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Microbial diversity and potential for arsenic and iron biogeochemical cycling at an arsenic rich, shallow-sea hydrothermal vent (Tutum Bay, Papua New Guinea)

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ABSTRACT

The shallow submarine hydrothermal systems of Tutum Bay, Papua New Guinea, are an ideal opportunity to study the influence of arsenic on a marine ecosystem. Previous reports have demonstrated that the hydrothermal vents in Tutum Bay release arsenic in reduced hydrothermal fluids into the marine environment at the rate of 1.5 kg of arsenic/day. Aqueous arsenite is oxidized and adsorbed onto hydrous ferric oxides [HFOs] surrounding the venting area. We demonstrate here that microorganisms are key in both the oxidation of Fe^{II} and As^{III} in the areas immediately surrounding the vent source. Surveys of community diversity in biofilms and in vent fluid indicate the presence of zeta-Proteobacteria, alpha-Proteobacteria, *Persephonella*, and close relatives of the archaeon *Nitrosocaldus*. The iron oxidizing zeta-Proteobacteria are among the first colonizers of solid substrates near the vents, where they appear to be involved in the precipitation of the hydrous ferric oxides (HFOs). Further, the biofilm communities possess the genetic capacity for the oxidation of arsenite. The resulting arsenate is adsorbed onto the HFOs, potentially removing the arsenic from the immediate marine system. No evidence was found for dissimilatory arsenate reduction, but the arsenate may be remobilized by detoxification mechanisms. This is the first demonstration of the genetic capacity for arsenic cycling in high temperature, shallow-sea vent communities, supporting recent culture-based findings in similar systems in Greece (Handley et al., 2010). These reports extend the deep-sea habitat of the zeta-Proteobacteria to shallow submarine hydrothermal systems, and together implicate biological oxidation of both iron and arsenite as primary biogeochemical processes in these systems, providing a mechanism for the partial removal of aqueous arsenic from the marine environment surrounding the vents.

1. Introduction

Shallow-sea hydrothermal vent systems (HVS) can be regarded as physical and geochemical transitions between deep-sea and terrestrial hydrothermal systems, and are typically associated with submarine volcanism, island and intra-oceanic arcs, ridge environments, areas of intraplate oceanic volcanism, and less commonly at continental margins and rift basins (Tarasov et al., 2005). These HVS are characterized by geochemical gradients formed by the transition between oxic seawater and anoxic vent fluid. It has also been demonstrated that fluid-mixing occurring between hydrothermal vents and lacustrine or marine fluids generates potential catabolic energy sources for chemolithotrophic and heterotrophic microorganisms (Amend et al., 2003; Amend et al., 2004; Rusch et al., 2005; Akerman et al., 2011). Therefore, shallow-sea HVS provide a unique insight into complex microbial communities at the interface between the aerobic surface and anaerobic subsurface biosphere communities.

Ambitle Island is one of the Tabar-Feni island chain, NE of New Ireland Province (Fig. 1a), occurring in the forearc region of the former ensimatic New Hanover–New Ireland–Bouganville island arc (Pichler et al., 1999b). Ambitle is part of a Quaternary stratovolcano, and several areas of shallow submarine venting exist submerged just west of the island in Tutum Bay, in 5–10 m of water (Fig. 1b). The Tutum Bay area hosts several high flux thermal vents (up to 400 L/min), which are surrounded by fields of more diffuse flow. The bay consists of areas of coral reefs surrounded by coarse-grained carbonate–volcanoclastic sands and sediments (Fig. 1b). The geochemistry of shallow-sea HVS in Tutum Bay has been extensively characterized (Meyer-Dombard et al., 2012; Pichler et al., 2000; Pichler and Dix, 1996; Pichler and Veizer, 1999a; Pichler et al., 1999b; Pichler et al., 1999c; Price et al., 2007; Price and Pichler, 2005). Of particular interest in this area are the effects of elevated arsenic concentrations on the surrounding marine ecosystem (Pichler et al., 2006). These HVS fluids discharge ~1.5 kg of arsenic/day into a small (~50×100 m) area. The

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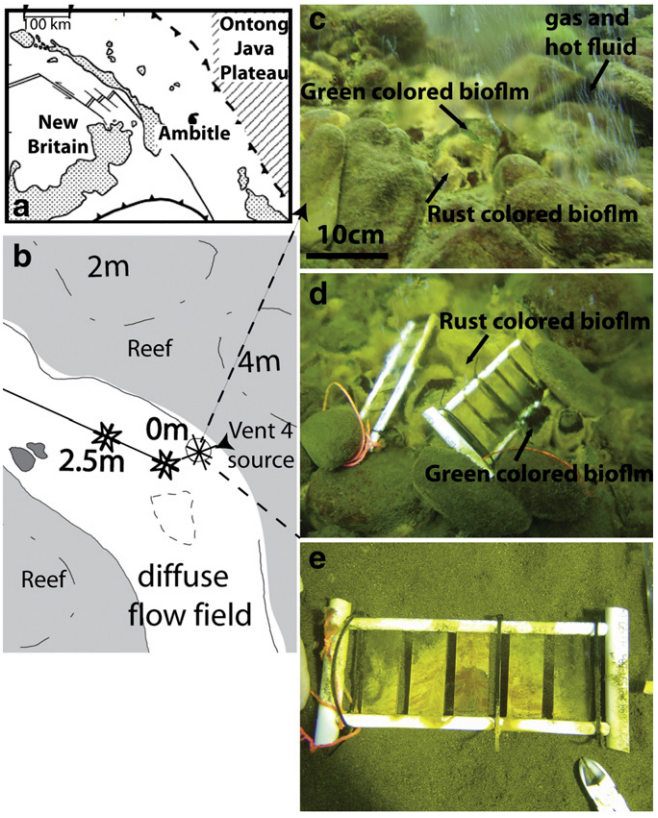

Fig. 1. The sampling location. [a] Regional map of New Ireland Province, Papua New Guinea showing the location of Ambitle Island (modified from Pichler and Dix, 1996). [b] Sketch map of the area surrounding the Vent 4 sample location in Tutum Bay, modified after Meyer-Dombard et al. (2012). Shown are the source of the Vent 4 fluids and the previously reported location 2.5 m away from the vent source. [c] A discrete vent source in the "Vent 4A" area of Tutum Bay. Rust colored and green colored biofilms are noted with arrows. Note the HFO-coating on rocks and corals surrounding the venting area. [d] Slide racks were placed directly on the vent source, in proximity to the rust and green colored biofilms. [e] A slide rack, removed after eight days incubation on vent 4A, showing microscope slides coated in HFO-biofilms. Microscope slides are 75×25 mm, for scale.

surrounding sediments and coral are coated by arsenate (As^V) co-precipitated with hydrous ferric oxides (HFOs); these HFO precipitates contain up to 7 wt.% arsenate (Pichler and Veizer, 1999; Pichler et al., 1999c). Recent work has revealed prokaryotic communities inhabiting thermal sediments near a high flux vent (Meyer-Dombard et al., 2012). However, prokaryotic communities in the vent fluids and in the biofilms forming directly at the high flux vents and surrounding diffuse flow areas have not been described. These biofilms may harbor the metabolic capability for arsenic and iron biogeochemical cycling.

