DIVERSITY OF CHEMOSYNTHETIC THIOSULFATE OXIDIZING BACTERIA FROM DIFFUSE FLOW HYDROTHERMAL VENTS AND THEIR ROLE IN MERCURY DETOXIFICATION

By

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ABSTRACT OF THE DISSERTATION

Diversity of Chemosynthetic Thiosulfate Oxidizing Bacteria from Diffuse Flow Hydrothermal Vents and Their Role in Mercury Detoxification

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Dr. Costantino Vetriani

The mixing of hydrothermal fluids with seawater creates chemical, temperature, and pH gradients that support chemosynthetic primary production at deep-sea vents. These fluids are enriched with reduced sulfur compounds and their oxidation under aerobic conditions is considered the main chemosynthetic process at the vents. The main objective of my research was to gain a better understanding of the aerobic chemosynthetic thiosulfate oxidation processes at deep-sea hydrothermal vents, by studying the abundance and diversity of chemosynthetic thiosulfate oxidizing bacteria, and their role on the detoxification of heavy metals, with an emphasis on mercury detoxification. Fluids, sediments, and biomass from microbial colonization experiments were collected during several expeditions to the East Pacific Rise (EPR) at "9º50'N, 104º17'W" and to the Guaymas Basin, Gulf of California. Microbial isolations were carried out from diluted and undiluted samples. Isolates were identified by 16S rRNA gene analysis. The isolates obtained in pure cultures were related to the genera

Thiomicrospira, *Halothiobacillus*, *Hydrogenovibrio, Thioclava, Thalassospira,* and *Pelagibaca,* as well as a new isolate EPR 70, which was described as a new species, *Salinisphaera hydrothermalis*. The isolates were further characterized, and their functional genes encoding enzymes for carbon fixation (RubisCO) and thiosulfate oxidation (SoxB) were analyzed. The Most Probable Number (MPN) technique was carried out in order to determine the abundance of chemosynthetic thiosulfate oxidizing bacteria, and the values obtained were compared with the total number of microorganisms per sample, estimated from microscopic direct counts. Our data show that this group of microorganisms represented from 10^3 to 10^7 cells per ml of sample, which accounts for about 0.002% to 14.1% of the total cell counts per sample. The chemical composition of the fluids was analyzed, and results indicated that hydrothermal fluids were enriched in mercury with concentrations comparable to the concentrations found in contaminated surface waters. MPN counts were done with the addition of mercury in order to determine the percentage of chemosynthetic thiosulfate oxidizing bacteria that were mercury resistant. Results indicated that from 0.2 to 24.6% of the chemosynthetic bacteria were resistant, suggesting an adaptation to life in the presence of this toxic metal.

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Dedication

I want to dedicate this thesis to my family in Puerto Rico, for all the time that we have not been together physically, although our hearts have always been next to each other, and to my husband Carlos Lopez-Maisonave, for all his support, love, and comprehension.

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Chapter 1 – Introduction

Hydrothermal vents and chemosynthetic primary production in vent ecosystems

Deep-sea hydrothermal vents are areas of the seafloor often associated with spreading centers along mid-ocean ridge systems (MOR). They were discovered in 1977 along the Galapagos Rift (Corliss *et al*., 1979; Lonsdale, 1977) and later more vents were discovered along the East Pacific Rise (EPR) (Spiess *et al*., 1980) and on the Mid-Atlantic Ridge (MAR) (Rona *et al*., 1984; Rona *et al*., 1986). At these sites, seawater is drawn into the crust, gets heated to over 1000ºC and reacts with crustal basalts, converting geothermal energy into chemical energy in the form of reduced inorganic compounds (Corliss *et al*., 1979; Edmond *et al*., 1979). When the hot water rises and reaches the sea floor, it mixes with cold bottom seawater resulting in the precipitation of reduced inorganic compounds, minerals, and metals. Hydrothermal fluids can occur as either low temperature diffuse flows, with temperature ranging from ~ 2 to 60ºC due to subsurface mixing with cold seawater, or as high temperature focused flows, often called black smokers, where temperature can be as high as 350ºC (Figure 1.1) (Jannasch & Mottl, 1985; McCollom & Shock, 1997).

The first photographs obtained from the hydrothermal vent sites revealed a discharge of a white-yellowish material that precipitated down into the adjacent basalt (Lonsdale, 1977) and dense communities of benthic macro-fauna populating the vicinities of active vents (Corliss *et al*., 1979; Lonsdale, 1977). This discovery led to an intense debate on what could be maintaining these ecosystems alive. The initial hypothesis was that the communities were sustained by the richness of the hydrothermal fluids released

from the ocean crust (Lonsdale, 1977). The second hypothesis was that these organisms may be feeding directly on bacterial cells, especially chemolithoautotrophic (chemosynthetic) bacteria (Lonsdale, 1977).

Chemosynthesis is a microbial process that refers to the activity of organisms that use chemical compounds to obtain energy for the fixation of CO_2 into organic matter, and although it is a process known for years, the possibility that an entire ecosystem relied on chemosyntetic primary production was not considered before the discovery of the deep-sea hydrothermal vents.

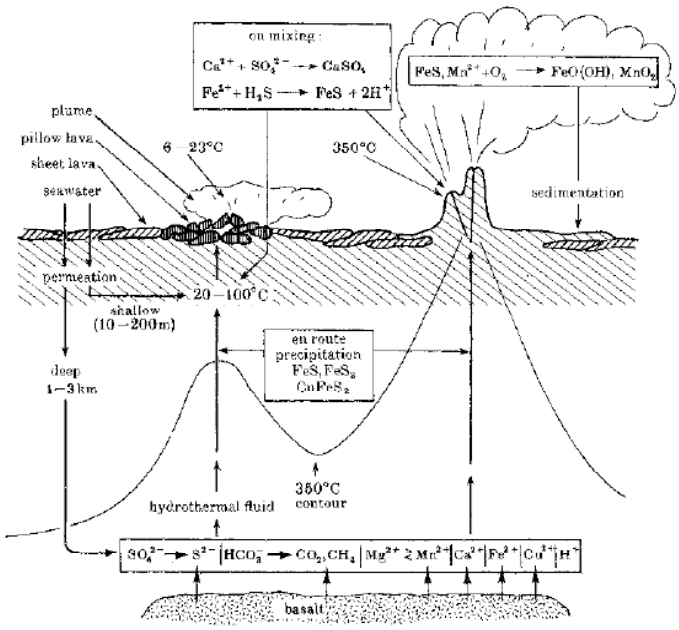

Figure 1.1 Hydrothermal fluids circulation. (Jannasch 1985)

The white particulate observed in hydrothermal fluid was soon identified as reduced sulfur compounds, mostly elemental sulfur (Jannasch & Wirsen, 1979). The

abundance of H_2S in the hydrothermal fluids was also confirmed, and these quickly led to the hypothesis that the precipitated sulfur was the product of the microbial oxidation of H_2S (Jannasch & Wirsen, 1979).

Initial studies estimated that the total microbial cell counts in hydrothermal fluids ranged from 10^8 to 10^9 cells mL^{-1} of fluid (Corliss *et al.*, 1979). When the fluids were collected about 1 meter above the source, the counts decreased to 5 x 10^5 to 10^6 cell mL^{-1} (Jannasch & Wirsen, 1979), which suggests that there is a dilution factor when the fluids are mixed with seawater at the time of collection. However, even the diluted values were at least two order of magnitude higher than what was previously reported for bottom seawater (Jannasch & Wirsen, 1979; Karl *et al.*, 1980). The isolation of over 200 strains of chemosynthetic sulfur-oxidizing bacteria from enrichments carried out using hydrothermal fluids (Jannasch & Wirsen, 1979), and the isolation of this group of microorganisms from the guts of filter feeding mussels collected on an oceanographic expedition (Rau & Hedges, 1979; Rau, 1981), confirmed the abundance of these bacteria and their importance in the hydrothermal vent environments.

Chemosynthetic bacteria have been also shown to occur in symbiotic relationships with the tubeworm *Riftia pachyptila*. These organisms lack a mouth, gut, or anus, however they have a specialized tissue called trophosome, which occupies most of their body mass. The trophosome was shown to be the site of CO_2 fixation and S^0 oxidation, and this led to the assumption that the tubeworms were autotrophic organisms themselves (Felbeck, 1981). The presence of a high number of symbiotic microbial cells inside the trophosome (up to 3.7 x 10^9 cells per gram of homogenate trophosome) (Cavanaugh *et al.*, 1981), and the presence of a highly developed circulatory system that was proposed

to deliver O_2, CO_2, and H_2S to the chemosynthetic microbial symbionts as energy and carbon sources, was later discovered (Arp & Childress, 1981). Symbiosis was also demonstrated to occur (although differently) in the polychete *Alvinella pompejana* (Campbell *et al*., 2003). This type of worms are associated with a dense layer of episymbiotic bacteria (Campbell *et al*., 2003).

After extensive research on the macro-benthic community surrounding hydrothermal vent sites, it was concluded that diffuse flow hydrothermal vents are the sites with the most rapid chemosynthetically sustained biomass production of any ecosystem on Earth (Lutz, 1994).

Chemosynthetic primary production at hydrothermal vents: Thiosulfate oxidizing bacteria

The mixing of the highly reduced hydrothermal fluids with cold oxygenated seawater creates steep gradients of chemistry, temperature, and pH that provide metabolic energy for chemosynthetic bacteria (McCollom & Shock, 1997). Under aerobic or micro-aerobic conditions, chemosynthesis can be carried out by bacteria which couple CO_2 fixation to sulfide oxidation:

$$H_2S + CO_2 + O_2 + H_2O \rightarrow [CH_2O] + H_2SO_4 \quad (1)$$

The electron acceptor in this process is O_2, while the electron donors can be a variety of oxidable substrates including forms of reduced sulfur other than sulfide, such as elemental sulfur or thiosulfate, as well as iron, manganese and ammonia (Jannasch & Mottl, 1985; Van Dover, 2000). Table 1.1 shows some of the most common aerobic chemosynthetic processes that occur at hydrothermal vents.

The abundance of H_2S over other reduced compounds (Corliss *et al.*, 1979), the frequent isolation of sulfur-oxidizing bacteria (Jannasch & Mottl, 1985; Ruby *et al.*, 1981), and the high energy yield that can be obtained from the oxidation of reduced sulfur compounds (Edmond *et al.*, 1979; McCollom, 2000) indicate that aerobic sulfur-oxidation is the primary chemosynthetic process in the vent ecosystem.

Table 1.1 Aerobic chemosynthetic processes at deep-sea hydrothermal vents. Modified from Jannasch 1985.

Electron donor	Electron acceptor	Organisms
S^{2-}, S^0, $S_2O_3^{2-}$	O_2	Sulfur oxidizing bacteria
NH_4^+, NO_2^-	O_2	Nitrifying bacteria
Fe^{2+}, Mn^{2+}	O_2	Iron and manganesse oxidizing bacteria
CH_4, CO	O_2	Methylotrophic and carbon monoxide-oxidizing bacteria

Sulfur oxidizing bacteria (SOB) include obligate and facultative autotrophs, as well as lithoheterotrophic bacteria (able to use inorganic electron donors), and heterotrophs (which oxidize sulfur compounds, but do not obtain energy from the process) (Sorokin, 2003). The existence of SOB in open oceans as well as in the deep-sea was known well before the discovery of hydrothermal vents (Tilton *et al.*, 1967; Tuttle & Jannasch, 1976). In 1976, Turtle and Jannasch showed the occurrence of thiosulfate oxidation at high pressure (530 atm) by previously isolated SOB, as well as by natural deep-sea microbial populations (Tuttle & Jannasch, 1976). The first sulfur oxidizing

chemolithoautotrophic bacteria isolated from hydrothermal fluids belonged to the genus *Thiomicrospira* (Ruby *et al*., 1981; Ruby & Jannasch, 1982).

The complete oxidation of thiosulfate can result in the production of sulfate (SO_4^{2}; equation 2) or tetrathionate ($S_4O_6^{2-}$; equation 3) (Robertson & Kuenen, 1999) based on the following equations:

$$Na_2S_2O_3 + 2O_2 + H_2O \rightarrow Na_2SO_4 + H_2SO_4 \qquad (2)$$

$$4Na_2S_2O_3 + O_2 + 2H_2O \rightarrow 2Na_2S_4O_6 + 4NaOH \qquad (3)$$

When SOB are cultured, using thiosulfate as source of inorganic reduced sulfur, the growth medium is usually supplemented with a pH indicator to monitor change in pH, and to identify the thiosulfate metabolism used by the organism. If SO_4 is the final product of the oxidation the pH of the culture medium decreases; in contrast if S_4O_6 is produced the pH of the medium will become basic (Figure 1.2).

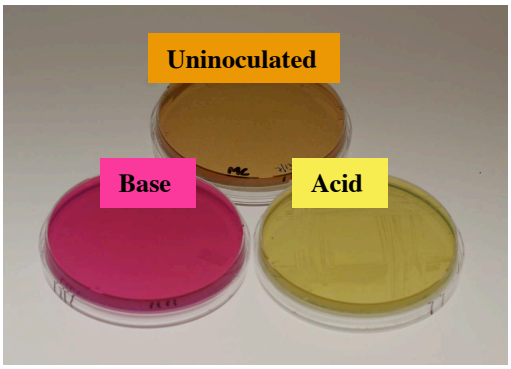

Figure 1.2 Energy metabolism by thiosulfate oxidizing bacteria results in the production of an acid or a base, which in turn lowers or raises the pH of the culture medium, respectively.

Pathways of sulfur oxidation in SOB

Sulfur is considered among the 10 most abundant elements in the Earth's crust. It occurs in living organisms mostly as sulfhydryl (-SH) groups in aminoacids. The most

common sulfur compounds used as electron donors are hydrogen sulfide (H_2S), elemental sulfur (S^0), and thiosulfate ($S_2O_3^{2+}$) (Figure 1.3). In SOB, the oxidation of these reduced sulfur compounds can occur through two different pathways (Figure 1.3). One involves a multienzyme complex, the Sox enzyme system, which catalyzes the complete oxidation of reduced sulfur compounds to sulfate. The second pathway requires sulfite and elemental sulfur as intermediates and can be carried out either by one of two enzymes, the sulfite-acceptor oxidoreductase (SOR), or the adenosine 5'-phosphosulfate reductase (APSR). Figure 1.3 shows the different pathways used by SOB for the oxidation of reduced sulfur compounds.

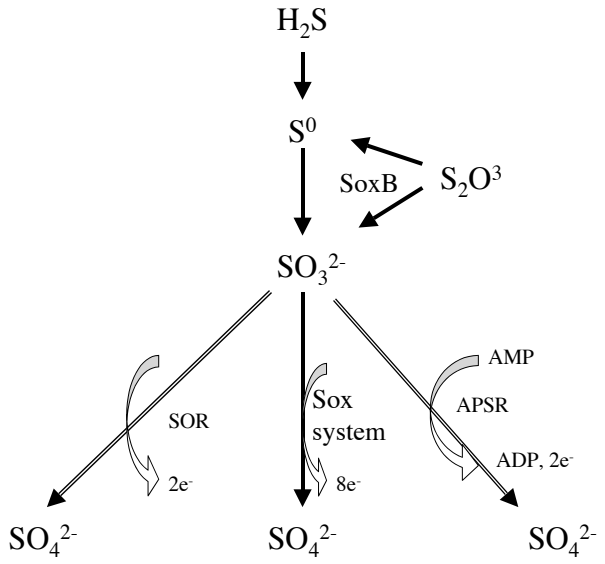

Figure 1.3 Oxidation of reduced sulfur compounds.

Four proteins are required for the oxidation of different reduced sulfur compounds trough the Sox multienzyme complex. These four proteins, located in the periplasmic space of SOB, are encoded in seven structural genes, *soxXA*, *soxYZ*, *soxB*, and *soxCD* (Friedrich et al 2001, and Friedrich et al 2000). In *Thiomicrospira cronogena,* the

soxZYXA genes are found in one cluster, while *soxCD* and *soxB* are located elsewhere in the genome (Scott et al. 2006).

The first stage in H_2S oxidation is the production of S^0, which in many microorganisms is deposited inside the cell and can be used as an energy reserve. When S^0 is provided externally as an electron donor, the organism must grow attached to the sulfur particle because of its insolubility. However, when thiosulfate is the electron donor, it is split into S^0 and SO_3^{2-}, both of which are eventually oxidized to SO_4^{2-}. This process is catalyzed by the sulfur thiol esterase enzyme, encoded by the *soxB* gene. SoxYZ is thought to bind thiosulfate and sulfite, SoxXA functions as a c-type cytochrome that may act as an electron mediator during all reactions, and SoxCD functions as a sulfur dehydrogenase.

In many organisms, especially heterotrophic organisms, thiosulfate is oxidized to tetrathionate. This occurs by the oxidative condensation of two thiosulfate anions, catalyzed by the enzyme thiosulfate dehydrogenase. The bacteria that use this pathway are usually chemoorganotrophic, and use thiosulfate as supplemental energy source (in contrast to chemolithotrophic bacteria, which use thiosulfate as their sole energy source).

Carbon dioxide fixation

Autotrophic organisms grow using carbon dioxide (CO_2) as their sole source of carbon and are located at the base of the food web while generating biomass that supports all other organisms. Among microorganism, five pathways of carbon fixation have been described, including (1) the Calvin-Benson-Bassham Cycle, (2) the Reductive citric acid cycle, (3) the Reductive acetyl-CoA cycle, (4) the 3-hydroxypropionate/malyl-CoA cycle,

and (5) the 3-hydroxypropionate/4-hydroxybutyrate cycle. These pathways differ in many ways, such as in their energy requirements, sensitivity to oxygen, usage of coenzymes, and metal cofactors. These differences are what determines the distribution of the pathways in autotrophic organisms in different habitats (Berg *et al.*, 2007).

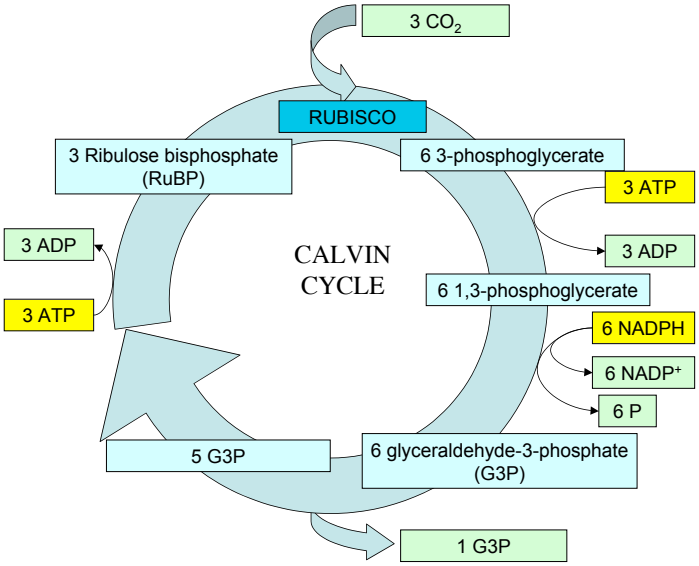

Figure 1.4 The Calvin-Benson-Bassham. From Brock Biology of Microorganisms

Among all these pathways, the Calvin-Benson-Bassham (CBB) Cycle (Figure 1.4) is the most widespread cycle, used by green plants, algae, cyanobacteria, and autotrophic *Proteobacteria*. The key enzyme in this pathway is the ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) enzyme. This enzyme is considered to be the most abundant enzyme in the planet (Ellis, 1979). In the CBB Cycle, RubisCO catalyzes the two major reactions in carbon dioxide fixation, the carboxylation and the oxygenolysis of Ribulose bisphosphate (RuBP; Figure 1.5).

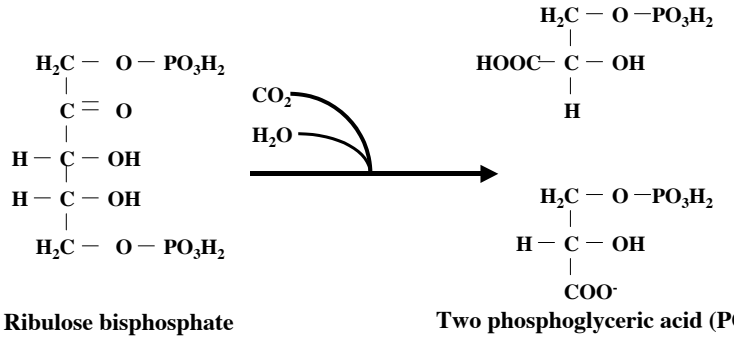

Figure 1.5 Reaction of the CBB Cycle catalyzed by the Ribulose 1, 5-bisphosphate carbozylase/oxygenase (RubisCO). From Brock Biology of Microorganisms.

RubisCO may be isolated in two forms, Form I and Form II, which possess both similarities and differences (Table 1.2, (Tabita, 1999)). Form II enzymes exhibits uniform catalytic features which includes low CO_2/O_2 substrate specificity and poor affinity for CO_2, while Form I exhibits greater variation in such parameters which varies depending on the source of the enzyme (Tabita, 1999).

Table 1.2 Comparison of Form I and Form II RubisCO (Tabita, 1999)

Property	Rubisco type	
	I	II
Quarternary structure	L_8S_8	(L_2)
X-ray structures available	Yes	Yes
Chaperonin-assisted folding	Yes	Yes
Regulated synthesis	Yes	Yes
Carbamate formation	Yes	Yes
Metal specificity for carboxylase acitivity	Mg^2 Mn^2 Ni^2 Co^2	Mg^2 Mn^2 (Co^2 inhibits)
Metal specificity for oxygenase activity	Mn^2 Mg^2 ?	Mn^2 Mg^2 Co^2
CO_2/O_2 specificity (Ω)	25–240	10–15
K_{CO_2} (μM)	5–175	100–250
Fallover	Yes and no	No
Inhibition by RuBP	Yes and no	No
Inhibition by sugar phosphates (activated enzyme)	Yes	Slight

Form I RubisCO is composed of eight large subunits and eight small subunits, which are encoded by the *cbbL* and *cbbS* genes, respectively (Watson & Tabita, 1997). This form can be subdivided into two major groups, the 'green' class, which includes the enzymes from green plants, green algae, and cyanobacteria, and the 'red' class, which includes the enzymes from red algae and purple bacteria (Tabita, 1999). Form II RubisCO is composed of large subunits encoded by the *cbbM* gene. This form is restricted to several phototrophic purple bacteria, aerobic and facultatively anaerobic chemoautotrophic bacteria, and dinoflagellates (Watson & Tabita, 1997). Form II is thought to be the most ancient form which possibly evolved from in organisms inhabiting anoxic environments (Watson & Tabita, 1997).

The RubisCO genes in *Proteobacteria* are often part of a large operon containing structural genes that encode for other enzymes in the CBB Cycle. This operon includes a single promoter, regulated by the positive transcriptional regulator CbbR. However, the intracellular levels of RubisCO exceeds by far the levels of the other CBB cycle enzymes (Gibson *et al.*, 1991), suggesting that structural modifications should occur that allow the stability of the RubisCO transcript following post-transcriptional processing of the large operon message (Gibson *et al.*, 1991; Tabita, 1999).

Phylogenetic neighbor-joining trees of the RubisCO enzyme show that the phylogeny of the enzyme does not coincide with the boundaries between species established on the basis of 16S rRNA phylogenetic analysis. Such analyses indicate the possibility that there has been horizontal gene transfer and/or gene duplication of RubisCO among bacteria and plastids (Delwiche & Palmer, 1996).

Microbial interaction with mercury in deep-sea hydrothermal vents

Metal resistance is common among microorganisms and is critical for the impact of metals in the environment. Although the evolutionary origin of microbial metal resistance is unknown, geothermal environments, where heavy metals concentrations may be comparable to those in contaminated sites, could represent ecological niches where metal-microbe interactions occurred early during microbial evolution (Nies, 1999). In these environments, metals are often complexed with reduced sulfur compounds (Dopson *et al*., 2003). A preliminary work, in which the optimal temperature of activity of the MerA (mercuric reductase, an enzyme that catalyzes the reduction of ionic mercury to elemental mercury) was determined, showed that the MerA from different mesophilic organisms had an optimal temperature of activity in the thermophilic range (40 to 70ºC, Figure 1.6). Previous work suggested that the Mer system evolved in geothermal environments and prompted us to investigate microbial interactions with mercury in geothermal environments, such as deep-sea hydrothermal vents.

