	+	+	Mk 119 Vent	37-45
Marinobacter sp. EPR 49	-	+	-	Mk 119 Plume	28
Marinobacter sp. EPR 80	+	+	+	Tica Vent	45
Marinobacter sp. EPR 81	+	+	+	Tica Vent	46
Marinobacter sp. EPR 108	+	+	+	Mk 89	30
Marinobacter sp. EPR 109	+	-	-	Mk 89	30
Marinobacter sp. EPR 229	+	+	-	from slurp (sediments, very oily)	35
Marinobacter sp. EPR 35	-	+	+	Mk 119 Plume	28
Halomonas sp. EPR 84	-	-	-	Mk 119 Plume	30
Marinobacter sp. EPR 59	Faint band near correct size	+	-	Mk 119 proper (Fish hole)	28
Rhodococcus sp. EPR 110	-	-	-	Microbial Mat, Bio9	30
Acinetobacter sp. EPR 111	-	-	-	Microbial Mat, Bio9	30
Kribella sp. EPR 178	+	+	-	Alvinella worm	30
Klebsiella sp. EPRN3	-	+	+	Crab spa	30
Klebsiella sp. EPRN2	-	+	+	bottom seawater	30
Shewanella sp. EPRN1	-	+	+	bottom seawater	30
Pseudomonas sp. P412-1	-	+	+	Mediterranean sea	30
Pseudomonas sp. P412-2	-	+	-	Mediterranean sea	30

Comparative RFLP analysis of the *narG* gene amplified from deep-sea vents natural microbial communities

narG clones obtained from the genomic DNA extracted from three environmental samples, the EPR East Wall diffuse flow fluids library (Dive 4102), the EPR bacterial filaments at Marker 89 library (Dive 4109) and the MAR sulfide library from Rainbow chimney (Dive 3678) were analyzed based on differences in their RFLP profiles. Comparative diversity of *narG* restriction profiles in the three sites is summarized in Table 5.2. The number of unique *narG* restriction patterns was 18 out of 59 screened clones in dive 4102, 21 out of 60 screened clones in dive 4109 and 6 out 57 screened clones in dive 3678. The diversity index, as calculated using the Shannon-Wiener Index for sample 4102 is 2.59, 2.56 for sample 4109 and 1.13 for sample 3678. Rarefaction curves for the *narG* restriction profiles (Figure 5.1) are in accordance with the Shannon-Wiener Index, were samples 4102 and 4109 have similar richness of *narG* restriction profiles, whereas sample 3678 showed a significantly lower richness in *narG* restriction profiles. Rank-abundance curves were also performed for each site, as shown in Figure 5.2. The long right-hand tail in the rank-abundance curves of samples 4102 and 4109 shows that a few *narG* restriction profiles are abundant, but most are rare. In comparison, sample 3678 *narG* restriction profiles indicate a lower diversity of the *narG* gene involved in nitrate reduction.

Table 5.2 *narG* restriction profiles for three environmental samples: Dive 4102 (EPR bacterial filaments at Marker 8), Dive 4109 (EPR East Wall diffuse flow fluids) and Dive 3678 (MAR sulfide from Rainbow chimney). *Bold numbers are cloned representatives

Restriction group	Dive 4102 (number of clones)	Dive 4109 (number of clones)	Dive 3678 (number of clones)
1	(0)	**A1**, **A4**, E10 (3)	(0)
2	**A5**, B1, B2, B7, D3, **D10** (6)	**A12**, B6, D2 (3)	**A10**, B7, C3, C9, **D7** (5)
3	(0)	D9, E2, **E9** (3)	**A12**, **C1** (2)
4	(0)	(0)	**D5** (1)
5	**E4** (1)	(0)	(0)
6	(0)	F3, **F5** (2)	(0)
7	(0)	**B2** (1)	(0)
8	(0)	**B11** (1)	(0)
9	**A1** (1)	**A10** (1)	(0)
10	A8, **B5**, **D7**, E2, E7, E10, **E12** (7)	**D4** (1)	(0)
11	(0)	**D5** (1)	(0)
12	**B12** (1)	**D1** (1)	(0)
13	(0)	**C10** (1)	(0)
14	(0)	A3, A7, B4, **E11**, E12, F1 (6)	(0)
15	**C3**, C4, C6, **D2**, D6, D9, D12, E1, E5 (9)	(0)	(0)
16	(0)	**A6**, A9, A11, **B12**, C4 (5)	(0)
17	(0)	**E5** (1)	(0)
18	(0)	A8, B1, **B5**, B8, **B9**, C2, C9, C11, D3, D7, E1, **E6**, E7, E8, F4 (15)	(0)
19	**C12**, D1 (2)	(0)	(0)
20	**A3**, **A6**, **D11** (3)	(0)	(0)
21	(0)	(0)	**A1**, A3, A4, A5, A6, A7, A8, **A11**, B1, B2, B4, B5, B6, **B8** (14)
22	(0)	**C7**, **D6** (2)	**A9**, B9, **B10**, B11, B12, C2, C4, C5, **C6**, C7, C8, C10, C11, C12, D1, D2, **D3**, D6, D8, **D10**, D11, D12, E1, E2, E3, E4, E5, E6, **E7**, E8, E9, E12, F2, F3 (34)
23	(0)	**B3**, **B10**, C3, C6, C8, **D11**, D12, E3, E4 (9)	(0)
24	**A4**, A12, C9, C11 (4)	**D10** (1)	(0)
25	(0)	**B7** (1)	(0)
26	**E11** (1)	(0)	(0)
27	**D8**, D9 (2)	(0)	(0)
28	**E6** (1)	(0)	(0)
29	**A10**, B3, **B6**, **B8** (4)	(0)	(0)
30	**A9**, **B9**, C2, F2 (4)	(0)	**D4** (1)
31	**A7** (1)	(0)	(0)
32	**A2**, B4, B10, **B11**, C5, C7, C8, **C10**, D4 (9)	(0)	(0)
33	**E8** (1)	**C5** (1)	(0)
34	**A11**, **F1** (2)	(0)	(0)
35	(0)	**F2** (1)	(0)
cloned	30	28	15
total	59	60	57

