1 Time series analysis of two hydrothermal plumes at 9˚50'N East Pacific Rise reveals distinct,

2 heterogeneous bacterial populations

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4 Jason B Sylvan[1*], Benjamin C Pyenson[2], Olivier Rouxel[3,4], Christopher R German[3] and Katrina J

5 Edwards[1]

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7 [1]University of Southern California, Department of Biological Sciences, Marine

8 Environmental Biology Section, 3616 Trousdale Parkway, Los Angeles, CA 90089

9 [2]Haverford College, 794 College Avenue, Haverford, PA 19041

10 [3]Woods Hole Oceanographic Institution, 266 Woods Hole Rd, Woods Hole, MA 02543

11 [4] Université Européenne de Bretagne, Université de Brest, Institut Universaire Européen de la

12 Mer (IUEM), UMR 6538, Insititut Français de Recherche pour l'Exploitation de la Mer

13 (IFREMER), BP 80 F- 29280 Plouzané, France

14 *email: jsylvan@usc.edu

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16 ABSTRACT

17 We deployed sediment traps adjacent to two active hydrothermal vents at 9°50'N on the

18 East Pacific Rise (EPR) to assess variability in bacterial community structure associated with

19 plume particles on the time scale of weeks to months, to determine if an endemic population of

20 plume microbes exists, and to establish ecological relationships between bacterial populations

21 and vent chemistry. Automated rRNA intergenic spacer analysis (ARISA) indicated there are

22 separate communities at the two different vents and temporal community variations between

23 each vent. Correlation analysis between chemistry and microbiology indicated that shifts in the

24 coarse particulate (>1 mm) Fe/(Fe+Mn+Al), Cu, V, Ca, Al, ^{232}Th, and Ti as well as fine-grained
25 particulate (<1 mm) Fe/(Fe+Mn+Al), Fe, Ca and Co are reflected in shifts in microbial
26 populations. 16S rRNA clone libraries from each trap at three time points revealed a high
27 percentage of Epsilonproteobacteria clones and hyperthermophilic Aquificae. There is a shift
28 towards the end of the experiment to more Gammaproteobacteria and Alphaproteobacteria, many
29 of whom likely participate in Fe and S cycling. The particle attached plume environment is
30 genetically distinct from the surrounding seawater. While work to date in hydrothermal
31 environments has focused on determining the microbial communities on hydrothermal chimneys
32 and the basaltic lavas that form the surrounding seafloor, little comparable data exists on the
33 plume environment that physically and chemically connects them. By employing sediment traps
34 for a time series approach to sampling, we show that bacterial community composition on plume
35 particles changes on time scales much shorter than previously known.
36
37 INTRODUCTION
38 Hydrothermal plumes form at deep-sea hydrothermal vents, where hot hydrothermal
39 fluids are injected into the water column and mix with ambient seawater. The hot, buoyant
40 hydrothermal fluids rise in the water column as they mix with cold, oxygenated seawater until a
41 level of neutral buoyancy is attained, typically after a time scale of ~1 hour and after dilution of
42 the vent-fluid ~10^4:1 by surrounding seawater (Lupton et al., 1985). At this point, neutrally
43 buoyant plumes become dispersed by local deep ocean currents and can readily be detected
44 through elevated particle concentrations as well as elevated hydrothermal constituents, such as
45 ^{3}He, Mn, CH_4 and NH_4^+ over distances spanning several kilometers away from the vent-source
46 (Cowen et al., 1998; Lilley et al., 1995).

47 The microbiology of hydrothermal plumes has received less attention than that of the
48 source fluids emanating from chimneys and active hydrothermal chimneys themselves. Given
49 that the entire volume of the oceans cycles through hydrothermal plumes on timescales of just a
50 few thousand years (comparable, for example, to the timescale for mixing of the entire volume of
51 the deep ocean through thermohaline circulation), this environment warrants greater attention
52 (Elderfield & Schultz, 1996; German & Von Damm, 2004). Cell counts in hydrothermal plumes
53 are consistently higher than background waters (Sunamura et al., 2004). Mn oxidation in
54 hydrothermal plumes is elevated compared with background seawater, up to 2 nM h^{-1} (Cowen et
55 al., 1986; Dick et al., 2009). Oxidation of NH_4^+ and CH_4 is also prevalent in hydrothermal
56 plume environments, whereas these processes are generally absent or occur at very low levels in
57 deep water masses outside of plumes (De Angelis et al., 1993; Lam et al., 2008). These
58 combined evidences for microbial processes in the plume environment indicate that it is a
59 potentially unique microbial biome.

60 The chemistry of hydrothermal plumes is directly related to the end-member chemistry of
61 the hydrothermal fluids from which the plumes originate. The spatial and temporal variability of
62 vent fluid chemistry at 9°50'N EPR is among the most thoroughly studied of any vent field.
63 Multi-year time series of annual to semi-annual sampling at this site have revealed both temporal
64 variability between samplings as well as differences between vents that are tens of meters apart
65 (Von Damm, 2004). It would be expected that the microbial community in the hydrothermal
66 plumes resulting from these variable end-member fluids may also vary on short temporal and
67 spatial scales, but less is known about the microbial communities at 9°50'N than the fluid
68 chemistry at any comparable resolution of study. Sampling of hydrothermal plumes is often
69 done using shipboard CTD rosettes, but sediment traps have recently been employed to collect

