MICROBIOLOGY OF SOME SULFIDIC HOT SPRINGS IN THE PHILIPPINES

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ABSTRACT

This paper surveys the results of our investigation of the microbial diversity and biogeochemistry of some Philippine hot springs. The thermophilic microbial communities inhabiting these springs are of extreme interest for their unique value as potential analogues for Earth's earliest life. Anaerobic, acidic, sulfidic, thermal springs represent potentially important settings for the earliest organisms on Earth, and the chemolithotrophs and phototrophs inhabiting them are possible candidates for Earth's earliest sustainable microbial communities. Thermostable enzymes from these thermophilic microbial communities are also of potential biotechnological value, specifically for medicinal and industrial use, hence there is also some practical interest in documenting and characterizing these communities.

We are presently examining the connection between biogeochemical cycling of sulfur, carbon and other nutrients and the diversity of metabolic activities in the thermophilic autotrophic communities from several hot springs historically monitored by the Philippine National Oil Company-Energy Development Corporation (PNOC-EDC). We will highlight one particular locality in detail (Lipayo #1, Negros Oriental, Philippines), where we have shown that the gradient of pO_2, pH and T is accompanied by consistent changes in the microbial community composition from those dominated by anaerobic, acidophilic and S oxidizing bacteria (e.g., Desulfurella multipotens, Hydrogenobacter acidophilus, Aquificales) to those dominated by cyanobacteria (e.g., Microcystis aerugosa), Cyanidium caldarium (red algae), Deinococcus geothermalis within a 10-meter flow interval. The same interval showed a monotonic decline of H_2S and increase in $SO_4^{=}$, fluctuation in $S_2O_3^{=}$, and no significant shift in $\delta^{34}S$ of either H_2S or $SO_4^{=}$. Despite the predominance of oxygenic photosynthesizers (i.e., cyanobacteria) in these sampled hot springs, the relative values of $\delta^{13}C$ of dissolved inorganic carbon, bulk biomass, and component fatty acids near the hot spring vent area are consistent with the significant presence of organisms that fix carbon using the reverse tricarboxylic acid cycle in these vents.

The authors dedicate this paper to the memory of Prof. Roger Datuin, a great mentor and friend.

1.0 INTRODUCTION

The Philippine archipelago is endowed with a tremendous diversity of active hot spring environments owing to its active island arc setting (Datuin, 1982; Datuin and Troncales, 1986; Malapitan and Reyes, 2000). Among the thermophilic environments now fully documented in these hot springs localities are anaerobic, acidic, sulfidic, thermophilic springs (AASTS) (Abrajano et al., 2004). Geochemical conditions exemplified by AASTS are not common in modern environments, but they are thought to represent important and prevalent habitats for the earliest organisms on Earth. It is this analogy that motivated the present study of microbial communities in Philippine AASTS environments. In the AASTS microbial communities described in this paper, we surmise that a very limited "geochemical reservoir" (e.g., biofilm) appears sufficient in sustaining the metabolic requirements of the anaerobic thermophilic microbiota. This strategy may well reflect an attempt by these microbial communities to buffer the effects of the present oxygenated atmosphere and the gradual or episodic geochemical changes in the effluent waters. Hence, future investigations of the interplay between abiotic and biotic controls in these systems must focus on quantification of biotic processes at the biofilm scale.