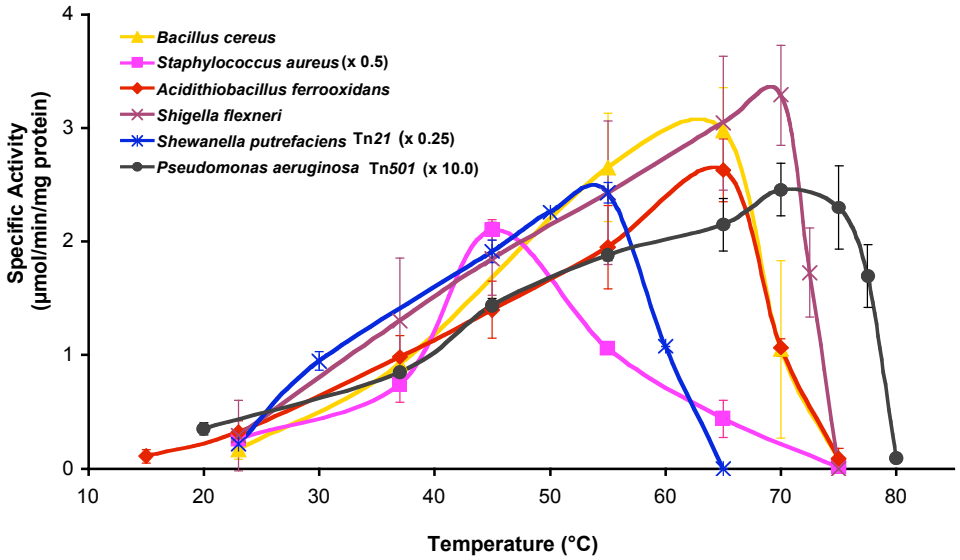

Figure 1.6 Thermophilicity of MerA enzyme from several mesophilic bacteria (Crespo-Medina, unpublished)

Mercury (Hg) is a toxic heavy metal that contaminates a wide variety of aquatic habitats due to either atmospheric deposition of by anthropogenic sources (Figure 1.7). In the open oceans, the concentration of Hg ranges from 2 – 50 ng L^{-1} (Cox, 1981). In geothermal environments Hg exists mostly as cinnabar: HgS (Varekamp & R, 1984), which may be solubilized by sulfur oxidizing microbes (Baldi & Olson, 1987). More recently, Hg has also been found in these environments as small droplets of its elemental form (Hg^0) (Dekov, 2007; Stoffers *et al.*, 1999). Measurements of Hg concentrations in sediments from the periphery of hydrothermal vents revealed values ranging from 1 to 9 x 10^5 ng L^{-1} (Cox, 1981). Hg accumulation in the tissue of invertebrates that inhabits hydrothermal vents have been shown to be very elevated, with concentrations ranging from 60 to 3760 ng Kg^{-1} of tissue (Ando *et al.*, 2002; Martins *et al.*, 2001).

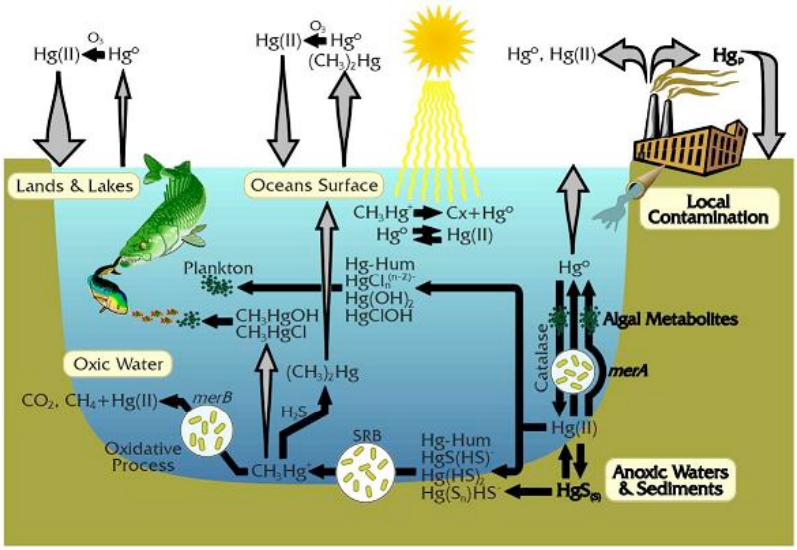

Figure 1.7 The Mercury Cycle (Barkay et al. 2003)

Although the notion that Hg is elevated around deep-sea spreading centers has been long known (Bostrom & Fisher, 1969; Varekamp & R, 1984), only recently were direct measurements of Hg in hydrothermal fluids taken. These measurements, which were obtained from high temperature focused fluids, i.e., black smoker chimneys, suggest elevated Hg concentrations, sometimes to levels that in surface natural waters are only observed in highly contaminated environments (Ekstrom, 2007; Lamborg *et al.*, 2006). To the best of our knowledge, this is the first study in which Hg concentrations in diffuse flow hydrothermal vents have been measured (see Chapter 4, Table 4.1), although previous work done in our laboratory demonstrated adaptation to Hg among moderately thermophilic heterotrophic bacteria from diffuse flow vents, and suggested exposure of vent microorganisms to elevated concentration of this toxic metal (Vetriani *et al.*, 2005).

The toxicity and mobility of Hg is greatly affected by microbial activity. Microorganisms are able to transform Hg between its three oxidation states (0, +1, and +2), as well as between organic and inorganic forms (Barkay *et al*., 2003). Microbial mercury resistance is conferred by a multi-enzyme system encoded in the *mer* operon. The enzyme responsible for the reduction of ionic mercury (Hg^{2+}) to elemental mercury (Hg^{0}), which is less toxic and volatile, is the mercuric reductase enzyme (MerA). Figure 1.8 shows the activity of the Mer multienzyme complex.

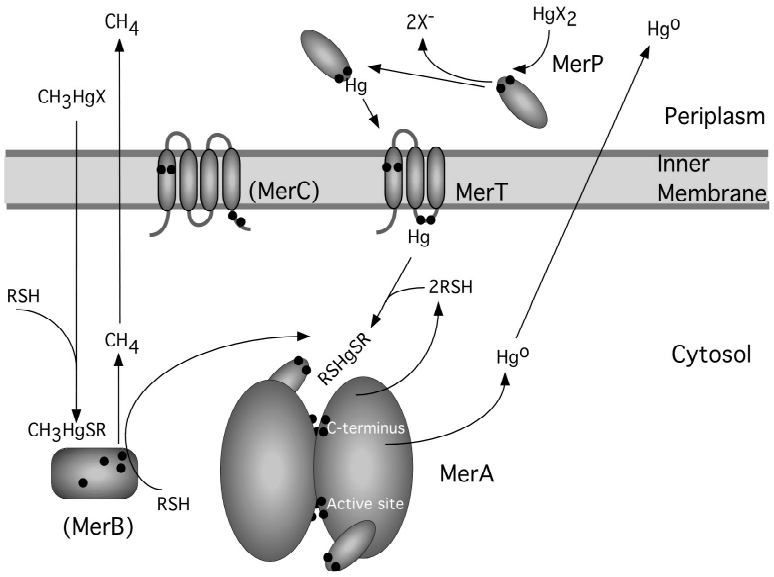

Figure 1.8 The activity of the Mer multienzyme complex, encoded in the *mer* operon is responsible for the microbially mediated mercury reduction. (Barkay et al. 2003)

Study scope and objectives

The main goal of this study is to gain a better understanding of the aerobic chemosynthetic thiosulfate oxidation process at diffuse flow hydrothermal vents, through (i) the enumeration of the organisms carrying out this process, their isolation and identification, and the study of selected functional genes encoded by these organisms, and

(ii) the assessment of their role in mercury detoxification in hydrothermal vent environments. To accomplish these goals, diffuse flow hydrothermal samples were collected along the EPR at 9ºN, over a 5 years period (2004 to 2008), and at Guaymas Basin (Gulf of California) during an oceanographic expedition in 2007.

Specific Objectives

1. To study the abundance and diversity of aerobic chemosynthetic thiosulfate oxidizing bacteria from diffuse flow fluid samples.
2. To isolate novel chemosynthetic thiosulfate oxidizing bacteria and assess their phylogenetic distribution by comparing isolates' 16S rRNA gene sequences to that of known organisms.
3. To identify which of the known carbon fixation cycles is used by the autotrophic thiosulfate oxidizing isolates
4. To study the phylogeny of their *sox*B gene, a gene essential for thiosulfate oxidation
5. To determine the total mercury concentration in the hydrothermal vents fluids
6. To estimate the abundance of mercury resistant chemosynthetic bacteria at diffuse flow hydrothermal vents and to assess their specific role in the detoxification of mercury at hydrothermal vents environments

Chapter 2: Diversity of chemosynthetic thiosulfate-oxidizing bacteria from deep-sea hydrothermal vents

To be submitted to Applied and Environmental Microbiology

Introduction

In hydrothermal vents, the mixing of highly reduced hydrothermal fluids with cold oxygenated seawater creates steep chemistry, temperature, and pH gradients that provide metabolic energy for chemosynthetic bacteria (McCollom & Shock, 1997). Under aerobic or micro-aerobic conditions, chemosynthesis can be carried out by bacteria that couple CO_2 fixation to reduced sulfur oxidation. The abundance of H_2S over other reduced compounds (Corliss *et al*., 1979), the frequent isolation of sulfur-oxidizing bacteria (Jannasch & Mottl, 1985; Ruby *et al*., 1981), and the high energy yield that can be obtained from the oxidation of reduced sulfur compounds (Edmond *et al*., 1979; McCollom, 2000) are strongly suggest that aerobic sulfur-oxidation is the primary chemosynthetic process in the vent ecosystem.

The first sulfur oxidizing chemolithoautotrophic bacteria isolated from hydrothermal fluids belonged to the genera *Thiomicrospira* (Jannasch *et al*., 1985; Ruby *et al*., 1981; Ruby & Jannasch, 1982) and *Halothiobacillus* (formerly called *Thiobacillus*) (Durand *et al*., 1993). More recently, several studies have been focused on the study of the diversity, distribution, and enumeration of sulfur-oxidizing microorganism from different hydrothermal vent systems (Brinkhoff *et al*., 1999; Sievert *et al*., 1999; Teske *et al*., 2000). These studies indicated that this group of organisms, and in particular

representatives of the genus *Thiomicrospira,* is abundant and relevant in hydrothermal vents ecosystems. The recent completion of the genome of *Thiomicrospira crunogena* (Jannasch *et al.*, 1985; Scott *et al.*, 2006), which was isolated from hydrothermal vent samples (Jannasch *et al.*, 1985) revealed new insights on adaptations that may have enabled this organism to thrive in different hydrothermal vent systems globally.

Fluids and sediment samples, as well as biomass recovered from microbial colonization experiments, were collected along the East Pacific Rise at 9ºN and in the Guaymas Basin, Gulf of California, with the objective of studying more in depth the chemosynthetic thiosulfate oxidizing bacterial communities within different hydrothermal vent sites. I carried out isolations from both direct enrichments and from dilutions to extinction, and isolated representatives of genera *Thiomicrospira*, *Thioclava*, *Halothiobacillus*, *Hydrogenovibrio*, *Thalassopira*, and *Salinisphaera*, among others. Isolates were further characterized metabolically, and the phylogeny of their RubisCO and *sox* genes, involved in carbon fixation and thiosulfate oxidation, respectively, was investigated. This work gives a more detailed and comprehensive analysis of the microbial chemosynthetic communities at deep-sea hydrothermal vents. The quantification of these organisms and the bacterial isolations from highly diluted samples suggests that these organisms play a very important role in the production of organic carbon and the maintainment of the ecosystem.

Materials and Methods

Sample collection- Hydrothermal fluid samples from diffuse flow vents were collected from the East Pacific Rise (EPR; 9º50'N, 104º17'W), at a depth of

approximately 2500 m during five oceanographic expedition aboard the R/V *Atlantis* on April 2004, May 2005, June 2006, January 2007, and January 2008. The fluids were collected using titanium samplers operated by the manipulator of the DSV *Alvin*, immediately above the venting source. Microbial biomass was also obtained from experimental microbial colonizers, which are made of a section of PVC pipe covered with stainless steel mesh and are used to study microbial colonization and attachment processes. The colonizers were deployed in diffuse flow vents on the EPR during the 2007 and 2008 expeditions. Sediment samples were collected from Guaymas Basin (GB) in the Gulf of California, at a depth of approximately 2000 m, during an expedition in October 2007. At the surface, samples were transferred promptly to the ship's laboratory and sub-samples were stored at 4ºC.

Isolations and quantifications- Primary enrichment cultures were initiated immediately after sample collection by serially diluting the sample to extinction, or by adding 1 mL of fluids to 10 mL of growth medium designed to enrich for chemolithoautotrophic thiosulfate oxidizing bacteria. The medium used was 142-A (Crespo-Medina *et al.*, In press-a and Chapter 4 of this thesis). Cultures were incubated at 30ºC in the dark. Growth was initially determined by the observation of a change in color of the pH indicator in the medium and then confirmed by direct microscopic counts after staining the cells with acridine orange. For isolation of single colonies, liquid cultures were inoculated on Petri dishes containing medium 142-A solidified with 15 g L^{-1} of noble agar (Sigma). Stocks for long-term storage were prepared by adding 150 µL of 100% sterile glycerol (Fisher Scientific) to 850 µL of overnight cultures, and were stored at –80ºC.

Quantification of chemosynthetic thiosulfate oxidizing bacteria was determined by the most probable number (MPN) technique as described previously (Crespo-Medina *et al.*, In press-a and Chapter 4 of this thesis). Bacterial isolations were carried out from the MPN dilutions showing growth. For direct counts, cells were stained with 0.1% acridine orange and visualized with an Olympus BX60 microscope with and oil-immersion objective lens (Uplan F1 100X/1.3).

DNA extraction, 16S rRNA gene amplification and sequencing – Total DNA was extracted from pure cultures using the UltraClean™ Microbial DNA isolation kit, according to the manufacturer's instructions (MoBio Laboratories, Solana Beach, CA, USA). Metagenomic DNA from environmental biomass, concentrated on a 0.22 μm polycarbonate filter, was extracted using a phenol chloroform-based extraction protocol (Voordeckers *et al*., submitted)

The full-length 16S rRNA gene was selectively amplified from the genomic DNA by PCR, sequenced and subjected to phylogenetic analysis as described previously (Vetriani *et al*., 2004; Voordeckers *et al.*, 2005).

Metabolic characterization- Heterotrophic growth was determined by transferring 100 μL of an overnight culture from medium 142-A to medium 142+A, which was depleted of $NaHCO_3$ and $Na_2S_2O_3$, and supplemented with 10 mmol L^{-1} sodium acetate (Sigma). Growth was also tested in Artificial Seawater (ASW) medium, in low strength ASW (LS ASW) medium, and in ASW minimal medium (ASW MM) supplemented with dodecane ($C_{12}H_{26}$) in the vapor phase as the only carbon and energy source (Crespo-Medina *et al*., In press-b and Chapter 3 of this thesis).

Analysis of functional genes- The genes encoding for the enzymes ribulose 1, 5-bisphosphate carboxylase/oxygenase (RubisCO) form I (*cbbL*/*rbcL*) and II (*cbbM*) and SoxB (involved in the oxidation of reduced sulfur compounds) were amplified from the genomic DNA of the new strains. An 800 bp fragment of the gene encoding for form I RubisCO (*cbbL*) was amplified as described previously (Elsaied & Naganuma, 2001), and its sequence was determined. The PCR protocol used to amplify the *cbbL* genes was: 2 min at 94ºC, followed by 30 cycles of 1 min at 94ºC, 1 min at 49ºC, and 3 min at 72ºC, and ending with a final extension of 15 min at 72ºC.

Internal primers thiocbbLF (5'-ATTCACCGCGCAATGCACGG - 3') and thiocbbLR (5' - GAAGTCTTGGTCGAACATGA - 3') were designed based on conserved regions from the *cbbL* gene sequence from *Thiomicrospira crunogena*, *T. thermophila*, and *Hydrogenovibrio marinus*, (from now on refered to as the *Thiomicrospira* group) using the IDT OligoAnalyzer 3.1 Program; (www.idtdna.com). These primers were used to amplify a 220 bp fragment of the *cbbL* gene form environmental genomic DNA extracted from fluids collected at EPR 9ºN at a site called TamTown. The PCR protocol used to amplify the short fragment of *cbbL* gene was: 5 min at 94ºC, followed by 30 cycles of 1 min at 94ºC, 1 min at 49ºC, and 45 sec at 72ºC, and ending with a final extension of 10 min at 72ºC. PCR product was gel purified using the QIAquick Gel Extraction Kit (Qiagen, Santa Clarita, CA), cloned into a pCR4-TOPO plasmid vector. Ligation products were transformed into competent *E. coli* One Shot cells (Invitrogen, Inc., Carlsbad, CA, USA). Ten clones were screened in order to verify the efficiency of the primers, and to validate the specificity of the primers for the *Thiomicrospira* group.

The PCR reaction for the amplification of a 400 bp fragment of the gene encoding for the form II RubisCO (*cbbM*) was carried out as previously described (Elsaied & Naganuma, 2001). The PCR protocol used to amplify the *cbbL* genes was: 2 min at 94ºC, followed by 30 cycles of 1 min at 62ºC, 1 min at 49ºC, and 45 sec at 72ºC, and ending with a final extension of 7 min at 72ºC.

The *soxB* gene was amplified using primers soxB432F and soxB1446R and PCR conditions as described previously (Petri *et al*., 2001). The sequences for *cbbL/rbcL, cbbM,* and of the *soxB* gene were determined for both strands and were translated into the respective amino acid sequences using EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq). The deduced amino acid sequences were aligned with ClustalX v 1.8 (Thompson *et al.*, 1997) and manually adjusted using Seaview (Galtier *et al.*, 1996). Phylogenetic distances were calculated using the Observed Divergence matrix, and the neighbor joining method was used to evaluate tree topologies. Phylo_win was utilized to plot tree topologies (Galtier *et al.*, 1996) and their robustness was tested by bootstrap analysis with 1,000 resamplings.

Results

Isolation and molecular characterization- Microbial isolations were done in selective medium 142-A, designed to enrich for chemosynthetic thiosulfate oxidizing bacteria, under aerobic conditions, and at 30ºC. A total of 90 acid or base producing isolates were obtained in pure culture from either direct enrichments or from serial dilutions of the original samples. The base producing strains were closely related to heterotrophic thiosulfate oxidizers from genera such as *Pseudomonas*, *Marinobacter*,

Halomonas, *Pseudoalteromonas* and *Vibrio* (data not shown). It has been demonstrated that members of these genera are able to oxidize reduced sulfur compounds as supplement, but do not depend on this reaction for growth (Durand *et al*., 1994; Sorokin, 2003) and thus, for the purpose of project, they were not considered for further characterization.

Fifty-three acid producing isolates were related to different genera of chemosynthetic organisms. These organisms obtain the energy required for the fixation of CO_2 into organic matter from the oxidation of thiosulfate. The growth of these isolates in medium 142-A decreased the pH of the medium by approximately 2.5 pH units (Figure 2.1). The isolates were identified by PCR amplification and sequencing of their 16S rRNA gene. The chemosynthetic isolates were determined to be closely related to the "*gammaproteobacteria*" *Thiomicrospira crunogena* (18 strains), *Thiomicrospira thermophila* (7 strains), *Halothiobacillus hydrothermalis* (7 strains), *Hydrogenovibrio marinus* (4 strains), *Salinisphaera hydrothermalis* (3 strains) (Crespo-Medina *et al*., In press-b, and Chapter 3 of this thesis), and to the "*alphaproteobacteria*" *Thioclava pacifica* (11 strains), *Pelagibaca bermudensis* (2 strains), and *Thalassospira* sp. (1 strain). Figure 2.2 shows the 16S rRNA phylogeny of representative isolates of each genus, whose full sequence was obtained. A subgroup of the isolates was selected for further characterization. A list of these representative isolates, along with information about their origin, is presented in Table 2.1.

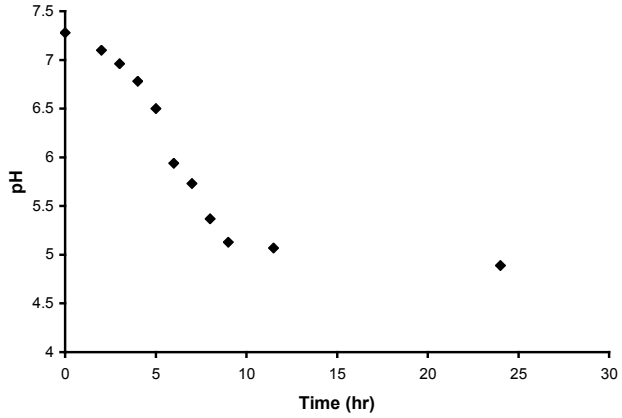

Figure 2.1 Acidification of medium 142-A by *Thiomicrospira* sp. EPR96

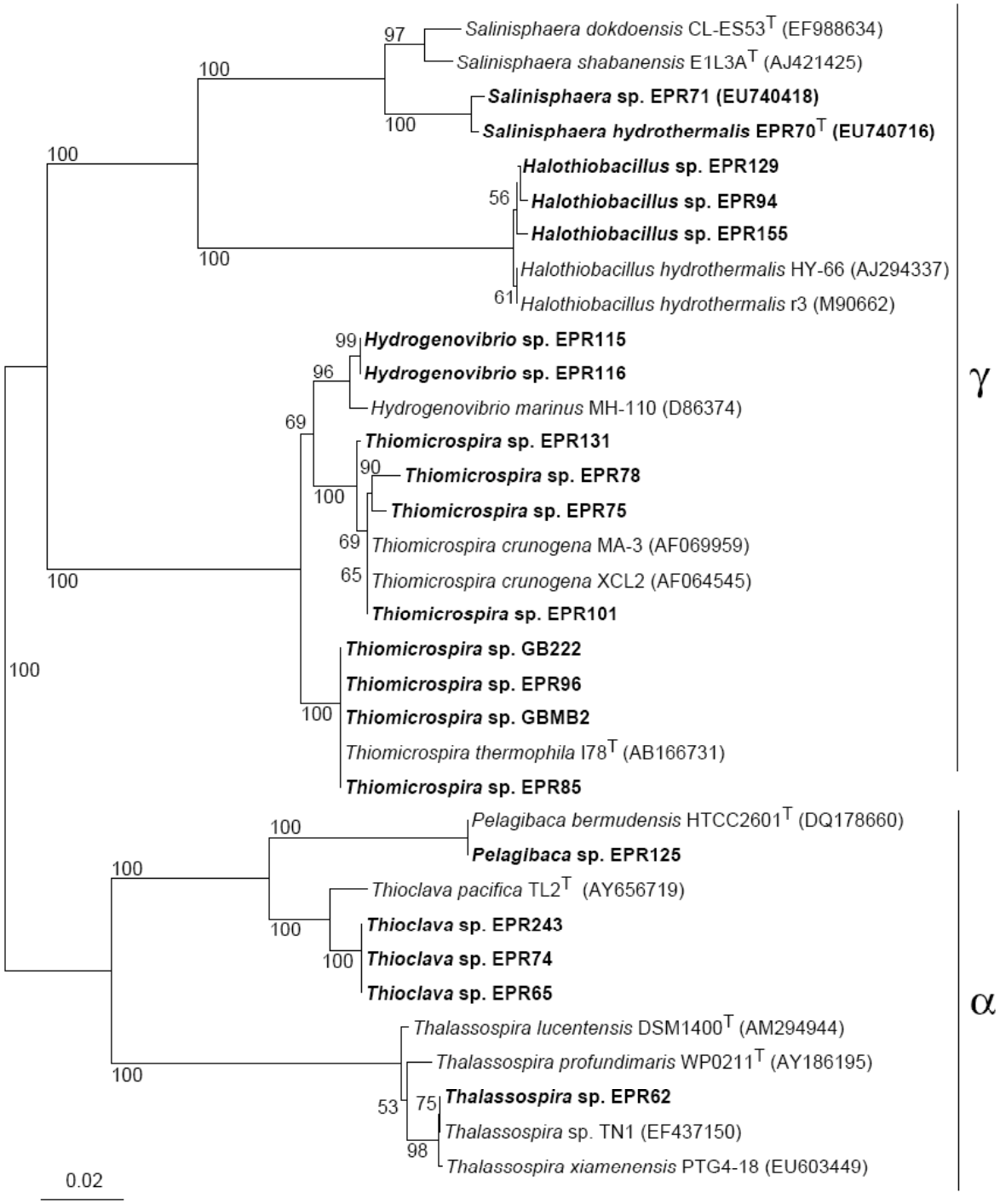

Figure 2.2 Phylogenetic position of the chemosynthetic isolates based on their 16S rRNA gene sequence. The neighbor-joining tree was constructed using Phylo_Win. Boostrap values higher than 50 are indicated. Bar indicate 2% estimated substitution.

