RESEARCH ARTICLE

Halomonas and *Marinobacter* ecotypes from hydrothermal vent, subseafloor and deep-sea environments

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Abstract

Moderately halophilic and euryhaline bacteria are routinely found in cool to warm hydrothermal vent and nearby cold, deep-sea environments. To elucidate the diversity of these microorganisms – with the goal of determining which among them constitute ecotypes specifically associated with hydrothermal vent and subseafloor habitats – PCR primers were designed to detect natural populations of euryhaline *Gammaproteobacteria* belonging to the cosmopolitan genera *Halomonas* and *Marinobacter*. The distribution patterns of 16S rRNA gene sequence data revealed that *Halomonas* group 2A comprised a subseafloor population at Axial Seamount on the Juan de Fuca Ridge. Complementary biogeographic and physiological data suggested that other *Halomonas* clades include members that are cold adapted (*Halomonas* group 2B) or associated with massive sulfide deposits (*Halomonas* group 2C). Similarly, a monophyletic *Marinobacter* clade may represent Fe^{2+}-oxidizing facultative chemoautotrophs based on the phylogenetic data presented here and previously reported phenotypic characterizations. The biogeographic distributions of *Halomonas* and *Marinobacter* isolates and clones reveal that these are cosmopolitan genera, commonly found in the deep sea and in hydrothermal vent settings. As such, they are good candidates for further laboratory investigations into the biogeochemical processes in these environments.

Introduction

Easily cultivated microorganisms from marine environments, such as *Pseudomonas* and *Vibrio* of the *Gammaproteobacteria*, are infrequently observed in bacterial phylogenies of community DNA based on the 16S rRNA gene (Rappé & Giovannoni, 2003). Less easily cultured microorganisms, including representatives of the ubiquitous SAR clades, comprise a significant proportion of 16S rRNA gene sequences in many pelagic marine habitats (Morris *et al.*, 2002; Rappé & Giovannoni, 2003), and, along with other numerically dominant bacterial species, have been enriched and characterized physiologically using novel cultivation techniques (Rappé *et al.*, 2002; Giovannoni & Stingl, 2007; Alain & Querellou, 2009).

The cultivation of ecologically and biogeochemically important taxa and culture-independent analyses of marine microbial community structure augment the importance and utility of each other. The combination of laboratory-determined physiologies and a map of the distribution of bacterial clades, such as the SAR groups in pelagic environments (Morris *et al.*, 2002; Rappé *et al.*, 2002) and the *Epsilonproteobacteria* in hydrothermal vent habitats (e.g. Campbell *et al.*, 2006), reveals where and how microbial metabolisms may be impacting the transformations of organic compounds and inorganic nutrients. There are still significant caveats, however, with regard to making inferences of specific physiological attributes from 16S rRNA gene sequences and when distinguishing the genotypic potential of a microorganism from the phenotype expressed *in situ*.

In between the rare, historically cultivated heterotrophic microorganisms and numerically dominant bacterial taxa in both pelagic and hydrothermal vent environments lie groups such as *Halomonas* and *Marinobacter* (Kaye & Baross, 2000). These genera have been isolated frequently from enrichments on media with elevated Fe^{2+} and Mn^{2+} at 2–20 °C (Kaye & Baross, 2000; Edwards *et al.*, 2003;

Tempelton *et al.*, 2005) and were the sole representatives obtained with quantitative enrichments using the same medium augmented with elevated salt (16% NaCl) and incubated at 20 °C (Kaye & Baross, 2000). Using quantitative enrichments, salt-tolerant microorganisms comprised 0.01 to > 10% of the total microbial community in 43 hydrothermal vent and water column samples from the North and South Pacific Oceans (Kaye & Baross, 2000). Six *Halomonas* strains isolated from hydrothermal vent habitats were characterized as psychrotolerant, heterotrophic, euryhaline, slightly to moderately halophilic, cadmium resistant and facultatively anaerobic, consistent with a phenotype suitable for growth in hypothesized low-temperature hydrothermal fluids (diffuse flow) and subseafloor environments (Butterfield *et al.*, 1997; Huber *et al.*, 2003; Mehta *et al.*, 2003; Kaye *et al.*, 2004). Partial characterization of novel *Marinobacter* strains from hydrothermal vent habitats (Edwards *et al.*, 2003) highlighted the ability of *Marinobacter* to oxidize Fe^{2+} from metal sulfide minerals and basalt at a low temperature and emphasized their role in the weathering of metal sulfide deposits. Complementary mineralogical and phylogenetic analyses revealed their numerical significance in clone libraries among extinct sulfides and metalliferous deposits at the seafloor (Rogers *et al.*, 2003). Some of these *Marinobacter* strains are also closely related to the numerically significant euryhaline strains isolated at 20 °C from seawater and diffuse flow environments (Kaye & Baross, 2000; Edwards *et al.*, 2003). These studies suggest a potentially important biogeochemical role for *Marinobacter* and *Halomonas*, and indicate that they may be quantitatively significant in certain habitats.