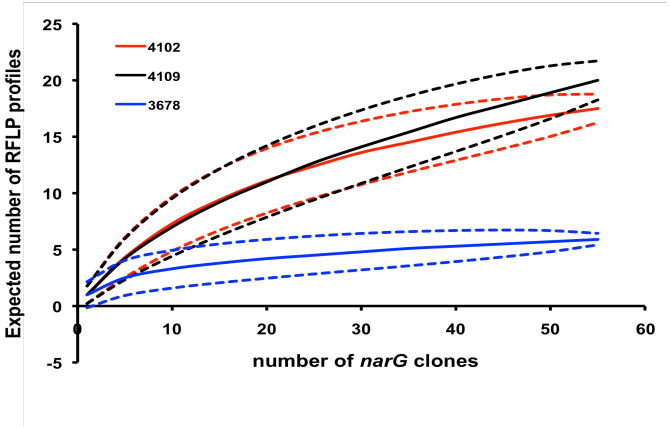


Figure 5.1 Rarefaction curves of observed *narG* restriction profiles for sampling sites 4102, 4109 and 3678. The error bars (dashed lines) are 95% confidence intervals.

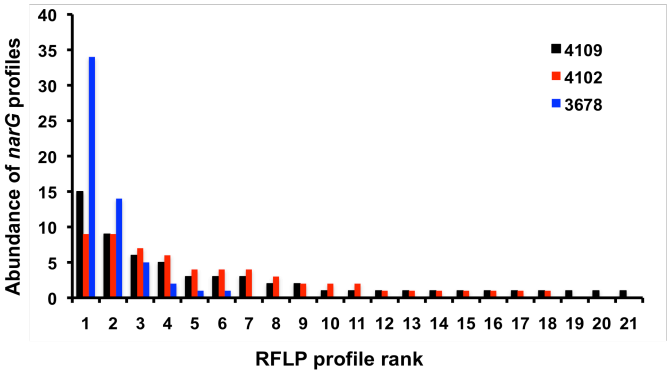


Figure 5.2 Rank-abundance curves for observed *narG* restriction profiles for sampling sites 4102 (n = 59), 4109 (n = 60) and 3678 (n = 57).

Comparative phylogenetic analyses of *narG* from deep-sea vents natural microbial communities