Table 2.1 Chemosynthetic isolates selected for further characterization

Isolate	Closest Relative (16S rRNA sequence similarity)	Site/sample	Sample Temperature and pH
EPR65	*Thioclava pacifica* TL2^T (97%)	Fluid sample from Tica Vent	15.5ºC
EPR74	*Thioclava pacifica* TL2^T (97%)	Fluid sample from East Wall 04/04 E Vent	11ºC, pH5.9
EPR75	*Thiomicrospira crunogena* XCL-2 (98%)	Fluid sample from Tica Vent	2.5ºC
EPR78	*Thiomicrospira crunogena* XCL-2 (99%)	Fluid sample from Tica Vent	15.5ºC
EPR85	*Thiomicrospira thermophila* I78^T (100%)	Fluid sample from East Wall 04/04 B Vent	50-70ºC
EPR96	*Thiomicrospira thermophila* I78^T (99%)	Fluid samples from Mk 89	8ºC
GB222	*Thiomicrospira thermophila* I78^T (99%)	Oily sediment, GB NE area, white microbial mat	4.9ºC
GBMB2	*Thiomicrospira thermophila* I78^T (99%)	Central area of a white mat	14ºC
EPR129	*Halothiobacillus hydrothermalis* r3 (99%)	Fluid sample from Crab Spa MkF	28ºC, pH 6.6
EPR155	*Halothiobacillus hydrothermalis* r3 (99%)	Experimental microbial colonizer Mk 19	
EPR125	*Pelagibaca bermudensis* HTCC2601^T (99%)	Experimental microbial colonizer at TamTown	12ºC
EPR115	*Hydrogenovibrio marinus* MH-110 (99%)	Experimental microbial colonizer at TamTown	12ºC
EPR62	*Thalassospira sp.* NT1 (99%)	Fluid samples from East Wall Mk 3 Vent	pH 5.95
EPR70	*Salinisphaera hydrothermalis* [1]	Fluid samples from Mk 119 Vent	13
EPR71	*Salinisphaera sp.*[1]	Fluid samples from Mk 119 Plume	2.5

[1]Crespo-Medina, et al. In press. IJSEM. (Chapter 3 of this thesis)

Metabolic characterization- Different growth media were used in order to assess the metabolic versatility of the representative isolates listed in Table 2.2. In order to test the ability of these isolates to grow on acetate, 100 µL of an overnight culture (in medium 142-A) were transfered into a test tube containing 10 mL of medium 142+A, which is

medium 142 deprived of bicarbonate and supplemented with 10 mmol L^{-1} sodium acetate. All the isolates grew in this medium, indicating that they are facultative autotrophs being able of heterotrophic growth on acetate. However, when overnight cultures were transfered from defined medium to a complex medium (ASW), only isolates related to *Thioclava pacifica* (EPR65 and EPR74), *Thalassospira* sp. (EPR62), *Salinisphaera hydrothermalis* (EPR70 and EPR71), *Pelagibaca bermudensis* (EPR125), and one of the isolates closely related to *Halothiobacillus hydrothermalis* (EPR155) grew (Table 2.2). The ability to grow on *n*-alkanes as the only carbon source was tested by transferring the isolates from medium 142-A to a defined medium containing dodecane in the vapor phase. Only isolates related to *Salinisphaera hydrothermalis* grew in this medium (Crespo-Medina *et al.*, In press-b and Chapter 3 of this thesis).

Since the concentration of oxygen in vents fluids fluctuates, resulting in conditions that range from mildly oxidizing to highly reducing, we tested the ability of the chemosynthetic isolates to grow under microaerobic conditions. For this, overnight cultures from fully aerated medium were transferred to defined medium under a CO_2/O_2 atmosphere (95:5). All strains related to the genus *Thiomicrospira* (EPR75, EPR78, EPR85, EPR96, GB222, and GBMB2), but no representative of other genera, grew under micoaerobic conditions (Table 2.2).

Table 2.2 Metabolic characterization of chemosynthetic isolates

Isolate	Closest relative	Autotrophic growth[1]	Heterotrophic growth on acetate[2]	Heterotrophic growth in complex medium[3]	Growth on n-alkanes[4]	Microaerobic growth (5% O_2) [5]
EPR65	*Thioclava pacifica*	+	+	+	-	-
EPR74	*Thioclava pacifica*	+	+	+	-	-
EPR75	*Thiomicrospira crunogena*	+	+	-	-	+
EPR78	*Thiomicrospira crunogena*	+	+	-	-	+
EPR85	*Thiomicrospira thermophila*	+	+	-	-	+
EPR96	*Thiomicrospira thermophila*	+	+	-	-	+
GB222	*Thiomicrospira thermophila*	+	+	-	-	+
GBMB2	*Thiomicrospira thermophila*	+	+	-	-	+
EPR129	*Halothiobacillus hydrothermalis*	+	+	-	-	-
EPR155	*Halothiobacillus hydrothermalis*	+	+	+	-	-
EPR125	*Pelagibaca bermudensis*	+	+	+	-	-
EPR115	*Hydrogenovibrio marinus*	+	+	-	-	-
EPR62	*Thalassospira* sp.	+	+	+	ND[6]	ND
EPR70	*Salinisphaera hydrothermalis*[7]	+	+	+	+	-
EPR71	*Salinisphaera* sp.[7]	+	+	+	+	-

[1] 142-A
[2] 142+Acetate (10mM)
[3] ASW (artificial seawater media) and LSASW (Low Strength ASW)
[4] ASW minimal medium + dodecane (vapor)
[5] HB1 minimal medium + thiosulfate, 5% oxygen
[6] ND, Not determined
[7] Crespo-Medina, et al. In press. IJSEM

Abundance of chemosynthetic thiosulfate oxidizers- Table 2.3 shows a comparison between most probable number counts (Crespo-Medina *et al*., In press-a and Chapter 4 of this thesis), carried out to estimate the number of chemosynthetic thiosulfate oxidizing bacteria, with the estimated number of cells per mL of samples obtained from acridine orange direct microscopic counts.

Results indicated that the number of chemosynthetic thiosulfate oxidizing bacteria ranged form 1.1 x 10^3 to 2.4 x 10^7 cells mL^{-1} (Crespo-Medina *et al*., In press-a and Chapter 4 of this thesis). When these numbers were compared to direct microscopic

counts, the percentage of thiosulfate oxidizers was estimated to range from 0.002 to 14.1%.

Table 2.3 Comparison between most probable number (MPN) counts of chemosynthetic thiosuflate oxidizing bacteria and total cells per mL of sample estimated by total microscopic counts after acridine orange staining.

Sample site	Total cell counts (cells mL^{-1})	MPN (cells mL^{-1})[1]	%
Mk119, 1 m above fluid, after mussel removal	1.6×10^7	2.4×10^5	1.5
Tica Vent	4.25×10^7	1.1×10^5	0.26
Tica, after mussel removal	7.0×10^7	3.3×10^4	0.005
Mk89	1.75×10^7	5.4×10^4	0.31
Microbial Mat, Bio9	2.41×10^8	1.1×10^4	0.005
Alvinella Pillar	4.88×10^7	1.4×10^5	0.29
Mk35	1.7×10^8	2.4×10^7	14.1
Mk16	1.1×10^8	2.7×10^4	0.02
Tica, Mk4	7.0×10^7	1.1×10^3	0.002

[1] Crespo-Medina, et al. In press. Limnol. Oceanogr. (Chapter 4 of this thesis)

Bacterial strains closely related to *Thiomicrospira crunogena* and to *Hydrogenovibrio marinus* were isolated from both primary enrichments (undiluted samples) and serially diluted samples. The occurrence of *T. crunogena* and *H. hydrothermalis* at dilutions as high as 10^5 and 10^4, respectively, suggested that these two genera are highly abundant in hydrothermal vents fluids (Table 2.4).

Table 2.4 Isolates obtained from serial dilutions

Isolate	Closest relative	Sample	Dilution
EPR75	*T. crunogena*	Fluid sample from Tica vent	10^{-2}
EPR76	*T. crunogena*	Fluid sample from Tica vent	10^{-2}
EPR77	*T. crunogena*	Fluid sample form Mk119, plume	10^{-3}
EPR78	*T. crunogena*	Fluid sample from Tica vent	10^{-4}
EPR79	*T. crunogena*	Fluid sample from Tica vent	10^{-3}
EPR102	*T. crunogena*	Fluid sample from *Alvinella* Pillar	10^{-4}
EPR103	*T. crunogena*	Fluid sample from *Alvinella* Pillar	10^{-5}
EPR116	*H. marinus*	Experimental microbial colonizer at Tica	10^{-2}
EPR118	*T. crunogena*	Alvinella worm	10^{-2}
EPR120	*H. marinus*	Experimental microbial colonizer at Tica	10^{-4}
EPR131	*T. crunogena*	Fluid sample Crab Spa MkF	10^{-2}
EPR151	*T. crunogena*	Colonization experiment Mk19	10^{-5}

Analyses of functional genes- The genes encoding for the From I (*cbbL/rbcL*) and Form II (*cbbM*) RubisCO enzyme were amplified from the total genomic DNA of the chemosynthetic isolates. Form I RubisCO was amplified from most of the isolates, with the exception of EPR125, which carried for Form II RubisCO. Some of the isolates, including close relatives to *T. thermophila* (EPR85 and EPR96) and to *H. hydrothermalis* (EPR129 and EPR155) had both forms of the gene. Figure 2.3 shows the phylogenetic position of the RubisCO enzyme from most of the isolates. Three distinctive clusters were identified for the Form I enzyme. One of the clusters includes the enzyme from the *S. hydrothermalis* strains, the second cluster includes the sequences of *Thioclava* related strains, and a third cluster includes the enzymes related to *T. crunogena*, *T. thermophila*, *H. hydrothermalis* and *H. marinus*. The latter cluster was defined as the *Thiomicrospira* cluster.

Internal primers designed to amplify a 220 bp fragment of the Form I RubisCO based on the conserved sequence of the representatives of the *Thiomicrospira* cluster

were designed in order to study the distribution of the this enzyme in the environment. A preliminary assessment indicated that the gene encoding the Form I RubisCO of *T. crunogena*, *T. thermophila* and *H. marinus* could be amplified from environmental genomic DNA extracted from a diffuse flow area at the EPR 9ºN (Figure 2.4) indicating their occurrence at at this site. Further studies involving a larger number of sites and are necessary to determine the distribution and occurrence of the *Thiomicrospira* group RubisCO enzyme in diffuse flow hydrothermal vents.

The gene encoding the sulfur thiol esterase enzyme, SoxB, which is an enzyme in the thiosulfate oxidation pathway, was amplified for some of the chemosynthetic isolates. Figure 2.5 shows the phylogeny of the enzyme.

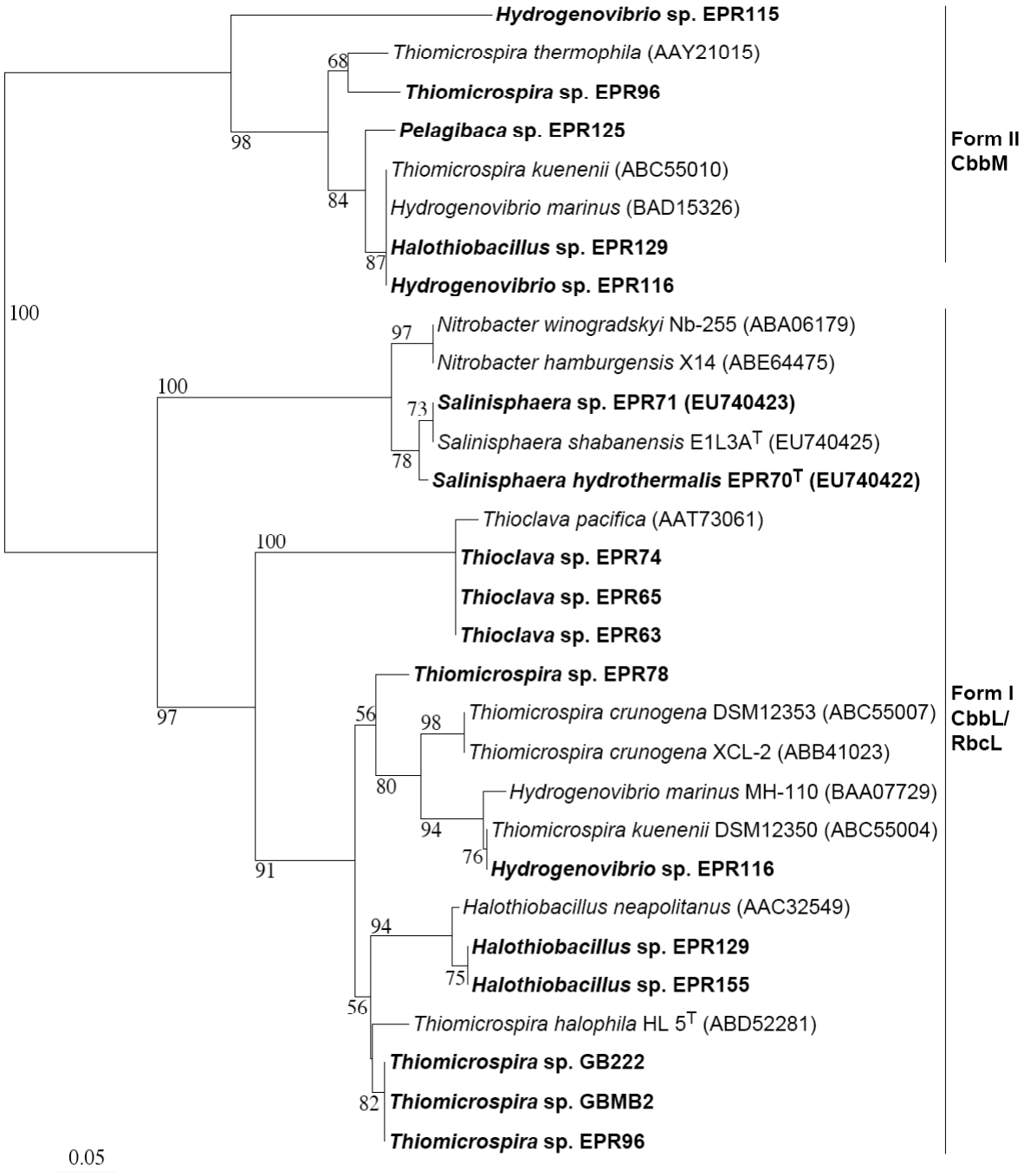

Figure 2.3 Phylogenetic analysis of the Form I and Form II of the protein RubisCO large from the chemosynthetic isolates. The neighbor-joining tree was constructed using Phylo_Win. Bootstrap values higher than 50 are indicated. Bar indicate 5% estimated substitutions.

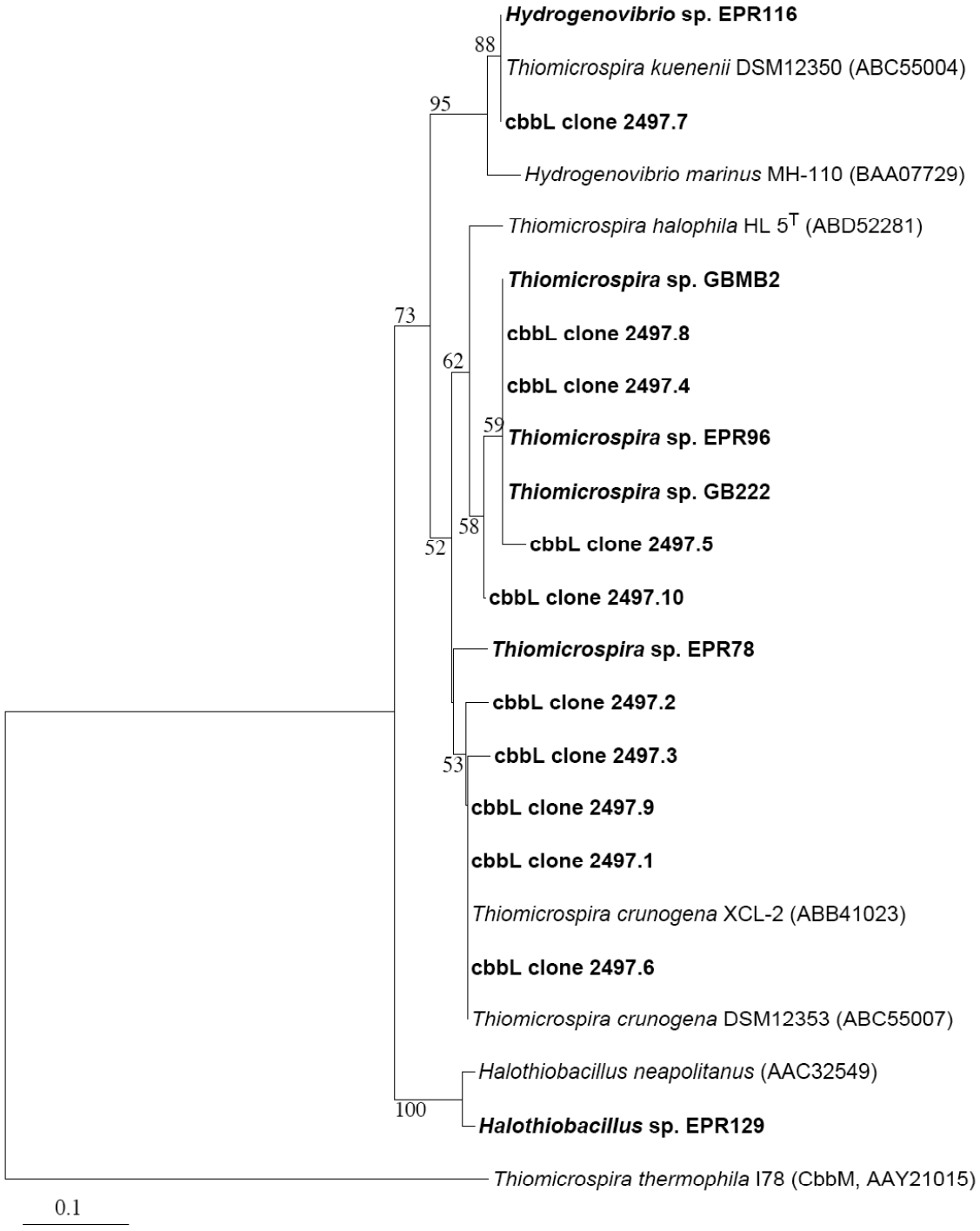

Figure 2.4 Phylogenetic analysis of the Form I of the protein RubisCO of chemosynthetic isolates and environmental clones. The neighbor-joining tree was constructed using Phylo_Win. Bootstrap values higher than 50 are indicated. Bar indicate 10% estimated substitutions.

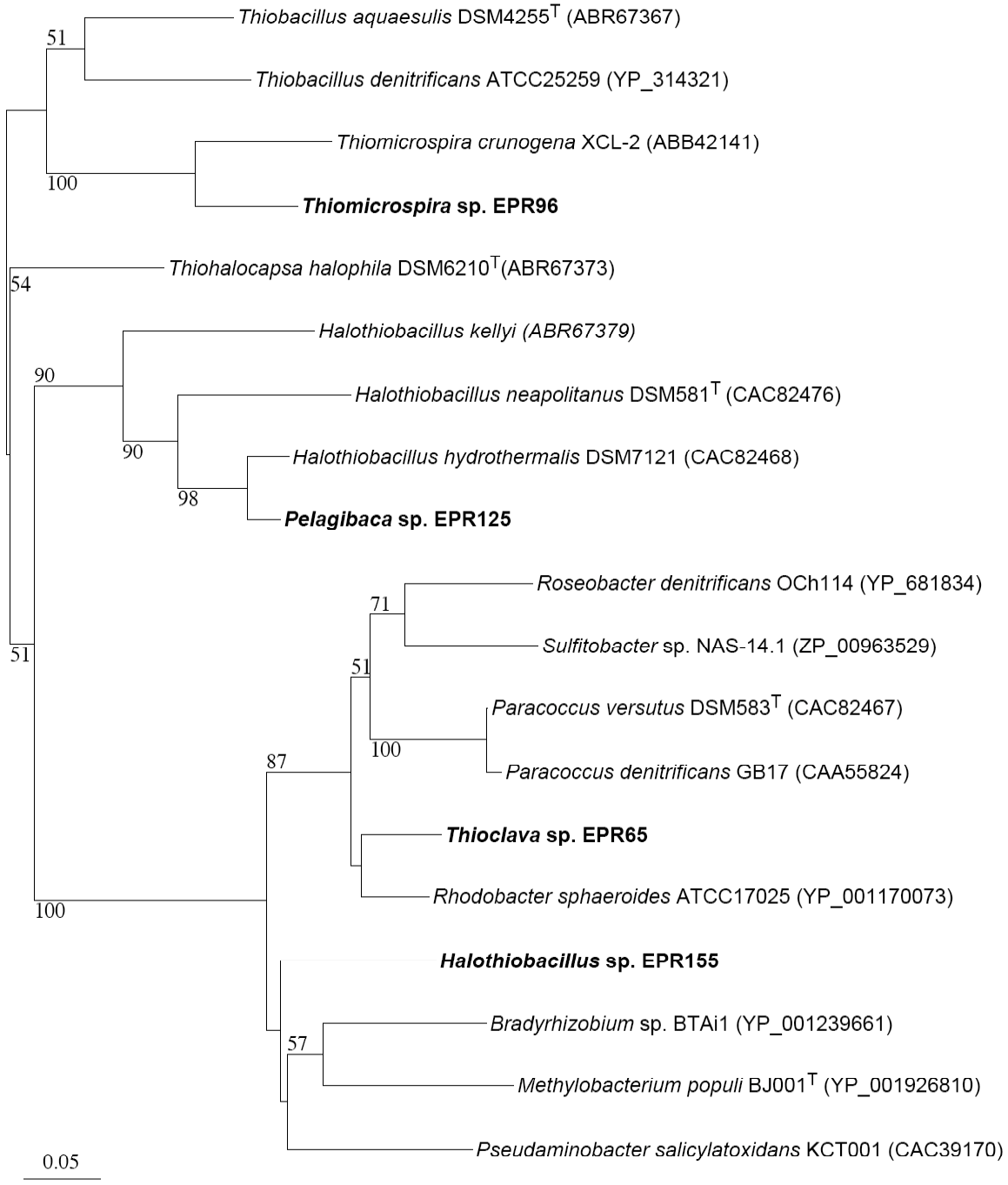

Figure 2.5 Phylogenetic analysis of the SoxB protein from the chemosynthetic isolates. The neighbor-joining tree was constructed using Phylo_Win. Bootstrap values higher than 50 are indicated. Bar indicate 5% estimated substitutions.

Discussion

Hydrothermal fluids are enriched in reduced sulfur compounds, which favors the activity of chemosynthetic sulfur oxidizing bacteria. In this study growth medium with thiosulfate as the only energy source was used in order to isolate chemosynthetic thiosulfate oxidizing bacteria and to assess their abundance at different diffuse flow samples collected along the East Pacific Rise at 9ºN and at the Guaymas Basin, the Gulf of California. Fifty-three strains of thiosulfate oxiding bacteria were isolated and were closely related to *Thiomicrospira crunogena, Thiomicrospira thermophila*, *Thioclava pacifica*, *Halothiobacillus hydrothermalis*, *Hydrogenovibrio marinus, Salinisphaera hydrothermalis, Pelagibacca bermudensis*, and *Thalassospira* sp. (Table 2.1 and Figure 2.2). Representative isolates were chosen for further metabolic characterization, including their ability to grow heterotrophically using a two carbon organic compound, as well as their ability to grow in complex medium (Table 2.2). All the isolates grew in medium supplemented with 10 mmol L^{-1} acetate. To the best of our knowledge this is the first study in which isolates related to *Thiomcrospira crunogena* are shown to grow heterotrophically in the presence of acetate. However, growth of *Thiomcrospira* spp. was inhibited by higher carbon concentration (e.g., in ASW medium). Our study also showed that *Pelagibaca* sp. strain EPR125, an isolate closely related to the chemoheterotrophic "*alphaproteobacterium*" *Pelagibaca bermudensis*, grew under strict autotrophic conditions and encoded for Form II RubisCO. Only isolates closely related to *Thioclava pacifica* (EPR65 and EPR74), *Pelagibaca bermudensis* (EPR125), *Salinisphaera hydrothermalis* (EPR70 and EPR71), *Thalassospira* sp. (EPR62), and one of the isolates closely related to *Halothiobacillus hydrothermalis* (EPR155), were able to grow in

complex medium. It is possible that the ability of these organisms to grow autotrophically and heterotrophically in a two carbon organic compound as well as in a complex medium, confers them an advantage over other bacterial groups to colonize and inhabit different microniches within the highly dynamic hydrothermal vent environment.