Our goal in the present study is to understand the biogeography and diversity of moderately halophilic and euryhaline *Halomonas* and *Marinobacter* in cool to warm hydrothermal vent and plume environments and the nearby cold deep sea in selected Juan de Fuca Ridge and associated ridge flank habitats to explore their significance, occurrence and possible contribution to transformations of organic carbon and iron. Herein, we designed taxonomically focused PCR primers to determine the strain and species diversity of these two genera at several Juan de Fuca Ridge environments and to ascertain whether certain subpopulations of these genera live in particular hydrothermal vent and deep-sea habitats. We propose that *Halomonas* and *Marinobacter* are ecologically important participants in hydrothermal vent settings, where they contribute to transformations of iron and carbon. Their ease of culturing makes them excellent candidates as model bacteria to use in laboratory experiments to further understand their ecological function in hydrothermal vent ecosystems.

Materials and methods

Sample collection

Low-temperature hydrothermal fluids and deep seawater were collected in the northeast Pacific Ocean (Fig. 1a; Table 1). Diffuse flow fluids were obtained at Axial Seamount and the Main Endeavour Field (MEF) on the Juan de Fuca Ridge using a suction sampler and either filtered on deck or *in situ* using a hydrothermal fluid and particle sampler mounted on the remote-operated vehicle *ROPOS* or the deep submergence vehicle *Alvin*. In some instances, fluids were filtered first through cellulose membranes (3.0-μm pore size; Millipore) and then Sterivex-GP filters (0.2-μm pore size; Millipore) and frozen in liquid nitrogen (Huber *et al.*, 2002). The former fraction was defined as 'particle-attached' and subsequently analyzed. Other diffuse

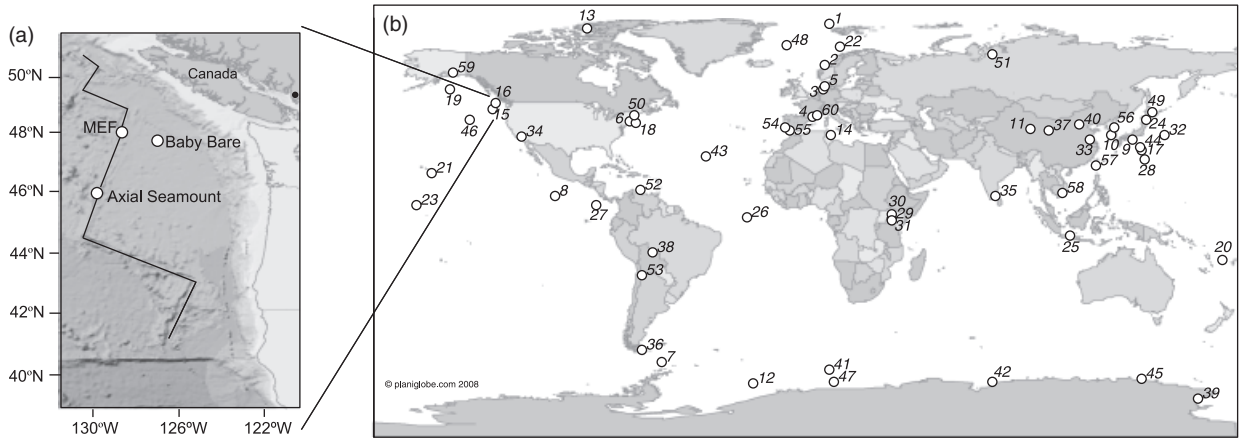

Fig. 1. (a) Map of the Juan de Fuca Ridge, associated ridge flanks, and sampling sites. (b) Based on data obtained from GenBank, locations of samples from which *Halomonas* or *Marinobacter* species were retrieved in clone libraries or isolated in culture. Species names, accession numbers and sample setting are listed in Tables S1 and S2.