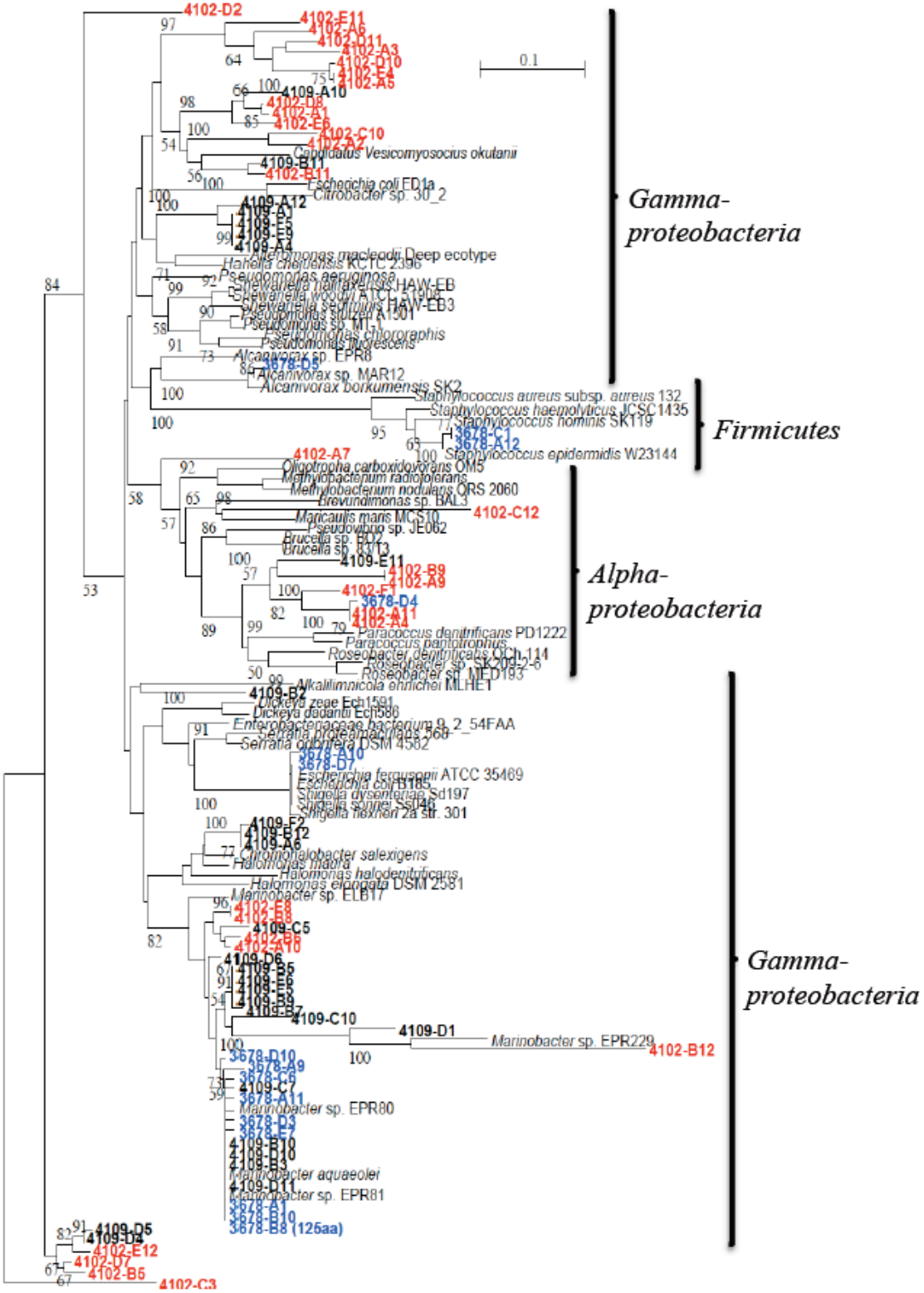
Comparative phylogenetic analyses were performed on the *narG* gene sequences generated from the laboratory isolates and environmental samples. The bacterial *narG* clones obtained from microbial communities from white bacterial filaments samples (Dive 4102) revealed that 78% (46/59) of the *narG* clones were related to gammaproteobacterial *narG* genes while 22% (13/59) were related to alphaproteobacterial phylotypes (Table 5.2, Figure 5.3). From the gammaproteobacterial phylotypes obtained, 52% (24/46) were related to the *narG* gene of *Candidatus Vesicomyosocius okutanii HA*. The remaining gammaproteobacterial clones were related to the *Marinobacter* group (33%; 15/46) and the *Pseudomonas* group (15%; 7/46). The alphaproteobacterial *narG* clones were composed of sequences related

to the *Paracoccus/Roseobacter* group (77%; 10/13), followed by the *Maricaulis* (15%; 2/13) and *Brevundimonas* groups (8%; 1/13).

The diversity of *narG* phylotypes from diffuse flow fluids community (Dive 4109) were almost entirely associated to the *Gammaproteobacteria* (90%, 54/60), with 10% (6/60) of the sequences related to alphaproteobacterial *narG* genes (Table 5.2, Figure 5.3). Most of gammaproteobacterial sequences were phylogenetically placed in association with the *Marinobacter* group (59%, 32/54), followed by related sequences to the *Hahella spp*. (20%, 11/54), *Halomonas spp.* (11%, 6/54) and *Candidatus Vesicomyosocius okutanii* (9%, 5/54). The small alphaproteobacterial fraction of sequences associated with sample 4109 clustered with the *Roseobacter spp*. (11%, 6/54).

The majority (95%; 54/57) of the bacterial *narG* clones retrieved from the Rainbow site (Dive 3678) were related to the *Gammaproteobacteria*, while 5% (3/57) were related to the *Alphaproteobacteria* and *Bacilli* (Table 5.2, Figure 5.3). Phylogenetic analyses revealed that 89% (48/54) of the gammaproteobacterial *narG* sequences, belonged to the *Marinobacter* group, 9% (5/54) belonged to the *Escherichia spp*. and 2% (1/54) was related to the *Alcanivorax narG* gene sequence. The remaining sequences clustered with the alphaproteobacterial group of *Roseobacter* (1.5%; 1/57) and with the *Firmicutes* (3.5%; 2/57).

Figure 5.3 Phylogenetic tree of the *narG* gene for samples 4102 (red), 4109 (bold/black) and 3678 (blue) **narG* sequences for strains EPR 7 and 178 are not included because they were too short.



Comparative RFLP analysis of the 16S rRNA gene amplified from deep-sea vent natural microbial communities

16S rRNA clone libraries for environmental samples 4102 and 4109 were analyzed based on the diversity of restriction profiles on the RFLP procedure and through phylogenetic methods performed on amplified 16S rRNA gene sequences. The comparative diversity of 16S rRNA restriction profiles in the two sites is summarized in table 5.3. The number of unique 16S rRNA restriction patterns was 15 out of 61 screened clones in site 4102 and 23 out of 60 screened clones in site 4109. The diversity index, as calculated using the Shannon-Wiener Index for sample 4102 is 2.04 and 2.66 for sample 4109. Rarefaction curves for the 16S rRNA restriction profiles (Figure 5.4) are in accordance with the Shannon-Wiener Index, were sample 4109 has a higher richness of 16S rRNA restriction profiles in comparison to sample 4102. Rank-abundance curves were also performed for each site, as shown in Figure 5.5. The long right-hand tail in the rank-abundance curves of samples 4102 and 4109 shows that a few restriction profiles are abundant, but most are rare.