Isolates closely related to *Thiomicrospira crunogena* and *Thiomicrospira thermophila* were able of microaerobic growth in 5% O_2, which also may confer them an advantage to colonize environments where oxygen concentration is limited. I tested the ability of the representative isolates to grow using *n*-alkanes, which are abundant throughout hydrothermal fluids (Brault *et al*., 1988), but only strains closely related to *Salinisphaera hydrothermalis* were able to use dodecane as their only carbon and energy source.

In a different study the the number of thiosulfate oxidizing bacteria was estimated to range from 1.1 x 10^3 to 2.4 x 10^7 cells mL^{-1}(Crespo-Medina *et al*., In press-a and Chapter 4 of this thesis). In this study such numbers were compared with acridine orange total cell counts, and the results indicate that the percentage of chemosynthetic thiosulfate oxidizing bacteria ranged from 0.002 to 14.1% of the total cell counted per mL of sample (Table 2.3). Most of the organisms obtained from serial dilutions were closely related to *Thiomicrospira crunogena* and *Hydrogenovibrio marinus*, indicating that these bacteria are abundant on the environment. However, representative of both these genera were also isolated from undiluted samples, suggesting that, in some cases, these bacteria are able to outgrow other organisms competing for the same resources (Table 2.4). Taken together, these results suggest that *Thiomicrospira crunogena* and *Hydrogenovibrio marinus* are both dominant and relatively fast growing species at deep-sea vents.

In an effort to gain a better understanding on the carbon fixation and thiosulfate oxidation metabolism in our representative organisms, the genes encoding for their RubisCO and SoxB enzymes were amplified. Phylogenetic analyses of the deduced amino acid sequences of the Form I RubisCO revealed three main clusters were (Figure 2.3); that the first cluster included the sequences for *Salinisphaera* sp. related isolates, the second included the sequences from *Thioclava pacifica* related isolates, and the third and larger cluster defined the *Thiomicrospira* group cluster, which included the sequences from isolates closely related to *Thiomicrospira* spp, *Hydrogenovibrio* spp. and *Halothiobacillus* spp. The position of isolates related to *Halothiobacillus hydrothermalis* (EPR129 and EPR155) within the *Thiomicrospria* cluster is not consistent with the phylogenetic position of these isolates based on the 16S rRNA gene (Figure 2.2), and might be an indication that a possible horizontal gene transfer event have occurred.

This study showed for the first time that an isolate (EPR125), related to the chemoheterotrophic marine bacterium *Pelagibaca bermudensis,* grew under autotrophic conditions and encoded for the Form II RubisCO. However, phylogenetic analysis of Form II RubisCO from EPR125, an "*alphaproteobacterium"*, showed that this enzyme clustered with the enzymes of several "*gammaproteobacteria*", suggesting another possible event of horizontal gene transfer. Furthermore, phylogenetic analyses showed that two strains closely related to *Hydrogenovibiro marinus* (EPR115 and EPR116) have divergent forms of Form II RubisCO, forming separate clusters in the phylogenetic tree. This might be yet an additional indication of horizontal gene transfer or gene duplication, both of which have been described for genes encoding for the RubisCO enzyme (Delwiche & Palmer, 1996).

I carried out a preliminary assessment of the occurrence of the Form I RubisCO enzyme of the *Thiomicrospira* group from a vent sample. Only 10 clones were screened so far to determine the ability of these newly designed primers to amplify *Thiomicrospira*-related RubisCO genes directly from vent microbial communities. The phylogenetic position of these environmental clones relative to other *Thiomicrospira*-related RubisCO sequences is shown in Figure 2.4. This preliminary experiment demonstrated that the new primers can be used to assess the environmental distribution of *Thiomicrospira*-related RubisCO genes in vent samples and possibly to estimate the abundance of these genes using q-PCR. The gene encoding the sulfur thiol esterase enzyme, SoxB, which is a central enzyme in the thiosulfate oxidation pathway, was amplified from some of the chemosynthetic isolates. Phylogenetic analysis of the translated sequences of *soxB* (Figure 2.5) also showed a very distinctive arrangement, where the sequence of *Pelagibaca* sp. EPR125, an "*alphaproteobacterium*", clustered together withthe "*gammaproteobacteria*", while the sequence of *Halothiobacillus* sp. EPR155, a "*gammaproteobacterium"* clustered with a group of "*alphaproteobacteria*". Therefore, the phylogenetic analysis of the *soxB* locus showed in Figure 2.5 may reveal yet another case of horizontal gene transfer.

Chapter 3 - *Salinisphaera hydrothermalis* sp. nov., a mesophilic, halotolerant, facultative autotrophic, thiosulfate oxidizing *"gammaproteobacterium"* from deep-sea hydrothermal vents, and emended description of the genus *Salinisphaera*.

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Introduction

Deep-sea hydrothermal vents are located in tectonically active areas of the sea floor and release either high temperature fluids (focused flow vents), or moderate temperature fluids (diffuse flow vents). At deep-sea vents, seawater interacts with volcanic rocks at high temperature and becomes enriched in a variety of reduced chemical species, which represent a constant flux of electron donors for microbial oxidations. In the absence of light, chemosynthetic microorganisms oxidize these reduced chemical species (predominantly sulfur compounds) and mediate the primary production of organic carbon (Jannasch, 1995; McCollom & Shock, 1997).

Typically, representatives of the genera *Thiomicrospira* and *Thiobacillus* have been isolated when diffuse flow hydrothermal fluids were inoculated in culture media for the enrichment of chemosynthetic aerobic, sulfide, sulfur, and thiosulfate oxidizing bacteria (Durand *et al.*, 1993; Jannasch *et al.*, 1985; Ruby *et al.*, 1981; Ruby & Jannasch, 1982; Takai *et al.*, 2004; Teske *et al.*, 2000). Heterotrophic bacteria of the genera *Marinobacter, Vibrio, Pseudoalteromonas,* and *Halomonas,* among others, were also

routinely isolated from hydrothermal vent samples (Kaye *et al.*, 2004; Raguenes *et al.*, 1997; Simon-Colin *et al.*, 2008; Teske *et al.*, 2000; Vetriani *et al.*, 2005) and their abundance in vent fluids collected from the Pacific Ocean was estimated to be up to a 28% of the total microorganisms per sample (Kaye & Baross, 2000).

Here I report the isolation of three new strains of mesophilic, halotolerant, facultatively chemolithoautotrophic, thiosulfate oxidizing *"gammaproteobacteria"* from deep-sea hydrothermal vents located on the East Pacific Rise (EPR) at 9° North, and the characterization of one of these strains as a new species.

Materials and methods

Hydrothermal fluid samples from diffuse flow vents were collected from the East Pacific Rise (EPR; 9º 50'N, 104º 17'W), at a depth of approximately 2500 m during an oceanographic expedition aboard the R/V *Atlantis* on April 2004. The fluids were collected using titanium samplers operated by the manipulator of the DSV *Alvin*, immediately above the venting source and one meter above the source. On the surface, samples were transferred promptly to the ship's laboratory and sub-samples were stored at 4ºC. Primary enrichment cultures were initiated immediately after sample collection in growth medium designed to enrich for chemolithoautotrophic thiosulfate oxidizing bacteria. The medium used was a modification of medium 142 (http://www.dsmz.de), called 142-A, which was composed of (L^{-1}): NaCl (25.0 g), $(NH_4)_2SO_4$ (1.0 g), $MgSO_4 \bullet 7H_2O$ (1.5 g), $CaCl_2 \bullet 2H_2O$ (0.42 g), KCl (0.64 g), $NaHCO_3$ (0.046g), K_2HPO_4 (0.05 g). Two ml of a 0.5% phenol red solution were added to 1 L of media as a pH indicator.

Following sterilization, the media was supplemented with 20 mmol L^{-1} $Na_2S_2O_3$, 1 µmol L^{-1} vitamin B_{12}, 1 mL of mixed vitamin solution 141 (www.dsmz.de), and 1 mL of trace element solution SL10 (http://www.dsmz.de). For isolation of single colonies, liquid cultures were inoculated on Petri dishes containing medium 142-A solidified with 15 g L^{-1} of Noble agar (Sigma). Stocks for long-term storage were prepared by adding 150 µL of sterile glycerol (Fisher Scientific) to 850 µL of cultures grown overnight, and were stored at –80ºC.

Heterotrophic growth was determined by transferring 100 µl of an overnight culture from medium 142-A to medium 142+A, which was depleted of $NaHCO_3$ and supplemented with 10 mmol L^{-1} sodium acetate (Sigma). Growth was also tested in Artificial Seawater (ASW) medium, composed of (L^{-1}): NaCl (24 g), KCl (0.7 g), $MgCl_2$ (7.0 g), yeast extract (3.0 g), and peptone (2.5 g); in low strength ASW (LS ASW) medium, which is a modified version of ASW containing 0.1 g L^{-1} of yeast extract and 0.5 g L^{-1} of peptone, and in ASW minimal medium (ASW MM), composed (L^{-1}) of: NaCl (23.6 g), KCl (0.64 g), $MgCl_2 \bullet 6\ H_2O$ (4.53 g), $MgSO_4 \bullet 7\ H_2O$ (5.94 g), $CaCl_2 \bullet 2\ H_2O$ (1.3 g), $Na_2HPO_4 \bullet 7H_2O$ (43.0 mg), $NaNO_3$ (0.22 g), NH_4Cl (0.65 g), and supplemented with dodecane ($C_{12}H_{26}$) in the vapor phase as the only carbon and energy source. After autoclaving, the ASW minimal medium was supplemented with 1 µmol L^{-1} Vitamin B-12, 1 mL of Trace Element Solution SL-10, and 1mL of mixed vitamin solution 141.

Growth rates (µ; h^{-1}) were estimated as previously described (Vetriani *et al.*, 2004). Unless differently specified, growth ranges and optimal growth conditions were determined in LS ASW medium. To determine the optimal growth temperature of the new isolate, cultures were incubated between 10 to 45ºC (at 5ºC intervals). All other

experiments were carried out at 35ºC, the optimal growth temperature for this strain. To determine optimal salt requirements, the concentration of NaCl was varied between 1.0 to 25% (w/v). The influence of pH on growth was determined between pH 4.5 to 8.0 by using the following buffers at a concentration of 10 mmol L^{-1}: acetate at pH 4.5 and 5.0; MES at pH 5.5 and 6.0; PIPES at pH 6.5, 7.0 and 7.5; and Tris at pH 8.0. Anaerobic growth with nitrate as an electron acceptor was tested in ASW medium supplemented with 7.3 mmol L^{-1} KNO_3, under a N_2 atmosphere.

Catalase activity was determined as previously described (Vetriani *et al.*, 2004), and the presence of cytochrome C, a component of the cytochrome oxidase system, was determined according to the protocol described in (Kovacs, 1956). *Escherichia coli* and *Pseudomonas aeruginosa* were used as negative and positive controls for the cytochrome oxidase test, respectively.

A Biolog GN2 MicroPlate™ test panel (BiOLOG, Hayward, CA) was used to comparatively characterize the carbon utilization/oxidation profiles of the new isolate and of the reference strain, *Salinisphaera shabanensis*. Confluent growth of both strains was obtained on ASW solid medium overnight. Cells were collected using a sterile cotton swab and resuspended in 15 ml of salt solution (23.5 g L^{-1} NaCl and 10.6 g L^{-1} $MgCl_2 \bullet 6H_2O$). The cell suspension was adjusted to an optical density of 0.3 ± 0.05, it was supplemented with 5 mmol L^{-1} Sodium thioglycolate, and it was dispensed (150 μL aliquots) to each well of two Biolog GN2 MicroPlates™, which were incubated at 35ºC. A change in color, indicative of the oxidation of the substrate, was monitored for 48 hours.

For direct counts, cells were stained routinely with 0.1% acridine orange and visualized with an Olympus BX 60 microscope with an oil-immersion objective lens (UplanF1 100x/1.3). Cells for ultrathin sections and for platinum shadowing were prepared as described previously (Vetriani *et al.*, 2004). Motility was determined by phase contrast microscopy. Gram staining was performed as described elsewhere (Holt *et al.*, 1994).

Genomic DNA was extracted from cells collected by centrifugation using the UltraClean™ Microbial DNA isolation kit, according to the manufacturer's instructions (MoBio Laboratories, Solana Beach, CA, USA). The full-length sequence of the16S rRNA gene was selectively amplified from the genomic DNA by PCR, cloned, sequenced and subjected to phylogenetic analysis as described previously (Vetriani *et al.*, 2004; Voordeckers *et al.*, 2005).

The genes encoding for the enzymes ribulose 1, 5-bisphosphate carboxylase/oxygenase form I and II (RubisCO) and alkane hydroxylase (AlkB, involved in the oxidation of hydrocarbons) were amplified from the genomic DNA of the new strains and *S. shabanensis*. A 500 bp fragment of the gene encoding for form I RubisCO (*rbcL*/*cbbL*) from *S. shabanensis* was amplified as described previously (Nanba *et al.*, 2004), and its sequence was determined. Internal primers ss rbcLF (5'-GGTCTATGAAAGCGCTCAAGG-3') and ss rbcLR (5'-ATCCATTTCGAGATCACGCGG-3') were designed based on the *rbcL* sequence from *S. shabanensis* (using the IDT OligoAnalyzer 3.1 Program; www.idtdna.com), and were used to amplify a 400 bp fragment of the *rbcL* gene from the three new strains. The PCR protocol used to amplify the *rbcL* genes was: 5 min at 94ºC, followed by 30 cycles of 45

sec at 94ºC, 1 min at 50ºC, and 45 sec at 72ºC, and ending with a final extension of 20 min at 72ºC. The PCR reaction for the amplification of a 1040 bp fragment of the gene encoding for the form II RubisCO (*cbbM*) was carried out as previously described (Elsaied *et al*., 2007). A 550 bp fragment of the gene encoding for the enzyme alkane hydroxylase (*alkB*) was amplified selectively from the genomic DNA of *S. shabanensis* and of two new strains by PCR, as previously described (Smits *et al*., 1999). The sequences for *rbcL* and of the *alkB* gene were determined for both strands and were translated into the respective amino acid sequences using EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq). The amino acid sequences were aligned with ClustalX v 1.8 (Thompson *et al.*, 1997) and manually adjusted using Seaview (Galtier *et al.*, 1996). Phylogenetic distances were calculated using the Observed Divergence matrix, and the neighbor joining method was used to evaluate tree topologies. Phylo_win was utilized to plot tree topologies (Galtier *et al.*, 1996) and their robustness was tested by bootstrap analysis with 1,000 resamplings. The determination of total DNA base composition (mol% G+C) was carried out by High Performance Liquid Chromatography (HPLC) (Mesbah *et al*., 1989).

Results

Enrichment cultures for mesophilic, chemolithoautotrophic, thiosulfate oxidizing bacteria were obtained by inoculating 10 mL of medium 142-A with 1 mL of fluids from three different samples collected on the East Pacific Rise at 9º N. The temperature of the fluids at the time of collection was: 2.5, 6 and 13ºC, respectively. Cultures were incubated at 30ºC. A change in color of the pH indicator present in the medium,

suggesting growth, was observed after one or two days from the beginning of the incubation, and then confirmed by direct counts of cells. Three independent cultures showed consistent growth after repeated transfers, and were purified by successive isolations of single colonies on solidified medium. The resulting pure cultures were designated as strains EPR70^T, EPR71, and EPR72. Preliminary phylogenetic analysis of the16S rRNA gene sequence indicated that the three strains were closely related (sequence identity: 99%). Strain EPR70^T was selected for further characterization.

Cells of EPR70^T were short rods, 0.8 to 1.0 μm in length and 0.6 μm in width, and divided by constriction (Figure 3.1a). Cells stained Gram-negative. The cell envelope of EPR70^T included a cytoplasmic membrane surrounded by a periplasmic space and an outer membrane (Figure 3.1b). The organism was motile and possessed one or more flagella which were observed in electron micrographs of platinum-shadowed cells (Figure 3.1c). The presence of endospores was not observed.

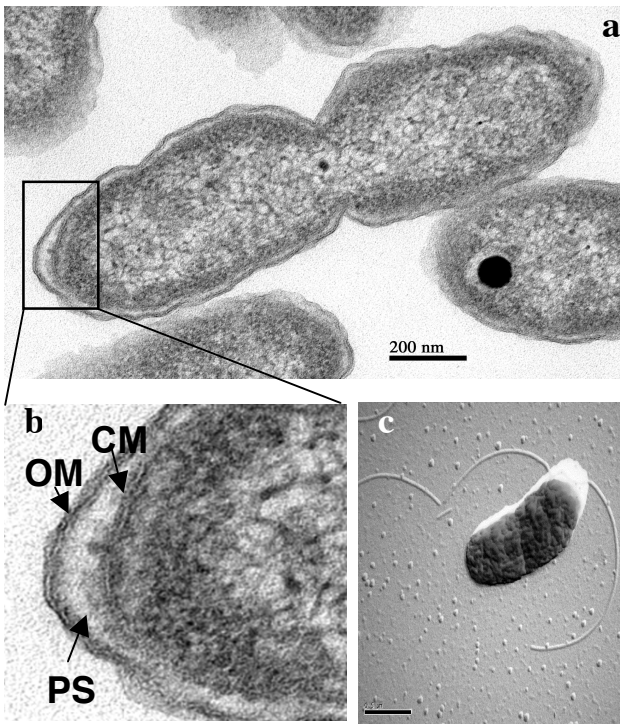

Figure 3.1 (a) Electron micrograph of a thin section of cells of strain EPR70^T. (b) Details of the cell envelope of strain EPR70. OM, outer membrane, PS, periplasmic space, and CM, cytoplasmic membrane. (c) Electron micrograph of a platinum-shadowed cell of strain EPR70 showing the presence of flagella.

Under strictly autotrophic conditions, EPR70^T, EPR 71 and EPR 72 oxidized $Na_2S_2O_3$ and acidified the culture medium. Heterotrophic growth occurred when medium 142 was depleted of $Na_2S_2O_3$ and $NaHCO_3$ and supplemented with 10 mmol L^{-1} acetate; under these conditions strain EPR70^T alkalified the culture medium. EPR70^T, EPR71 and EPR72 also grew well heterotrophically in ASW, LS ASW and ASW MM supplemented with dodecane as the sole carbon and energy source. Strain EPR70^T grew at temperatures between 20 to 40ºC, with optimal growth at 35ºC. No growth was detected at 10 or 45 ºC. EPR70^T grew at NaCl concentrations between 1.0 to 25.0% (w/v) with an optimum at 2.5 % (w/v). Growth occurred between pH 5.0 to 7.5 with a pH optimum of 5.5. No growth was observed at pH 4.5 or 8.0. The shortest generation time of strain EPR70^T in LS ASW

was 42 min (Figure 3.2). For comparison, the generation time of strain EPR70^T grown under optimal conditions of temperature, salinity and pH and with CO_2 as the sole carbon source (in medium 142) was 231 min. Strain EPR70^T was a fully aerobic organism, and growth did not occur in medium 142 supplemented with 5% oxygen (v/v) or in ASW medium with 7.3 mmol L^{-1} nitrate.

Phylogenetic analysis of the 16S rRNA gene sequences, carried out using the neighbor joining method, placed EPR70^T, EPR 71, and EPR72 (98 to 99% sequence identity between each sequence) in a unique cluster within the class *"gammaproteobacteria"* (Figure 3.3).

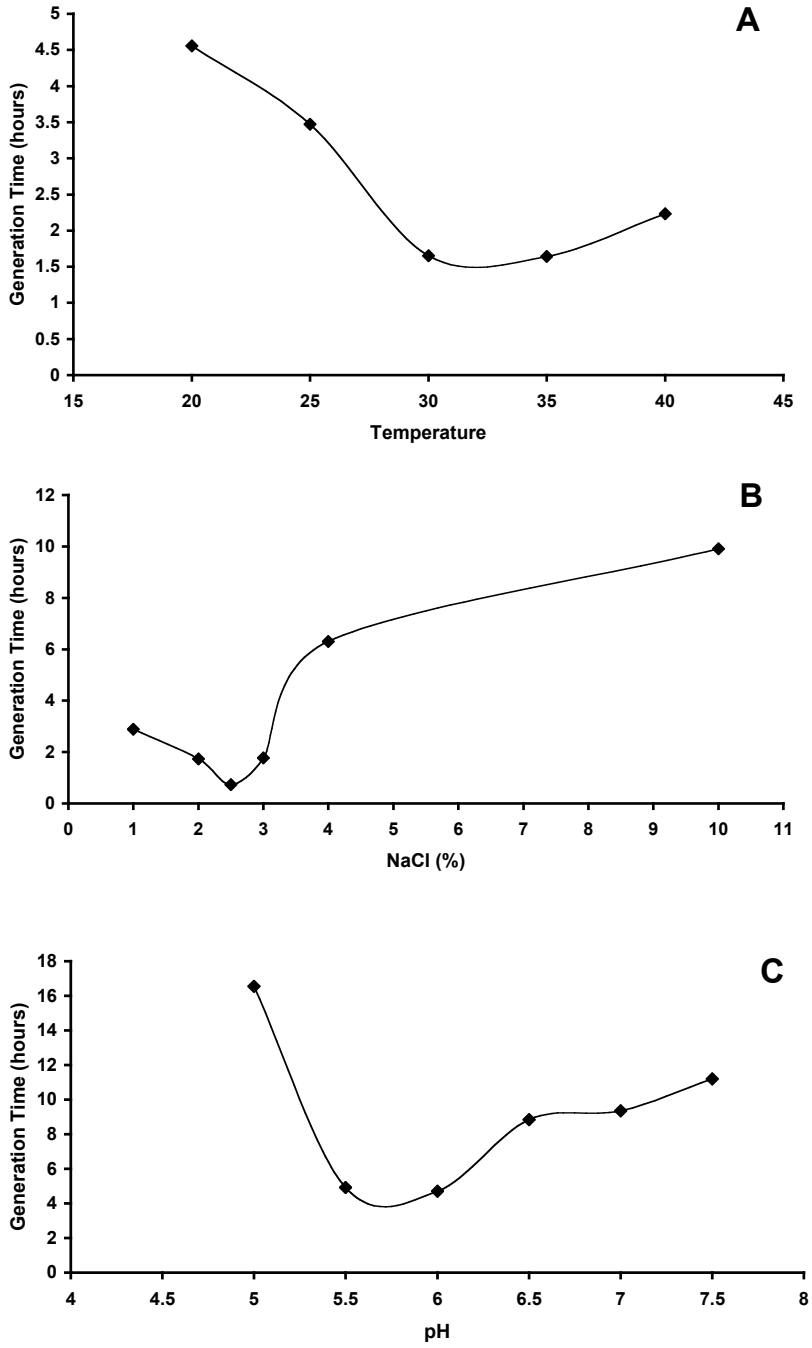

Figure 3.2 Generation time (in h) of *Salinisphaera hydrothermalis* sp. nov. EPR70^T under different growth conditions. (a) Temperature; (b) NaCl concentration; (c) pH.

The closest relatives to these strains were *Salinisphaera shabanensis*, a moderate halophile which was isolated from the brine-seawater interface in the Shaban Deep, in the Red Sea (94% sequence identity to the 16S rRNA gene of strain EPR70^T), and the only formally described member of this new genus (Antunes *et al*., 2003), *Salinisphaera* sp. strain ARD M17, isolated from deep-sea water from the Knipovich Ridge, in the Arctic Ocean (94% sequence identity to strain EPR70^T; Okamoto, and Naganuma, unpublished; accession number: AB167073), and "*Salinisphaera dokdoensis"*, isolated from the East Sea of Korea (95% sequence identity to strain EPR70^T; Cho et al. unpublished; accession number: EF988634). While the type strain for the genus *Salinsphaera, S. shabanensis*, was originally described as an heterotroph (Antunes *et al*., 2003), our study demonstrated that this organism also grew chemolithoautotrophically by thiosulfate oxidation and with *n*-alkanes as the sole carbon and energy source (Table 3.1). Interestingly, 16S rRNA gene sequences related to *Salinisphaera* spp. were retrieved from the microbial community attached to hydrocarbon-contaminated rocks along the Spanish shoreline, represented in Figure 3.3 by clones RC23, RC32 and RC62 (Alonso-Gutierrez et al, unpublished).

High bootstrap values supported the branching topology of the EPR strains relative to the other strains (Figure 3.3). The G + C content of the genomic DNA of strain EPR70^T, determined by HPLC analysis of the deoxyribonucleosides, was 64.0 mol%, while that of *S. shabanensis* was 61.8 mol% (Antunes *et al*., 2003).