flow samples were filtered only using Sterivex-GP filters. Background deep seawater (without a detectable hydrothermal vent signal) was collected near Axial Seamount, in the axial valley near the MEF and near Baby Bare with Niskin bottles jointly mounted on a rosette with a conductivity–temperature–depth–transmissometry package. These deep seawater samples were filtered only through Sterivex-GP filters on deck. Finally, sections of sulfide structures measuring several meters in length were retrieved hot and fresh from the Mothra vent field on the Endeavour Segment during the Edifice Rex sulfide recovery program, subsampled by the mineralogical zone and frozen in liquid nitrogen (Schrenk *et al*., 2003).

Molecular analyses

Community DNA was extracted from filters (Huber *et al*., 2002) and rocks (Schrenk *et al*., 2003) as described previously. Direct cell counts were reported previously (Kaye & Baross, 2000; Huber *et al*., 2002; Mehta *et al*., 2003; Schrenk *et al*., 2003) or performed for this study according to a standard technique (Porter & Feig, 1980). PCR primers were designed to amplify *Halomonadaceae* and *Marinobacter* by aligning 46 full-length 16S rRNA gene sequences from members of these groups: 115F (5′-GAG TAA TGC ATA GGA ATC TGC C) and 1446R (5′-TAG GCT AAC CAC TTC TG) (see Appendix S1 for details). The annealing temperature for each primer was optimized using temperature-gradient PCR with genomic *Halomonas* DNA from three species as a template using the following combinations of primers: 115F and the universal bacterial primer 1492R (5′-GGT TAC CTT GTT ACG ACT T) (Lane, 1991); 1446R and the universal bacterial primer 8F (5′-AGA GTT TGA TCC TGG CTC AG); and 115F and 1446R. The PCR cocktail for each 20 μL reaction contained 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.25 μM of each primer, 1 × PCR buffer (Promega), 1 U *Taq* DNA polymerase (Promega) and 1 μL template. The profile had an initial denaturation at 94 °C for 5 min, followed by 28 cycles of denaturation at 94 °C for 30 s, annealing at 54–61 °C for 45 s and extension at 72 °C for 2 min, with a final 10-min extension at 72 °C. Optimal amplification occurred using an annealing temperature of 56 °C. Environmental DNA was amplified using the primers 115F and 1446R over a temperature gradient to confirm that optimal performance was still at 56 °C. Diffuse flow, sulfide rock and deep seawater samples were optimized separately for cycle number if a band was obtained (Table 1). Multiple PCR reactions were run with each sample using a cycle number that indicated that product saturation had not yet been reached (Suzuki & Giovannoni, 1996; Polz & Cavanaugh, 1998). Each product was reconditioned by running a three-cycle PCR on a 1 : 10 dilution of the initial product using the same cocktail and profile (Thompson *et al*., 2002). The reconditioned products were pooled and cleaned using Qiaquick PCR purification columns (Qiagen) and then further purified by running 10 μL on a 1% w/v agarose gel and using the Qiaquick Gel Extraction Kit (Qiagen). Most libraries were assembled from multiple independent cloning procedures (see Appendix S1 for further details).

Phylogenetic analysis

Sequences were confirmed as nonchimeric using the CHIMERA CHECK function of the RDP-II website (Cole *et al*., 2003; Hugenholtz & Huber, 2003), consistent with their seamless fit into a master alignment of closely related taxa. Variable stem-loops at bases 201–216 and 1135–1139 (*Escherichia coli* numbering) were excised to facilitate the alignment. Unique sequences were aligned in ARB (Ludwig *et al*., 2004) against the Greengenes curated ARB database (DeSantis *et al*., 2006). All sequences retained in the final tree were exported from ARB and aligned in MEGA 4 (Tamura *et al*., 2007), followed by tree construction using neighbor joining with 1000 bootstraps. The accession numbers for clone libraries D, E, F, G and H are, respectively, AY687565–AY687590, AY687539–AY687564, AY687520–AY687538, AY687591–AY687627 and AY687475–AY687519.