Data from previous phylogenetic analyses performed by Voordeckers *et al.*, 2008 on the microbial community of sample 3678 (Table 5.4, Figure 5.4) indicate an overall lower diversity of the microbial community associated to black smoker environments. This is also reflected by a lower diversity index of 1.27.

Table 5.3 16S rRNA restriction profiles for for the environmental samples: Dive 4102 (EPR bacterial filaments at Marker 8) and Dive 4109 (EPR East Wall diffuse flow fluids).*Bold numbers are cloned representatives

Restriction group	Dive 4102 (number of clones)	Dive 4109 (number of clones)
1	B7, **B8** (2)	(0)
2	B3, **B5**, B6 (3)	**D6**, D11 (2)
3	**B10** (1)	(0)
4	(0)	**E10** (1)
5	(0)	**F1** (1)
6	**B1**, B2 (2)	(0)
7	**A3**, A5, A12 (3)	B12, C2, **C3**, **C7**, C10, C12, D3, **D5**, D12, **E3**, E4, E7 (12)
8	(0)	**E11** (2)
	(0)	**F3**
9	A6, A8, A9, **D12** (4)	**A11**, **A12**, B1, B2 (4)
10	**A7**, E11 (2)	**C5**, A1 (2)
11	**E2** (1)	(0)
12	**A4** (1)	(0)
13	(0)	**A2** (1)
14	B9, B11, B12, **C1**, C2, C5, C10, C11, C12, D1, D2, **D3**, D5, D7, **D11**, E3, E4, **E5**, E9, E10, E12, F5 (22)	**E1**, E9 (2)
15	F1, **F4** (2)	(0)
16	**A2**, A10, A11, C3, C4, **C6**, C7, C8, D6, D8, D9, D10, E1, E7, **E8** (15)	B11, **E6** (2)
17	(0)	**C9** (1)
18	**F3** (1)	(0)
19	(0)	A3, A6, B9, B10, **C1**, D7, **D8**, D9, **E5**, F2 (10)
20	(0)	A4, **B6** (2)
21	(0)	D4, **D10**, E8 (3)
22	(0)	**A7** (1)
23	(0)	A9, **C4**, D10, E8 (4)
24	(0)	**B8**, C6 (2)
25	(0)	**A5**, C11 (2)
26	(0)	**D2** (1)
27	(0)	**B7** (1)
28	(0)	**A8** (1)
29	(0)	**B3**, B4 (2)
sequenceed	19	31
total	59	59

Table 5.4 16S rRNA restriction profiles, dive 3678 (data from Voordeckers *et al.*, 2008)

Restriction group	Dive 3678 (number of clones)
1	1, 7, 9, 22 (4)
2	19 (1)
3	11, 14, 21 (3)
4	3 (1)
5	2, 4, 5, 6, 8, 12, 13, 16, 18, 20, 10, 17, 23, 24 (14)
6	15 (1)
7	25 (1)
total	25

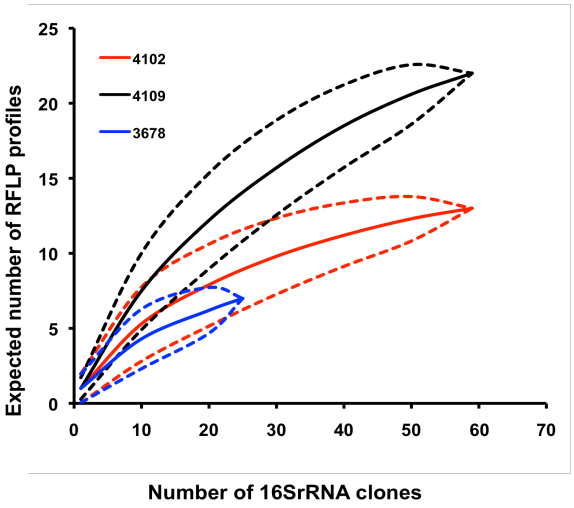


Figure 5.4 Rarefaction curves of observed 16S rRNA restriction profiles for sampling sites 4102, 4109 and 3678. The error bars (dashed lines) are 95% confidence intervals.

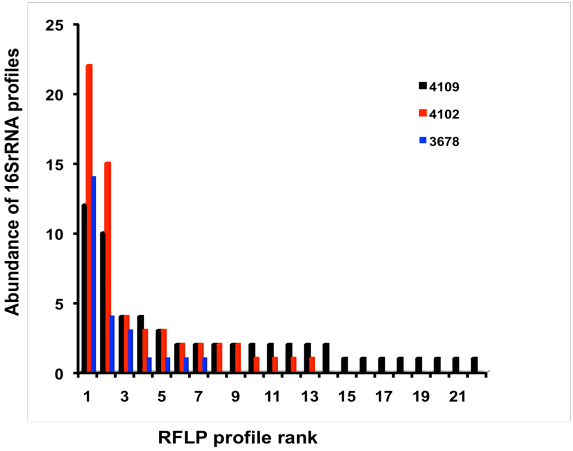


Figure 5.5 Rank-abundance curves for observed *narG* restriction profiles for sampling sites 4102 (n = 59), 4109 (n = 59) and 3678 (n=25).