Comparative analyses of strain EPR70^T and *S. shabanensis* revealed morphological (rods and cocci, respectively) and physiological differences (Table 3.1). In particular, EPR70^T had lower salinity and pH optima than *S. shabanensis* (2.5% vs. 10% NaCl and pH 5.5 vs. pH ~7.0, respectively), suggesting specific adaptations to the

slightly acidic vent fluids, and it could not grow anaerobically in the presence of nitrate as a terminal electron acceptor (Table 3.1). The metabolic fingerprints of strain EPR70^{T} and that of *S. shabanensis*, determined using a Biolog assay on a GN2 MicroPlate^{TM}, showed that both bacteria were able to grow on a wide range of carbon sources, but some differences were evident (Table 3.2). For instance, EPR70^{T} preferentially grew on sugars and sugar-alcohols, while *S. shabanensis* preferred amino acid derivates.

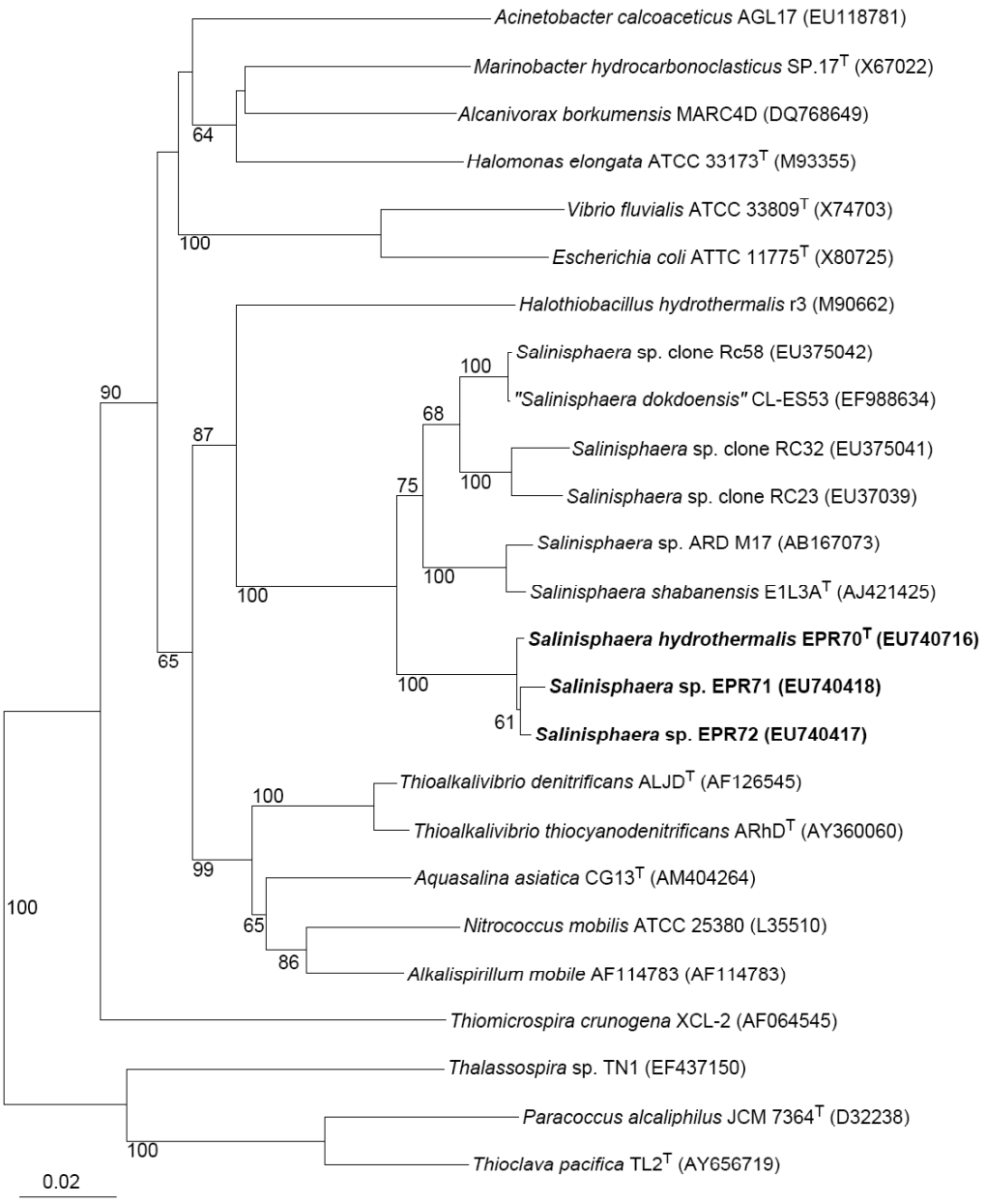

Figure 3.3 Phylogenetic position of *Salinisphaera hydrothermalis* sp. nov. EPR70^T, *Salinisphaera* sp. strains EPR71, and EPR72, according to their 16S rRNA gene sequence. The neighbor-joining tree was constructed using Phylo_Win. Bootstrap values higher than 50 are indicated. Bar indicate 2% estimated substitution.

Table 3.1 Differentiating features of *Salinisphaera hydrothermalis* sp. nov. EPR70^T and *Salinisphaera shabanensis*.

Feature	***Salinisphaera hydrothermalis* EPR70^T**	***Salinisphaera shabanensis* E1L3A^T**
Morphology	Small rods	Cocci
Size	0.8-1.0 μm long, 0.3-0.5 μm wide	0.7-1.2 μm in diameter
Catalase	Positive	Positive
Oxidase	Negative	Positive
Temperature range (ºC)	20-40	5-42
Optimal temperature (ºC)	30-35	30-37
Salinity range (% NaCl)	1-25	1-28
Optimal salinity (% NaCl)	2.5	10
pH range	5.0-7.5	4.0-8.0
Optimal pH	5.5	6.5-7.5
Chemolithoautotrophic growth	Yes	Yes[1]
Growth on *n*-alkanes (dodecane)	Yes	Yes[1]
Anaerobic growth in ASW[2] + nitrate	No	Yes
mol% G+C	64.0 %	61.8 %

[1]Data for *S. shabanensis* that were obtained in this study
[2]ASW, Artificial Sea Water

Table 3.2 Comparative metabolic profiles[1] of *Salinisphaera hydrothermalis* sp. nov. EPR70^T and *Salinisphaera shabanensis* E1L3A^T

Carbon Source	*Salinisphaera hydrothermalis* EPR70^T	*Salinisphaera shabanensis* E1L3A^T
Glycogen	-	+
Adonitol	+	-
D-Arabitol	+	-
i-Erythritol	+	-
D-Fructose	+	-
D-Galactose	+	-
α-D-Glucose	+	-
L-Rhamnose	+	-
D-Sorbitol	+	-
Cis-Aconitic Acid	-	+
Formic Acid	-	+
α-Ketobutyric Acid	+	-
α-Ketoglutaric Acid	-	+
Bromosuccinic Acid	-	+
Succinamic Acid	+	-
L-Alanyl-glycine	-	+
L-Asparagine	-	+
L-Aspartic Acid	-	+
L-Glutamic Acid	-	+
Glycyl-L-Aspartic Acid	-	+
Glycyl-L-Glutamic Acid	-	+
L-Proline	+	-
γ-Aminobutyric Acid	+	-
Glycerol	+	-

[1]Both species grew in: Tween 20, Tween 80, D-Mannitol, D-Mannose, Xylitol, Pyruvic Acid, Methyl Esther, Succinic Acid Mono Methyl Ester, γ-Hydroxybutyric Acid, D, L-Lactic Acid, Succinic Acid, L-Alanine.

In order to investigate the carbon fixation pathway in the EPR strains and in *S. shabanensis*, PCR amplification of the genes encoding for both RubisCO forms I (*rbcL*/*cbbL*) and II (*cbbM*) for all isolates were carried out, and a product was obtained only for the *rbcL*/*cbbL* gene (encoding for RubisCO form I). The amino acid identity among the RubisCO sequences of the three EPR strains was 98 to 99%, while the

sequence identity between these enzymes and the RubisCO from *S. shabanensis* ranged from 94 to 95%. Phylogenetic analysis of the amino acid sequence of the form I RubisCO of strains EPR70^T, EPR71, EPR72 and *S. shabanenesis*, carried out using the neighbor joining method, showed that these sequences formed a unique cluster related to other form I, type C enzymes (Figure 3.4; (Xu & Tabita, 1996). The closest relatives to the RubisCO from the EPR strains and *S. shabanenesis* were the enzymes of the methylotrophic bacterium *Methylibium petroleiphilum* and of the ammonia-oxidizing bacteria *Nitrosospira multiformis* and *Nitrosococcus oceani* (Figure 3.4). It is worth noting that the RubisCO enzymes from the EPR strains were also related to a group of sequences retrieved from natural microbial communities associated with the plumes of black smokers located in the Western Pacific arc hydrothermal vent system, represented in Figure 3.4 by clones ICS1 and ICP1 (Elsaied *et al*., 2007).

Since both the EPR strains and *S. shabanensis* can use *n*-alkanes as their sole carbon and energy sources, the presence in these organisms of the *alkB* gene, was investigated. This gene encodes for the alkane hydroxylase (AlkB), an enzyme that catalyzes the first step in the oxidation of hydrocarbons. Phylogenetic analysis of the amino acid sequence of the AlkB enzyme from strains EPR70^T, EPR71 and *S. shabanensis* placed these enzymes in a discrete cluster related to the alkane hydroxylase of *Nocardia farcinia* (Figure 3.5). The amino acid identity between the AlkB sequence of EPR70^T and EPR71 was 90 %, while the sequence identities of the enzymes from the two EPR strains and the alkane hydroxylase from *S. shabanensis* were 62 and 63%, respectively.

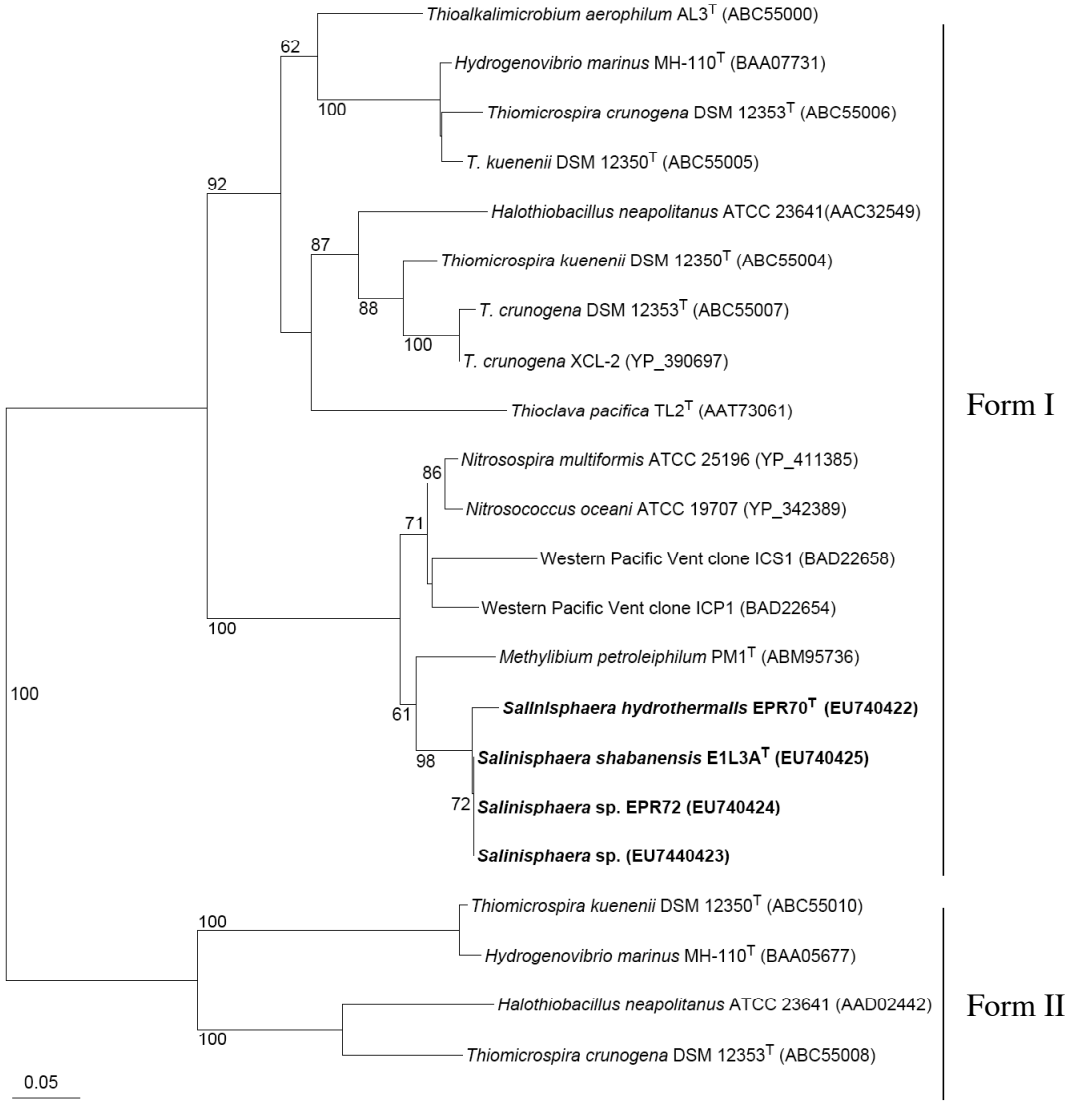

Figure 3.4 Phylogenetic analysis of the large subunit of the protein RubisCO form I from *Salinisphaera hydrothermalis* sp. nov. EPR70^T, *Salinisphaera* sp. strains EPR71, EPR72, and *S. shabanensis*. The neighbor-joining tree was constructed using Phylo_Win. Bootstrap values higher than 50 are indicated. Bar indicate 5% estimated substitutions.

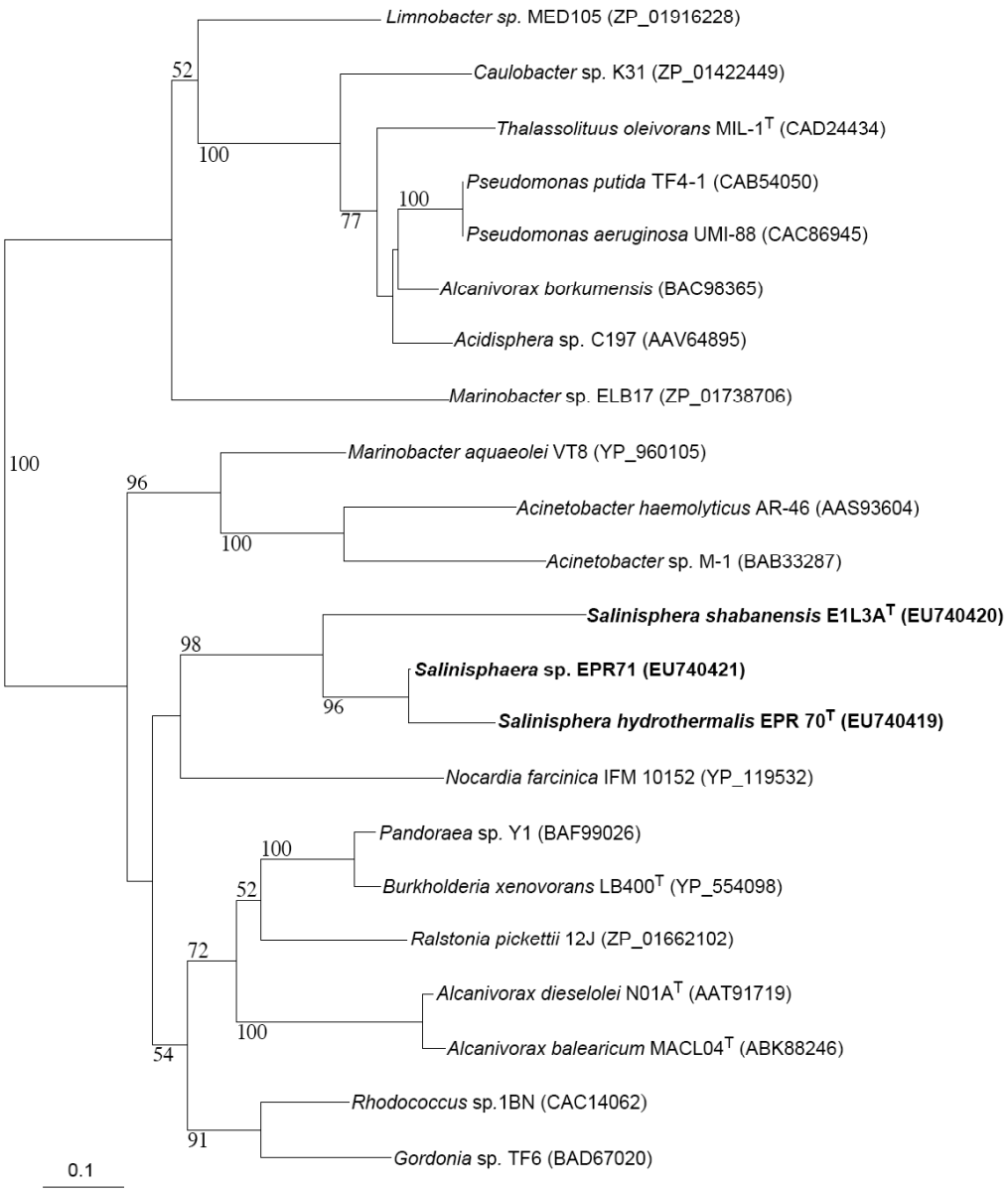

Figure 3.5 Phylogenetic analysis of protein alkane hydroxylase (AlkB) from *Salinisphaera hydrothermalis* sp. nov. EPR70^T, *Salinisphaera* sp. strain EPR71, and *S. shabanenensis*. The neighbor-joining tree was constructed using Phylo_Win. Bootstrap values higher than 50 are indicated. Bar indicate 10% estimated substitutions.

Physiological and phylogenetic analyses indicated that strain EPR70^T and *S. shabanensis* are not related at the species level, and therefore EPR70^T represents a new

species within the genus *Salinisphaera*, for which we propose the name *Salinisphaera hydrothermalis*.

At deep-sea hydrothermal vents, microorganisms must adapt to highly dynamic environmental conditions, where there are fluctuations in temperature, salinity and nutrient availability (Karl, 1995). The presence of the *rbcL*/*cbbL* gene, which encodes for RubisCO form I, in all the *Salinisphaera* strains, strongly suggests that these bacteria use the Calvin-Benson-Bassham cycle to fix CO_2. The metabolic versatility of *S. hydrothermalis*, which can fix CO_2 and use a wide range of organic carbon sources, may be an advantage for its growth and survival in these environments. In particular, the ability of *S. hydrothermalis* to grow autotrophically, to oxidize *n*-alkanes (which are enriched in hydrothermal fluids; (Brault *et al*., 1988) and to grow optimally at a slightly acidic pH suggests that this bacterium is particularly well suited to thrive in the moderate temperature fluids that are typically emitted by diffuse flow deep-sea hydrothermal vents.

Emended description of the genus *Salinisphaera*. Cells are Gram-negative cocci or short rods, possessing one or more flagella. Mesophilic, halotolerant, catalase and oxidase positive. Aerobic or facultatively anaerobic. Growth occurs chemolithoautotrophically with thiosulfate as an electron donor and oxygen as an electron acceptor, heterotrophically on *n*-alkanes (dodecane) as the sole carbon and energy source, or on complex medium. Found in marine environments, including the brine-seawater interface of the Shaban deep in the Red Sea and the deep-sea hydrothermal vents on the East Pacific Rise. The type strain is *Salinisphaera shabanensis* (Antunes *et al*., 2003).

Description of *Salinisphaera hydrothermalis* sp. nov. *Salinisphaera hydrothermalis* (hy.dro.ther.mal'is N.L. adj. *hydrothermalis*, pertaining to a hydrothermal vent). Cells are small rods (0.8-1.0 μm long, 0.3-0.5 μm wide), which are motile by means of one or more flagella. Obligate aerobe. Gram-negative. Catalase positive. Oxidase negative. Growth occurs between 20 and 40°C (optimum 30-35ºC), 1 and 25% NaCl (optimum 2.5%) and pH 5.0 and 7.5 (optimum pH 5.5) The shortest generation time observed was 42 min. Growth occurs under aerobic, chemolithoautotrophic conditions in the presence of thiosulfate and CO_2. Heterotrophic growth occurs with acetate or *n*-alkanes as sole carbon and energy sources, and in complex Artificial Seawater (ASW) medium. Nitrate is not used as an electron acceptor. The DNA G+C content of *S. hydrothermalis* is 64.0 mol% as determined by the HPLC method. The type strain is EPR70^T (= DSM 21483 = JCM 15514), which was isolated from diffuse flow hydrothermal vent fluids collected from the East Pacific Rise at 9º 50' North.

Chapter 4 – Adaptation of chemosynthetic microorganisms to elevated mercury concentrations in deep-sea hydrothermal vents

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Introduction

Metal resistance is common among microorganisms and is a critical factor modulating the effects of metals in the environment (Nies, 1999). Although the evolutionary origin of microbial metal resistance is unknown, geothermal environments, such as deep-sea hydrothermal vents, where heavy metal concentrations may be comparable to those in contaminated sites, could represent ecological niches where metal-microbe interactions occurred early during microbial evolution. In these environments, metals often form complexes with reduced sulfur compounds (Dopson *et al.*, 2003). Deep-sea hydrothermal vents are associated with spreading centers along mid-oceanic ridge systems, with hydrothermal fluids occurring as either low temperature diffuse flows (where cold, oxygenated seawater mixes with hydrothermal fluids prior to their emission at the seafloor) or as high temperature focused flows. Diffuse flow vents are sites characterized by the most rapid biomass production of any ecosystem on Earth (Lutz, 1994); among other organisms, tube worms, mussels, clams, crabs, and zoarcid fishes populate these vents. The rapid biomass production in this ecosystem is sustained by the activity of chemosynthetic microorganisms, which obtain energy, for the most part, by the oxidation of sulfur compounds (McCollom & Shock, 1997; Van Dover, 2000). An important aspect of the biology and ecology of deep-sea hydrothermal vents,

which has been scarcely investigated, is how microorganisms interact with metals and how they influence their toxicity.

Since mercury (Hg) in geothermal environments exists mostly as HgS complexes (Varekamp & R, 1984), and since the oxidation of sulfur compounds is considered the most efficient energy generating process at hydrothermal vents (Edwards *et al.*, 2005; McCollom & Shock, 1997) I initiated a study on the interaction between Hg and sulfur-oxidizing bacteria from these environments. The rationale for this choice is that the activity of sulfur-oxidizing bacteria may mobilize Hg and increase its bioavailability (Baldi & Olson, 1987), as has been shown for other metals (Rohwerder *et al.*, 2003). It has been long known that Hg is elevated around deep-sea spreading centers (Bostrom & Fisher, 1969; Varekamp & R, 1984), but only recently have direct measurements of Hg in hydrothermal fluids been made. These measurements, all obtained from high temperature focused flows, i.e., black smoker chimneys, suggest elevated Hg concentrations, sometimes to such high levels as those observed in surface natural waters in highly contaminated environments (Ekstrom, 2007; Lamborg *et al.*, 2006). While, to the best of our knowledge, Hg concentrations in diffuse flow hydrothermal vents have not been measured, previous work, demonstrated adaptation to Hg among moderately thermophilic, heterotrophic bacteria from such vents, thus suggesting that these microorganisms are exposed to elevated concentration of this toxic metal (Vetriani *et al.*, 2005). Here I report that Hg concentrations are elevated (relative to seawater concentrations) in deep-sea diffuse flow vents, and that Hg resistant thiosulfate oxidizing bacteria are enriched in these environments.

Materials and methods

Sampling site and sample treatment - Samples of hydrothermal fluids from both focused and diffuse flow vents were collected from the East Pacific Rise (EPR) at 9º50'N, 104º17'W during three oceanographic expeditions on board of the R/V *Atlantis* in April 2004, May 2005, and January 2007. Samples for microbiological analysis were collected and stored as described previously (Vetriani *et al.*, 2005). For this work, samples were collected immediately above the vents and diffuse flow areas, as well as one meter above them, both before and after the removal of colonizing mussels, using a titanium sampler operated by the manipulator of the DSV *Alvin.* Fluid temperatures were measured in-situ using the Inductive Coupled Link (ICL) devices associated with the titanium samplers.

To minimize contamination, all samples for total Hg (THg) and trace metal analysis were the first to be retrieved from the titanium samplers immediately upon arrival on board ship. The first volume extracted from the samplers was discarded and then bottles for the preservation of samples were filled. Common clean protocols for the handling of samples with trace Hg concentrations were followed (Gill and Fitzgerald, 1987). However, at the time of sampling I had no control on the degree of "cleanliness" of the titanium samplers. Therefore, I realized that some of the measurements, especially those of diffuse flow vents with low THg concentrations may over estimate true THg concentrations. This issue needs to be addressed during future cruises. Samples from 2004 were stored in Teflon tubes and frozen shipboard at –80°C for later analysis. In 2007, samples for THg analysis were collected using the titanium samplers, stored in I-Chem Certified™ 300 series bottles, fixed with 0.5% HCl, and kept at 4ºC for later

analysis. Reference seawater samples were collected at 60 m and at 2400 m depth using Niskin samplers, were stored in 1 L Teflon bottles, fixed with 0.5% HCl, and stored at 4ºC. Samples for THg analysis were not collected in 2005.