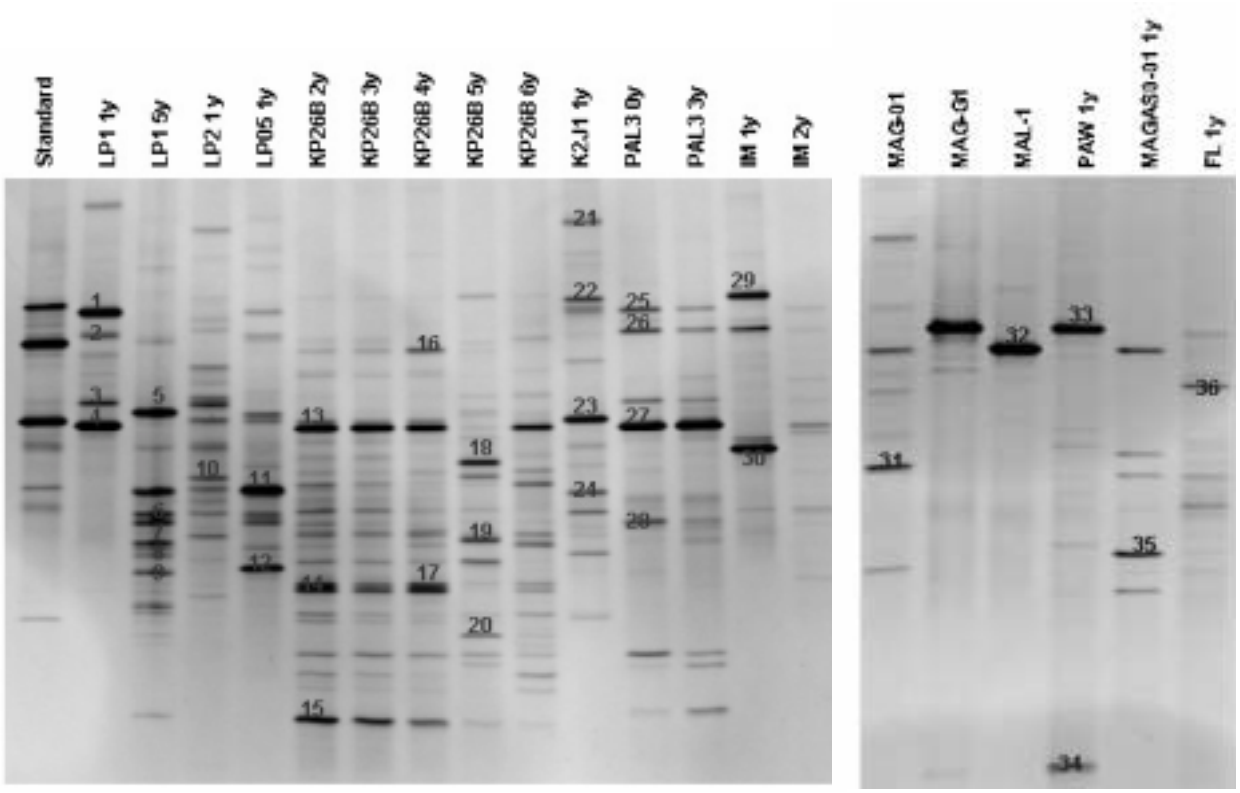

Figure 1. DGGE gel image of amplimers from a conserved region of bacterial 16S rDNA. Banding patterns and relative intensities of the recovered bands provide a measure of differences among the communities. Dominant species must constitute at least 1-2% of the total bacterial community to form a visible band. Labeled bands were excised and sequenced. Results from sequencing can be found in Table 1.

2.0 SAMPLED LOCALITIES AND METHODS

Numerous hot spring localities from Tongonan (Leyte), Palinpinon (Negros Oriental), Bac-Man (Sorsogon), Mt. Apo (North Cotabato), and Northern Negros (Negros Occidental) were visited and sampled in 2001 as part of a collaborative effort between Rensselaer Polytechnic Institute (RPI) and PNOC-EDC. The geology of these geothermal localities was recently summarized by Malapitan and Reyes (2000), who pointed out that all these fields are associated with Quaternary to Recent volcanism.