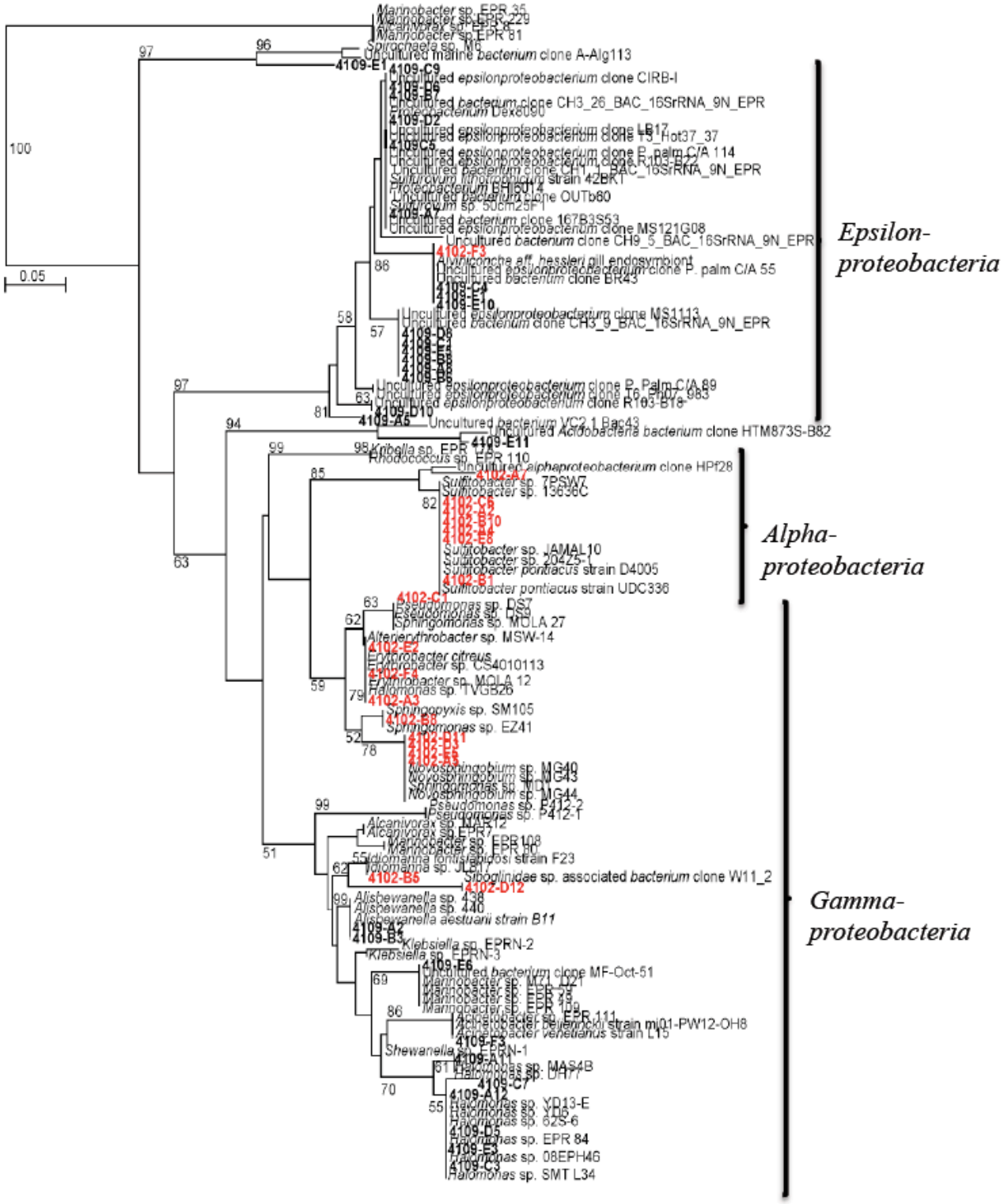
Phylogenetic analysis of the 16S rRNA gene in deep-sea vents microbial communities

The bacterial 16S rRNA gene phylogeny (Figure 5.6) from samples obtained from the white filaments was dominated by *Alphaproteobacteria* (60%; 18/30), while 30% (9/30) belonged to the *Gammaproteobacteria* and 10% (3/30) belonged to unknown bacterial species. The majority of the 16S rRNA alphaproteobacterial clones were related to the *Sulfitobacter* spp. (55%; 10/18), a group that represent a substantial part of the heterotrophic population in litoral marine environments (Sorokin, 1995) and to the *Novosphingobium* spp. (39%; 7/18), an aromatic compound-degrading group of bacteria (Takeuchi *et al.*, 2001).