Chemical composition of diffuse flow fluids - Samples from the 2004 cruise were analyzed at Frontier GeoScience Inc., Seattle, Washington. Total mercury was analyzed after bromine monochloride oxidation and stannous chloride reduction using dual amalgamation cold vapor atomic fluorescence spectrometry (CVAFS), as described previously (Bloom & Fitzgerald, 1988; Fitzgerald & Gill, 1979). Methylmercury was analyzed after distillation and aqueous-phase derivation using dual amalgamation CVAFS (Bloom, 1989; Horvat *et al.*, 1993). Samples from 2007 were analyzed for THg concentration at Rutgers University (New Brunswick, New Jersey), as previously described (Bloom & Crecelius, 1983) using a Tekran® CVAFS mercury detector (Tekran Instruments Corporation, Knoxville, Tennessee). Precision of THg analyses varied from 1% to 6% (RSD of replicate analyses) for diffuse flow samples (with the exception of Mk35) and from 0.4% to 7% for focused flow samples. The detection limit for THg, calculated as three times the standard deviation of the blanks, was 2.5 pmol L^{-1} for the 2004 samples and 0.35 pmol L^{-1} for the 2007 samples.

Sulfide data were obtained mostly in-situ, using an electrochemistry probe that was attached to the mechanical arms of the submarine (Luther *et al.*, 2001). In some occasions, sulfide data and pH were measured shipboard. To obtain sulfide data, samples were preserved by fixing 1 mL of the sample with 1 mL of 1 mol L^{-1} NaOH and 0.1 mol L^{-1} $Zn(CH_3COO)_2$. Samples were mixed well and stored frozen until analysis (Rozan &

Luther, 2002).

Growth medium - Medium 142 from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, www.dsmz.de), modified by the elimination of acetate, was used for culturing chemosynthetic thiosulfate oxidizing bacteria. The medium had a pH of 7.0 and was composed of: 430 mmol L^{-1} NaCl, 7.5 mmol L^{-1} $(NH_4)_2SO_4$, 6.0 mmol L^{-1} $MgSO_4 \bullet 7H_2O$, 2.8 mmol L^{-1} $CaCl_2 \bullet 2H_2O$, 8.6 mmol L^{-1} KCl, 0.5 mmol L^{-1} $NaHCO_3$, 0.3 mmol L^{-1} K_2HPO_4. Two mL of a 0.5% phenol red solution was added per L of the growth medium as a pH indicator. Following sterilization, the media was supplemented with 20 mmol L^{-1} thiosulfate ($Na_2S_2O_3$), 1.0 μmol L^{-1} of vitamin B_{12}, 1 mL of mixed vitamin solution 141 (www.dsmz.de), and 1 mL of trace element solution SL10 (www.dsmz.de). To select for Hg resistant microbes, $HgCl_2$ was added to the same medium to a final concentration of 10 μmol L^{-1}.

Modeling of Hg speciation - Because reduced sulfur affects Hg bioavailability (Benoit *et al.*, 1999; Benoit *et al.*, 2001), the speciation of Hg in both the growth medium and the *mer-lux* assay buffer (*see* below) was determined using the chemical equilibrium speciation model, MINEQL+ (version 4.5) (Schecher & McAvoy, 1994), using input parameters obtained from the MINEQL+ and the National Institute of Standards and Technology (NIST) database (Martell *et al.*, 1998). Dissociation constants used as input parameters in the modeling can be requested from the authors. Hg speciation in medium 142 was modeled with 10 μmol L^{-1} $HgCl_2$, the concentration added in order to select for Hg resistant microbes.

***mer-lux* bioassays** - To determine the bioavailability of the negatively charged Hg-thiosulfate complexes, that were formed in the growth media upon addition of 10 μmol L^{-1} $HgCl_2$, I used a *mer-lux* biosensor assay (Selifonova *et al.*, 1993). This biosensor contains a gene fusion in which the regulatory system of the mercury resistance (*mer*) operon controls the expression of bioluminescence (*lux*) from *Vibrio fischeri*. Thus, when Hg^{2+} is present inside the cell, bacterial luminescence is induced and since the response of the *mer* promoter to Hg^{2+} is quantitative, the amount of light that is emitted corresponds to the amount of bioavailable Hg^{2+}, i.e., Hg^{2+} that has been taken into the cell from the surrounding medium (Barkay et al. 1998). The assay was performed using *Escherichia coli* HMS174/pRB28 and its constitutive derivative strain HMS174/pRB27 as described previously (Barkay *et al.*, 1998), at a cell density of 10^8 mL^{-1}. The bioassay medium, containing: 5 mmol L^{-1} pyruvate, 67 mmol L^{-1} PO_4^{-3}, 34 mmol L^{-1} Na^+, 33 mmol L^{-1} K^+, and 91 μmol L^{-1} $(NH_4)_2SO_4$, at pH 7.0, was modified by the addition of thiosulfate at 1 to 10 mmol L^{-1} to simulate the Hg-thiosulfate speciation in the growth medium. The MINEQL+ model was used to examine the effect of thiosulfate addition on Hg speciation. Mercury concentration was kept at a constant of 10 nmol L^{-1} $Hg(NO_3)_2$, which is below the toxicity threshold for biosensor cells, in the MINEQL+ simulations. Light production was determined using a Beckman LS 6500 Scintillation System (Beckman Coulter, Inc. Fullerton, California) operated at the single photon count mode. The number of photons emitted per minute were converted to quanta per minute as described previously (Selifonova *et al.*, 1993).

Most probable number counts - Most probable number (MPN) counts were used to estimate the number of chemosynthetic, thiosulfate oxidizing microbes, and the proportion of Hg resistant microbes among them. The counts were carried out by setting up serial ten fold dilutions of the hydrothermal fluid samples under autotrophic conditions using medium 142, with and without the addition of 10 μmol L^{-1} $HgCl_2$. Five replicate tubes were inoculated for each dilution and incubated aerobically at 28ºC for up to ten days in the dark, without shaking. Growth was monitored by observing a change in color of the pH indicator and was confirmed by direct microscopic counts of acridine orange stained cells visualized with an Olympus BX60 microscope with an oil-immersion objective lens (UplanF1 100X/1.3). The number of cells mL^{-1} and the 95% confidence intervals were determined using an MPN index (Alexander, 1982).

Results

Total Hg in diffuse flow fluids - Mercury concentrations measured in diffuse flow hydrothermal fluid samples ranged from 13.9 to 445 pmol L^{-1}, and most of the samples were enriched relative to THg concentrations in reference seawater (Table 4.1). The sample with the highest Hg concentration, 445 pmol L^{-1}, similar to that of highly contaminated surface waters (Hines *et al*., 2000; Schaefer *et al*., 2004), was collected at the Tica site following mussel removal, in 2004. The moderate temperature of 12ºC and the sulfide concentration of 55.3 μmol L^{-1} indicated weak venting activity and highly diluted vent fluids. In 2007, another fluid sample was collected from Tica, which had a higher temperature (29ºC), and THg concentration (422±15 pmol L^{-1}) that was consistent with the previous measurement. In contrast, the East Wall sample with the highest

temperature (65ºC) had elevated, but lower, concentration of THg (75 pmol L^{-1}). Methylmercury concentrations in all 2004 samples were below the limit of detection, < 1.5 pmol L^{-1}. Methylmercury was not analyzed in samples collected in 2007.

Concentrations of THg measured in non-vent seawater samples collected at various depths in 2007, using Niskin bottles, ranged from 1.0 to 2.0 pmol L^{-1} (Table 4.1), and are consistent with previously reported Hg concentrations in the North Pacific Ocean, which ranged from 0.3 to 1.9 pmol L^{-1} (Gill & Fitzgerald, 1988; Nozaki, 2001).

Total Hg in high temperature focused flow fluids – The measurements of THg in focused flow hydrothermal fluids from the EPR at 9ºN show Hg concentrations ranging from 3.5 ±0.1 to 11.0 ± 0.8 nmol L^{-1} (Table 4.2). As expected temperatures and sulfide concentrations in focused flow samples were much higher than those of diffuse flow samples (Table 4.1) and may have been mostly due to particulate Hg as indicated by the dark color of the samples. Yet, a correlation between these factors and THg was not found. For example, the Bio vent sample with the highest THg concentration (11.0$\pm$0.8 nmol L^{-1}), had the lowest temperature (245ºC) and one of the lower sulfide concentrations (4703.9 μmol L^{-1}), while Bio9 with the highest temperature (378ºC) had the lowest THg concentration (3.5 ±0.1 nmol L^{-1}).

Table 4.1 Physical chemical parameters of diffuse flow water samples from the EPR at 9ºN

Sample site	Year of collection	T[1] (ºC)	THg (pmol L^{-1}) [2]	Sulfide (μmol L^{-1})	pH
Mk119, source above mussels	2004	12.0	15	27.4	ND[3]
Mk119, source after mussel removal	2004	13.0	40	57.2	ND
Mk 119, 1 m above mussels	2004	2.5	15	4.7	ND
Mk119, 1 m above after mussel removal	2004	2.5	35	8.1	ND
Tica, top of mussels	2004	9.5	35	13.2	ND
Tica, after removing mussels	2004	12.0	445	55.3	ND
MkB, top of mussels	2004	ND	170	ND	ND
East Wall, 04/04/B source	2004	65.0	75	157.6	ND
Tica, Mk4	2007	29.0	422 ± 15	ND	7.1
Mk16	2007	2.0	31	NA[4]	7.8
Mk15	2007	21.0	118 ± 2	204.0	6.9
MkF	2007	28.0	63.7 ± 3	398.2	6.7
Mk35	2007	26.0	13.9 ± 7	118.1	7.0
Mk28	2007	36.0	195.7 ± 12	160.1	6.7
Mk27	2007	20.0	244.8 ± 5	167.2	7.0
Fish hole	2007	2.0	12	NA	7.4
Water column (depth 60 m) [5]	2007	2.6	1.2 ± 0.05	NA	ND
Bottom seawater (depth 2400 m) [5]	2007	1.8	1.8 ± 0.02	NA	ND
Bottom seawater (depth 2400 m) [5]	2007	1.8	1.4± 0.05	NA	ND

[1] T= Temperature
[2] When available, means and ranges of duplicate analyses are provided
[3] ND = not determined
[4] NA = not applicable, when T = 2ºC (ambient temperature), sulfide concentration is expected to be zero.
[5] Seawater samples that were used as references

Table 4.2 Physical chemical parameters of focused flow fluid samples collected in 2007 on the EPR at 9ºN

Sample Description	Temperature (ºC)	THg (nmol L^{-1})[1]	Sulfide (μmol L^{-1})	pH
Bio9	378	3.5 ± 0.1	8070.1	3.0
Alvinella Pillar	369	5.0 ± 0.0	11458.9	2.9
P vent	333	5.1 ± 0.2	4296.6	3.8
Bio vent	245	11.0 ± 0.8	4703.9	3.3

[1]Means and ranges of duplicate analyses

The interactions of chemosynthetic vent bacteria with Hg – The observation that THg concentrations are elevated in hydrothermal fluids from diffuse flow vents raised the question of Hg toxicity to chemosynthetic microbes, the primary producers in the vent environment. In Hg contaminated surface waters, an increased proportion of Hg resistant counts relative to control surface water indicates that microorganisms are adapted to life in the presence of Hg (Barkay *et al*., 2003). I therefore estimated the proportion of Hg resistant chemosynthetic sulfur oxidizing bacteria in diffuse flow vent waters with varying concentrations of THg.

To accurately assess Hg resistance among vent sulfur-oxidizing bacteria, I first tested if the Hg added to medium 142 was bioavailable to chemosynthetic prokaryotes. This step was necessary as Hg readily forms ligands with media constituents and these interactions control its bioavailability and thus toxicity (Farrell *et al*., 1990). It has been previously shown that metal toxicity to hyperthermopholic sulfur reducing *Archaea* was mitigated by sulfides (Edgcomb *et al*., 2004) and because this study focused on thiosulfate as a sole energy source, the known affinity of Hg to sulfur (Varekamp and Buseck 1984), necessitated (i) a delineation of the speciation of Hg in the growth medium

and (ii) an assessment of how this speciation affected bioavailability relative to speciation in the absence of thiosulfate. I achieved this goal by combining the determination of Hg speciation in the growth medium using MINEQL+ with in vivo determinations of Hg bioavailability using our *mer-lux* biosensor system (Barkay et al. 1998). Thus, I first determined Hg speciation in the growth medium, then modified the biosensor assay buffer by the addition of thiosulfate to simulate the same molar ratio of Hg complexes as present in the growth medium, and finally used the biosensor to determine the bioavailability of these complexes. This approach verified that the Hg added to the growth medium was bioavailable under the experimental conditions and was useful in comparing resistance levels among various diffuse flow vent samples.

Mercury speciation in growth medium - MINEQL+ modeling results (dissociation constants used are available upon request) showed that when 10 μmol L^{-1} $HgCl_2$ was added to medium 142, which contained 20 mmol L^{-1} thiosulfate, all Hg(II) was present as Hg-thiosulfate complexes, with the -2 charged dithiosulfate species representing 60% of the Hg[II], and the -4 charged species trithiosulfate complex making up the remaining 40% (Figure 4.1).

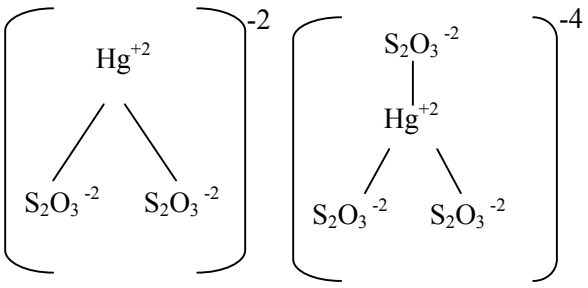

Figure 4.1 The two dominating forms of Hg(II) as identified by MINEQL+ in Medium 142 (thiosulfate at 20 mmol L^{-1} and $HgCl_2$ at 10 μmol L^{-1}), and in the *mer-lux* bioassay medium (thiosulfate at 2 mmol L^{-1} and $Hg[NO_3]_2$ at 10 nmol L^{-1}). Hg-dithiosulfate ($Hg[S_2O_3]_2^{-2}$) represented 60%, while Hg-trithiosulfate ($Hg[S_2O_3]_3^{-4}$) made up the remaining 40% (a list of association coefficients is available upon request from the authors).

Bioavailability of Hg-thiosulfate complexes - The *mer-lux* bioassay (Barkay *et al*., 1998) was used to determine how the complexation of Hg(II) with thiosulfate in the microbial growth medium affected Hg bioavailability. The speciation of Hg(II) in the bioassay buffer was adjusted to match the ratio of di- and trithiosulfate Hg complexes, i.e., 6 to 4, respectively, in the growth medium. MINEQL+ modeling of the bioassay medium (association coefficients are available upon request) showed that, as the thiosulfate concentration increased, so did the negative charge of the Hg(II)-thiosulfate complexes (Table 4.3). At 2 mmol L^{-1} thiosulfate and 10 nmol L^{-1} $Hg(NO_3)_2$, the ratio of di- to trithiosulfate Hg(II) complexes in the assay buffer was the same as that in the growth medium. When thiosulfate was not added to the assay buffer, 99.3% of the Hg was present as $Hg(NH_3)_2^{+2}$. This form is not expected to affect Hg(II) bioavailability relative to that of $Hg(NO_3)_2$ (Golding *et al*., 2002).

Table 4.3 Results of MINEQL+ modeling for the effect of thiosulfate ($[S_2O_3]_2^{-2}$) on Hg(II) speciation in the *mer-lux* biosensor assay medium[1].

Hg(II) speciation in assay medium	$(S_2O_3)_2^{-2}$ concentration in *mer-lux* assay media (mmol L^{-1})			
	1	2	3	10
% $Hg(S_2O_3)_2^{-2}$	75	59.9	50	25
% $Hg(S_2O_3)_2^{-4}$	25	40.1	50	75

[1]Medium contained 10 nmol L^{-1} $Hg(NO_3)_2$

Biosensor assays showed a decrease in *mer-lux* expression with increasing thiosulfate concentrations, suggesting that the formation of Hg-thiosulfate complexes at thiosulfate concentrations higher than 1mmol L^{-1} decreased the bioavailability of Hg(II) (Figure 4.2A). When the data were used to calculate expression factors, i.e., the slope of the exponential portions of the curves describing light emission over time (Barkay et al., 1998), a decline of 55% was observed at 2 mmol L^{-1} thiosulate, as compared to the control without thiosulfate (Figure 4.2B). Assays performed with the *lux* constitutive strain *E. coli* HMS174/pRB27 (Barkay *et al*., 1998) showed no effect of increasing thiosulfate concentrations on biosensor performance (data not shown), indicating that the effect of thiosulfate on the response of strain HMS174/pRB28 was due to a decline in the Hg(II)-dependent induction of *lux* expression.

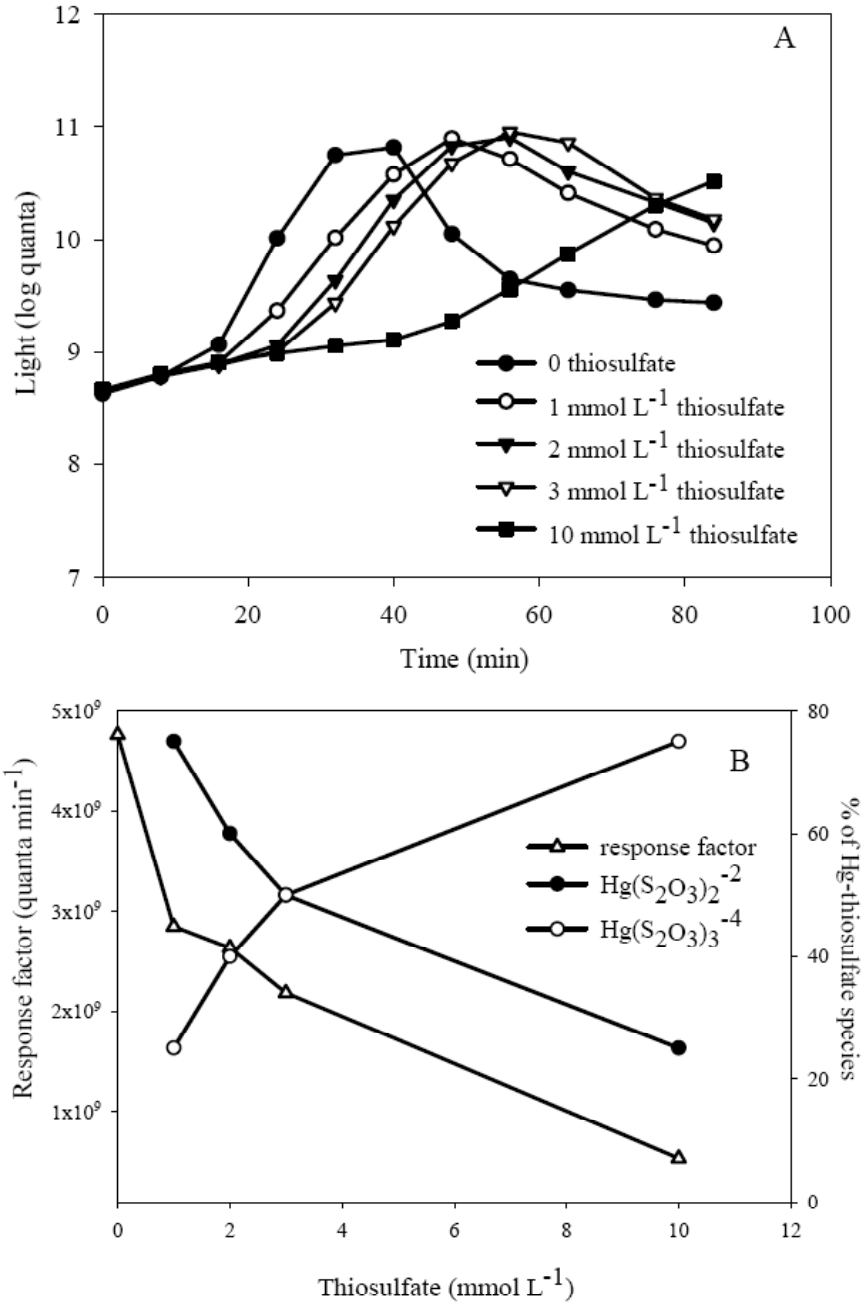

Figure 4.2 The effect of thiosulfate on Hg(II) bioavailability as indicated by the *mer-lux* biosensor. (A) Light induction with time in biosensor assays containing 10 nmol L^{-1} $Hg(NO_3)_2$ and increasing concentrations (in nmol L^{-1}) of thiosulfate. (B) Effect of Hg-thiosulfate speciation on Hg(II) availability as revealed by the relationship of the response factor (the maximal rate of light increase as calculated from the slopes of curves presented in [A]) and the proportion of the two major Hg-thiosulfate complexes as calculated by MINEQL+.

Abundance of Hg resistant chemosynthetic microbes in diffuse flow vents - The MPN estimates showed that the number of chemosynthetic thiosulfate oxidizing bacteria in our samples ranged from 1.1 x 10^3 (95% confidence interval: 3.3 x 10^2 - 3.6 x 10^3) to 2.4 x 10^7 (95% confidence interval: 7.3 x 10^6 - 7.9 x 10^7) cells mL^{-1} (Figure 4.3). These values are consistent, though higher, than those reported in shallow-water hydrothermal vents where the number of chemosynthetic bacteria ranged from 10^3 to 10^6 cells mL^{-1} (Brinkhoff *et al*., 1999). The Hg supplemented MPN estimates were, in general, at least an order of magnitude lower than those without Hg, at the 10^2 to 10^4 cells mL^{-1} range (Figure 4.3). Together, MPN estimates showed that 0.2 to 24.6% of the chemosynthetic microorganisms were Hg resistant. The samples that contained the highest THg concentrations (Tica, at 445 pmol L^{-1} and 425$\pm$15 pmol L^{-1}, for 2004 and 2007, respectively) also contained the highest percentage of resistant cells (13.9% and 24.6%, respectively) as compared to samples with lower THg concentrations, such as Mk35 (15 pmol L^{-1}), which contained only 0.2% resistant cells (Figure 4.3).

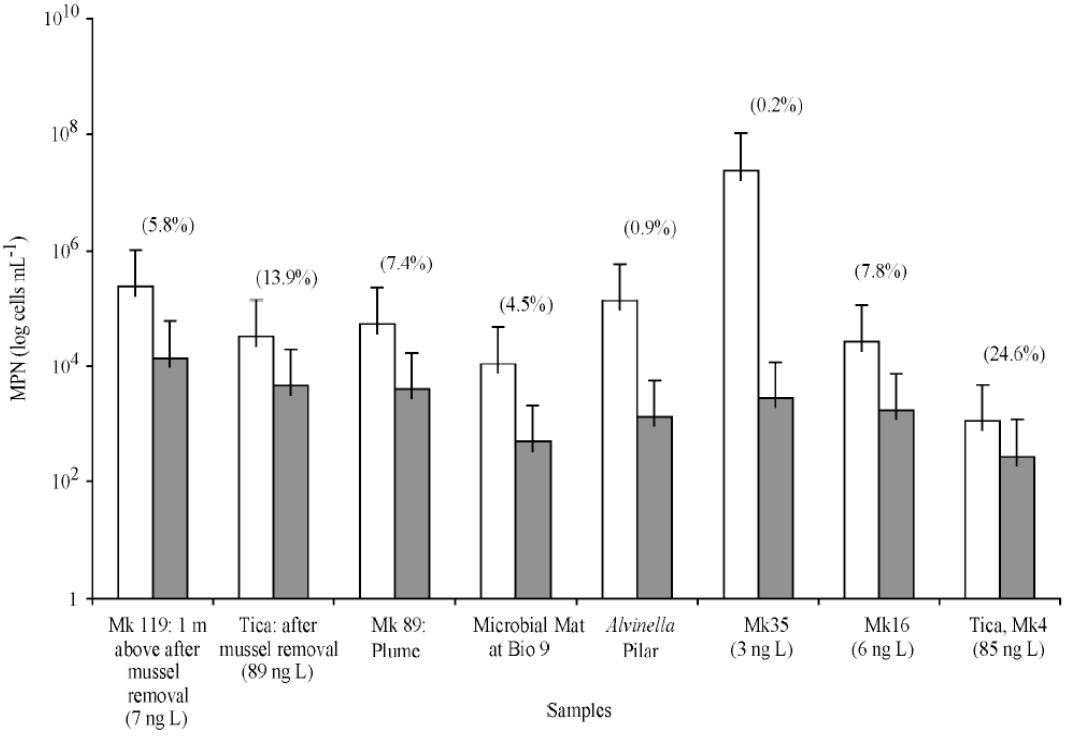

Figure 4.3 MPN estimates of total and Hg resistant chemosynthetic thiosulfate oxidizing microorganisms. Estimations were performed in the presence and absence of 10 μmol $HgCl_2$ L^{-1}, which was present in the medium in di- and tri-thiosulfate complexes (*see* Fig. 1). White and gray bars present MPN estimates of total and Hg resistant chemosynthetic thiosulfate oxidizing microorganisms, respectively. Error bars indicate 95% confidence intervals. Percent chemosynthetic microorganisms that were resistant to Hg are provided above the bars. THg concentrations in pmol L^{-1} are presented when available.