The role of Bacteria and Archaea in the biogeochemical cycling of arsenic is of broad interest, and has direct implications for public health (Silver and Phung, 2005a). The genes and enzymes involved in the key steps of arsenate reduction and arsenite oxidation are thus of particular interest, and recent advances have supplied reliable genetic markers for these processes (Mukhopadhyay et al., 2002; Malasarn et al., 2004; Silver and Phung, 2005b; Stolz et al., 2006; Oremland et al., 2009; Bini, 2010; Stolz et al., 2010). Arsenate is reduced by a wide range of bacterial and archaeal phyla (Silver and Phung, 2005b). Anaerobic arsenate respiration is found in phylogenetically diverse organisms, and a reliable genetic marker, the respiratory arsenate reductase (*arrA*) gene (Saltikov and Newman, 2003; Saltikov et al., 2003; Malasarn et al., 2004) has recently been identified in the genetically tractable strain (*Shewanella* ANA-3). Additionally, there is evidence to indicate that the ArrAB arsenate reductase complex can be operated in reverse and serve as a means to oxidize arsenite anaerobically (Oremland et al., 2009; Richey et al., 2009; Handley et al., 2010). Arsenate resistance/detoxification as conferred by arsenate reduction via the *ars* operon is also widespread among Prokaryotic phyla, and is thought to have an ancient origin that has diverged into several classes today (Mukhopadhyay et al., 2002; Jackson and Dugas, 2003). Capacity for the chemolithotrophic, anaerobic oxidation of arsenite (*aro* genes) and heterotrophic, periplasmic detoxification of arsenite (*aso*/*aox* genes) has been found in a variety of Proteobacteria, Aquificales, Thermales, and Crenarchaeota, to name a few (Santini et al., 2000; vanden Hoven and Santini, 2004; Stolz et al., 2006; Inskeep et al., 2007).

In this report, we characterize the community and functional diversity present in the Tutum Bay vent fluids, and biofilms originating at the discrete focused vent site (Fig. 1c, d). These biofilms include both *in situ* biofilms collected on site, and biofilms colonized on microscope slides incubated directly in the vent orifice (Fig. 1c–e). Extracted DNA was screened for 16S rRNA diversity and the presence/absence of functional genes associated with arsenic metabolism (*aoxB*, *aroA*, *arsC*, and *arrA*).

2. Materials and methods

2.1. Sample location and related reports

The Tutum Bay sample site (previously described in Pichler and Dix, 1996; Pichler et al., 1999a,b; Price and Pichler, 2005; Price et al., 2007), is the focus of a collaborative effort to investigate the effect of arsenic on a marine ecosystem. Companion studies regard the geochemistry, macrofauna, and meiofauna along two transects (A and B) originating at one vent source (Vent 4) (Karlen and Garey, 2005; Price and Pichler, 2005; Pichler et al., 2006; Karlen et al., 2007a; Karlen et al., 2007b; Price et al., 2007; Akerman and Amend, 2008; Akerman, 2009; McCloskey, 2009; Akerman et al., 2011; Amend et al., 2012; Meyer-Dombard et al., 2012). This report is concerned with biofilms, sediments, and vent fluids at Vent 4, collected by SCUBA in Tutum Bay. The area is shown in maps given in Fig. 1.

2.2. Sample collection

In situ "native" biofilms growing on rocks at Vent 4A were collected via sterile implements and placed in sterile containers (Fig. 1c, d). These consisted of both rust colored, and green colored biofilms growing in and around the vent source, which were collected separately. These *in situ* biofilms grew in close contact to each other, and were directly in the flow of vent fluid; currents were often strong enough to shift the vent fluid flow path for short periods of time.

Fresh biofilms were induced to colonize on microscope slides that were sterilized and placed into a hand-made frame; the frame was placed directly over the vent source (Fig. 1e). After 8 days of incubation, the slides were removed from the frame. While still underwater, each slide was placed in a separate 50 mL conical vial for safe transport to the surface; vials were filled with air and held upside down while the slide was placed inside to prevent disturbance of the biofilms.

Fluid from Vent 4 was collected via a stainless steel cone fitted over the vent. Teflon tubing attached to a sample container with a 3-way valve allowed thorough flushing of the sample container before collection, and sample was brought shipboard for filtration. Approximately 25 L of vent fluid was pushed by syringe through filters (Sterivex 0.22 μm, Millipore). Soft sediment near the vent site was cored and collected as previously reported in Meyer-Dombard et al. (2012).

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2.3. Sample storage

Native rust and green colored biofilms were split while on board the research vessel and subsamples were kept refrigerated and frozen at 4 °C and −20 °C, respectively, until analysis. The 50 mL conical vials containing the colonized microscope slides were filled with EtOH and stored at −20 °C. Sterivex filters holding vent fluid particulates and organisms were filled with a preservation solution [40 mM EDTA, 50 mM Tris–HCl, 0.7 M sucrose], placed in sterile whirlpac bags, and frozen at −20 °C until analysis. Sediment was scooped from the interior of the retrieved cores as previously reported (Meyer-Dombard et al., 2012), and stored at −20 °C.

2.4. DNA extraction

DNA was extracted with several methods, depending on the type of sample. Two methods were applied to rust and green colored biofilms collected *in situ*, and stored refrigerated and frozen, at 4 °C and −20 °C, respectively. We used both a commercially available kit utilizing a bead-beating technique that was modified to maximize the quality of DNA extracted (FastDNA spin kit for soils, MP Biomedicals, with a modification to the manufacturer's instructions of milling the sample at 2500 rpm for 120 s., per recommendations in Miller et al., 1999), and a chemical extraction method using a pretreatment of 1% polyvinlypyrolidone (PVP) and 24 μL polyadenic acid (polyA), followed by lysis with SDS, lysozyme, and proteinase K (see also Miller et al., 1999). Both methods are previously described (Meyer-Dombard et al., 2005; Meyer-Dombard et al., 2012).

Two methods were adapted for extraction of DNA from the colonized microscope slides. One slide was placed into a sterile whirlpac bag, and subjected to the chemical extraction method described in Meyer-Dombard 2005; the slide was rubbed vigorously through the bag during the lysing stages of the extraction to loose cells from the slide surface. The biofilm on a second slide was scraped off with a sterile razor blade, and placed into a 1.5 mL centrifuge tube. DNA was then extracted from this biofilm using a commercial kit suited for cell cultures, following the manufacturer's instructions (QIAGEN DNA kit 51306). DNA extracted from both methods was pooled prior to PCR.

DNA was extracted from the filtered vent fluid using a technique first described in Massana et al. (1997), and modified for use with hydrothermal fluids (Massana et al., 1997; Rogers and Amend, 2005).

DNA from the soft sediment cores was extracted as previously described in Meyer-Dombard et al. (2012).

2.5. PCR, DNA sequencing, and phylogenetic inference

The polymerase chain reaction (PCR) was used to amplify partial 16S rRNA genes from the biofilms and vent fluid as described above, and as previously reported in Meyer-Dombard et al. (2012), the soft sediments cored near the vent Archaeal and bacterial specific primer sets 21F/1391R and 27F/1492R, respectively, were applied to the *in situ* native biofilms as previously described (Meyer-Dombard et al., 2005; Meyer-Dombard et al., 2012). DNA extracted from the colonized microscope slides and vent fluid was amplified using archaeal primer sets 344F/958R, and 344F/1391R, and bacterial primer sets 338F/907R, and 8F/1492R (344F:5′-ACG GGG CGC AGC AGG CGC GA-3′, 338F: 5′-ACT CCT ACG GGA GGC AGC-3′, 8F:5′-TCC GGT TGA TCC TGC C-3′, 958R: 5′-YCC GGC GTT GAM TCC AAT T-3′, 907R: 5′-CCG TCA ATT CCT TTG AGT TT-3′) (Lane, 1991; Barns et al., 1994; Summit, 2000; Osburn and Amend, 2010). PCR reaction recipes were as described previously, modifying the annealing temperature of the program for each of the primer sets above as needed (reported in Table 1).

In addition, extracted DNA was screened by the PCR using primers that target the arsenite oxidase genes, *aoxB* and *aroA*, and the arsenate reductase genes, *arsC* and *arrA*. Primers, source references, and PCR conditions are given in Table 1. We screened the rust colored biofilm, the slide biofilm, and two sediment samples cored from soft sediment ~2.5 m from the vent source (see Meyer-Dombard et al., 2012) for arsenic cycling functional genes. Unfortunately, the extracted DNA from the vent fluid and the green colored biofilm was completely consumed in the 16S rRNA PCRs, so these samples were not surveyed for arsenic cycling functional genes.