International transport and import of collected microbial specimens to the US were conducted with all necessary USDA permit. The collaboration between RPI and PNOC-EDC dates back to 1999, when several hot spring localities Tongonan and Palinpinon were sampled for preliminary microbial characterization, but only the bacterial communities from a more extensive sampling campaign in 2001 are described in the present paper (Fig. 1, Table 1). The sampling sites are labeled "**LP**" for Lipayo (Palinpinon), "**KP**" for Kapakuhan (Tongonan), "**IM**" for Inang Maharang (Bac-Man), "**MA**" – for Mag-aso (Palinpinon), "**MAL**" for Malaunay (Tongonan), "**PAW**" for Pawa (Bac-Man) and "**FL**" Flortam (Mt. Apo). For each site, a wide array of field measurements was also conducted including temperature, pH, dissolved oxygen, and sulfur speciation. Water samples were likewise collected for each sampling location. Each site was also carefully photo documented including the exact location of sampling points in the spring. Microbial samples for 16S rRNA gene analysis were collected as mini-cores and bulk (using sterilized spatula into sterile cryovials), wrapped in foil, immediately frozen, transferred to sealed vials, and stored in dry ice for transport.

Samples for sulfur speciation were collected using a resin-collection technique recently described by Schoonen and co-workers

Table 1. Sequence results from bands excised from Figure 1. Identifications and percent matches are based upon GenBank database.

Band	Closest Match	Percent Match	Phylogenetic Affiliation
1LP1	Novel Sequence	-	Affiliated with Chloroplasts
2LP1	Unsequenceable	-	-
3LP1	Desulfurella multipotens	97%	δ Proteobacteria purple
4LP1	Hydrogenobacter acidophilus(DB)	93%	eubacteria
5 LP5	Cyanidium caldarium	95%	Affiliated with Chloroplasts (most thermophilic known algae) Red Algae
6 LP5	Thiobacillus Acidobacter	96%	DProteobacter S/Fe oxidizer
7 LP5	Deinococcus geothermalis	96%	Thermus/Deinococcus group
8 LP5	Aquifex	97%	-
9 LP5	? Proteobacteria	96%	-
10	Failed	-	-
11	Microcystis aeruginosa	91%	Cyanobacteria
12	Thiomonas sp.	95%	Beta Proteobacteria
13	Synechococcus sp.	93%	Cyanobacteria
14	Thermus silvanus sp./ Meiothermus silvanus sp.	100%	Thermus/Deinococcus group
15	Novel Sequence	-	Affiliated with *Chlorobium tepidum*
16	Novel Sequence	-	Affiliated with Cytophaga-Flexibacter-Bacteroides (CFB), GSB phylogenetic link
17	Thermus silvanus sp./ Meiothermus silvanus sp.	97%	Thermus/Deinococcus group
18	Novel Sequence	-	Affiliated with Aquificales
19	Novel Sequence	-	Affiliated with Aquificales
20	Failed	-	-
21	Novel Sequence	-	Affiliated with unclassified anaerobic bacterium
22	Failed	-	-
23	Pleurocapsa sp.	97%	Cyanobacteria
24	Failed	-	-
25	Chlorogloeopsis	96%	Cyanobacteria
26	Oscillatoria sancta	96%	Cyanobacteria
27	Uncultured cyanobacteria	88%	Cyanobacteria
28	Failed	-	-
29	Novel sequence	-	-
30	Novel sequence	-	-
31	Microcoleus sp.		Cyanobacteria
32	Stanieria cyanosphaera	97%	Cyanobacteria
33	Acidimicrobium ferrooxidans	99%	Firmicutes
34	Failed	-	
35	Synechococcus sp.	99%	Cyanobacteria
36	Failed	-	-

(Schoonen, pers. comm.). Briefly, the sampling procedure involves stabilization of H_2S by sulfide precipitation with $ZnCl_2$ (although field measurement of H_2S concentrations will also be made) and field collection of water in 5-60 ml sterile plastic syringes, filtration through 0.45 μm filter, passing the filtrate through an anion-exchange resin Bio-Rad™ AG1-X8 at 3 mL/minute and flushing the remaining solution in the column with nanopure water. In the laboratory, sequential elution of SO_4^{2-}, $S_2O_3^{2-}$ and $S_xO_6^{2-}$ with 0.1 M KCl, 0.5 M KCl and 9 M HCl, respectively, using a multi-channel peristaltic pump were performed. Quantitation was achieved using an HPLC.