70 samples in time-series of hydrothermal plume fallout at the Endeavour Segment of the Juan de
71 Fuca Ridge (JdFR) (Cowen et al., 2001; Roth & Dymond, 1989), 13˚N EPR (German et al.,
72 2002) and 9˚50'N (Adams et al., 2011; Bennett et al., 2011). While those studies examined the
73 impact of hydrothermal plumes on deep-sea geochemistry, they did not study plume
74 microbiology. However, their time-series approach to sampling hydrothermal plumes did reveal
75 that particulate organic carbon is elevated in plumes at distances of a few km from the vent sites
76 (Cowen et al., 2001; Roth & Dymond, 1989) and that plumes are important to the cycling of
77 hydrothermally derived metals (Fe, Cu, Zn) and seawater derived oxyanions (P, V, As) as well as
78 particle-reactive elements (REE, Th) (German et al., 2002).
79 Here, we have explored the use of sediment traps for a time-series approach to
80 understanding spatial and temporal effects on the bacterial communities within hydrothermal
81 plumes at 9°50'N EPR following the eruption in Winter 2005-06 (Tolstoy et al., 2006). That
82 eruption resulted in elevated dissolved CH_4 in hydrothermal plumes at 9°50'N EPR when
83 compared to typical hydrothermal plume concentrations, one month prior to our deployment
84 (Cowen et al., 2007). Our study, spanning July-November 2006, included a collaboration with
85 both larval biologists, who have already reported evidence for a very disrupted contemporaneous
86 larval supply compared to what was previously present at this site (Mullineaux et al., 2010), and
87 geochemists who have investigated chemical flux variations at both sites using major and trace
88 element analyses, stable and radiogenic isotope measurements and organic carbon measurements
89 (Bennett et al., 2011). Furthermore, the present study builds on previously published
90 mineralogical and bio-inorganic chemical investigations of similar vent-trap samples to those
91 that are studied here which hypothesized that microbial populations in plumes at 9°50'N EPR

92 produce the organic ligands that bind hydrothermally produced Fe^{2+}, inhibiting its oxidation
93 (Toner et al., 2009).
94

95 MATERIALS AND METHODS

96 *Site description and sample collection* - The two sediment traps (McLane Parflux Mark
97 78H-21McLane Research Laboratories) with a sampling aperture of 0.5 m^2 (80 cm diameter at
98 top, 2.5 cm diameter at bottom) and 21 cups were deployed during the RESET06 cruise AT15-6
99 aboard the R/V Atlantis II, June/July 2006, as described previously (Mullineaux et al., 2010).
100 Briefly, before deployment, cups were filled with a solution of 20% dimethylsulfoxide (DMSO)
101 in ultrapure water saturated with NaCl and buffered to pH 9.0 ±0.5. DMSO was used as a
102 preservative for biological samples and the saturated NaCl solution ensured that particles that fell
103 into the solution, which was denser than seawater, would remain in the sampling cups. The
104 combination of DMSO and saturated NaCl in sediment trap collection cups has been proven
105 successful previously to prevent biological activity while preserving DNA for later study
106 (Comtet et al., 2000). The sampling schedule for both traps was 6 days per bottle, starting on 01
107 July 2006 (samples R1-1 and R2-1) and ending on 03 November 2006 (Trap R1, sample R1-21)
108 or 01 November 2006 (Trap R2, sample R2-21). Trap-moorings were anchored such that the
109 opening of each trap was located 4 m above the seafloor. Following retrieval of Trap R2, it was
110 discovered that a jellyfish had become lodged in the opening at the bottom of the trap during the
111 collection of sample 11 (which began collection on 30 August 2006), and remained there for the
112 duration of the experiment.
113 Trap R1 was located ~30 m southwest of the Bio9 vent complex within the axial summit
114 caldera in an area with a series of small chimneys, black smokers and spires, that hosts *Riftia*

115 *pachyptila* and *Tevnia* tubeworm populations. Trap R2 was located outside the axial summit
116 caldera, 115 m southwest of Ty and Io vents and approximately 370 m southwest of Trap R1. Ty
117 and Io are black smokers located ~350 m southeast of Bio9 and are home to populations of
118 *Alvinella* worms.

119 Samples were retrieved from the traps, 15 ml was subsampled for microbiology, and all
120 samples were stored at 4°C until DNA extraction.

121 *DNA extraction and ARISA* - 7.5 ml of each sample was filtered onto a 0.2 μm Nuclepore
122 filter. DNA was extracted using a CTAB phenol/chloroform procedure (Ausubel et al., 1999).
123 ARISA PCR was carried out according to Hewson et al. (2006). 10 ng DNA was added to each
124 DNA reaction of 2.5 units of RedTaq (Sigma-Aldrich), 10X reaction buffer, 2.5 mM MgCl, 0.2
125 mM dNTPs and 0.2 μM of each primer in 50 μl reactions. ARISA primers 78F (5'-
126 GYACACACCGCCCGT-3') and 79R (5'-[TET]GGGTTBCCCCATTCRG-3') were used. The
127 amplification cycle used was: denaturing cycle for 5 min at 95°C, followed by 30 cycles of 95°C
128 for 40 sec, 56°C for 40 sec and 72°C for 90 sec, and a final extension for 7 min at 72°C. PCR
129 reactions were treated with Clean and Concentrate Kit-5 (Zymo Research). 6 ng of cleaned
130 DNA per sample was loaded in duplicate into an ABI 377XL Running Slab Gel and bands were
131 analyzed using ABI Genescan software. Initial analysis of bands was carried out using
132 Microsoft Excel and statistical analyses of band ARISA profiles were carried out using Primer6
133 (PrimerE).

134 *Chemical analyses of plume particulates* - Trap samples were split into fine size fractions
135 (<1mm) and coarse fractions (>1mm) using nylon sieves. Each fraction was later filtered through
136 47 mm filters, dried under class-100 laminar flow hood, and weighed to determine mass flux.
137 The remaining <1mm fraction was passed through a 10-port rotating wet sediment splitter.

138 Between 5 to 10 mg dried subsamples were analyzed for Al, Ca, Fe, Mg, Si, Ti and Mn using an
139 inductively coupled plasma optical emission spectrometer using standard methods established for
140 sediment trap analyses (Honjo et al., 1995).