Amplicons of 16S rRNA and arsenic cycling genes were cloned into *Escherichia coli*, sequenced, and analyzed for phylogenetic relationships as previously described (Meyer-Dombard et al., 2005; Meyer-Dombard et al., 2012). Sequences were screened for chimeric anomalies before inclusion in phylogenetic analysis. For each sample, the following number of 16S rRNA clones was sequenced (numbers in parentheses): native green colored biofilms (93 bacterial and 50 archaeal clones), native rust colored biofilms (80 bacterial and 66 archaeal clones), slide-colonized biofilms (130 bacterial and 51 archaeal clones), and vent fluids (59 bacterial and 88 archaeal clones). 16S rRNA cloning and sequencing for the soft sediments have been previously reported in Meyer-Dombard et al. (2012).

PCR products of targeted arsenic cycling functional genes were sequenced (24–36 clones each) from the rust colored biofilms (*aroA* and *arsC*), the slide-colonized biofilms (*aroA* and *arsC*), and the two soft sediment samples (*aroA* only).

Representative clones of each OTU for each sample were submitted to GenBank using the following naming convention; "PNG_TB" denotes that the sequences are from Papua New Guinea, Tutum Bay, "R", "G", "SL", and "V4" indicate sample origins as rust colored, green colored, and slide biofilms, and Vent 4, respectively. GenBank accession numbers are JN881568-JN881723. Sediment community clones were named according to the convention given in Meyer-Dombard et al. (2012).

2.6. Calculation of community richness and evenness

Community richness for each sample was determined by comparison of sequenced 16S rRNA genes with available data deposited with the National Center for Biotechnology Information (NCBI), via the BLAST (Basic Local Alignment Search Tool) function, using the "nr" database and searching for highly similar sequences. Community evenness (E) was quantified as given by (Pielou, 1977), written as

$$E = \frac{-\ln D}{\ln S}, \tag{1}$$

where S stands for the number of taxonomic groups in the sample, and D represents a dominance index, defined as

$$D = \sum_{i=1}^{S} P_i^2, \tag{2}$$

where p_i is defined as

$$p_i = x_i / \sum_{i=1}^{S} x_i, \tag{3}$$

and x_i refers to the abundance of the i^{th} taxonomic group. In this formulation, the evenness ranges from 0 (minimum evenness) to 1 (maximum evenness).

3. Results

3.1. Colonization of growth surfaces

The glass slides that were incubated directly on top of the vent source were quickly colonized by biofilms. Within one day of

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Table 1
Results of PCR-based screens of extracted DNA for arsenic cycling functional genes.

Gene[a]	Primer names	"Rust" *in situ* biofilm	Slide biofilm	4A05-2.5H1[b]	4A05-2.5H4[b]	Primer sequences 5′–3′	PCR conditions/ (Primer concentration)[c]	Ref.
aoxB	aoxB 69 F/ aoxB1374R	−	−	−	−	TGYATYGTNGGNTGYGGNTAYMA/ TANCCYTCYTGRTGNCCNCC	As in Rhine et al. (2007)/(2.5 μM)	1
aroA	aroAF1/R1	+	+	+	+	GTSGGBTGYGGMTAYC/	As in Inskeep et al. (2007)/	2
	aroAF2/R2	+	+	+	+	TTGTASGCBGGNCGRTTRTGRAT GTCGGYTGYGGMTAYCABGYCTA/ YTCDGARTTGTAGGCYGGBCG	(both sets, 0.5 μM)	2
arsC	arsC F1/R1	−	[+]	−	−	ATGAGCAACATYACCAT/	For both sets	3
	[gram −]	+	+	−	−	TTATTTCAGTCGTTTACC	94 °C 5 min; 35 cycles of 94 °C 45 s,	3
	arsC F2/R2 [gram +]					ATTTAYTTTATATGYACAG/ GATCATCAAAACCCCAAT	37 °C 45 s, 72 °C 45 s/(both sets, 2.5 μM)	
arrA	ArrAfwd/ ArrArev	−	−	−	−	AAGGTGTATGGAATAAAGCGTTTgtbgghgaytt/ CCTGTGATTTCAGGTGCCcaytyvggngt	94 °C 5 min; 30 cycles of 94 °C 30 s, 52 °C 30 s, 72 °C 90 s/(1 μM)	4

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4] Malasarn, D., C. W. Saltikov., K. M. Campbell, J. M. Santini, J. G. Hering and D. K. Newman (2004). "*arrA* is a reliable marker for As(V) respiration". Science 306: 455.

[a] *aoxB* and *aroA* are arsenite oxidase genes, and *arsC* and *arrA* are arsenate reductase genes (as reviewed in the introduction).

[b] Sediment cored from transect 4A, as reported in Meyer-Dombard et al. (2012). Sample name indicates transect (4A), year of collection (2005), distance from vent 4 (2.5 m), and depth within retrieved core (H1 = 3–7 cm, H4 = 33–37 cm).

[c] Primer concentration is given for final concentration in the reaction mix. References, 1: (Rhine et al., 2007), 2: (Inskeep et al., 2007), 3: (Macur et al., 2004), and 4: (Malasarn et al., 2004).

incubation, an iridescent film coated the slides. After three days, a rust colored coating lightly covered the slides, and when the slides were removed after eight days, they were covered with a thick mineral-biofilm (Fig. 1). This coating resembled the rust colored biofilms forming *in situ* at the vent source, previously shown to be co-formed with HFOs and adsorbed As^{V} (Pichler and Veizer, 1999; Pichler et al., 1999c). At the time of sampling, temperature was recorded using a water-resistant probe to be >90 °C in the vent source, ~80 °C at the rust colored biofilms, and ~40 °C at the green colored biofilms.

3.2. DNA extraction and PCR

Not all of the DNA extraction methods used on the native rust and green colored biofilms yielded amplifiable DNA. Both frozen and refrigerated samples of native biofilms were subjected to a bead-beating and a chemical extraction method. For the refrigerated, green colored native biofilm, DNA extracted by the bead-beating method was not amplifiable using any primer combination. DNA extracted from the rust colored biofilm was much more difficult to amplify, and PCR products were only obtained from DNA extracted by bead-beating on frozen sample (both bacterial and archaeal specific primer sets), and by the chemical method on refrigerated sample (only the bacterial specific primer set), after numerous attempts.

The conventional 21F/1391R and 27F/1492R primer sets for archaeal and bacterial 16S rRNA gene amplification did not yield PCR products for DNA extracted from the colonized slide biofilms or vent fluids. Primer sets targeting bacterial 16S rRNA that were successful on these samples were 8F/1492R, and 338F/907R, yielding near complete and ~500 bp gene products, respectively. Archaeal 16S rRNA was successfully amplified from the slide biofilms using primer sets 344F/958R and 21F/907R (~600–880 bp amplicons) and from the vent fluids using 344F/958R and 344F/1391R (~600–900 bp amplicons).

3.3. 16S rRNA gene surveys

Bacterial and archaeal diversity, richness, and evenness of each sample are shown in Fig. 2. In order of decreasing diversity, with bacterial and archaeal richness at the genus-level shown respectively in parentheses, are the vent fluid (11; 5), rust colored biofilm (11; 2), and green colored and slide-colonized biofilms (both 7; 2). In general, archaeal diversity is low compared to bacterial diversity in all samples, with as few as two genus level taxa found in three out of the four samples. The dominant group found in the vent fluid bacterial clone library is comprised of uncultured Bacteria. The rust colored biofilm has no apparent dominant taxon. The bacterial clone libraries of the green colored and slide-colinized biofilms are dominated by alpha-Proteobacteria and *Persephonella*, respectively. The archaeal clone libraries of vent fluid, rust colored and slide colonized biofilms are dominated by *Nitrosocaldus*, and the green biofilm is dominated by uncultured marine "group I" taxa. In all clone libraries, the archaeal communities are only represented by groups of uncultured Archaea, or divisions with only one or a few cultured representatives such as the *Nitrosocaldus*, *Caldiarchaeum*, and Korarchaeota.