Bacterial lipids from mat samples were extracted using ultrapure chloroform and methanol in a simplification of the Bligh and Dyer (1959) procedure (Abrajano et al., 1994). Fatty acid methyl esters (FAME) were produced from methonolysis products by transesterification using boron trifluoride in methanol. FAMEs were analyzed using a gas chromatograph equipped with FID or MS detectors. $\delta^{34}S$ and $\delta^{13}C$ (from CO_2 collected by acidifying water collected into 10 ml vacutainers) analyses were performed using continuous flow isotope ratio mass spectrometry. The isotopic ratios of individual fatty acid biomarkers were determined using a Micromass Optima system coupled to a Hewlett Packard 6890 gas chromatogram with electronic pressure controller (after correction for derivatizing BF3-methanol). Results are reported in standard δ notation: $\delta = (R_S/R_{std} - 1)1000$ where R represents the ratio $^{34}S/^{32}S$ or $13C/^{12}C$, and the subscripts $_S$ and $_{std}$ refer to the sample and standard respectively.

As a way to detect and identify organisms from a whole community of organisms, a technique called denaturing gradient gel electrophoresis (DGGE) analysis is used (Muyzer et al., 1993). The DGGE approach directly determines the species composition of complex microbial assemblages based on the amplification of conserved gene sequences (16S rDNA fragments for prokaryotes, 18S or 28S rDNA for eukaryotes). In DGGE analysis, differences in gene sequences among organisms allow DNA from various organisms to be physically separated in a denaturing gradient gel, thereby allowing one to generate profiles of numerically dominant bacterial community members for a sample. The profiles are visible as bands (or lines) in a gel (Fig. 1). The banding patterns and relative intensities of the bands provide a measure of difference among the communities. Gel bands from dominant species, which constitute at least 1% of the total bacterial community, can be excised and sequenced. Sequence analysis of individual bands is used to infer the identity of the source organism based on database searches and phylogenetic methods. Phylogenetic affiliations are determined by comparing the DNA sequences retrieved from samples to DNA sequences of known bacterial sequences in national databases, such as the RDP or GenBank). Nucleic acid extraction was performed using a bead-beating method (Stephen et al., 1999). PCR amplification of 16S rRNA gene fragments was performed as described in Muyzer *et al*. with modifications (1). Thermocycling consisted of 35 cycles of 92°C for 45 sec., 55°C for 30 sec., and 68°C for 45 sec. Using 1.25 units of Expand High Fidelity polymerase and 10 pmole each primer (forward primer contained a 40 bp GC-clamp) in a total volume of 25 μL, thermocycling was performed using a "Robocycler™" PCR block. The primers targeted eubacterial 16S rDNA regions corresponding to *E. coli* positions 341-534. A portion (20%) of each PCR product was analyzed by agarose gel electrophoresis (1.5% agarose, 1x TAE buffer) and ethidium bromide fluorescence. DGGE employed a D-Code 16/16 cm gel system maintained at a constant temperature of 60°C in 6L of 0.5 x TAE buffer (20mM Tris actate, 0.5mM EDTA, pH 8.0). Gels were electrophoresed at 35V for 16 hr. Gels were stained with ethidium bromide (0.5 mg/L) and destained twice in 0.5 x TAE for 15 min. each. Gel images were captured using an Alpha Imager™ system (Fig. 1). Sequence identifications were performed using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/Blast) and the "Sequence Match" facility of the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.html).