141 For each sample, another subsample fraction was dissolved in concentrated acid to
142 determine trace element composition (Ba, Sr, Ga, P, V, Co, Cu, Pb, Zn, ^{232}Th and ^{238}U) by
143 inductively coupled plasma mass spectrometry (ICP-MS). Subsamples were leached overnight
144 with 10 mL of 8 mol l^{-1} distilled HNO_3 in 15 ml closed Teflon vials on a hot plate at 80°C. The
145 solutions were then slowly evaporated to dryness. A second dissolution step using 0.5 ml of
146 concentrated ultrapure HF and 3 ml of concentrated distilled HNO_3 was then used to obtain a
147 total digestion of the particles. The solid residue was dissolved in 10ml of 0.28 mol l^{-1} HNO_3
148 (Optima grade) and an aliquot was further diluted for multi-elemental ICP-MS analyses. Multi-
149 elemental analysis of the digests was carried out on a high-resolution ICP-MS Thermo-Electron
150 Element 2 after appropriate dilution (typically 25- to 200-fold dilution depending on the amount
151 of digested materials). The detection limit was calculated from repeat analysis of blank filter
152 digests. Indium was added to each sample as an internal standard to correct for changes of
153 instrument sensitivity. Stock 1000 μg L^{-1} standards (Specpure, Spex) of each element of interest
154 were diluted in preparation for instrument calibration (ranging from 5 to 1000 ppb). A number of
155 geo-reference standards (BHVO-1 and IFG) were also analyzed along with the samples to
156 confirm analytical accuracy with is better than 5% for all elements reported.

157 *Statistical analysis of ARISA results with vent chemistry* - Spearman correlation
158 coefficients between chemical measurements and ARISA profiles were calculated using the
159 BIOENV method in Primer6 with Euclidean distances and 99 permutations. This analysis
160 compares the individual sample chromatograms (input as a grid of samples versus band size and

161 intensity) from ARISA with the individual chemical variables measured (38 in total) and
162 determines what combination of chemical variables best correlates to the observed ARISA
163 patterns. The BIOENV calculation takes all combinations of the 38 total elemental analyses,
164 from single variables through all possible combinations of each variable, and reports the
165 individual or combination of elemental analyses that yield the highest correlation with the
166 ARISA profiles, regardless of the number of variables that correlate best (therefore, if Al+Ca
167 together have a higher correlation to the ARISA profiles than Al alone and/or Ca alone, it will
168 report the value for Al+Ca). We here report the correlations with the highest values.
169 *Clone library construction and analysis* - Universal bacterial primers 29F and 1492R
170 (Lane, 1991) were used to construct near full-length 16S rRNA gene libraries. PCR products
171 were run on an agarose gel, cut out and extracted using QIAquick Gel Extraction Kit (Qiagen)
172 according to the manufacturer's instructions. Fragments were cloned into the pCR 4 TOPO
173 vector using the TOPO TA Cloning Kit (Invitrogen) and transformants plated on LB+100μg ml^{-1}
174 ampicillin according to the manufacturer's instructions. Colonies were randomly selected and
175 grown in liquid culture followed by sequencing at the University of Florida Sequencing Services
176 Facility. Forward and reverse sequences were joined and edited using Bioedit v7.0.5 and
177 checked for chimeras using the Bellerophon tool of Greengenes (DeSantis et al., 2006b). The
178 resulting sequences were aligned using the Greengenes NAST server (DeSantis et al., 2006a) and
179 imported into ARB (Ludwig et al., 2004). The most similar cultured strains as well as some of
180 the nearest neighbors from ARB for uncultured clones were exported with the sequences from
181 this study and aligned using MEGA5 (Tamura et al., 2011). Phylogenetic trees were constructed
182 following manual adjustment of this alignment and maximum likelihood analysis based on the
183 Tamura-Nei model with a Gamma distribution and 500 bootstrap replicates using MEGA5.

184 Calculation of rarefaction curves and diversity estimates, as well as comparison between clone
185 libraries and those of other studies, was carried out using the software MOTHUR (Schloss et al.,
186 2009).
187 Clones sequenced in this study were deposited in the NCBI database with accession
188 numbers JN873927-JN874385.
189
190 RESULTS
191 ARISA was used to provide a broad view of major differences in the bacterial community
192 composition from sediment trap samples. Samples R2-11 through R2-21 (66-126 days) were
193 considered compromised by the jellyfish caught in the mouth of the sediment trap, therefore
194 these samples have not been analyzed by ARISA. All other samples from each sediment trap
195 were subjected to DNA extraction and ARISA PCR (n=31). Successful ARISA PCR
196 amplification was obtained from 13 samples. ARISA results for the bacterial communities from
197 Trap R1 group almost entirely separately from those in Trap R2 (Fig. 1). Sample R2-8 (42-48
198 days) is the only exception; it is most similar to sample R1-1 (1-6 days). Sample R1-21 (120-
199 126 days) is unique compared to all other samples. Bacterial communities in samples R1-2, R1-
200 8, R1-9, R1-13, R1-17 and R1-19 (6-12, 42-48, 48-54, 72-78, 96-102 and 108-114 days,
201 respectively) all group together, as do samples R2-2, R2-4 and R2-5 (6-12, 18-24 and 24-30
202 days), indicating that the samples from each trap display a characteristic genetic signature.
203 We used Spearman correlation analysis to determine which environmental variables,
204 measured here as elemental concentrations, best explain the distribution of bacterial communities
205 seen in the ARISA dendrogram (Fig. 1). Both size fractions of each of the 18 elemental
206 concentrations measured in addition to the ratio Fe/(Fe+Mn+Al) were compared to the ARISA

207 analysis using the BIOENV method in Primer6. Multiple combinations of only 11 chemical
208 parameters (the coarse grained Fe/(Fe+Mn+Al), Cu, V, Ca, Al, ^{232}Th, and Ti and the fine-grained
209 particulate (<1 mm) Fe/(Fe+Mn+Al), Fe, Ca and Co) correlate best with differences in the
210 observed distribution of bacterial communities, explaining 71% of the variation (Table 1).
211 Fe/(Fe+Mn+Al) and Ca are the only variables for which a correlation existed for both size
212 fractions.