The bacterial 16S rRNA gene library from diffuse flow fluids communities (Dive 4109) was entirely represented by unkown phylotypes mostly related to uncultured *Epsiloproteobacteria*. However, some of the unknown phylotypes obtained from this particular library clustered with the gammaproteobacterial taxa of *Halomonas* and *Marinobacter* (Figure 5.6). Thus, this phylogenetic data is in accordance to the rarefraction data suggesting a higher community diversity.

Previous studies on the 16S rRNA phylotypes from the Rainbow black smoker community showed that they were mostly associated to the *Epsilonproteobacteria* (97%; 34/35), while only 3 % (1/35) were related to the *Deltaproteobacteria* (Voordeckers *et al.*, 2008). Sequenced epsilonproteobacterial clones were mostly placed in the *Caminibacter/Nautilia* group (Voordeckers *et al.*, 2008).

Figure 5.6 16S rRNA phylogenetic tree for samples 4102 (red) and 4109 (bold/black)



Discussion

16S rRNA-based phylogenetic analysis of deep-sea vents microbial communities

Phylogenetic analyses of the 16S rRNA genes obtained from the microbial communities from the MAR black smoker, the white filaments and diffuse flow fluids from EPR showed differences in terms of composition and richness of the microbial communities surveyed. The highest diversity based on the 16S rRNA restriction profiles was the diffuse flow fluid microbial community (Dive 4109) with most of the16S rRNA bacterial clones related the *Epsilonproteobacteria* and bacterial phylotypes that clustered within the *Gammaproteobacteria*. The bacterial community of the white filaments in "Mk 89" (Dive 4102) showed an intermediate diversity from the communities surveyed, with a microbial phylogenetic composition of *Alpha-* (60%) and *Gamma-proteobacteria* (30%). Previous work of the Rainbow black smoker community showed a very limited diversity, with 97% of the16S rRNA bacterial clones related the *Epsilonproteobacteria* (Voordeckers *et al.*, 2008). Overall, the communities from diffuse flow fluids (in-situ temperature of 25°C) was more diverse that those communities inhabiting the MAR sulfide (temperature of fluid at the chimney orifice:158°C) and the EPR white filaments (in situ temperature:2°C). In general, a correlation exists between temperature and redox state of hydrothermal fluids, in that higher temperature fluids tend to be more reduced that lower temperature ones (Karl, 1995; Lopez-Garcia *et al.*, 2003). This explain the differences in the phylogenetic composition of the three microbial communities, where the community directly associated to hydrothermal fluid venting (Dive 3678) showed a lower diversity due to the restrictive nature of the physico-chemical conditions, in comparison to lower temperature environments. Therefore, in terms of microbial community composition, the high temperature environment was dominated by *Epsilonproteobacteri*a, while the diffuse flow and white filaments communities showed a shift in the composition of bacterial phylotypes. In these two lower temperature communities we observed the appearance of *Gamma-* and *Alpha-proteobacteria*, usually associated to low temperature background seawater environments (Kaye & Baross, 2000; Kaye *et al.*, 2011).

Diversity of the NarG nitrate reductase in deep-sea vent microbial communities and representative pure cultures

Culture-dependent data shows that mostly members of the *Marinobacter* and *Alcanivorax* genera are able to perform dissimilatory nitrate reduction (Singer *et al.*, 2011; Wang *et al.*, 2010). We demonstrated that strains belonging to *Marinobacter* and *Alcanivorax* spp. isolated from deep-sea hydrothermal vents grew under nitrate-reducing conditions in the laboratory, and we detected the *narG* gene by PCR in these strains. Overall, these results indicate that the ability to reduce nitrate in these strains is correlated with the presence of the *narG* gene.

narG was also detected in the microbial communities associated with three different deep-sea vent habitats: a black smokers, diffuse flow fluids and microbial filaments. WE were able to sample of *narG* environmental sequences from all communities, as shown with the rarefaction and the rank-abundance curves, can be attributed to the high conservation of this functional gene. Phylogenetic analyses on the *narG* gene showed that for all three microbial communities studied, the genes retrieved were mostly related to gammaproteobacterial loci. The percent of the gammaproteobacterial-like Nar enzymes obtained for each community was: 78% in microbial mats community, 90% in diffuse flow communities and 95% in the black smoker microbial community.

The richness of *narG* restriction profiles was consistently lower in the microbial community associated with the black smoker environment, compared to the diffuse flow and microbial mats communities, which was evident through both rarefraction curves and diversity indexes. In terms of temperature and reducing state, the black smoker was the most extreme of the three habitats examined in this study, and it was dominated by *Epsilonproteobacteria* (Voordeckers et al., 2008). These bacteria are typically associated with high to moderately reducing habitats and their nitrate-reducing ability depends on the periplasmic nitrate reductase Nap, rather than NarG (Voordeckers et al, in preparation). Therefore, it is likely that the stringent environmental conditions associated with the black smoker do not provide a niche compatible with a wide diversity of Nar-dependent nitrate-reducing bacteria, such as *Gamma-* and *Alphapreobacteria*.