3.0 RESULTS AND DISCUSSION

3.1 Microbial Communities

The LP samples (Fig. 2) showed noticeable changes within the bacterial communities as a function of distance from the spring vent. Four bands were excised from the sample closest to the vent (LP1 1y), of which three produced useable sequences. Band 1 represented a rather novel organism which was associated with two uncultured and unpublished bacteria; uncultured proteobacterium V5 (GenBank

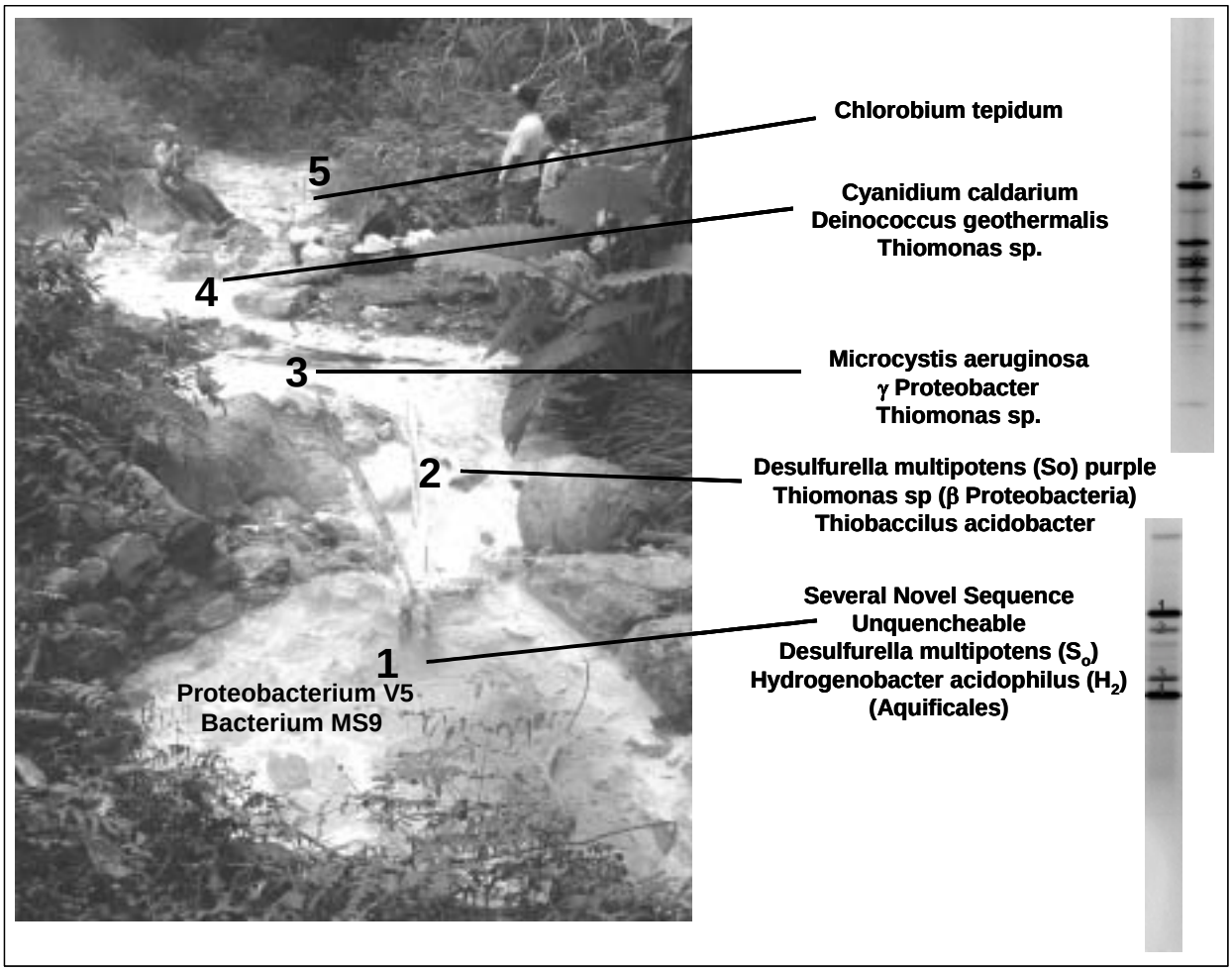

Figure 2. Microbial diversity and distribution in Lipayo #1 (Palinpinon, Negros Oriental). Examples of DGGE gel image of amplimers from a conserved region of bacterial 16S rDNA for sampling locations 1y and 5y are shown. More details on the organisms identified can be found in Table 1.)