213 We cloned and sequenced the near full length bacterial 16S rRNA gene from traps R1
214 and R2 at time points 8, 13 and 21 (42-48, 72-78 and 120-126 days, respectively). While
215 samples R2-13 and R2-21 are too compromised for quantitative comparison to the other samples,
216 we use the results generated here to determine Bacteria that may be present in the plume at these
217 locations. To avoid artifacts from the experimental disruption, clones from samples R2-13 and
218 R2-21 were included in the analysis of phylum level diversity (Fig. 2) and phylogenetic trees
219 (Figs. 3 and 4) only if they met at least one of two following criteria: (1) 97% similarity to at
220 least one clone from samples R1-8, R1-13, R1-21 or R2-8 (not compromised), or (2) were
221 Epsilonproteobacteria, Zetaproteobacteria or Aquificales, classes of Bacteria well represented at
222 hydrothermal vents. These clone libraries allow for comparison between traps, and also at each
223 trap over time.

224 Proteobacteria are the most common clones. Epsilonproteobacteria dominated samples
225 R1-8, R1-13 and R2-8, but are present in all clone libraries. Gammaproteobacteria are the most
226 common clones sequenced in samples R1-21, R2-13 and R2-21. Planctomycetes are present in
227 samples from both traps at time points 8 and 13, but not 21. Other commonly occurring bacterial
228 phyla are Bacteroidetes, Verrucomicrobia and Cyanobacteria. Clones related to Tenericutes are

229 recovered only from samples R1-8 and R1-13. Clones related to Aquificae are recovered only
230 from samples R1-13 and R2-8.

231 Due to the high number and diversity of clones sequenced, we demarcated clones by 97%
232 similarity to each other to define representative operational taxonomic units (OTUs) using the
233 program MOTHUR (Schloss et al., 2009). OTUs and even entire classes of Bacteria are not
234 evenly distributed between the two sediment traps (Table S1). For example, Firmicutes and
235 Actinobacteria are found in Trap R1, but not Trap R2. OTUs 113F02 (Planctomycetes), 18F04,
236 18E03, 18H12 and 18D03 (all Epsilonproteobacteria) and 18B02 (Alphaproteobacteria) are all
237 recovered from samples R1-8 and R1-13, but not from Trap R2. OTU 18B04
238 (Epsilonproteobacteria) is present in all three samples from Trap R1, but none from Trap R2.
239 OTU 28G07 (Alphaproteobacteria) is recovered only from samples R2-8 and R2-13. These
240 examples all occur in at least two samples; there are many OTUs that are recovered from single
241 samples. It must be noted, however, that the number of clones sequenced per sample was not
242 sufficiently high to draw strong conclusions about the absence of a particular group or OTU, but
243 can be used to discuss general trends in bacterial distributions.

244 There are also many OTUs recovered from both traps (Table S1). OTU 121A02, a
245 *Marinobacter*, is the most abundant OTU, recovered from samples R1-21 and R2-21. While no
246 OTUs were recovered from all six samples, or even from 5 out of 6, many are recovered from 3
247 or 4 samples including at least one sample from each trap. Only 9 OTUs are shared by three
248 samples - one Alphaproteobacterium, six Epsilonproteobacterium, one Gammaproteobacterium
249 and one cyanobacterium (Table S1). Two of these OTUs, both Epsilonproteobacteria (18G01
250 and 18H01), are shared by four samples (Table S1).

251 Sample R1-8 and R1-13 share the most OTUs with each other, followed by R1-8 and R2-
252 8 (Table 2 and Fig. S1). R1-8 has the lowest percentage of unique sequences, 23%. Sample R1-
253 21 has the highest (79%); it shares only one OTU with R1-8, two with and R1-13, four with R2-
254 21, and none with samples R2-8 and R2-13. Samples R1-8, R1-13 and R2-8 share the most
255 OTUs by absolute number.

256 By far, the Epsilonproteobacteria represent the most diverse class of Bacteria, with 65
257 OTUs (Figure 3). Most OTUs group within the orders Sulfurovumaceae or Sulfuricurvaceae, but
258 members of Arcobacteraeceae, Hydrogenimonaceae and Nautillaceae are all present as well.
259 Several Aquificae clones are detected, related to *Aquifex* and *Desulfurobacterium*. Many of the
260 Gammaproteobacterial OTUs recovered (Fig. 4) are most closely related to pyschrophiles
261 (*Colwellia*), symbionts (*Photobacterium leiognathi* str. RM1, *Umboniibacter marinipuniceus* str.
262 KMM 389, *Calyptogena phaseoliformis* symbiont str. KT-2 and *Codakia orbicularis* gill
263 symbiont), organisms isolated from sediment (*Haliea* sp. str. SY02, *Cycloclasticus pugetii* str.
264 PS-1 and *Kangiella koreensis* str. DSM16069) and known sulfur oxidizers (*Thioalkalviobrio* sp.
265 str. HL-EbGR7). *Marinobacter* are particularly diverse, forming 18 different OTUs as defined
266 by the 97% cutoff (Fig. S2). Nearly all of the Alphaproteobacterial OTUs fall within the
267 Rhodobacteraceae. OTUs that fell within the Acidobacteria are most closely related to
268 uncultured clones from Loihi Seamount and Kazan Mud Volcano.