Results

Site temperatures and cell counts

Diffuse flow samples ranged between 5 and 100 °C and background seawater was 2 °C (Table 1). Cell concentrations in the diffuse flow samples ranged from 1.7×10^4 cells mL^{-1} at Milky vent to 1.7×10^5 cells mL^{-1} near Puffer vent. Background seawater cell concentrations were $4.0–4.9 \times 10^4$ cells mL^{-1}. The sulfide rock sample collected from the Finn sulfide chimney contained 1.7×10^8 cells g^{-1}.

Primer construction and preliminary PCR amplification

Two primers were ultimately selected for use in the PCR amplification of *Halomonas* and *Marinobacter* 16S rRNA genes after testing a combination of five different primers (details in Appendix S1). The novel primers 115F (5′-GAG TAA TGC ATA GGA ATC TGC C) and 1446R (5′-TAG GCT AAC CAC TTC TG) were selected for optimization with the notion that using two novel primers would focus amplification on the desired taxa (because fewer undesired taxa matched both primers) and because the combination of these two primers provided maximal coverage of the 16S rRNA gene. Overlapping fully characterized taxa matched by both of these primers with zero mismatches included only certain *Halomonas* spp. With one base mismatch, selected members of the genera *Acinetobacter*, *Flavobacterium*,

Table 1. Source of DNA extracts amplified with primers designed for the moderately halophilic, euryhaline and mesophilic *Halomonadaceae* and *Marinobacter*

Region	Sample	Site	Sample type	T (°C)	Depth (m)	Cell concentration (mL^{-1})*	Size fraction (μm)	Volume filtered (L)	Date	Dive or cast	PCR†
Axial Seamount	SS001	Marker 33 vent	Diffuse flow	37‡	1524	$9.7 \pm 2.9 \times 10^4$	> 3.0	1.00	August 1998	*ROPOS* 462	G
	SS002	Milky vent	Diffuse flow	5	1531	$1.7 \pm 0.5 \times 10^4$	> 3.0	1.25	September 1998	*ROPOS* 463	+
	FS004	Marshmallow vent	Diffuse flow	100	~1520	$5.9 \pm 1.8 \times 10^4$	> 3.0	0.60	September 1998	*ROPOS* 469	+
	FS009	Easy vent	Diffuse flow	10	~1520	$1.2 \pm 0.3 \times 10^5$	> 3.0	1.00	September 1998	*ROPOS* 473	H
	FS024	Magnesia vent	Diffuse flow	5.0	~1520	$5.0 \pm 0.3 \times 10^5$	> 3.0	1.02	July 1999	*ROPOS* 488	−
	FS045	Cloud pit, marker N6	Diffuse flow	15.9	~1520	$1.2 \pm 0.2 \times 10^5$	> 3.0	1.00	July 2000	*ROPOS* 547	−
	FS047	Marker 33 vent	Diffuse flow	31.3	1524	$1.2 \pm 0.2 \times 10^5$	> 3.0	1.00	July 2000	*ROPOS* 551	−
Near Axial Seamount	CTD003§		Deep seawater	2	1295	$4.9 \pm 0.8 \times 10^4$	> 0.2	1.00	July 1999	CTD‡	F
Mothra vent field	G3Fe/Si¶	Finn	Iron and zinc sulfide, silica	ND	2270	$1.7 \pm 0.2 \times 10^8$ g^{-1}	Total cells	NA	June 1998	NA	+
MEF	MEF166	Near Puffer vent	Diffuse flow	39	~2200	$1.7 \pm 0.3 \times 10^5$	> 0.2	1.00	September 2000	*Alvin* 3618	+
Near MEF	MEF131		On-axis deep seawater‖	2	2350	ND	> 0.2	3.00	September 2000	CTD	E
Near Baby Bare	CTD005		Deep seawater	2	2540	$4.0 \pm 0.6 \times 10^4$	> 0.2	3.00	September 2002	CTD	D

* ± 95% confidence limit. mL^{-1} unless noted as g^{-1}.