Taxa found in the vent fluid but not in the biofilms include the Cyanobacteria, delta-Proteobacteria, Desulfurococcales, Uncultured Crenarchaeal group I, Euryarchaeota and Korarchaeota. Conversely, taxa found in the biofilms but not in the vent fluid include Thermotogales, Thermales, Chloroflexales, group OP11, Planctomycetales, *Caldiarchaeum*, marine "group I", and the Thermoproteales. The *Caldiarchaeum* and bacterial thermophilic taxa Thermotogales and Thermales are unique to the rust colored biofilm, while marine "group I" and uncultured Thermoproteales are unique to the green colored and colonized slide biofilms, respectively.

Clones from the biofilms and vent fluid are typically not closely related to cultured representatives (Table 2). Fig. 3 shows a phylogenetic analysis of *alpha-*, *beta-*, *gamma-*, and zeta-Proteobacteria relatives found in the 16S rRNA clone libraries. Identified zeta-Proteobacteria are most closely related to other clonal examples, and only distantly related to the cultured *Mariprofundus ferrooxydans* (Fig. 3). The environmental clones shown as reference are from environments rich in Fe^{II}, including many marine hydrothermal vent fields. Relatives of *alpha-* and *beta-Proteobacteria* in the Tutum Bay clone libraries are similarly distant from most cultured representatives.

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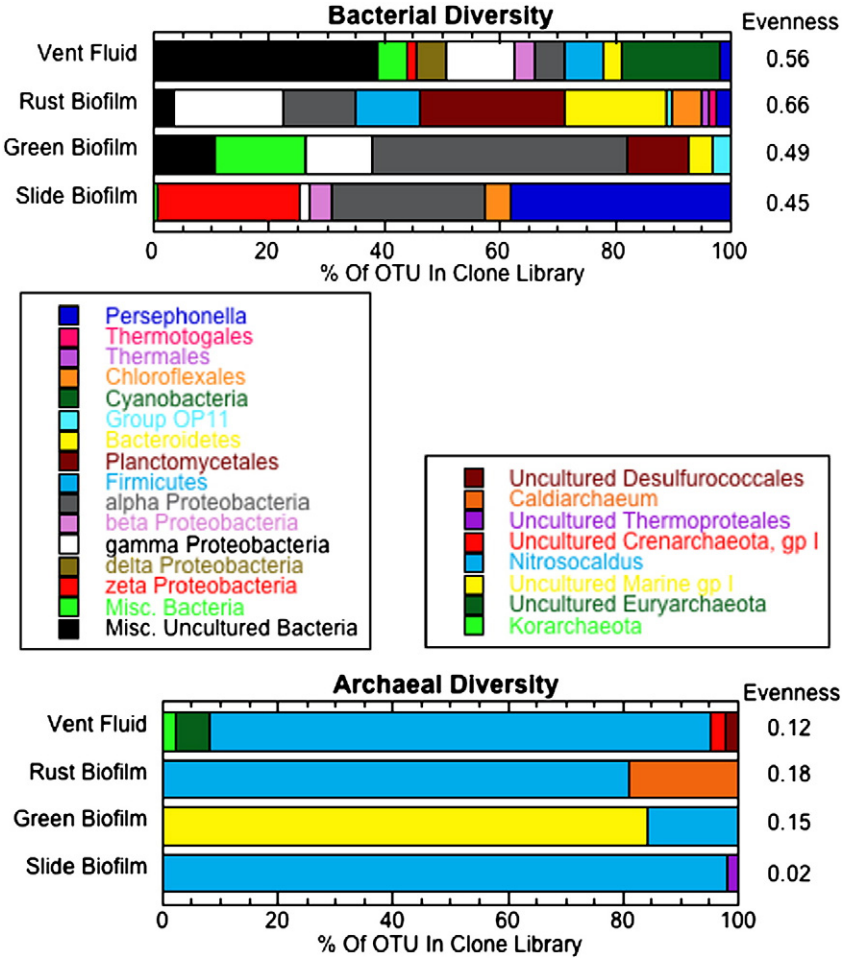

Fig. 2. Microbial diversity, richness (as measured by 16S rRNA gene surveys), and evenness (as calculated by Eq. (1)) for biofilms and vent fluid samples.

The evenness of each bacterial and archaeal 16S rRNA library is also shown in (Fig. 2), to the right of the richness histograms. The most even community in both bacterial and archaeal clone libraries was in the rust colored biofilm (0.66 and 0.18, respectively), and the least even communities were both in the slide biofilms (0.45 bacterial and 0.02 archaeal libraries).

3.4. Arsenic cycling functional gene surveys

Results of presence/absence surveys of arsenic functional genes, *aoxB*, *aroA*, *arsC*, and *arrA* are given in Table 1. The chemolithotrophic and anaerobic arsenite oxidase gene, *aroA*, was found in all samples examined, using two different primer sets. These primer sets, shown in Table 1, were designed as degenerate primers for use with environmental and hydrothermal samples, and may also amplify the *aso* or *aox* genes, so these results may be best viewed as "*aroA*-like" amplicons (Inskeep et al., 2007). The *arsC* gene was amplified from the slide-colonized biofilm DNA, using primers targeted to both gram-positive and gram-negative Bacteria, and the gram-positive primer set also produced an amplicon in the rust colored biofilm DNA. No amplicons were obtained using the *aoxB* or *arrA* primers, with repeated attempts under various PCR conditions.

Phylogenetic analyses of arsenite oxidase and arsenate reductase amplicons are given in Figs. 4 and 5, respectively. It can be seen in Fig. 4 that *aroA*-like genes retrieved from sediments and biofilms near Vent 4 are unique in all cases from *aroA* or *aoxB* genes of cultured representatives. The *aroA*-like sequences analyzed belong to relatives of alpha- and beta-Proteobacteria, *Thermus*, and *Pyrobaculum*, but are only 71–80% similar to other *aroA*-like sequences available in GenBank (Table 2). Phylogeny of sequenced *arsC* genes, shown in Fig. 5, suggests that several *arsC* genes from the slide biofilm can be attributed to *Bacillus*-like organisms. One clone, TBSL_arsC7, is only distantly similar to an *aroA* gene of an uncultured organism (74%). Further, sequenced *arsC* PCR products from the rust colored biofilm did not identify with any similar sequences in GenBank. These sequences were too divergent to maintain a stable tree topology, and thus it is unknown if they are unique *arsC* genes, or hypothetical genes amplified with the *arsC* primer pair used (Table 1).

4. Discussion

4.1. Microbial diversity in biofilms and vent fluids

As can be seen in Fig. 1c, the small vent source bathes the immediate surrounding surfaces in hydrothermal fluid, but is quickly mixed with seawater. Further, the path of this fluid is highly variable with the influence of localized currents and tides. The rust and green colored biofilms lay within a 10 cm zone in and around the vent source, existing in intimate proximity to each other.

The microscope slides that were placed directly on the vent source maximized the contact with the vent fluid and allowed what can be considered a primary colonization experiment. Therefore, the low diversity of the incubated slide community likely represents the first organisms to colonize these substrates over an eight day period. It can be seen in Fig. 2 that the colonized

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Table 2
Tutum Bay 16S rRNA, aroA, and arsC gene clones. Shown are representative clones for each sample, and closest GenBank BLAST relatives. Percent similarity is given in parentheses.