269 Rarefaction curves for the six clone libraries show that the three samples with the highest
270 proportion of Epsilonproteobacteria, R1-8, R1-13 and R2-8, display the steepest rarefaction
271 slopes (Fig. S3). Chao1, ACE and Inverse Simpson's diversity indices all indicate R2-8 to be the
272 most diverse sample, followed by R1-13 and R1-8, in agreement with the rarefaction analysis

273 (Table S2). All clones recovered were used for this analysis, therefore diversity may have been
274 higher in samples R2-13 and R2-21 if there had not been a disruption in sampling.
275 To test the hypothesis that hydrothermal plume particles comprise a unique deep ocean
276 habitat with endemic bacterial communities, we compared 16S rRNA gene clone libraries from
277 this study with those from basalts (Mason et al., 2009; Santelli et al., 2008; Sudek et al., 2009),
278 deep seawater (Dick & Tebo, 2010; Pham et al., 2008; Santelli et al., 2008), direct hydrothermal
279 fluids (Huber et al., 2006) and hydrothermal plumes (Dick & Tebo, 2010; Nakagawa et al.,
280 2005a). We restricted our selection of studies to those in which universal 16S rRNA primers
281 were used on uncultured samples, and for whom clone frequency was reported. We included all
282 sequences from samples R2-13 and R2-21 for this analysis. We used the Morisita-Horn index of
283 similarity because it accounts for proportion of OTUs in a sample and is not biased by sampling
284 effort, which was different between the studies in this analysis.
285 Samples from time period 8 and 13 form their own clade (Fig 5), as do basalts from the
286 EPR, Loihi Seamount and JdFR. Despite partial blockage of sample R2-13, it still groups with
287 samples R1-8, R1-13 and R2-8. As with the ARISA dendrogram (Fig. 1), analysis of the clone
288 libraries indicates that the communities in the two traps were different from each other- samples
289 R1-8 and R1-13 are most similar to each other as are R2-8 and R2-13. One basalt rock from
290 Vailulu'u Seamount, near Samoa, is more similar to a hydrothermal plume also from the western
291 Pacific. Samples from background water and hydrothermal plumes in the Guaymas Basin look
292 very similar to each other (Dick & Tebo, 2010).
293
294 DISCUSSION

295 Sampling for this study occurred following an eruptive event at the site in January 2006
296 (Tolstoy et al., 2006), during which lava reached as far as 2 km away from the ridge axis (Soule
297 et al., 2007). Methane concentrations measured in the hydrothermal plume at 9°50' N EPR
298 during May 2006 revealed concentrations up to 350 nM, 100X greater than typical plume
299 measurements for stable hydrothermal plumes and >4X greater than measurements made after
300 the 1991 eruption (Love et al., 2008). The eruptions killed off much of the resident
301 macrobiological communities, and post-eruption larval studies found that resident species had
302 been replaced by vent communities from 300 km away (Mullineaux et al., 2010). Here we
303 describe bacterial community composition in descending particles from two different active
304 hydrothermal vents at 9°50' N EPR during a 4 month time series from August-November 2006.
305 Our samples are the same as those collected by Mullineaux and colleagues (2010).
306 ARISA was used to investigate variability between 13 of the samples collected by the
307 sediment traps. The same methodology has been successfully used to investigate microbial
308 diversity across a deep-sea halocline (Daffonchio et al., 2006), to illustrate a predictable
309 seasonality in surface water bacterial communities collected during a time series experiment
310 (Fuhrman et al., 2006), and to investigate correlations between bacterial communities and
311 environmental variables in a time series study (Steele et al., 2011). Here, ARISA shows that
312 bacterial community composition is variable on the time scale of weeks and also the spatial scale
313 between the two vents. Bottles sampled consecutively in the same trap are sometimes less
314 similar than those taken a month or longer apart (Fig. 1). As an example, in Fig. 1, sample R1-2
315 is much less similar to sample R1-1 than samples R1-9 and R1-13. Similarly, sample R1-8 is
316 much more similar to samples R1-18 than it is to R1-9, which was sampled immediately
317 following R1-8. This is not specific to samples from Trap R1. Sample R2-5 is more similar to

318 sample R2-2 than it is to sample R2-4. The chemistry of hydrothermal vents is known to be
319 variable from year to year, a topic studied extensively at this study site (Von Damm, 2004), and
320 recent work indicates that it is also variable on much shorter time scales (Bennett et al., 2011).
321 While our samples represent particles falling out of a hydrothermal plume, and therefore the
322 observed changes in the microbial community are a result of changes in both the hydrothermal
323 environment (chemistry and microbiology) and also the overlying water column (manifested as
324 particles falling into the traps of a non-hydrothermal origin, as discussed below), the ARISA
325 results show that bacterial communities on hydrothermal plume particles are also variable over
326 short time scales.

327 Overall, there is also a difference between the two particle sample sets. Three of four
328 samples from Trap R1 form their own branch on the cladogram (Fig. 1), and seven of nine
329 samples from Trap R2 form a separate branch of the cladogram. This is strong evidence for
330 different communities in the two plumes. There are multiple likely causes for this. First,
331 individual vents appear to harbor unique microbial communities. This is evidenced here and also
332 at Axial Seamount, on the Juan de Fuca Ridge (Opatkiewicz et al., 2009), where individual
333 vents, while variable over a six-year study period, harbor prokaryotic communities unique to
334 each vent. Also, samples of direct hydrothermal fluid collected from within the vent orifice
335 during the time period of this study revealed that Bio9', which is part of the Bio9 vent complex,
336 had similar Fe concentrations to Ty vent, but only ~40% of the H_2S content, yielding higher
337 Fe/H_2S (339 at Bio9', 132 at Ty) ratios for the Bio9 vent complex (and resultant plumes) than the
338 Ty/Io area (Bennett et al., 2011). Therefore, we should expect that plumes emanating from
339 different vents potentially contain different bacterial communities.