Discussion

Hg emissions from deep-sea vents have two important implications. First, vent emissions may impact the marine Hg mass balance (Lamborg et al., 2006), and second, the toxicity of Hg may impact the biota in the vent's unique ecosystem. Our data showing elevated THg concentrations in diffuse and focus flow fluids along with the response of the vent microbiota to Hg toxicity suggest that increased levels of resistance are likely related to the high input of Hg to the vent ecosystem.

This study revealed, for the first time, that diffuse flow hydrothermal vent fluids contained elevated THg concentrations spanning a broad range of concentrations, from 12 to 445 pmol L^{-1} (Table 4.1). Analyses of all the data showed a positive relationship between temperature and sulfide concentration, as expected, yet these parameters were not positively related to THg concentrations. Currently, it is not clear how the sub seafloor circulation of hydrothermal fluids, and their mixing with seawater, affects the behavior of Hg in diffuse flow vents. However, due to the high sulfide concentrations in the fluids, it is likely that most of the Hg precipitates upon contact with oxygenated seawater (Varekamp and Buseck 1984). In addition, interpretations of chemical data in diffuse flow vents is complicated by the mixing of the hydrothermal fluids with seawater, changes in fluid flux, and frequent fluctuations in currents (Johnson *et al.*, 1988; Le Bris *et al.*, 2006; Luther *et al.*, in press), preventing the establishment of unequivocal correlations among temperature and the concentrations of Hg and sulfide. Furthermore, a recent seafloor eruption (2005-06) altered the distribution of the vent sites along the ridge crest at 9°N, and prevented us from replicating the 2004 sampling scheme in 2007. However, samples collected at Tica, with the highest THg concentrations (Table 4.1), were in the same range of concentrations in 2004 and 2007, 445 and 422±15 pmol L^{-1}, respectively. Clearly, more research is needed to gain an understanding of Hg mobility and toxicity in diffuse flow vents.

The THg concentrations reported here for focused flow fluids, ranging from 3.5 ± 0.1 to 11.0 ± 0.8 nmol L^{-1} (Table 4.2), are on average thirty-fold higher than those measured in diffuse flow fluids (Table 4.1), and are the highest that have been reported to date for any vent fluids. A recent report showed that the average THg concentration in

focused flow vent fluids from the Gorda Ridge was much lower, < 15 to 10 pmol L^{-1} (Lamborg *et al.*, 2006) than the nmol L^{-1} concentrations I found in the fluids from the EPR. The difference may be due to the fact that my samples, which were dark in color, likely included a high concentration of precipitated metals while Lamborg et al (2006) reported analyzing clear samples likely depleted of precipitated metals. One should note that the samples that were analyzed in the later study had been stored for an extended period of time at 4 ºC prior to analysis. Other data on focused flow vents from both the EPR and the Juan de Fuca Ridge showed concentrations of THg as high as 1.7 nmol L^{-1} (Ekstrom, E., pers. comm.), which is 2 to 7 fold lower than our measurements.

Using the approach of Lamborg et al. (2006) and a THg concentration of 1 nmol L^{-1}, which is within the range of concentrations reported here and by E. Ekstrom for the EPR, I calculated an input of Hg to the deep ocean of 10 Mmol yr^{-1}. This value is several fold higher than the estimated yearly input of Hg to the deep ocean, 2.4 Mmol yr^{-1} (Mason & Sheu, 2002), and thus unrealistic, suggesting that most of the geothermal THg is precipitated, likely as mercury sulfide, upon mixing with cold oxygenated sea-water in the vicinity of the vents. Hence, questions regarding the stability of these Hg deposits and the processes that may mobilize them into the oceanic water column come to the fore.

My observation of high tolerance to Hg among chemosynthetic microorganisms (Figure 4.3) suggests adaptation to Hg and a possible role for these microbes in Hg mobilization from solid metal sulfides. Previous observations that suggested that toxic metals such as copper and cadmium were released during the oxidative solubilization of reduced sulfur in near surface deep-sea sediments (Severmann *et al.*, 2006) point to the

possibility that chemosynthetic microbes that attach to the surface of metal sulfides and obtain energy from the oxidation of solid phase sulfur, and possibly iron (Edwards et al. 2005; (Eberhard *et al*., 1995), may release Hg during the oxidation of Hg-sulfide deposits. Deep-sea solid deposits contain $\mu g\ g^{-1}$ concentrations of total Hg (unpublished results and Lamborg et al. 2006). Demonstrations that pure bacterial cultures reduce (Baldi & Olson, 1987) and methylate (Benoit et al. 2001) Hg from cinnabar suggest that solid phase Hg may be bioavailable. Thus, activities of chemosynthetic prokaryotes may mobilize the solid phase Hg present in cinnabar as ionic Hg, a highly bioavailable form, which subsequently may enter further biogeochemical cycling. This consideration and the demonstration of elevated Hg resistance among vent bacteria (Figure 4.3 and Vetriani et al. 2005) brings up the scenario of Hg cycling following the emission and initial deposition of Hg with metal sulfides. According to this scenario, chemosynthetic microbes that oxidize metal sulfides release soluble ionic Hg, which is then reduced to its elemental form by microorganisms that express the *mer* system, a detoxifying mechanism that could be critical for the settlement of metazoan larvae and for the subsequent colonization of deep-sea vents.

While sulfur oxidizing bacteria can use different forms of reduced sulfur (hydrogen sulfide, sulfite, sulfur and thiosulfate), for the purpose of this study I used thiosulfate because of the ease of preparing microbial growth media with this reduced sulfur species, relative to sulfide or elemental sulfur. It should be noted that thiosulfate or its polysulfide precursors have not been consistently detected in fluids from the EPR (Luther *et al*., 2001), while they were readily detected in other deep-sea vents (Mullaugh *et al*., in press). Since the detection limit for thiosulfate using our in-situ system is 30

μmol L^{-1}, this sulfur species could go undetected if present at lower concentrations. Furthermore, if the thiosulfate is complexed to metals, its peak would shift to positive voltage and it would be out of the working voltage range for our system.

Previous work in deep-sea hydrothermal vents showed that the incidence of Hg resistant heterotrophic bacteria was directly related to their proximity to the vent source (Vetriani *et al*., 2005). The work reported here expands this observation to autotrophic thiosulfate oxidizers. A correlation between the percentage of Hg resistant MPN estimates and the THg concentration in the corresponding diffuse flow fluids suggests that vent chemosynthetic microorganisms are adapted to life in the presence of Hg. However, it is not clear how this resistance affects microbial life in-situ. Sulfide concentrations in the samples that were analyzed for THg, showing a million-fold excess of sulfide over THg concentrations (μmol vs. pmol L^{-1}, Table 1), suggest that complexation with sulfide, known to limit Hg bioavailability (Benoit, et al. 2001) and metal toxicity (Edgcomb *et al*., 2004), likely dominated Hg speciation in-situ. In addition to sulfide, Hg speciation and bioavailability is modulated by interactions with DOC (Barkay et al. 1997; Ravichandran, 2004) and iron (Rytuba, 2000). Measurements of these factors are needed for the evaluation of the impact of the emitted Hg on deep-sea vent biota. Nevertheless, the presence of Hg resistant bacteria in vent fluids (see above and Vetriani et al., 2005) strongly, though indirectly, suggests that Hg is in fact toxic to the vent microbiota in-situ.

The approach used here to assess the level of Hg resistance included the determination of the dominant Hg chemical form in the growth medium and its bioavailaibilty relative to neutral and positively charged forms (Table 4.3 and Figure 4.2).

This analysis showed that the bioavailability of Hg(II)-thiosulfate complexes in medium 142 was reduced by 55% relative to the $Hg(NH_3)_2^{+2}$, the dominating Hg species in medium 142 in the absence of thiosulfate. Hence, the level of toxicity expected in the thiosulfate and 10 μmol L^{-1} Hg(II) is similar to that of 4.5 μmol L^{-1} Hg(II) added to medium 142 without thiosulfate. There is little doubt that growth in the presence of μmol L^{-1} concentrations of Hg under aerobic conditions is only possible for resistant microorganisms (Barkay et al. 2003), even if the issue of Hg bioavailability in growth media is a complicated one (Farrell *et al.*, 1990). Results showed that Hg bioavailability decreased as the proportion of the negatively charged Hg-trithiosulfate increased (Figure 4.2B). It is likely that the negative charge of this Hg-thiosulfate complex reduced its bioavailability. Previous observations suggest that negatively charged chloride (Barkay *et al.*, 1997) or sulfide (Benoit *et al.*, 1999) complexes of Hg(II) have a reduced bioavailability relative to neutrally charged complexes. The mechanism by which negative charge reduces bioavailability is not known. However, the apparent higher bioavailability of the –2 charged dithiosulfate, as compared to the –4 charged trithiosulfate, may result from a higher rate of complex dissociation of the former within the cell's diffusive boundary layer, which could enhance transport of Hg into the cell (Hudson, 1998) by either diffusion of uncharged forms, e.g., Hg-monothiosulfate, or of various charged complexes through sulfate and thiosulfate transporters as was shown for the transport of silver thiosulfate complexes in algae (Fortin and Campbell, 2001).

In summary, this study shows that THg concentrations are elevated in focused and diffuse flow hydrothermal vent fluids and that Hg resistant chemosynthetic microorganisms are enriched in these fluids. The major implications of these finding are:

(i) Hydrothermal vent emissions may contain a wide range of THg concentrations suggesting variable sources. Additional measurements of Hg in its various chemical forms in high temperature hydrothermal fluids from a range of different vent systems are needed to facilitate an assessment of the contribution of hydrothermal emissions to the input and biogeochemistry of Hg to the deep ocean. (ii) In the vent ecosystem, microorganisms may play a role in the mobilization of Hg from Hg sulfide deposits, which are likely the most abundant forms of Hg at hydrothermal vents (Dopson *et al.*, 2003; Varekamp & R, 1984), and subsequently in the detoxification of the released ionic Hg. The ability of vent microorganisms to transform aqueous and solid phase Hg–sulfur complexes may be critical to the mobility, cycling, and toxicity of Hg in vent ecosystems.

Chapter 5 – Conclusion

Deep-sea hydrothermal vents have been defined as the sites of most rapid biomass production on Earth (Lutz, 1994). The energy required for chemosynthetic primary production is derived from the steep chemical, temperature, and pH gradients that form upon mixing of highly reduced hydrothermal fluids with cold oxygenated seawater (McCollom & Shock, 1997). Hydrothermal fluids are highly enriched in reduced sulfur compounds, such as H_2S (Corliss *et al*., 1979), and the oxidation of such compounds under aerobic conditions (O_2 as terminal electron acceptor) is a highly efficient process, resulting in a high energy yield (Edmond *et al*., 1979; McCollom & Shock, 1997; McCollom, 2000). This, and the frequent isolation of sulfur oxidizing bacteria from hydrothermal fluids, suggests that aerobic oxidation of reduced sulfur compounds is the primary chemosynthetic process in vent ecosystem.

The main objective of this thesis dissertation was to gain a better understanding of the aerobic chemosynthetic processes at deep-sea hydrothermal vents. Specifically I was interested in studying the abundance, diversity, and metabolic versatility of thiosulfate oxidizing chemosynthetic bacteria and their role in mercury detoxification in hydrothermal vent environments.

I used most probable number (MPN) counts to estimate the number of chemosynthetic thiosulfate oxidizing bacteria per mL of samples. According to my results, the abundance of this group of organisms is estimated to range between 1.1×10^3 to 2.4×10^7 cells mL^{-1}. These values are consistent, but higher, than those previously reported from shallow-water hydrothermal vents in which the number of chemosynthetic bacteria ranged from 10^3 to 10^6 cells mL^{-1} (Brinkhoff *et al*., 1999). I compared these

values with the total number of cells per mL of samples, counted microscopically after acridine orange staining, and estimated that the chemosynthetic thiosulfate oxidizing community represents approximately from 0.002 to 14.1% of the total cells counted microscopically, which ranged from 10^7 to 10^8 cells mL^{-1}.

Enrichment of chemosynthetic thiosulfate oxidizing bacteria from undiluted, as well as from serially diluted samples resulted in the isolation of fifty-three organisms that belonged to different genera within the "*alpha"*- and "*gammaproteobacteria"*. The isolates were closely related to *Thiomicrospira crunogena* (18 isolates)*, Thiomicrospira thermophila* (7 isolates), *Halothiobacillus hydrothermalis* (7 isolates), *Hydrogenovibrio marinus* (4 isolates), *Thioclava pacifica* (11 isolates), *Pelagibaca bermudensis* (2 isolates), and *Thalassospira* sp. (1 isolate). During the isolation process I obtained three isolates, EPR70, EPR71, and EPR72, which shared only 94% sequence similarity to their closest described relative, *Salinisphaera shabanensis* (Antunes *et al*., 2003). I characterized EPR70 in depth and described it as a new species, *Salinisphaera hydrothermalis,* a mesophilic, halotolerant, facultatively autotrophic organism (Crespo-Medina *et al*., In press-b, and Chapter 4 of this thesis).

The abundance of "*alpha"*- and "*gammaproteobacteria"*, specifically the isolation of several representatives of the genus *Thiomicrospira*, is consistent with previous studies of the microbial diversity of hydrothermal vents (Jannasch *et al*., 1985; Ruby *et al*., 1981). Representatives of this genus are very abundant, growing up to 10^5 dilution, and in some cases can outgrow other organisms in primary enrichments. The same is true for representatives of the genus *Hydrogenovibrio*, which were isolated both

from primary enrichments and form 10^4 dilutions. These findings suggest the importance of these genera (*Thiomicrospira* and *Hydrogenovibrio*) at this ecosystem.

In order to further understand the metabolism of these organisms, I tested their ability to grow in different substrates. All isolates grew in defined medium where CO_2 had been replaced with 10 mmol L^{-1} acetate, revealing their ability to grow heterotrophically with a two-carbon organic compounds. However, only *Thioclava* sp. EPR65, *Thioclava* sp. EPR74, *Halothiobacillus* sp. EPR129, *Pelagibaca* sp. EPR125, *Thalassospira* sp. EPR62, *Salinisphaera hydrothermalis* EPR70, and *Salinisphaera* sp. EPR71 were able to grow in complex medium, suggesting that the high carbon concentration in the complex medium might inhibit the growth of most of these organisms. Previously *Thiomicrospira crunogena* had been described as a strict autotroph (Jannasch *et al*., 1985), and this is, the first study that demonstrates the ability of isolates closely related to *T. crunogena* to use acetate as a carbon source. Furthermore, I demonstrated that *Pelagibaca* sp. EPR125 and *Salinisphaera hydrothermalis* EPR70, closely related to the chemoheterotrophic marine bacteria *Pelagibaca bermudensis* and *Salinisphaera shabanensis*, respectively, can grow autotrophically and encode for the RubisCO gene, suggesting that they use the Calvin-Benson- Bassaham cycle for CO_2 fixation.

Only *Salinisphaera hydrothermalis* strain EPR70^T, *Salinisphaera* sp. EPR71 and *Salinisphaera* sp. EPR72 were able to grow on *n*-alkanes as only carbon source, and the sequence of their *alkB* gene, encoding the enzyme alkane hydroxylase was determined. The description of these new isolates and the detailed characterization of EPR70, revealed new characteristics of the genus *Salinisphaera*, such as the ability to oxidize

hydrocarbons and the amplification and phylogenetic analysis of the *alkB* gene (encoding alkane hydroxylase enzyme), and to grow autotrophically (and the amplification and phylogenetic analysis of the genes encoding the RubisCO enzyme). These findings led to a taxonomic revision that resulted in the emended description of the genus *Salinisphaera*.

The isolates that were closely related to *Thiomicrospira crunogena* and *Thiomicrospira thermophila* were able to grow under microaerobic conditions in defined medium under a CO_2/O_2 (95:5) atmosphere. The physiological and metabolic versatility described for these sulfur oxidizing organisms not only represents an advantage to thrive in different microenvironments within the hydrothermal vent ecosystems, but it might be the key to explain the level of chemosynthetic primary production at hydrothermal vents. The efficiency of this process is what supports the highly dense biological communities at hydrothermal vents.

All the isolates fix CO_2 using the Calvin-Benson-Bassaham Cycle (CBB). This was suggested by the amplification of their RubisCO enzyme, key enzyme in the CBB cycle. There are two main form of this enzyme, Form I, which is encoded by the genes *rbcL*/*cbbL* and Form II, encoded by the gene *cbbM*. Some of the isolates had both forms of the enzymes, while others had only Form I RubisCO. In the case of isolate EPR125, which is closely related to *Pelagibaca bermudensis*, only Form II RubisCO was amplified.

Phylogenetic analyses were carried out using the deduced amino acid sequence of the genes encoding Form I and Form II RubisCO. The phylogeny of the Form I RubisCO showed three main clusters. One cluster included the sequences of *Salinisphaera* related isolates, the second cluster included the sequence of *Thioclava* related isolates, and the

third cluster included the sequences of the *Thiomicrospira*, *Hydrogenovibrio*, and *Halothiobacillus* related isolates. The position of *Halothiobacillus* related sequences within the *Thiomicrospira* cluster is not consistent with the position of this isolates based on 16S rRNA phylogeny. Similarly, the phylogeny of the Form II RubisCO shows the sequence of an "*alphaproteobacterium"* (*Pelagibaca* sp. EPR125) clustering together with the sequences of several "*gammaproteobacteria*". These inconsistencies between the RubisCO phylogeny and the 16S rRNA phylogeny might be indications of horizontal gene transfer (HGT) events.

The gene encoding the SoxB enzyme, a sulfate thiol esterase essential for thiosulfate oxidation, was amplified from representatives of most of the isolates. The partial sequence of the gene was selectively amplified and the deduced amino acid sequence was used to carry out phylogenetic analyses. SoxB phylogenetic analysis also showed some inconsistencies when compared to the 16S rRNA phylogeny of the isolates. The sequence of an "*alphaproteobacterium"* (*Pelagibaca* sp. EPR125) clustered together with the sequences of "*gammaproteobacteria*", while the sequence of a "*gammaproteobacterium*" (*Halothiobacillus* sp. EPR155) clustered within the group of "*alphaproteobacteria*". These phylogenetic differences might be explained as possible gene duplications or horizontal gene transfer events. HGT has been widely documented for RubisCO enzyme (Delwiche & Palmer, 1996) and, more recently, it has been suggested for the SoxB enzyme phylogeny (Petri *et al*., 2001; Scott *et al*., 2006)

This study also provides insights into the role of this group of organisms in heavy metal detoxification at hydrothermal vents. Since mercury (Hg) in geothermal environments exists mostly as HgS complexes (Varekamp & R, 1984), and the oxidation

of reduced sulfur compounds is considered the most efficient energy-generating process at hydrothermal vents (McCollom & Shock, 1997), I hypothesized that the activities of thiosulfate-oxidizing bacteria may be critical in the mobilization of Hg. Therefore, I decided to use mercury (Hg) as a model to investigate the interactions between thiosulfate oxidizing bacteria and Hg, and their possible detoxification activities, at hydrothermal vents. Previous studies demonstrated elevated total mercury (THg) concentrations at focused flow vents, but this is the first study that reports THg concentrations from diffuse flow hydrothermal fluids. The THg concentrations at various diffuse flow fluids ranged between 12 to 445 pmol L^{-1}, which were highly elevated in comparison to bottom seawater samples (THg = 1.8 pmol L^{-1}). There was no obvious correlation between temperature, sulfide concentrations, and THg concentrations in the fluid samples, which indicates the highly variability of diffuse flow fluid emissions. However, the THg concentrations at focused flow fluids were higher that the concentrations measured in diffuse flow fluids, ranging from 3.5 to 11.0 nmol L^{-1}. These results implicate that hydrothermal vent emissions are enriched in mercury, and that these emissions are highly variable. Our observations raise the question of how relevant is the input of Hg generated at deep-sea vents to the global ocean mercury cycle.

With the goal of investigating the role of chemosynthetic microorganisms in the detoxification of Hg in hydrothermal fluids, I used MPN counts to estimate the percentage of thiosulfate oxidizing organisms that were resistant to mercury. To do this I supplemented medium 142-A (designed to enriched for chemosynthetic thiousulfate oxidizing bacteria) with 10 μmol L^{-1} $HgCl_2$. To establish what Hg-thiosulfate complex was available to the microorganisms tested, I used chemical speciation modeling and a

mer-lux biosensor strain. As a result I determined that all the Hg in the growth medium was complexed with thiosulfate, forming two negatively charged complexes, and that these complexes were bioavailable, although in a reduced way, to the biosensor strain. The number of Hg-resistant chemosynthetic thiosulfate oxidizing organisms ranged from 10^2 to 10^4, which is from 0.2 to 24.6% of the total chemosynthetic thiosulfate oxidizers per sample. As expected, the samples that contained the highest number of resistant organisms were also the samples that contained highest THg concentration, suggesting a possible adaptation of the microbial communities to elevated Hg concentrations.

Taken together, the data from this study provided insights into the abundance, diversity, and versatility of aerobic chemosynthetic thiosulfate oxidizing bacteria form deep-sea hydrothermal vents. The data presented in this thesis also provided insights into the ecological importance of this group of bacteria, and specifically into their possible role in the detoxification of heavy metals in the deep-sea vent environment. This work suggests that the basic metabolic activities of these microorganisms are critical for the establishment of the vent ecosystem, not only in terms of chemosynthetic primary production, but also because of their possible role in the reduction and mobilization of mercury - and possibly of other heavy metals - which are enriched in hydrothermal fluids.

The further characterization of some of the isolates, specifically the ones related to *T. crunogena*, will help elucidate the differences between the isolates obtained in this study and the ones previously described. Further studies that can be done to establish, more clearly, the role of these organisms in the environment, includes the study of the distribution and occurance of the genes encoding the RubisCO enzyme from the *Thiomicrospira* group at different diffuse flow hydrothermal vent sites. This will

establish the importance of the genus in the organic carbon production at hydrothermal vents. To study the specific role of the chemosynthetic isolates on the detoxification of mercury in the environments, studies such as mercury volatilization assays can be done to determine if the isolates are able to volatilize mercury from ionic mercury to elemental mercury. Another experiment that could be done to determine the role of the isolates in mobilization of mercury is to test their growth in the presence of cinnabar (HgS). The analysis of mercury concentration left in the medium after bacterial growth might suggest the role of the organisms in moblization of mercury from HgS to Hg^{2+}. The stablishment of the specific role of the chemosynthetic isolates in the detoxification of mercury at hydrothermal vents is important in order to determine the contribution of these organisms in the mercury cylce in the oceans.

References

Alexander, M. (1982). Most probable number methods for microbial populations. In *Methods of soil analysis, Part2: Chemical and Microbiological Properties*, pp. 815-820. Madison, Wisconsin: American Society of Agronomy, Inc., Soil Science Society of America, Inc.

Ando, T., Yamamoto, M., Tommiyasu, T., Hashimoto, J., Miura, T., Nakano, A. & Akiba, S. (2002). Bioaccumulation of mercury in a vestimentiferan worm living in Kagoshima Bay, Japan. *Chemosphere* **49**, 447-484.

Antunes, A., Eder, W., Fareleira, P., Santos, H. & Huber, R. (2003). *Salinisphaera shabanensis* gen. nov., sp nov., a novel, moderately halophilic bacterium from the brine-seawater interface of the Shaban Deep, Red Sea. *Extremophiles* **7**, 29-34.

Arp, A. J. & Childress, J. J. (1981). Blood Function in the Hydrothermal Vent Vestimentiferan tube Worm. *Science* **213**, 342-343.

Baldi, F. & Olson, G. (1987). Effects of cinnabar on pyrite oxidation by *Thiobacillus ferrooxidans* and cinnabar mobilization by a mercury-resistant strain. *Appl Environ Microbiol* **53**, 772-776.