Gene	Taxa	Representative clones[a]	Closest BLAST relative
16S rRNA			
	Aquificales (*Persephonella*)	PNG_TBSL_B19	Clone PNG_War_B70; JF935227 (98%)
		PNG_TBV4_B2	"*Hydrogenivirga caldilitoris*"; AB120294
		PNG_TBR_B3	
	Thermotogales	PNG_TBR_B35	YNP clone EM3; EEU05660
	Thermales	PNG_TBR_B18	*Thermus* sp. SRI-248; AF255591
	Chloroflexales	PNG_TBSL_B141	Clone 110S; AB576167 (96%)
		PNG_TBSL_B33	Clone pLM5B-22; AB247862
		PNG_TBR_B5	Clone H1.43.f; AF005749
	Cyanobacteria	PNG_TBV4_B22	*Synechococcus* sp. CC9605; CP000110 (99%)
		PNG_TBV4_B55	Clone HOT157_125m67; JN166246 (99%)
	Bacteroidetes	PNG_TBV4_B48	Clone TKTMmvp-B7; AB611535 (99%)
		PNG_TBV4_B49	Clone B1-78; FJ175516 (89%)
		PNG_TBR_B79	Clone VHS-B5-17; DQ395037
		PNG_TBG_B39	Clone Hyd24-40; AJ535257
	Planctomycetes	PNG_TBR_B32	Hot spring clone PK60; AY555789
		PNG_TBG_B85	Lost City clone LC1446B-77; DQ270660
	Alpha proteobacteria	PNG_TBSL_B6	*Albidivulum* sp. S1K1; FJ222605 (96%)
		PNG_TBV4_B12	Clone PNG_Kap3_B99; JF935205 (80%)
		PNG_TBG_B27	
		PNG_TBR_B49	
	Beta proteobacteria	PNG_TBSL_B73	Clone PNG_War_B31; JF935224
		PNG_TBV4_B4	
	Gamma proteobacteria	PNG_TBR_B16	*Methylothermus subterraneus*; AB536747
		PNG_TBG_B35	Clone CLEAR-1; AF146236 (93%)
		PNG_TBSL_B118	*Methylothermus thermalis*; AB536747 (88%)
		PNG_TBV4_B9	Clone HF0010_01E20; GU474841 (99%)
	Delta proteobacteria	PNG_TBV4_B47	Clone UHAS6; JN037982 (99%)
	Zeta proteobacteria	PNG_TBSL_B14	Clone ELSC-TVG13-B2, GU220734 (90%)
		PNG_TBV4_B7	
	Caldiarchaeum	PNG_TBR_A27	*Caldiarchaeum subterraneum*/PNG_TB_4A2.5H4; EF100631
	Uncult. Desulfurococcales	PNG_TBV4_A79	YNP_SBC_BP2B_A1/PNG_TB_4B140H1_A057; HM448084/ GU137388
	Uncult. Thermoproteales	PNG_TBSL_A42	Clone PNG_TB_4A2.5H2_A64; EF100627
	Uncultured Crenarchaea	PNG_TBV4_A10	Clone PNG_TB_4B300H1_A036; GU137394
	Nitrosocaldus	PNG_TBV4_A60	Clone PNG_TB_4B300H1_A019; GU137393 (99%)
		PNG_TBSL_A14	Clone PNG_TB_4B300H1_A019; GU137393 (99%)
		PNG_TBR_A17	Clone PNG_TB_4B300H1_A002; GU137390
		PNG_TBG_A52	Clone PNG_TB_4B60H1_A060; GU137378
	Uncultured Marine Gp. I	PNG_TBG_A20	Clone PNG_TB_4B7.5H1_A059; GU137360
	Uncult. Euryarchaeota	PNG_TBV4_A15	Clone HF10_90C09; DQ156400 (99%)
	Korarchaeota	PNG_TBV4_A25	Clone PNG_TB_4B140H1_A053; EF100633 (99%)
aroA-like			
	N/A	PNG_TBSL_aroA1	YNP clone PS-1j; DQ380691 (78%)
	N/A	PNG_TBR_aroA19	Beta proteobacterium NT-14, aoxB; DQ412672 (71%)
	N/A	PNG_TBR_aroA12	Clone ZSAOX30, aroA, HQ316547 (82%)
	N/A	PNG_TBR_aroA11	Clone LYC4 arsenite oxidase, aroA, DQ412689 (74%)
	N/A	PNG_TBR_aroA3	*Thermus thermophilus* HB8 (71%)
	N/A	PNG_TBR_aroA4	*Pyrobaculum arsenaticum* DSM 13514, (71%)
	N/A	PNG_TB_4A05_2.5H4_aroA14	*Hydrogenovirga* sp. 128-5-R1-1, (78%)
	N/A	PNG_TB_4A05_2.5H1_aroA28	Clone 22 AC, HQ449590 (74–80%)
	N/A	PNG_TB_4A05_2.5H1_aroA12	Strain NT-26, AAR05656 (80%)
	N/A	PNG_TB_4A05_2.5H1_aroA21	Uncultured *Agrobacterium*, e.g. FJ151051 (80%)
arsC			
	N/A	PNG_TBSL_arsC1	*Bacillus subtilis* (99%)
	N/A	PNG_TBSL_arsC7	Uncultured bacterium, clone AS-P9-F9 (74%)
	N/A	PNG_TBR_arsC6	No direct match in BLAST

[a] Sediment cored from transect 4A, as reported in Meyer-Dombard et al. (2012). Sample name indicates transect (4A), year of collection (2005), distance from vent 4 (2.5 m), and depth within retrieved core (H1 = 3–7 cm, H4 = 33–37 cm).

microscope slides share several taxa with the vent fluid community that are not found in the other biofilm communities, such as the beta-, and zeta-Proteobacteria. The dominant bacterial taxa in the slide clone libraries are hyperthermophilic *Persephonella* (Aquificales) relatives, which are also found in smaller proportions in both the vent and rust biofilm communities. Of particular interest, in the context of primary colonizers in this ecosystem, is the presence of the zeta-Proteobacteria that colonized the slide surfaces. The role that these organisms may play in this ecosystem is discussed further below, in Sections 4.2 and 4.3. The zeta-Proteobacteria in the vent and colonized slide communities appear to be unique when compared to other cloned and cultured representatives (Fig. 3). The archaeal community found on the colonized slides is overwhelmingly composed of *Nitrosocaldus*-like organisms, a group of recently described thermophilic ammonia oxidizers (de la Torre et al., 2008). The presence of these Archaea is suggestive with respect to nitrogen cycling in the Tutum Bay HTV, and may indicate potential for hydrothermal nitrification in these systems. These organisms also dominated the vent fluid and rust colored biofilm clone libraries, which either indicates that they are highly