340 Additionally, our particle traps were placed at different distances from the vents. Trap
341 R1 was located just 30 m from Bio9 and, hence, would have underlain the most particle-laden
342 lower reaches of the buoyant hydrothermal plume. By contrast, Trap R2 was deployed 115 m
343 from Ty/Io and, hence, may only have underlain the uppermost fringes of the buoyant plume as
344 well as the non-buoyant plume plume. Consequently, it is to be expected that Trap R1 would
345 receive a more significant hydrothermal component of its total input than Trap R2. To examine
346 differences in hydrothermal input between the two traps, we use the ratio of Fe to (Fe+Mn+Al).
347 Fe, Mn and Al are all elevated in hydrothermal fluids relative to seawater, but Fe/Al and Mn/Al
348 ratios in hydrothermal fluids are well above detrital values (German & Von Damm, 2004). Since
349 Mn and Al generally remains in the dissolved phase in the near-vent non-buoyant plume, purely
350 hydrothermal samples contain nearly no particulate Al or Mn but high particulate Fe, so should
351 exhibit Fe/(Fe+Mn+Al) ratios of ~1. The greater the Mn and Al component, by contrast, the
352 lower the Fe/(Fe+Mn+Al) should be, consistent with a lower hydrothermal input (Boström et al.,
353 1969; German et al., 1990). For this study, Fe/(Fe+Mn+Al) values reveal that Trap R1 contains
354 almost exclusively hydrothermal input (Fe/(Fe+Mn+Al) ~0.90) whereas Trap R2, as was to be
355 predicted since it was located further from the active vent site, was less influenced by
356 hydrothermal input, (Fe/(Fe+Mn+Al) ~0.66; Table S3).

357 Finally, mineralogical changes can also occur as particles travel down-plume, away from
358 their hydrothermal source. Previous work has shown that sediment traps placed 1.5-20 m and
359 300 m away from active hydrothermal chimneys at the Totem vent site, 13°N EPR, collected
360 particles with very different compositions- the nearer traps, placed within the summit caldera,
361 collected 4-7 times higher Fe fluxes (derived from hydrothermal input) than the trap placed 300
362 m away, outside the caldera (German et al., 2002). As hydrothermal particles travel away from

363 their vent of origin, sulfide and sulfate minerals tend to be deposited rapidly while oxyhydroxide
364 materials disperse further afield; this may potentially influence the bacterial community (Cowen
365 & German, 2003).

366 In this study, we find a significant correlation between the >1mm size fraction of
367 Fe/(Fe+Mn+Al), Cu, V, Ca, Al, Ti and ^{232}Th, the <1mm size fraction of Fe/(Fe+Mn+Al), Fe, Ca
368 and Co, and bacterial community composition. It is interesting that Fe/(Fe+Mn+Al) and Ca are
369 the only elements for which both size fractions are significant - the former is an indication of the
370 relative proportion of hydrothermal influence in a sample while the latter is likely an indicator of
371 both hydrothermal input (in the form of anhydrite) and, more probably, biogenic (detrital) input
372 from the overlying surface ocean. Al, Ti and ^{232}Th, by contrast, are all clear indicators of detrital
373 input, presumably from eolian deposition which, like any Ca-rich biogenic input, must have
374 settled from the surface ocean. Given the fresh bare-rock settings of these axial vent-sites,
375 surrounded by fresh lava-flows (Soule et al., 2007), we do not anticipate that local sediment
376 resuspension is a likely candidate for biogenic or detrital inputs to these traps. Rather, the
377 correlation between microbial communities and indicators of non-hydrothermal (biogenic and
378 detrital) input to Trap R2 most likely reflect the fact that the total hydrothermal input to Trap R2
379 is lower than to Trap R1 (because it is further from the vent orifice), resulting in a higher degree
380 of dilution of the hydrothermal flux by particles settling from the overlying water column, which
381 can be considered relatively constant, over time, at both sites.

382 Our results from the cloning and sequencing of 16S rRNA from three concurrent samples
383 in both traps revealed a succession in the bacterial community during the course of the
384 experiment. Epsilonproteobacteria were present in all the samples, as is indicative of
385 hydrothermal systems (Campbell et al., 2006; Nakagawa et al., 2005b; Sunamura et al., 2004).

386 Rarefaction curves were steepest for the three samples with the highest proportion of
387 Epsilonproteobacteria (R1-8 was 52%, R1-13 was 60%, and R2-8 was 44%
388 Epsilonproteobacteria). This suggests that the diversity within the Epsilonproteobacteria drives
389 overall diversity within these samples. In other studies, Epsilonproteobacteria are similarly
390 found to be the drivers of inter-vent diversity at Axial Seamount (Opatkiewicz et al., 2009), and
391 specifically, the presence or absence of *Sulfurovum* appears to be a major driver for this diversity
392 (Huber et al., 2007).

393 Clones from the genera *Sulfurovum*, *Sulfurimonas* and *Caminibacter* dominated our
394 samples (Fig. 3 and Table S1). *Sulfurimonas autotrophica* and *Sulfurimonas denitrificans*, the
395 closest cultured representatives to the clones recovered here, are known to
396 chemolithoautotrophically oxidize reduced sulfur compounds under microaerophilic conditions
397 using O_2 as an electron acceptor (Inagaki et al., 2003; Sievert et al., 2008). *S. denitrificans* can
398 additionally use H_2 and NO_3^- as an electron donor and acceptor, respectively. *Sulfurovum*
399 *lithotrophicum* also chemolithoautotrophically oxidizes reduced sulfur, but not H_2, while
400 reducing O_2 or NO_3^- (Inagaki et al., 2004; Takai et al., 2005). Members of the *Caminibacter*
401 genus are strictly H_2 oxidizers that use O_2, S^0 or NO_3^- as electron acceptors (Alain et al., 2002;
402 Campbell et al., 2006; Miroshnichenko et al., 2004) and can grow autotrophically (*C. profundus*
403 and *C. mediatlanticus*) or mixotrophically (*C. hydrogeniphilus*). Members of *Sulfurimonas* and
404 *Sulfurovum* can grow at mesophilic temperatures, while members of the *Caminibacter* genus are
405 moderately thermophilic. Given the lifestyles of these Epsilonproteobacteria, the hydrothermal
406 plume particles collected here, rich in reduced sulfur compounds and containing microniches
407 where microaerophilic conditions can be maintained, are ideal substrates, especially for members
408 of *Sulfurimonas* and *Sulfurovum*.