Accession No. AF339745) and bacterium MS9 (AF232923). Proteobacterium V5 was isolated from acidic, saline waters of Vulcano (in the Aeolian Islands) whereas bacterium MS9 was found in acidic, geothermal springs of Montserrat. Band 3 was related to the genus *Desulfurella*, which is a sulfur-respiring thermophilic bacteria that has also been found in other thermal environments. Band 4 was related to *Hydrogenobacter acidophilus* which is a thermoacidophilic, obligately chemolithoautotrophic, hydrogen-oxidizing bacteria. The bacterial profile for sample LP2 1y immediately down flow from LP1 1y appeared to have one band, which aligned with band 3 from LP1. One additional band from LP2 1y was cut but failed to produce a useable sequence. Two bands were sequenced from the effluent LP5 (bands 11 and 12) of which band 11 also appeared to represent a rather novel organism. Band 11 was loosely associated with the cyanobacteria group whereas band 12 showed affiliation with the genus *Thiomonas.* A faint band approximating Band 15 (see below) is also apparent in LP1 5y, suggesting the presence of *Chlorobium tepidum*.

Six samples were collected from Kapakuhan (KP26B 2y – 6y, and K2-J1 1y). Comparison of the bacterial community from KP26B shows similar community structure in all but the sample closest to the spring effluent (KP26B 5y). Bands 13, 14, 15, and 17 were dominant in all but the 5y sample. Band 13 was closely related to the genus *Synechococcus*. Members of the species *Synechococcus* are found in planktonic cyanobacterial blooms, which are common in freshwater bodies, especially where eutrophic conditions exist. Band 15 represented a novel organism which was loosely associated with a

cloned sequence obtained from the thermophilic green sulfur bacterium *Chlorobium tepidum*. Band 16 also represented a rather novel organism but was more loosely affiliated with uncultured *cytophagales* found in Yellowstone Hot Springs. Bands 14 and 17 were affiliated with *Meiothermus silvanus* which are found wide spread in hot aqueous environments. Within the KP26 5y sample, two prominent bands produced useable sequences and were representative of novel organisms. Both bands were loosely associated with the genus *Aquificales.* Samples collected from Palinpinon showed very similar bacterial communities to those in Kapakuhan. Bands 25-28 were excised from the PAL samples and produced a useable sequence from all but band 28. Bands 25 through 27 were all associated with genera within the Cyanobacteria group (see Table 1 for closest matches).

Bacterial profiles from the two samples collected from Inang Maharang (IM 1y and 2y) showed little similarity between one another. Sample 1y contained two dominant bands (29 and 30), which were found to be representative of novel organisms. Band 29 was associated with other novel sequences found in carbon leader ore samples collected from Eat Driefontein gold mine in South Africa (unpublished source). Band 30 also represented a rather novel organism with no close relatives in the available databases. Samples MAG-01, MAL-1 and PAW –1Y contained essentially one dominant band within each profile. Band 33 appeared to be present in both MAG-01 and PAW 1y and was closely related to the genus *Acidimicrobium*. Band 32 isolated from MAL-1 was closely related to the *Stanieria cyanosphaera*, which is classified as a thermophilic cyanobacteria. This band (32) also appeared to be present in sample MAG-01 1y. Sample FL-1y produced a faint banding pattern of which sequence information is most closely matched with *Thermus aquaticus*.

3.2 Microbial Geochemistry

Detailed discussion of C and S geochemistry is the subject of another paper (Abrajano et al., 2004), and only a brief summary is offered here. The dominance of oxygenic photosynthesizers (i.e., cyanobacteria) appears to belie the reduced nature of the sampled hot springs, but we offer several observations that support the importance of anoxygenic metabolism in these systems. Anoxygenic bacterial photosynthesis uses a single photosynthetic reaction center (usually RC-1), and the following overall reaction:

$$0.5\,H_2S + H_2O + CO_2 \rightarrow CH_2O + H^+ + 0.5\,SO_4^{2-} \quad (1)$$

The coupling of C and S cycles represented by reaction 1 will manifest only if the hot spring in consideration is entirely anaerobic. In contrast, oxygenic photosynthesis, which occurs in cyanobacteria and eukaryotic chloroplasts, requires the linkage of two photosynthetic reaction centers (RC-1 and RC-2). This linkage of reaction centers gave primordial cyanobacteria the ability to use H_2O as an electron donor and provided them a distinct advantage over anoxygenic phototrophs (Pierson and Olson, 1989). Oxygenic photosynthesis is described by the reaction:

$$2H_2O + CO_2 \rightarrow CH_2O + H_2O + O_2 \quad (2)$$

The importance of organic fixation represented by reaction 2 in the Philippine hot springs is exemplified by the ubiquity of cyanobacterial species in virtually all the hot springs sampled (Table 1). Nevertheless, the observed occurrence of organisms that autotrophically fix carbon using the reverse tricarboxylic acid cycle also points to their importance in these hot spring systems. We can therefore surmise that large gradients in oxidation states must exist in these systems to support the co-existence of oxygenic and anoxygenic organisms.

We also examined compound-specific C and S isotope signatures in these microbial systems to evaluate the importance of alternate C fixation pathways. Based on expected isotopic fractionations of both S and C stable isotopes, we conclude that microorganisms that fix carbon using the reverse tricarboxylic acid cycle are important in these thermophilic systems, but only on the scale of microbial biofilms (Abrajano et al., 2004). Transformation of S and C species is often accompanied by isotope fractionation effects that in turn provide clues as to the S and C sources, specific pathways utilized or, under favorable conditions, the extent of substrate conversion. For example, dissimilatory sulfate reduction to sulfide results in lowering of $\delta^{34}S$ by an average of 18 ‰ in the product sulfide,

although depletion of ^{34}S by as much as 46 ‰ have been observed (e.g., Canfield and Teske, 1996; Hibicht et al., 1998). Variable degree of isotopic fractionation can also arise from differing degree of substrate (e.g., SO_4) limitation. Thiosulfate oxidation to sulfate or tetrathionate apparently does not result in any measurable sulfur isotope fractionation (Fry et al., 1986), but thiosulfate disproportionation to sulfide and sulfate results in 7 to 10 ‰ variation in either direction. No spatially systematic changes in $\delta^{34}S$ of either H_2S or $SO_4^{=}$ or $\delta^{13}C$ in dissolved inorganic carbon (DIC) in the thermal waters was evident in any of the Philippine hot springs examined thus far, effectively ruling out biological or abiotic overprint of disproportionation and $SO_4^{=}$ reduction in the bulk fluids. Comparison of $\delta^{13}C$ values for DIC and fatty acid methyl esters extracted from the "bulk" microbial biomass, however, implicates the importance of reverse tricarboxylic acid cycle fixation, especially in microbial communities that are closest to the hot spring vents (e.g., Lipayo, Fig. 2) (cf. Abrajano et al., 2004). Specifically, the parameter $\delta^{13}C$ (DIC) - $\delta^{13}C$ (fatty acids) showed values between 7 to 11 ‰ suggesting that a major portion of the thermophilic biomass near the spring effluent utilizes reverse tricarboxylic acid cycle fixation (Abrajano et al., 2004). Fixation dominated by the TCA cycle should have yielded $\delta^{13}C$ (DIC)-$\delta^{13}C$ (fatty acids) values in excess of 15 ‰.

We also modeled the expected spatial stability of various aqueous sulfur species in Lipayo #1 by combining *Geochemical Workbench*® calculations with simplified momentum equations for fluid flow (Mason, unpublished). We solved these equations subject to typical boundary conditions and obtained results that further affirmed that $\delta^{34}S$ values for both H_2S and $SO_4^{=}$ are completely governed by hydrothermal reactions that are unperturbed by thermophilic S metabolism. Finally, the implications of the present work to practical biotechnological issues including "mining" of thermostable enzymes, wastewater treatment and energy production will be discussed.

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