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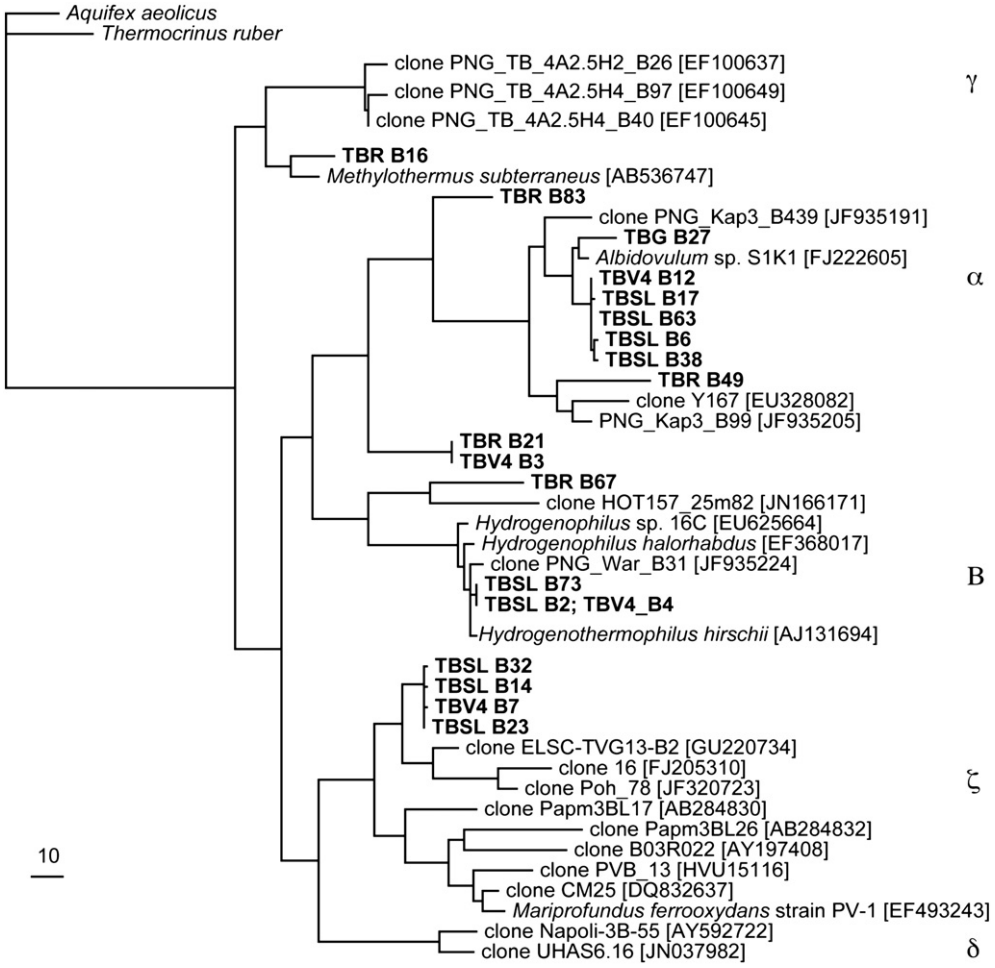

Fig. 3. Phylogenetic analysis of Proteobacterial clones, as identified by 16S rRNA surveys, for vent fluid and biofilm samples. Shown is a neighbor joining tree, built from nt sequences of the 16S rRNA gene — maximum parsimony and maximum likelihood trees resulted in similar topologies. Bootstrap values indicate 100–1000 replicates. Rust colored biofilm (TBR), green colored biofilm (TBG), slide biofilm (TBSL), and vent fluid (TBV4) clones are shown in bold type, with abbreviated clone names, along with closest relatives as identified by BLAST analysis, including 16S rRNA clones from previous PNG reports. GenBank accession numbers are JN881568-JN881687.

competitive in this ecosystem, or there is methodological bias affecting the results. The likelihood of systematic extraction or PCR-based bias diminishes when considering the DNA extraction techniques were different between the vent, biofilm, and sediment samples, and several primer sets were used (see Section 2.4).

If the colonized slide surfaces represent freshly formed biofilms, then the rust colored biofilms may represent a "mature" state of these biofilms. The rust colored biofilms were the most diverse of all studied, having the highest richness and evenness. Here, we see the presence (although in small percentages in the clone library) of other thermophilic and hyperthermophilic bacterial taxa, such as Thermotogales, Thermales, Chloroflexales, and the aforementioned *Persephonella*. There are also a greater diversity of a well-known biofilm forming aquatic organisms, such as varieties of Planctomycetes, Firmicutes, and Bacteroidetes. As outlined above, the archaeal clone library for the rust colored biofilms is largely composed of *Nitrosocaldus* relatives, however, nearly 20% of the library identified with *Caldiarchaeum*-related organisms. Both *Nitrosocaldus* and *Caldiarchaeum* are genera within the newly proposed Thaumarchaeota, and *Caldiarchaeum* is thought to be a thermophilic chemoautotroph with a carbon fixation scheme similar to that of *Pyrobaculum* (Nunoura et al., 2010).

Lastly, the green colored biofilm may represent areas surrounding the immediate vent source that receive less direct, or more intermediate, hydrothermal influence. The deep green color, reminiscent of algal chlorophyll pigmentation, may in fact be due to Eukaryotic members of the community (which we did not examine in this report). The bacterial diversity was low relative to the vent fluid and rust colored biofilms, and was dominated by alpha-Proteobacteria, with other major members represented by miscellaneous uncultured Bacteria, gamma-Proteobacteria, and Planctomycetes. No obvious thermophilic bacterial strains were identified in the green biofilm. In addition, the archaeal community was unique compared to other samples surveyed, being dominated by uncultured "Marine Gp.I" members. Like the other samples, the *Nitrosocaldus*-relatives were also present in the green colored biofilms.

4.2. Potential for iron and arsenic biogeochemical cycling in Tutum Bay

Previous reports have documented the chemistry of the Tutum Bay vent fluids (Pichler and Dix, 1996; Pichler et al., 1999b; Price et al., 2007; Meyer-Dombard et al., 2012), as well as the mineralogy of materials and sediments surrounding the vents (Pichler et al., 2006; Pichler and Veizer, 1999a; Pichler et al., 1999d; Price and Pichler, 2005). It has thus been demonstrated that the vent fluids contain ~1 mg/L Fe^{II} and ~900 μg/L As^{III}, and that HFOs (2-line ferrihydrite) with >7 wt.% adsorbed arsenate dominate the sediments for hundreds of meters around the vents, with higher concentrations nearest the vent source (Price and Pichler, 2005; Price et al., 2007; Meyer-

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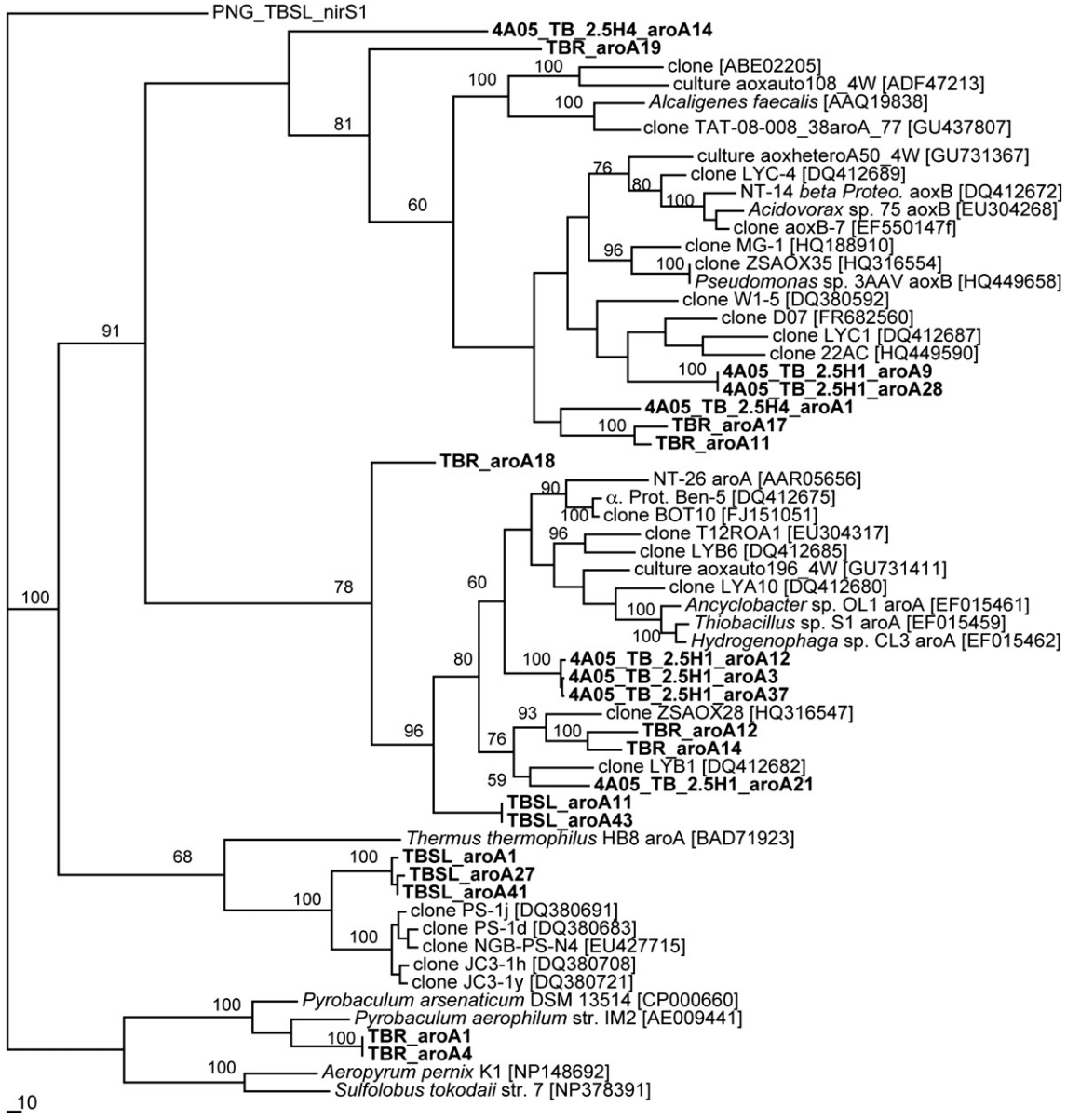