409 The biggest difference between time point 8 and time points 13 and 21 was an increase in
410 proportion of Gammaproteobacteria and Alphaproteobacteria while the proportion of
411 Epsilonproteobacteria diminished. Many of the Gammaproteobacteria clones from time points
412 13 and 21 samples were related to known heterotrophic species (Table S4) common to
413 hydrothermal environments. In particular, samples R1-21 and R2-21 are dominated by
414 *Marinobacter*, a genera known to be common in hydrothermal environments (Edwards et al.,
415 2003; Kaye & Baross, 2000; Kaye et al., 2011). The presence of these *Marinobacter* clones in
416 both traps (and therefore not likely due to the jellyfish caught in only Trap R2), indicates a shift
417 in bacterial communities in the plume during this period. The diversity of clones within the
418 *Marinobacter* genus (Figs. S2) indicates these different OTUs may have different ecological
419 niches. Known niches among cultured representatives of the *Marinobacter* include Fe-oxidation,
420 hydrocarbon degradation and a variety of organic degradation pathways (Antunes et al., 2007;
421 Edwards et al., 2003; Gauthier et al., 1992). Similar niche partitioning among species with
422 nearly identical 16S rRNA genes is well documented in marine Bacteria (Acinas et al., 2004;
423 Rocap et al., 2002) and may be the case here.

424 Also present during this last sampling period were clones that fall within *Halomonas*
425 Group 2A and Group 2C. Members of *Halomonas* Group 2A appear to form a clade of
426 subsurface Halomonads, while members of *Halomonas* Group 2C appear associated with
427 massive sulfide deposits (Kaye et al., 2011), and an iron-oxidizing *Halomonas* was recently
428 isolated from hydrothermally influenced subsurface crust (Smith et al., 2011). The presence of
429 these organisms is therefore indicative of hydrothermal input, although a shift from the earlier
430 samples.

431 Some of the Gammaproteobacteria recovered from later samples are indicative of water
432 column populations. These include the clones related to *Colwellia psycherythraea*,
433 *Photobacterium leiognathi* and *Pseudoalteromonas* sp. str. JL-S1, which are all residents of the
434 deep ocean water column (Table S3). These clones were primarily recovered from Trap R2,
435 which was less hydrothermally influenced than Trap R1 (Table S3).

436 Most of the Alphaproteobacteria clones recovered fall within the Rhodobacteraceae
437 family, and all of these fall within the Roseobacter clade. A recent survey of 32 Roseobacter
438 genomes revealed that 72% contained the *soxB* gene, which codes for the SoxB component of
439 the periplasmic thiosulfate-oxidizing Sox enzyme complex and is therefore indicative of the
440 capability for S-oxidation (Newton et al., 2010). Therefore, while not all Roseobacters are
441 capable of S-oxidation, many are and it is possible that those found here, in an environment with
442 elevated concentrations of reduced S compounds, are among those participating in S-oxidation.
443 In particular, 27 clones related to *Sulfitobacter dubious* str. KMM 3554 were recovered from
444 sample R2-21. Although only recovered from R2-21, *S. dubious* is known to oxidize reduced S,
445 and therefore is likely a resident of the plume environment.

446 Few investigations have focused specifically on hydrothermal plume microbiology since
447 the introduction of molecular methods in oceanography. A recent study found that, while clones
448 indicative of aerobic methanotrophy were enriched in non-buoyant plume samples from
449 Guaymas Basin versus background samples, plume and background samples were similar at the
450 class level (Dick & Tebo, 2010). These authors found few Epsilonproteobacteria in the non-
451 buoyant plumes above the hydrothermal field, despite their abundance on the chimneys and
452 sediments on the seafloor in this area. However, they recovered representatives from the SUP05
453 clade of Gammaproteobacteria, which are known sulfur oxidizers common in other hydrothermal

454 plumes and oxygen minimum zones (German et al., 2010; Sunamura et al., 2004; Walsh et al.,
455 2009). Perhaps the niche filled by Epsilonproteobacteria at 9°50'N EPR is filled by SUP05
456 Gammaproteobacteria in the Guaymas plumes.

457 Another study found Beta- and Gammaproteobacterial ammonia oxidizers in plumes over
458 the Endeavour Segment of the Juan de Fuca Ridge (Lam et al., 2008). It is possible that our lack
459 of detection of bacterial ammonia oxidizers and methanotrophs results from their lack of
460 association with particles. The studies cited above used Niskin bottles to collect their samples at
461 plume depth. Therefore, hydrothermal particles and planktonic cells were both sampled,
462 whereas we collected almost exclusively sinking particles in our sediment traps. Previous work
463 has shown that while there is some overlap between the membership of particle attached and free
464 living Bacteria in hydrothermal environments, differences do exist, including the elevated
465 presence of Epsilonproteobacteria in the particle attached population (Huber et al., 2003). Our
466 work confirms the association of Epsilonproteobacteria with hydrothermal particles, even at
467 >100 m away from the venting source of the particles.