†Letters correspond to clone library constructed (strong amplification); +, preliminary or inconsistent amplification; −, no amplification.

‡55 °C measured on a later dive during the cruise.

§CTD, Conductivity–temperature–depth package that was attached to a rosette of Niskin bottles for water sampling.

¶Zone FZ2b, 2–7 cm within a sulfide structure (Schrenk *et al.*, 2003).

‖Hydrothermal plume not detected. Cell concentration assumed to be ~5.5×10^4 (Mehta *et al.*, 2003).

ND, not determined; NA, not applicable; MEF, Main Endeavour Field, Endeavour Segment, Juan de Fuca Ridge.

Marinobacter, *Methylomonas*, *Moraxella*, *Pseudomonas*, *Thioalkalimicrobium* and *Vibrio*, symbiont bacteria and additional *Halomonas* spp. also matched. Temperature-gradient PCR using each primer in concert with a universal bacterial primer and with each other (using genomic *Halomonas* and *Marinobacter* DNA as template) revealed that the optimum annealing temperature for each primer was 56 °C. The primer pair amplified a DNA fragment of approximately 1300 base-pairs length from pure-culture genomic extracts of numerous strains of *Halomonas*, *Marinobacter* and *Cobetia*, but not of *Marinococcus* (Gram-positive), *Desulfurobacterium* and *Thermococcus* (archaeon) or other strains of *Gammaproteobacteria*, including *E. coli*, *Salinisphaera* and *Vibrio*.

Amplification of environmental DNA extracts

The primers 115F and 1446R were used to amplify 16S rRNA genes from community DNA extracts from various diffuse flow, sulfide rock and deep seawater samples from 12 sites in the northeast Pacific Ocean (Fig. 1a; Table 1). Amplification of some samples did not produce a band, while others did so inconsistently. Presumably, the samples for which amplification was not successful did not contain sufficient numbers of organisms detectable with the primer pair used. Five samples were selected for cycle optimization (31–35 cycles each) and cloning, resulting in 17–45 16S rRNA gene sequences per sample of approximately 1200–1250 base-pairs length.

Clone libraries

One hundred and fifty-one 16S rRNA gene sequences were compiled, belonging exclusively to the genera *Halomonas* (96% of clones) and *Marinobacter* (4% of clones; Table 2). The *Halomonas* 16S rRNA gene sequences fell among only three phylogenetic branches, all within 16S rRNA group 2 of the genus (Arahal *et al.*, 2002; de la Haba *et al.*, 2010) with

Table 2. Composition of clone libraries based on phylogenetic clades within *Halomonas* and *Marinobacter* as measured by the number of clones per clade

	Halomonas group 2A	*Halomonas* group 2B	*Halomonas* group 2C	*Marinobacter*
> 3.0 μm fraction				
Marker 33 vent, G	25	6	6	0
Easy vent, H	45	0	0	0
> 0.2 μm fraction				
Near Baby Bare, D	4	25	0	0
MEF axial valley, E	2	0	24	0
Near Axial Seamount, F	7	0	4	6

MEF, Main Endeavour Field, Endeavour Segment, Juan de Fuca Ridge.
Libraries G and H derived from the > 3.0 μm filter fraction of cool to warm diffuse flow hydrothermal fluids, whereas libraries D, E and F were constructed from cold, deep seawater samples (> 0.2 μm filter fraction) located near the sites mentioned.

high bootstrap values (Figs 2 and 3). Over the region of the 16S rRNA gene used to construct phylogenetic trees, a small number of clones contained sequences identical to other clones or to previously reported sequences; most sequences were unique, however. Identical matches included: F1 and *Marinobacter* spp. 1802c.Splume1; H21, H32, H37 and *Halomonas meridiana*; and H28 and *H. meridiana* strain Slthf1. Within each of the three *Halomonas* (groups 2A, 2B and 2C) and one *Marinobacter* clade, clone sequences shared > 99% sequence similarity to each other and to the isolates included in the given group (except in the case of outliers such as *Halomonas magadiensis*). The similarity between a given group and its closest-neighboring group was > 98%. The source of isolation of both named and incompletely characterized *Halomonas* and *Marinobacter* taxa included in the phylogenetic analysis is summarized in Table S2.