Fig. 4. Phylogenetic analysis of arsenite oxidase (*aroA*-like) genes. Shown is a neighbor joining tree, built from alignment of amino acid residues (other tree methods and nt comparisons revealed similar topologies). Bootstrap values indicate 100–1000 replicates. Rust colored biofilm (TBR), slide biofilm (TBSL), and sediment profile (4A05_2.5H1 and H4) clones are shown in bold type, with abbreviated clone names, along with closest relatives as identified by BLAST analysis. GenBank accession numbers JN881688-JN881715.

Dombard et al., 2012). In contrast, pore fluids in sediments 2.5 m from Vent 4 contain only 0.02 mg/L Fe^{II} and ~100–600 μg/L arsenic, primarily as As^{V} and total iron in the sediments drops by an order of magnitude within the first 1 m from the vent source (Price and Pichler, 2005; Meyer-Dombard et al., 2012). These previously reported data demonstrate that reduced iron is quickly oxidized and precipitated near the source fluids, and that while As^{III} may be sorbed onto the HFO precipitates, there is also possibility that As^{V} is released in the sediments. However, the role of microorganisms in these processes has only been speculative (Akerman et al., 2011; Meyer-Dombard et al., 2012).

The presence of the zeta-Proteobacteria in the Vent 4 fluids and the colonized slide community strongly suggests that these organisms have a direct role in the oxidation of Fe^{III} from the vent fluids, and potentially the precipitation of the HFOs that coat the sediments, coral, and rocks around the vents. While it is typically considered inconclusive to attribute metabolic capacity of environmental clones based on similarity to cultured organisms, to date the zeta-Proteobacteria have been identified primarily in HFO-rich, Fe^{II} venting environments (Emerson and Moyer, 2002; Edwards et al., 2004; Emerson, 2009; Rassa et al., 2009; Sudek et al., 2009; Handley et al., 2010), with one notable exception of nearshore and estuarine environments (McBeth et al., 2011). Examples are various deep-sea HFO/biofilm mats, where it has been shown that the zeta-Proteobacteria actively precipitate the HFOs as a function of their Fe^{II}-oxidizing chemolithotrophic metabolism (Emerson and Moyer, 2002; Emerson et al., 2007; Rassa et al., 2009). While these organisms are typically found in deep-sea hydrothermal vent systems, there has been one other report identifying their involvement in iron cycling in a shallow-sea hydrothermal vent system similar to Tutum Bay (Handley et al., 2010). It is therefore reasonable to implicate the zeta-Proteobacteria found in the freshly deposited biofilms on the colonized slides and in the vent fluid with the role of precipitation of HFOs via the oxidation of Fe^{II} from the vent fluid. Indeed, the oxidation of Fe^{II} in porewaters from the Vent 4 area is energetically favorable for both aerobic and anaerobic organisms (Akerman

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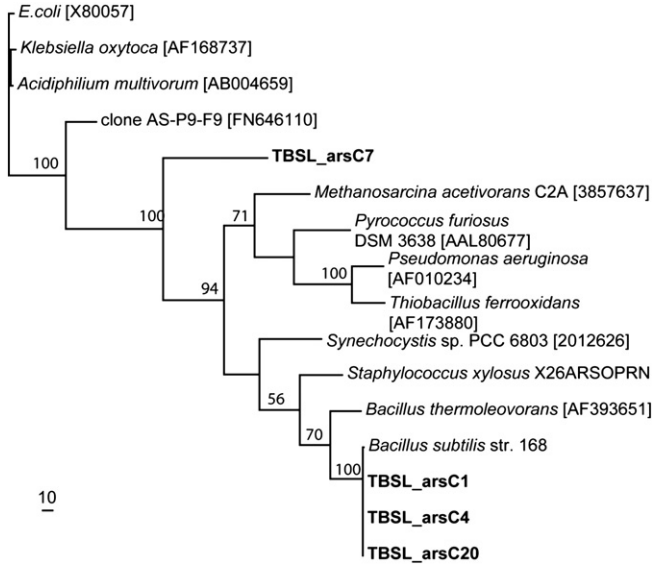

Fig. 5. Phylogenetic analysis of arsenate reductase (*arsC*) genes. Shown is a neighbor joining tree, built from alignment of amino acid residues (other tree methods and nt comparisons revealed similar topologies). Bootstrap values indicate 100–1000 replicates. Slide biofilm (TBSL) clones are shown in bold type, with abbreviated clone names, along with closest relatives as identified by BLAST analysis. Genbank accession numbers JN881716-JN881723.

et al., 2011). The zeta-Proteobacteria were not found in the rust or green colored biofilms, potentially indicating that this role is restricted to the initial colonization of biofilms around the vents. This hypothesis is further supported by the lack of zeta-Proteobacteria in sediment profiles in transects extending away from the vents and low Fe^{II} in sediment pore waters (Akerman, 2009; Meyer-Dombard et al., 2012).

It has been previously proposed that the more labile As^{III} is immobilized in this marine ecosystem by oxidation to As^{V} and adsorption onto the HFO surfaces (Pichler et al., 1999c). Previously, it was unknown if the vent communities had the capacity to oxidize arsenite in these sytems. Here, we show that the rust colored and colonized slide biofilms and two sediment communities from a previously investigated sediment core have the genetic capacity for arsenite oxidation. PCR amplicons were obtained using degenerate primer sets designed to amplify arsenite oxidase genes (*aroA*-like genes) from hydrothermal environmental samples. Subsequent sequencing of those amplicons shows arsenite oxidases related to those of various alpha-Proteobacteria, cultured and uncultured representatives from continental hydrothermal systems (Inskeep et al., 2007), and possibly *Pyrobaculum arsenaticum* (Table 2 and Fig. 4). Phylogenetic analysis confirms that *aroA*-like genes are novel compared to other *aroA*-like genes, including those from other hydrothermal systems (Inskeep et al., 2007), and have only 71–80% sequence similarity with other *aroA*-like genes in GenBank (Fig. 4 and Table 2). This indicates that the Tutum Bay community may have a unique arsenite oxidase gene. Akerman et al. (2011) found that a geochemical mixing model of hydrothermal vent fluid and seawater did not account for the As^{III}:As^{V} ratios in pore fluids near Vent 4, consistent with the possibility that there is substantial microbial oxidation of arsenic (Akerman et al., 2011). In addition, arsenite oxidation in this system is calculated to yield 30–80 kJ mol^{-1} e^{-} of energy transferred—sufficient potential energy for microbial metabolism (Akerman et al., 2011). While the presence of a functional gene does not guarantee activity of that gene, the genetic capacity for arsenite oxidation and the supporting geochemical evidence that microorganisms can obtain energy by mediating arsenite oxidation, make a compelling argument that biofilm and sediment communities are actively removing arsenic from this marine ecosystem by oxidizing As^{III} to As^{V} with subsequent sorption of As^{V} to the HFOs present in the venting areas.