468 It is also possible that the short duration of time the samples could have traveled within
469 the plumes from any vent source to these traps would not be long enough to allow methane and
470 ammonia oxidizing microbes to bloom. At the slow lateral current speeds present above the EPR
471 near 9˚50'N (~0.01m sec^{-1}; Thurnherr et al., 2011) and 115m from Ty and Io vents to Trap R2, it
472 would take at least 3 hours to travel the horizontal distance (vertical travel time is not considered
473 because the plume is likely traveling horizontally with the currents as it rises). In that case,
474 because only a few hours might have elapsed from when the fluids left the vent until the particles
475 fell into our sampling devices, the populations reported here may be comprised largely of
476 organisms entrained into the buoyant plume as opposed to those that have developed *in situ*

477 within the dispersing non-buoyant plume. We might especially expect this to be the case for Trap
478 R1, but to evaluate this further would require additional study involving the deployment of traps
479 farther afield from the EPR 9°50'N vent-site and at a greater height off bottom so that only
480 particles settling from the *non-buoyant* hydrothermal plume (together with surface ocean
481 biogenic and detrital inputs) could settle into those traps, without any buoyant hydrothermal
482 plume input.

483 Our analysis of bacterial communities from hydrothermal and deep seawater
484 communities (Fig. 5) revealed that samples from EPR plume particles are unique (Time Points 8
485 and 13) from the other communities. They are dissimilar to hydrothermal plume samples
486 collected in Guaymas Basin (Dick & Tebo, 2010), likely due to the differences in host
487 environments (bare-rock versus sediment hosted vents) and sampling methods discussed above.
488 Samples R1-21 and R2-21 are most similar to each other, and most closely related to background
489 seawater from the deep EPR. The high numbers of *Marinobacter* and Alphaproteobacteria
490 clones in these samples likely drives this similarity - 19% of the clones in the background deep
491 EPR sample are *Marinobacter*.

492

493 SUMMARY

494 We found bacterial communities on hydrothermal plume particles at 9°50'N to be
495 heterogeneous on the time scale of weeks. This work illustrates the use of a time-series approach
496 to studying hydrothermal plumes, an environment that is difficult and expensive to sample. By
497 deploying sediment traps to study this environment, we were able to observe bacterial
498 community dynamics on a timescale previously unattainable.

499 ARISA revealed that bacterial communities can shift on weekly time scales and that the
500 plume particle bacterial communities were unique at each vent. There is a statistically
501 significant ($p<0.01$) relationship between elemental concentrations of Fe/(Fe+Mn+Al), Al, Ca,
502 Cu, ^{232}Th, Ti, V, Fe and Co and bacterial community structure. 16S rRNA clone libraries
503 mirrored the ARISA distributions in that sample R1-21 was dissimilar from the other samples
504 and showed a progression in bacterial communities during the time scale of this experiment.
505 Earlier clone libraries are dominated by sulfur oxidizing Epsilonproteobacteria, which
506 diminished in proportional importance in later samples, where more Gammaproteobacteria and
507 Alphaproteobacteria were recovered. The diversity of these samples with Epsilonproteobacteria
508 is higher than those without. These hydrothermal plume particle samples appear to form their
509 own group when compared to other environmental 16S rRNA clone libraries, indicating that the
510 hydrothermal plumes at 9°50'N host a unique community.
511

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FIGURE LEGENDS

Figure 1 - Dendrogram analysis of ARISA profiles from traps R1 (filled triangles) and R2 (open circles) as calculated using the Bray-Curtis similarity index.

Figure 2 - Bacterial distribution, by class, in each 16S rRNA gene clone library. Samples in the left column are all from Trap R1, those on the right are from Trap R2. The top row is time period 8, the middle time period 13, and the bottom time period 21. Data from a background deep seawater sample (non-plume) is included for comparison. This sample was collected in the same area as our study site in 2004 (Santelli et al., 2008). The number of clones per library is indicated next sample name.

Figure 3 - 16S rRNA phylogenetic tree of Epsilonproteobacteria and Aquificae clones retrieved in this study. Clones in tree are all <97% similar with all others in this study, as determined by MOTHUR. For a full reporting of all clones, see Table S1. Alignments of sequences from this study and their nearest cultured organisms in the Greengenes database as well as some close uncultured clones from the ARB database were constructed using MEGA5. The tree was constructed using Maximum Likelihood algorithm with 500 bootstraps. Bootstrap values >50% are reported. Aquificae are used as an outgroup. NCBI accession numbers for sequences from cultured representatives and environmental clones are given following the species/clone name.

Figure 4 - 16S rRNA phylogenetic tree of Alphaproteobacteria, Gammaproteobacteria, Zetaproteobacteria, Acidobacteria and Deferribacteres clones retrieved in this study. Clones in tree are all <97% similar with all others in this study, as determined by MOTHUR. For a full

reporting of all clones, see Table S1. Alignments of sequences from this study and their nearest cultured organisms in the Greengenes database as well as some close uncultured clones from the ARB database were constructed using MEGA5. The tree was constructed using Maximum Likelihood algorithm with 500 bootstraps. Bootstrap values >50% are reported. Acidobacteria and Deferribacteres are used outgroups. The *Marinobacter* branch is expanded in Fig. S1. NCBI accession numbers for sequences from cultured representatives and environmental clones are given following the species/clone name.

Figure 5 - (A) Cluster analysis of samples from this study with 16S rRNA gene clone libraries from basalts, deep seawater, hydrothermal plumes and end member hydrothermal fluids. All clone libraries were constructed using universal bacterial 16S rRNA primers with environmental samples. Environments are color coded by squares next to each sample. Samples were first reported in: 1 - Dick and Tebo (2010), 2 - Huber et al. (2006), 3 - Mason et al. (2009), 4 - Nakagawa et al. (2005a), 5 - Pham et al. (2008), 6 - Santelli et al. (2008) and 7 - Sudek et al. (2009). (B) Map of sample locations included in the cluster analysis.