Discussion

Taxonomically focused PCR primers enabled the detection of the slightly to moderately halophilic and euryhaline genera *Halomonas* and *Marinobacter* in the cold deep sea and in cool to warm hydrothermal fluids. Using universal bacterial PCR primers, *Halomonas* 16S rRNA gene sequences have been identified previously in a clone library from Baby Bare deep seawater (from which library D was also created), where five of 84 clones belonged to *Halomonas* group 2A (Huber *et al*., 2006). In addition, *Halomonas* and *Marinobacter* were detected using 454 pyrosequencing of the V6 hypervariable region of the 16S rRNA gene in certain deep-sea hydrothermal vent samples (Huber *et al*., 2007). In that study, *Halomonas* comprised < 1% of all sequence tags in two samples from Axial Seamount, and *Marinobacter* comprised 12% of the total bacterial community in one of those samples, which was taken in diffuse flow in a community of tubeworms. However, the detection of these groups across hydrothermal vent habitats has been spotty; for example, other clone libraries generated with universal bacterial PCR primers from hydrothermal vent and deep-sea samples collected atop and near Axial Seamount by Huber *et al*. (2003) did not produce any *Halomonas* or *Marinobacter* signals. Hence, the taxonomically focused primers applied here for *Halomonas* and *Marinobacter* appear to have increased our ability to detect these groups in hydrothermal fluids.

Although the ability to recover *Marinobacter* and *Halomonas* sequences in clone libraries that are generated from hydrothermal fluids using universal bacterial PCR primers is less than predicted by most probable number assays, a number of recent studies have recovered them without such a targeted approach from other hydrothermal vent sample types, most notably from solid mineral surfaces. For example, in one study of the East Pacific Rise (EPR), *Halomonas* and *Marinobacter* were detected on basalt and in the overlying seawater (Santelli *et al*., 2008), as indicated by the presence of sequences in *Halomonas* groups 2A and 2B and *Marinobacter* that begin with 'EPR' in Figs 2 and 3. Rogers *et al*. (2003) also recovered a high proportion of *Marinobacter* from extinct sulfide and metalliferous sediments from Juan de Fuca Ridge hydrothermal samples; *Marinobacter* overwhelmingly dominated some samples in that study. These observations suggest that *Marinobacter* – and possibly *Halomonas* – proliferate in biofilms associated with mineral particles and as such they may be missed in samples and studies that do not consider the particle-associated fraction of the community. This inference is supported by some of the sampling carried out as part of this study. For example, *Halomonas* group 2A dominated in the 'particle-attached' fraction of diffuse fluids (> 3.0 μm size fraction; Fig. 3). Variations between particle-attached and free-living taxa from hydrothermal habitats have been observed similarly in other studies (Huber *et al*., 2002, 2003; Cowen, 2004).

We infer that the 16S rRNA gene sequences from *Halomonas* and *Marinobacter* detected in this study reflect an indigenous diversity and indicate clear population delineations among taxa. The topology of the phylogenetic trees (Figs 2 and 3) reveals distinct, analytically robust branches