Comparative analyses of the 16S rRNA and *narG* clonal libraries

There is some correlation between the NarG and the 16S rRNA phylogenetic trees, suggesting that in many cases the gene histories of the NarG subunits represent the phylogeny of the organisms (Philippot, 2002; Philippot, 2005). This potentially suggests that the taxonomic composition of the nitrate-reducing community relying on the membrane-bound NarG enzyme is similar in different vent-microbial niches. We have shown a clear abundance of gammaproteobacterial *narG*-related sequences in different vent from the EPR and the MAR. Thus, there is a possibility that *Gammaproteobacteria* related to *Marinobacter s*pecies dominate the NarG-dependent nitrate-reducing bacteria in different deep-sea vent communities. However, our 16S rRNA surveys have shown that *Gammaproteobacteria* are more abundant in low temperature deep-sea environments, in comparison to higher temperature microbial niches, which show a shift in the microbial composition to members of the *Epsilonproteobacteria*. Thus our results suggest that nitrate-reduction through the *narG* enzymatic pathway appears to be relevant to low and moderate temperature communities such as those associated to the white filaments and diffuse flow, where the *Gamma-* and *Alphproteobacteria* were well represented both in the 16S rRNA and *narG* clonal libraries.

It is interesting to note that while gamma- and alpha-proteobacterial phylotypes were not obtained from the 16S rRNA library of the black smoker and diffuse flow communities, we were able to detect *narG* sequences related to *Marinobacter* spp. Evidence from previous surveys carried out on hydrothermal vent microbial communities and cultured vent *Epsilonproteobacteria*, revealed that a NapA catalyzed respiratory nitrate reduction to either dinitrogen gas or to ammonium is mediated by chemolithotrophic *Epsilonproteobacteria* (Voordeckers *et al.,* unpublished). Thus, it is likely that in these higher temperature, more reducing habitats, where *Epsilonproetobacteria* dominate the microbial communities, nitrate-reduction is being performed by the dominant community through the periplasmic Nap nitrate reductase.

Implications on the role of the "rare biosphere"

While the detection of organisms that correspond to the most abundant phylotypes requires minimal sampling, as shown in this work (figures 5.1 and 5.4) the recovery of the "rare biosphere" in these communities demands surveys that are many orders of magnitude larger that those reported in this work

(Sogin *et al.*, 2006). We hypothesize that, because of the changing conditions inherent to hydrothermal fluids at deep-sea vents, these undetected "rare phylotypes", which are subjectively defined as "rare" depending on the local environmental conditions, will reappear and possibly dominate under new physico-chemical regimes. Specifically to this work, the microbial communities involved in the process of nitrate reduction have the potential to shift from those communities catalyzing a Nap-based nitrate respiration (epsilonproteobacterial phylotypes in high temperature environments), to a Nar-based nitrate respiration (gamma- and alphaproteobacterial phylotypes at mesophilic and low temperature environments).

The observed differential richness and distribution of 16S rRNA and *narG* genes obtained in this survey is of ecological significance to our understanding of the biogeography of nitrate-reducers at vent and deep-sea environments, providing new insights into the distribution of nitrate reduction pathways under various physico-chemical conditions associated to deep-sea geochemical processes.

Conclusion

In this study we used the α subunit of the Nar nitrate reductase (encoded by the *narG* gene) as a molecular marker to target the microbial community genetically capable of reducing nitrate through this enzymatic pathway in different microbial communities from deep-sea hydrothermal vents. The PCR protocol used allowed the amplification of *narG* sequences from taxonomically diverse bacteria, from the *Gamma-* and *Alpha-proteobacteria* as well as *Bacillus* spp. and sequences of yet-unknown phylotypes. A similar approach has previously been developed for genes encoding the periplasmic nitrate reductase (Grove *et al.,* 1996) in vent environments and amplification of sequences from *Epsilonproteobacteria* was achieved (Voordeckers *et al.,* unpublished). Even if these results don't give any insight on *in situ* expression, given that nitrate reduction and denitrification are facultative processes (Philippot, 2005), this study furthers our understanding of the spatial distribution of bacteria carrying nitrate reductase genes in vent communities. Results obtained from all *narG* and 16S rRNA gene clone libraries are consistent with the possibility that although not necessarily metabolically active, viable bacteria of a particular functional type can be recovered from almost any environment, using appropriate tools for detection, even where that environment cannot support their growth, as is the case of gammaproteobacterial phylotypes in black smoker associated

environments (Falkowski *et al.*, 2008). This ubiquity of the core nitrate-reducing gene set, whether by vertical or horizontal gene transfer, allows a wide variety of microorganisms to simultaneously, but temporarily, become guardians of nitrate-reduction under different conditions in the deep-ocean (Falkowski *et al.*, 2008).

Chapter 6

Conclusion

Our current environment reflects the historically integrated outcomes of microbial experimentation on a tectonically active planet endowed with a thin film of liquid water (Falkowski *et al.*, 2008; Vernadsky, 2007). Through abiotic geochemical reactions based on acid/base chemistry and through the chemistry of life based on redox reactions, biogeochemical cycles have been - and keep - evolving on a planetary scale (Falkowski *et al.*, 2008). The metabolic processes that evolved exclusively in microorganisms are of special importance given that they have altered the chemical speciation of virtually all elements on the surface of the planet.