We found no evidence that the As^{V} could be remobilized from the HFO via reduction by respiring organisms (e.g. dissimilatory arsentate reduction), using the primer sets indicated in Table 1, as found in other environments (Langner and Inskeep, 2000; Zobrist et al., 2000; Handley et al., 2010). However, the potential for remobilization as a result of arsenate reduction by microbial detoxification processes does exist. Surveys for the *arsC* gene in the rust colored and slide biofilms revealed that the biofilms have the genetic capacity for arsenate reduction as a defense mechanism. The same primer sets did not produce amplicons from the sediment community DNA (Table 1). The *arsC* operon is found in widespread bacterial and archaeal genera, but may be confined to aerobic environments (Jackson and Dugas, 2003). A BLAST search of the *arsC* PCR products revealed that the closest match is to an arsenate reductase from *Bacillus*; clones from the rust colored biofilm did not match any known sequences in GenBank. The distant relationship of the *aroA* and *arsC* clones to GenBank sequences suggests that many of the Tutum Bay arsenic cycle functional genes are unique. In addition, the lack of amplification using the *arrA* primers might simply suggest that the primer sets were not a close enough match to the communities in Tutum Bay; additional attempts with revised primer sets may yet show dissimilatory arsenate reduction capability.

4.3. Comparison with other reports of arsenic cycling in hydrothermal environments

Previous descriptions of microbial diversity in the Tutum Bay area have been reported. These include sediment samples along two profiles extending from the Vent 4 source, and sediments from on-land systems on the nearby Ambitle Island (4.08°S, 153.62°) (Akerman, 2009; Amend et al., 2012; Meyer-Dombard et al., 2012). The Vent 4 area biofilm and fluid communities share some common members with the broader Tutum Bay/Ambitle Island hydrothermal ecosystem (Table 2), including previously identified clones of uncultured *Planctomycetes*, *Thermus*, *Thermotoga*, *Persephonella*, *Nitrosocaldus*, *Caldiarchaeum*, and *Korarchaeota*, to name a few. In addition, there is similarity in the order-level diversity and richness of these related ecosystems, although proportions of each OTU in the clone libraries are dissimilar. These previous reports did not identify the key iron oxidizers, the zeta-Proteobacteria, again supporting the evidence that these may be primary colonizers and aren't prevalent in mature biofilms or sediment communities.

One other report has also investigated iron and arsenic cycling in a shallow-sea hydrothermal system. Handley et al. (2010) investigated ferruginous sediments in the Santorini, Greece hydrothermal vent system, via 16S rRNA and RISA surveys and enrichment culturing of sediments retrieved by push cores. These hydrothermal systems are of lower temperature (20–40 °C) than the Tutum Bay systems reported here, and cultures were incubated at 25 °C. The authors identified *Mariprofundus ferroxidans* relatives in the near surface sediments, by 16S rRNA survey. They were also able to culture relatives of *M. ferroxidans* in an anaerobic, Fe^{II} enriched growth media — previously cultured strains of *M. ferroxidans* are microaerobes. Potential for arsenic cycling was demonstrated by the culturing of a *Marinobacter koreensis*-like organism [a gamma-Proteobacterium], in both As^{V} and As^{III} enriched growth media, indicating that this organism is capable of both dissimilatory arsenate reduction and arsenite oxidation (see also: Richey et al., 2009). There is one other known report of demonstrated arsenic metabolism in a marine hydrothermal setting, in the deep sea vent of the Suiyo Seamount (Takai et al., 2003). This trio of reports points to the potential for large scale influence of deep- and shallow-sea HTV in iron-arsenic biogeochemical cycling.

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Perhaps the best studied example of iron-arsenic cycling in a hydrothermal system is a continental hot spring in Yellowstone National Park. It has been shown in the acidic (pH 3.1) hot springs of Norris Geyser Basin (NGB), that microbially-mediated As^{III} oxidation can be rapid in hydrothermal systems. In fact, the fastest arsenic oxidation rate in any natural aquatic system was recorded in one Norris spring (NHSP 106) (Jackson et al., 2001; Inskeep et al., 2004). Unlike the Tutum Bay hydrothermal system, in the NGB springs, arsenite oxidation, ferrihydrite precipitation, and biogenesis of arsenate-rich HFO mats appear to be directly influenced by the presence of sulfide and its consumption by archaeal and bacterial taxa (Donahoe-Christiansen et al., 2004; Inskeep et al., 2004; Macur et al., 2004). In Fig. 4, it can be seen that several of the Tutum Bay colonized slide *aroA* sequences are most closely related to *aroA* clones from the NGB (e.g. clone JC3-1y; DQ380721), sharing however, only a 78% similarity in nucleotide sequence. The NGB arsenite oxidation has been attributed to species of *Thiomonas, Hydrogenobaculum* (Macur et al., 2004), and *Thermus* (Inskeep et al., 2007), while Tutum Bay arsenite oxidase genes are related to those from alpha- and beta-Proteobacteria, *Thermus*, and *Pyrobaculum*. There is therefore, little similarity in the capability for arsenite oxidation in these two hydrothermal systems.

5. Conclusions

The Vent 4 fluid and immediately surrounding *in situ* and induced biofilms are shown here to have distinct microbial communities. Further, there is a clear relationship between the vent fluid, the freshly colonized biofilms, and the more mature biofilms of indeterminate age. We identified a promising candidate for microbially mediated iron oxidation at the venting area, relatives of the zeta-Proteobacteria, which are found in the vent fluid and colonized slide biofilms, but not in the mature biofilms or previously reported sediment communities. Our results have facilitated the development of a preliminary model of iron-arsenic biogeochemical cycling in the Tutum Bay vent field. We propose that the zeta-Proteobacteria relatives from the vent fluid and fresh biofilms near the vent oxidize the Fe^{II}, and are likely directly responsible for the precipitation of the HFOs in the venting area. Unidentified relatives of alpha-Proteobacteria, beta-Proteobacteria, *Thermus*, and *Pyrobaculum* are genetically capable of arsenite oxidation, using a version of arsenite oxidase (*aroA*, *aoxB*, or *asoA*), and produce As^{V}. This is adsorbed onto the HFOs; active vs. passive involvement in the adsorbtion is still unknown. *Bacillus* relatives and other unidentified taxa within the biofilms (but not the sediment communities) are capable of releasing As^{V} from the HFOs, as part of a detoxification process, using a putative *arsC* genetic system. Future work using enrichment culture methods will aim to reveal the identity of the arsenite oxidizers and potential reducers in these systems.

To date, this is the only direct genetic-based evidence of arsenic cycling in a shallow submarine hydrothermal system, showing the genetic capacity for arsenic cycling in relevant communities. The evidence given here is complimentary to culture-based approaches reported previously in the Santorini, Greece hydrothermal field (Handley et al., 2010). Further, this is the only report to consider arsenic biogeochemistry in high temperature biofilms in a shallow-sea hydrothermal system. We are confident that additional investigation will further expose the widespread microbiological influence on iron–arsenic cycling in shallow submarine hydrothermal systems.

Acknowledgments

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