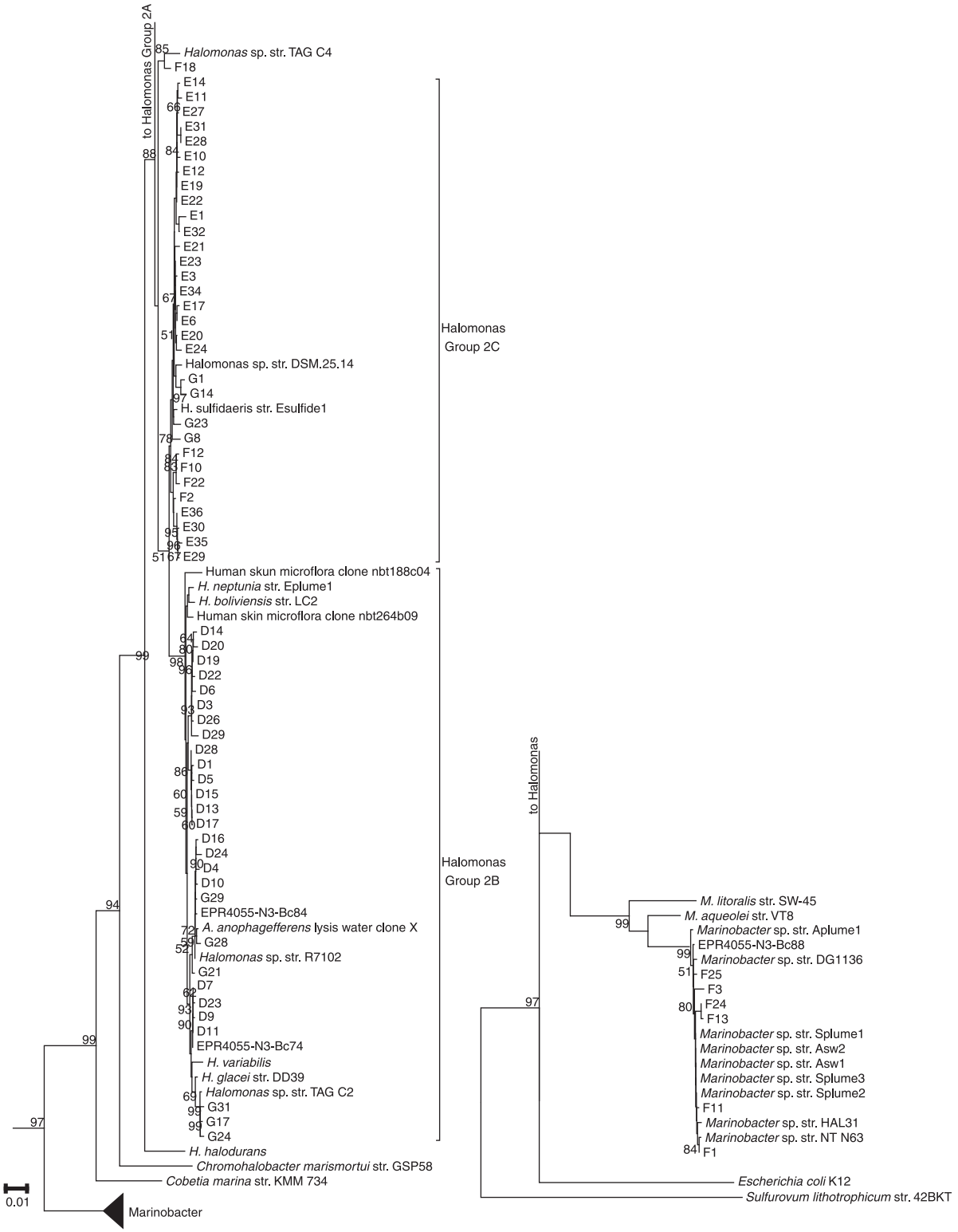

Fig. 2. Neighbor-joining phylogenetic tree highlighting *Halomonas* groups 2B and 2C (left) and *Marinobacter* (right). Clone sequences from this study are denoted by a letter followed by a number (e.g. "F11"). Bootstrap values > 50% are indicated. *Escherichia coli* and *Sulfurovum lithotrophicum* (epsilonproteobacterium) are outgroups.