The unique physico-chemical characteristics of Mid-Ocean Ridges may bear resemblance to conditions that existed on early Earth (Pace, 1991; Reysenbach *et al.*, 2000). Based on evidence for anaerobic metabolic processes in 3.5 billion year deposits (Canfield & Raiswell, 1999)**,** it is likely that the earliest organisms on Earth coupled oxidants with chemically reduced compounds delivered from Earth's interior (Canfield *et al.*, 2010). In this context, it is fascinating to learn that modern microorganisms living at deep-sea vents also posses many characteristics that represent putative early traits (Battistuzzi *et al.*, 2004), such as anaerobic chemosynthetic metabolism (Jannasch, 1995; Russell & Hall, 1997), thermophily (Russell & Hall, 1997), carbon fixation through the rTCA cycle and other alternative pathways (Pereto *et al.*, 1999; Wachtershauser, 1990), and biofilm formatiom (Davey & O'Toole G, 2000; Stoodley *et al.*, 2002). This evidence show that the involvement of microorganisms in the transformation of rocks and minerals at and below the seafloor is presumably very old and that they have been involved in these processes for most of life's history on Earth (Edwards *et al.*, 2005; Falkowski *et al.*, 2008; Fredrickson & Zachara, 2008).

In this thesis dissertation, I presented the isolation and characterization of two novel anaerobic, chemolithoautotrophic, thermophilic and hyperthermophilic bacteria from deep-sea hydrothermal vents. In accordance to previously discussed work, microbial representatives associated to the reduced, higher temperature deep-sea environments belong to the *Epsilonproteobacteria* and the *Aquificae*. Both strains described in this work, *Natutilia nitratireducens* and *Phorcys thermohydrogenephilus* represent novel species

within these groups, thus providing further information on the diversity, physiology and metabolism of microbial communities at deep-sea vents. The two bacteria discussed in this thesis, along with most cultured anaerobic, chemolithoautotrophic and thermophilic microorganisms from deep-sea vents, have been shown to couple both nitrate- and sulfur-reduction to hydrogen oxidation as their main respiratory pathways (Table 1.1). Under laboratory conditions, optimal growth consistently occurs when microorganisms are grown under nitrate reducing conditions. This, together with bioenergetic considerations, points to the possibility that nitrate reduction might be a much more relevant metabolic process than previously tought. My work, along with that of others in the Vetriani laboratory and elsewhere, provides evidence for the widespread presence of the nitrate-reduction pathway in different hydrothermal vent environments. Through a *narG* based -nitrate reductase gene survey in different seafloor environments, we determined that micoorganisms capable of this pathway are widespread in different environments on the seafloor. Thus, this data supports the hypothesis that nitrate-reduction can be carried out in environments were nitrate reduction was previously believed to not occur (such as oxidized, low temperature environments).

This work also provides insights into the role of quorum sensing in deep-sea vent microbial communities. As it has been pointed out throughout this dissertation, microorganisms at deep-sea vents (and most natural environments) preferably grow as biofilm communities. Microorganisms within a consortium obtain "mutlicellular and macrobial" characteristics that allow them to impact their environment in ways that would be impossible for individual cells (Nadell *et al.*, 2009). The biofilm life strategy appears to have had a crucial role for the survival of organisms in the early Earth (Reysenbach & Cady, 2001; Stetter, 1996) as well as in modern times (reviewed in Stoodley *et al.*, 2002). In the deep-sea, these communities appear to sustain themselves from the extreme hydrothermal vents and subseafloor environments. Moreover, these extensive deep-sea vent associated biofilms, which are constantly interacting with the surrounding chemistry, have an intrinsic effect on global ocean chemistry. My work focuses on mechanisms that could potentially regulate biofilm formation, as well as other potential group behaviors in deep-sea microorganisms. We have aimed to explore the *luxS* AI-2 based quorum sensing mechanism in deep-sea vent *Epsilonproteobacteria*, a well-represented taxa in hydrothermal vents. In clinical epsilonproteobacterial relatives, *luxS* is used as a

quorum sensing mechanism that regulates virulence, adhesion and biofilm formation. We have found that *luxS* is differentially expressed during growth of the cultured deep-sea chemosynthetic *Epsilonproteobacteria*, *Caminibacter mediatlanticus* and *Sulfurovum lithotrophicum*. This pattern of expression is correlated to the detection of a functional AI-2 molecule that is able to induce bioluminescence, a quorum sensing response, in a *Vibrio harveyi* reporter strain. Moreover, we have been able to detect the expression of *luxS* in natural *Epsilonproteobacteria*-dominated vent biofilms, suggesting that AI-2 signalling molecules are being synthesized *in situ*.

The realization that deep ocean microbial biofilms could influence global biogeochemistry has been a major revelation in the geosciences (Schrenk *et al.*, 2010) given their strength in numbers and outstanding metabolic versatility (Nadell *et al.*, 2009). Thus, learning about the physiological and metabolic characteristics of cultured vent microorganisms can help us make inferences about the physiological and metabolic potential of uncultured microbes with related phylogeny (Jannasch, 1995). Moreover, understanding the mechanisms that microorganisms evolved to respond and interact with abiotic conditions at the molecular and microscopic scales, has great implications on our understanding of their tremendous past and present influence over large scales on global biogeochemical cycling of C, Fe, N, and other elements (Fredrickson & Zachara, 2008).

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