Barkay, T., Gillman, M. & Turner, R. R. (1997). Effects of dissolved organic carbon and salinity on bioavailability of mercury. *Appl Environ Microb* **63**, 4267-4271.

Barkay, T., Turner, R. R., Rasmussen, L. D., Kelly, C. A. & Rudd, J. W. (1998). Luminescence facilitated detection of bioavailable mercury in natural waters. *Methods Mol Biol* **102**, 231-246.

Barkay, T., Miller, S. M. & Summers, A. O. (2003). Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiol Rev* **27**, 355-384.

Benoit, J. M., Gilmour, C. C., Mason, R. P. & Heyes, A. (1999). Sulfide controls on mercury speciation and bioavailability to methylating bacteria in sediment pore waters. *Environ Sci Technol* **33**, 951-957.

Benoit, J. M., Gilmour, C. C. & Mason, R. P. (2001). The influence of sulfide on solid-phase mercury bioavailability for methylation by pure cultures of *Desulfobulbus propionicus* (1pr3). *Environ Sci Technol* **35**, 127-132.

Berg, I. A., Kockelkorn, D., Buckel, W. & Fuchs, G. (2007). A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in archaea. *Science* **318**, 1782-1786.

Bloom, N. (1989). Determination of picogram levels of methylmercury by aqueous phase ethylation, followed by cryogenic gas chromatography with cold vapour atomic fluorescence detection. *Can J Fish Aq Sci* **46**, 1131-1140.

Bloom, N. S. & Crecelius, E. A. (1983). Determination of mercury in seawater at subnanogram per liter levels. *Mar Chem* **14**, 49-59.

Bloom, N. S. & Fitzgerald, W. F. (1988). Determination of volatile mercury species at the picogram level by low-temperature gas chromatography with cold vapour atomic fluorescence detection. *Anal Chim Acta* **208**, 151-161.

Bostrom, K. & Fisher, C. R. (1969). Distribution of mercury in East Pacific sediments. *Geochim Cosmochim Acta* **33**, 743-745.

Brault, M., Simoneit, B. R. T., Marty, J. C. & Saliot, A. (1988). Hydrocarbons in waters and particulate material from hydrothermal environments at the East Pacific Rise, 13°N. *Org Geocem* **12**, 209-219.

Brinkhoff, T., Sievert, S. M., Kuever, J. & Muyzer, G. (1999). Distribution and diversity of sulfur-oxidizing *Thiomicrospira* spp. at a shallow-water hydrothermal vent in the Aegean Sea (Milos, Greece). *Appl Environ Microbiol* **65**, 3843-3849.

Campbell, B. J., Stein, J. L. & Cary, S. C. (2003). Evidence of chemolithoautotrophy in the bacterial community associated with *Alvinella pompejana*, a hydrothermal vent polychaete. *Appl Environ Microbiol* **69**, 5070-5078.

Cavanaugh, C. M., Gardiner, S. L., Jones, M. L., Jannasch, H. W. & Waterbury, J. B. (1981). Prokaryotic Cells in the Hydrothermal Vent Tube Worm *Riftia pachyptila* Jones: Possible Chemoautotrophic Symbionts. *Science* **213**, 340-341.

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

Cox, M. E. (1981). Vertical distribution of mercury in sediments from the East Pacific Rise. *Nature* **289**, 789-792.

Crespo-Medina, M., Chatziefthimiou, A. D., Bloom, N. S., Luther, G. W., III, Wright, D. D., Reinfelder, J. R., Vetriani, C. & Barkay, T. (In press-a). Adaptation of chemosynthetic microorganisms to eleveted mercury concentrations in deep-sea hydrothermal vents. *Limnol Oceanogr*.

Crespo-Medina, M., Chatziefthimiou, A. D., Cruz-Matos, R., Perez-Rodriguez, I., Barkay, T., Lutz, R. A., Starovoytov, V. & Vetriani, C. (In press-b). *Salinisphaera hydrothermalis* sp. nov, a mesophilic, halotolerant, facultative autotrophic, thiosulfate oxidizing gammaproteobacterium from deep-sea hydrothermal vents, and emended description of the genus Salinisphaera. *Int J Syst Evol Microbiol.*

Dekov, V. M. (2007). Native Hg_{liq} in the metalliferous sediments of the East Pacific Rise (21 ^{O}N). *Mar Geol* **238**, 107 - 113.

Delwiche, C. F. & Palmer, J. D. (1996). Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plastids. *Mol Biol Evol* **13**, 873-882.

Dopson, M., Baker-Austin, C., Koppineedi, P. R. & Bond, P. L. (2003). Growth in sulfidic mineral environments: metal resistance mechanisms in acidophilic micro-organisms. *Microbiol* **149**, 1959-1970.

Durand, P., Reysenbach, A. L., Prieur, D. & Pace, N. (1993). Isolation and characterization of *Thiobacillus hydrothermalis* sp. nov., a mesophilic obligately chemolithotrophic bacterium isolated from a deep-sea vent in Fiji Basin. *Arch Microbiol* **159**, 39-44.

Durand, P., Benyagoub, A. & Prieur, D. (1994). Numerical Taxonomy of Heterotrophic Sulfur-Oxidizing Bacteria Isolated from Southwestern Pacific Hydrothermal Vents. *Can J Microbiol* **40**, 690-697.

Eberhard, C., Wirsen, C. O. & Jannasch, H. W. (1995). Oxidation of polymetal sulfides by chemolithoautotrophic bacteria from deep-sea hydrothermal vents. *Geomicrobiol J* **13**.

Edgcomb, V. P., Molyneaux, S. J., Saito, M. A., Lloyd, K., Boer, S., Wirsen, C. O., Atkins, M. S. & Teske, A. (2004). Sulfide ameliorates metal toxicity for deep-sea hydrothermal vent archaea. *Appl Environ Microbiol* **70**, 2551-2555.

Edmond, J. M., Measures, C., McDuff, R. E., Chan, L. H., Collier, R., Grant, B., Gordon, L. I. & Corliss, J. B. (1979). Ridge Crest Hydrotehrmal Activity and the Balances of the Major and Minor Elements in the Ocean: The Galapagos Data. *Earth Planet Sc Lett* **46**, 1-18.

Edwards, K. J., Wolfgang, B. & McCollom, T. M. (2005). Geomicrobiology in oceanography: microbe-mineral interactions at and below the seafloor. *TRENDS Microbiol* **13**, 449-456.

Ekstrom, E. B. (2007). Ph. D. dissertation: Investigations into the mechanisms of biotic and abiotic mercury methylation. Princeton, NJ: Princeton University.

Ellis, R. J. (1979). The most abundant protein in the world. *Trends Biochem Sci* **4** 241-244.

Elsaied, H. & Naganuma, T. (2001). Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Appl Environ Microbiol* **67**, 1751-1765.

Elsaied, H. E., Kimura, H. & Naganuma, T. (2007). Composition of archaeal, bacterial, and eukaryal RuBisCO genotypes in three Western Pacific arc hydrothermal vent systems. *Extremophiles* **11**, 191-202.

Farrell, R. E., Germida, J. J. & Huang, P. M. (1990). Biotoxicity of mercury as influenced by mercury(II) speciation. *Appl Environ Microbiol* **56**, 3006-3016.

Felbeck, H. (1981). Chemoautotrophic Potential of the Hydrothermal Vent Tube Worm, *Riftia pachyptila* Jones (Vestimentifera). *Science* **213**, 336-338.

Fitzgerald, W. F. & Gill, G. A. (1979). Subnanogram determination of mercury by two-stage gold amalgamation and gas pahse detection applied to atmospheric analysis. *Anal Chem* **51**, 1714-1720.

Galtier, N., Gouy, M. & Gautier, C. (1996). SeaView and Phylo_win, two graphic tools for sequence alignment and molecular phylogeny. *Comput Applic Biosci* **12**, 543-548.

Gibson, J. L., Falcone, D. L. & Tabita, F. R. (1991). Nucleotide-Sequence, Transcriptional Analysis, and Expression of Genes Encoded within the Form-I Co2 Fixation Operon of Rhodobacter-Sphaeroides. *J Biol Chem* **266**, 14646-14653.

Gill, G. A. & Fitzgerald, W. F. (1988). Vertical mercury distributions in the oceans. *Geochim Cosmochim Acta* **52**, 1719-1728.

Golding, G. R., Kelly, C. A., Sparling, R., Lowen, P. C., Rudd, J. W. & Barkay, T. (2002). Evidence for facilitated uptake of Hg(II) by *Vibrio anguillarum* and *Escherichia coli* under anaerobic and aerobic conditions. *Limnol Oceanogr* **47**, 967-975.

Hines, M. E., Horvat, M., Faganeli, J. & other authors (2000). Mercury biogeochemistry in the Idrija river, Slovenia, from above the mine into the Gulf of Trieste. *Environ Res* **83**, 129-139.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (1994). *Bergey's Manual of Determinative Bacteriology*, Ninth Edition edn. Baltimore, Maryland: Williams and Wilkins.

Horvat, M., LIang, L. & Bloom, N. S. (1993). Comparison of distillation with other current isolation methods for the determination of methyl mercury ocompounds in low level environmental samples: PartII. Water. *Anal Chim Acta* **282**, 153-168.

Jannasch, H. W. & Wirsen, C. O. (1979). Chemosynthetic Primary Production at East Pacific Sea Floor Spreading Centers. *BioScience* **29**, 592-598.

Jannasch, H. W. & Mottl, M. J. (1985). Geomicrobiology of Deep-Sea Hydrothermal Vents. *Science* **229**, 717-725.

Jannasch, H. W., Wirsen, C. O., Nelson, D. C. & Robertson, L. A. (1985). *Thiomicrospira crunogena* sp. nov., a colorless sulfur-oxidizing bacterium from a deep-sea hydrothermal vent. *Intl J Syst Bacteriol* **35**, 422-424.

Jannasch, H. W. (1995). Microbial interactions with hydrothermal fluids. In *Seafloor hydrothermal systems: Physical, chemical, biological and geological interactions*, pp. 273-296. Edited by S. E. Humphris, R. A. Zierenberg, L. S. Mullineaux & R. E. Thomson. Washington, DC: American Geophysical Union.

Johnson, K. S., Childress, J. J. & Beehler, C. L. (1988). Short-term temperature variability in Rose Garden hydrothermal field: an unstable deep-sea environment. *Deep-Sea Res* **35**, 1711-1721.

Karl, D. M., Wirsen, C. O. & Jannasch, H. W. (1980). Deep-Sea Primary Production at the Galapagos Hydrothermal Vents. *Science* **207**, 1345-1347.

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

Kaye, J. Z. & Baross, J. A. (2000). High incidence of halotolerant bacteria in Pacific hydrothermal-vent and pelagic environments. *FEMS Microbiol Ecol* **32**, 249-260.

Kaye, J. Z., Marquez, M. C., Ventosa, A. & Baross, J. A. (2004). *Halomonas neptunia* sp. nov., *Halomonas sulfidaeris* sp. nov., *Halomonas axialensis* sp. nov. and *Halomonas hydrothermalis* sp. nov.: halophilic bacteria isolated from deep-sea hydrothermal-vent environments. *Int J Syst Evol Microbiol* **54**, 499-511.

Kovacs, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature* **178**, 703.

Lamborg, C. H., Von Damm, K. L., Fitzgerald, W. F., Hammerschmidt, C. R. & Zierenberg, R. (2006). Mercury and monomethylmercury in fluids from Sea Cliff submarine hydrothermal field, Gorda Ridge. *Geophys Res Lett* **33**, L17606.

Le Bris, N., Govenar, B., Le Gall, C. & Fisher, C. R. (2006). Variability of physico-chemical conditions in 9 degrees 50 ' N EPR diffuse flow vent habitats. *Mar Chem* **98**, 167-182.

Lonsdale, P. (1977). Clustering of Suspension-Feeding Macrobenthos Near Abyssal Hydrothermal Vents at Oceanic Spreading Centers. *Deep-Sea Res* **24**, 857-863.

Luther, G. W., 3rd, Glazer, B. T., Hohmann, L., Popp, J. I., Taillefert, M., Rozan, T. F., Brendel, P. J., Theberge, S. M. & Nuzzio, D. B. (2001). Sulfur speciation monitored in situ with solid state gold amalgam voltammetric microelectrodes: polysulfides as a

special case in sediments, microbial mats and hydrothermal vent waters. *J Environ Monitor* **3**, 61-66.

Luther, G. W., 3rd, Glazer, B. T., Ma, S. & other authors (in press). Use of voltammetric solid-state (micro) electroddes for studying biogeochemical processes: laboratory measurements to real time measurements with an in situ electrochemical analyzer (ISEA). *Mar Chem.*

Lutz, R. A. (1994). Rapid growth at deep-sea vents. *Nature* **371**, 663-664.

Martell, A. E., Smith, R. M. & Motekaitis, R. J. (1998). *NIST* Critical Stability Constants of Metal Complexes Database. *US Department of Commerce: Gaithersburg, MD.*

Martins, I., Costa, V., Porteiro, F., Cravo, A. & Santos, R. S. (2001). Mercury concentrations in invertebrates from Mid-Atlantic Ridge hydrothermal vent fields. *J Mar Biol Ass U K* **81**, 913-915.

Mason, R. P. & Sheu, G.-R. (2002). Role of the ocean in the global mercury cycle. *Global Biogeochem Cy* **16**, 1093-2007.

McCollom, T. M. & Shock, E. L. (1997). Geochemical constraints on chemolithoautotrophic metabolisms by microorganisms in seafloor hydrothemal systems. *Geochim Cosmochim Acta* **61**, 4375-4391.

McCollom, T. M. (2000). Geochemical constraints on primary productivity in submarine hydrothermal vent plumes. *Deep-Sea Research I* **47**, 85-101.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J System Bacteriol* **39**, 159-167.

Mullaugh, K. M., Luther, G. W., III, Ma, S. & other authors (in press). Voltammetric (micro)electrodes for the in situ study of Fe2+ oxidation kinetics in hot springs ans S2O32- production at hydrothermal vents. In *Electroanalysis*

Nanba, K., King, G. M. & Dunfield, K. (2004). Analysis of facultative lithotroph distribution and diversity on volcanic deposits by use of the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase. *Appl Environ Microbiol* **70**, 2245-2253.

Nies, D. H. (1999). Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* **51**, 730-750.

Nozaki, Y. (2001). Elemental Distribution, Overview. In *Encyclopedia of Ocean Sciences*, pp. 840-845. Edited by J. H. Steele. Oxford: Academic Press.

Petri, R., Podgorsek, L. & Imhoff, J. F. (2001). Phylogeny and distribution of the soxB gene among thiosulfate-oxidizing bacteria. *FEMS Microbiol Lett* **197**, 171-178.

Raguenes, G., Christen, R., Guezennec, J., Pignet, P. & Barbier, G. (1997). Vibrio diabolicus sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, Alvinella pompejana. *Int J Syst Evol Microbiol* **47**, 989-995.

Rau, G. H. & Hedges, J. I. (1979). Carbon-13 Depletion in a Hydrothermal Vent Mussel Suggestion of a Chemosynthetic Food Source. *Science* **203**, 648-649.

Rau, G. H. (1981). Hydrothermal Vent Clam and Tube Worm 13C/12C: Further Evidence of Nonphotosynthetic Food Source. *Science* **213**, 338-339.

Ravichandran, M. (2004). Interactions between mercury and dissolved organic matter--a review. *Chemosphere* **55**, 319-331.

Robertson, L. A. & Kuenen, J. G. (1999). The Colorless Sulfur Bacteria. In *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*. New York: Springer-Verlang.

Rohwerder, T., Gehrke, T., Kinzler, K. & Sand, W. (2003). Bioleaching review part A: Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. *Appl Microbiol Biotechnol* **63**, 239-248.

Rona, P. A., Thompson, G., Mottl, M. J., Karson, J. A., Jenkins, W. J., Graham, D., Mallette, M., Von Damm, K. L. & Edmond, J. M. (1984). Hydrothermal Activity at the TAG Hydrothermal Field, Mid-Atlantic Ridge Crest at 26ºN. *J Geophy Res* **89**.

Rona, P. A., Klinkhammer, G., Nelsen, T. A., Trefry, J. H. & Elderfield, H. (1986). Black Smokers, Massive Sulphides and Vent Biota at the Mid-Atlantic Ridge. *Nature* **321**, 33-37

Rozan, T. F. & Luther, G. W. (2002). Voltammetric evidence suggesting Ag speciation is dominated by sulfide complexation in river water. In *Environmental Electrochemistry: Analyses of Trace Element Biogeochemsitry ACS Symposium Series*, pp. 371-378. Edited by M. Taillefert & T. F. Rozan: American Chemical Society, Washington, DC.

Ruby, E. G., Wirsen, C. O. & Jannasch, H. W. (1981). Chemolithotrophic Sulfur-Oxidizing Bacteria from the Galapagos Rift Hydrothermal Vents. *Appl Environ Microbiol* **42**, 317-324.

Ruby, E. G. & Jannasch, H. W. (1982). Physiological characteristics of *Thiomicrospira* sp. Strain L-12 isolated from deep-sea hydrothermal vents. *J Bacteriol* **149**, 161-165.

Rytuba, J. J. (2000). Mercury mine drainage and processes that control its environmental impact. *Sci Total Environ* **260**, 57-71.

Schaefer, J. K., Yagi, J., Reinfelder, J. R., Cardona, T., Ellickson, K. M., Tel-Or, S. & Barkay, T. (2004). Role of the bacterial organomercury lyase (MerB) in controlling methylmercury accumulation in mercury-contaminated natural waters. *Environ Sci Technol* **38**, 4304-4311.

Schecher, W. D. & McAvoy, D. (1994). MINEQL+: A Chemical Equilibrium Program for Personal Computers. *Environmental Research Software: Hollowell, ME*.

Scott, K. M., Sievert, S. M., Abril, F. N. & other authors (2006). The genome of deep-sea vent chemolithoautotroph *Thiomicrospira crunogena* XCL-2. *Plos Biology* **4**, 2196-2212.

Selifonova, O., Burlage, R. & Barkay, T. (1993). Bioluminescent sensors for detection of bioavailable Hg(II) in the environment. *Appl Environ Microbiol* **59**, 3083-3090.

Severmann, S., Mills, R. A., Palmer, M. R., Telling, J. P., Cragg, B. & Parkes, R. J. (2006). The role of prokaryotes in subsurface weathring of hydrothrmal sediments: A combined geochemical and microbiolgical investrigation. *Geochim Cosmochem Acta* **70**, 1677-1694.

Sievert, S. M., Brinkhoff, T., Muyzer, G., Ziebis, V. & Kuever, J. (1999). Spatial heterogeneity of bacterial populations along an environmental gradient at a shallow submarine hydrothermal vent near Milos Island (Greece). *Appl Environ Microbiol* **65**, 3834-3842.

Simon-Colin, C., Raguenes, G., Cozien, J. & Guezennec, J. G. (2008). Halomonas profundus sp. nov., a new PHA-producing bacterium isolated from a deep-sea hydrothermal vent shrimp. *J Appl Microbiol* **104**, 1425-1432.

Smits, T. H. M., Röthlisberger, M., Witholt, B. & van Beilen, J. B. (1999). Molecular screening for alkane hydroxylase genes in Gram-negative and Gram positive strains. *Envion Microbiol* **1**, 307-317.

Sorokin, D. (2003). [Oxidation of inorganic sulfur compounds by obligatory organotrophic bacteria]. *Mikrobiologiia* **72**, 725-739.

Spiess, F. N., Macdonald, K. C., Atwater, T. & other authors (1980). East Pacific Rise: Hot Springs and Geophysical Experiments. *Science* **207**, 1421-1433.

Stoffers, P., Hannington, M., Wright, I., Herzig, P. & de Ronde, C. (1999). Elemental mercury at submarine hydrothermal vents in the Bay of Plenty, Taupo volcanic zone, New Zeland. *Geology* **27**, 931-934.

Tabita, F. R. (1999). Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A different perspective. *Photosynth Res* **60**, 1-28.

Takai, K., Hirayama, H., Nakagawa, T., Suzuki, Y., Nealson, K. H. & Horikoshi, K. (2004). *Thiomicrospira thermophila* sp. nov., a novel microaerobic, thermotolerant, sulfur-oxidizing chemolithomixotroph isolated from a deep-sea hydrothermal fumarole in the TOTO caldera, Mariana Arc, Western Pacific. *Int J Syst Evol Microbiol* **54**, 2325-2333.

Teske, A., Brinkhoff, T., Muyzer, G., Moser, D. P., Rethmeier, J. & Jannasch, H. W. (2000). Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. *Appl Environ Microbiol* **66**, 3125-3133.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876-4882.

Tilton, R. C., Cobet, A. B. & Jones, G. E. (1967). Marine thiobacilli. *Can J Microbiol* **13**, 1521-1528.

Tuttle, J. H. & Jannasch, H. W. (1976). Microbial Utilization of Thiosulfate in the Deep-Sea. *Limnol Oceanogr* **21**, 697-701.

Van Dover, C. L. (2000). *The Ecology of Deep-Sea Hydrothermal Vents*. Princeton, NJ: Princeton University Press.

Varekamp, J. C. & R, B. P. (1984). The speciation of mercry in hydrothermal systems, with applications to ore depositation. *Geochim Cosmochim Acta* **48**, 177-185.

Vetriani, C., Speck, M. D., Ellor, S. V., Lutz, R. A. & Starovoytov, V. (2004). Thermovibrio ammonificans sp nov., a thermophilic, chemolithotrophic, nitrate-ammonifying bacterium from deep-sea hydrothermal vents. *Int J Syst Evol Microbiol* **54**, 175-181.

Vetriani, C., Chew, Y. S., Miller, S. M., Yagi, J., Coombs, J., Lutz, R. A. & Barkay, T. (2005). Mercury adaptation among bacteria from a deep-sea hydrothermal vent. *Appl Environ Microbiol* **71**, 220-226.

Voordeckers, J. W., Starovoytov, V. & Vetriani, C. (2005). Caminibacter mediatlanticus sp nov., a thermophilic, chemolithoautotrophic, nitrate-ammonifying bacterium isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge. *Int J Syst Evol Microbiol* **55**, 773-779.

Voordeckers, J. W., Crespo-Medina, M., Lutz, R. A. & Vetriani, C. (submitted). Detection and phylogenetic analysis of the periplasmic nitrate reductase (NapA) in

chemolithotrophic Epsilonproteobacteria and microbial communities from deep-sea hydrothermal vents. *Appl Environ Microbiol.*

Watson, G. M. F. & Tabita, F. R. (1997). Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A molecule for phylogenetic and enzymological investigation. *FEMS Microbiol Lett* **146**, 13-22.

Xu, H. H. & Tabita, F. R. (1996). Ribulose-1,5-bisphosphate carboxylase/oxygenase gene expression and diversity of Lake Erie planktonic microorganisms. *Appl Environ Microbiol* **62**, 1913-1921.

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Lutz, R.A., T.M. Shank, G.W. Luther III, C. Vetriani, M.Tolstoy, D.B Nuzzio, T.S. Moore, F. Waldauser, **M. Crespo-Medina**, A. Chatziefthimiou, E.R. Annis, and A.J. Reed. (2008). Interrelationships between vent fluid chemistry, temperature, seismic activity and biological community structure at a mussel-dominated, deep-sea hydrothermal vent along the East Pacific Rise. *Journal of Shellfish Research*. 27 (1): 177-190.

Chatziefthimiou, A.D., **Crespo-Medina, M.,** Wang, Y., Vetriani, C., and Barkay, T. (2007). The Isolation and Initial Characterization of Mercury Resistant Chemolithotrophic and Thermophilic Bacteria from Mercury Rich Geothermal Springs. *Extremophiles*. 11: 469-479.

Crespo-Medina, M., A. Chatziefthimiou, R. Cruz-Matos, I. Pérez-Rodríguez, T. Barkay, R. A. Lutz, V. Staravoytov, and C. Vetriani. *Salinisphaera hydrothermalis* sp. nov, a mesophilic, halotolerant, facultative autotrophic, thiosulfate oxidizing *"gammaproteobacterium"* from deep-sea hydrothermal vents, and emended description of the genus *Salinisphaera*. In press. *International Journal of Systematic and Evolutionary Microbiology*.

Voordeckers, J.W., **M. Crespo-Medina**, and C. Vetriani. Detection and phylogenetic analysis of the periplasmic nitrate reductase (NapA) in chemolithoautotrophic *Epsilonproteobacteria* and microbial communities from deep-sea hydrothermal vents. (Revised version submitted, *Applied and Environmental Microbiology*).

Crespo-Medina, M., A. Chatziefthimiou, T. Barkay, G.W. Luther, R.A. Lutz, and C. Vetriani. Diversity of chemosynthetic thiosulfate-oxidizing bacteria from deep-sea hydrothermal vents. (In preparation to be submitted to *Applied and Environmental Microbiology*).