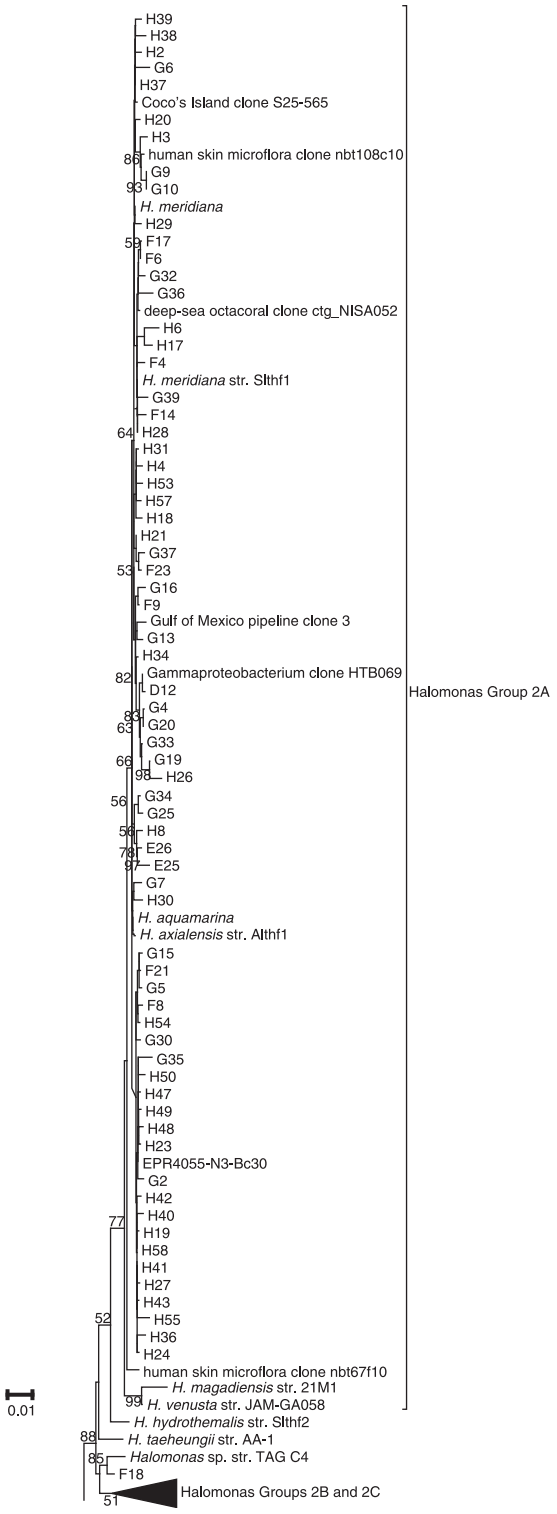

Fig. 3. Neighbor-joining phylogenetic tree highlighting *Halomonas* group 2A. Clone sequence notation and other features as in Fig. 2 The lower part of the tree was removed to save space, but remains the same as in Fig. 2.

with broad, bushy tips that contain 16S rRNA gene sequences of > 99% sequence identity. The tips, including *Halomonas* groups 2A, 2B and 2C, appear to be similar to the 'microdiverse clusters' that have been observed in other settings (Acinas *et al.*, 2004). The clusters defined by Acinas *et al.* (2004) grouped at the level of > 99% 16S rRNA gene sequence identity and were hypothesized to function as ecotypes that resulted from selective sweeps during their evolutionary history (Acinas *et al.*, 2004; Koeppel *et al.*, 2008). We propose that marine representatives of the genus *Halomonas* evolved in a similar fashion, resulting in the ecotypes described here.

Members of group 2A were detected in all the samples analyzed, but their enrichment in low-temperature vent fluids suggests growth in this niche. The maximum recorded temperature for growth within the genus *Halomonas* is 40–50 °C (Mata *et al.*, 2002), although the maximum temperature for the growth for most members of the *Halomonas* 16S rRNA group 2 is 30–40 °C, including *H. meridiana*, *Halomonas axialensis*, *Halomonas aquamarina*, *Halomonas variabilis*, *Halomonas neptunia*, *Halomonas glaciei* and *Halomonas sulfidaeris* (Mata *et al.*, 2002; Reddy *et al.*, 2003; Kaye *et al.*, 2004). However, the maximum temperature for growth may increase by several degrees under hydrostatic pressures equivalent to mid-ocean-ridge depths (Yayanos & DeLong, 1987; Takai *et al.*, 2008). While the marker 33 hydrothermal vent may be too warm (37–55 °C) to allow the growth of certain *Halomonas* spp., members of groups 2A, 2B and 2C may be growing in cooler nearby subseafloor habitats and flushed into the water column during the vigorous hydrothermal flow associated with the vent (Huber *et al.*, 2002). At 10 °C, Easy vent is a more hospitable environment for the growth of *Halomonas*, and indeed, each of the 45 clones from library H collected there belonged to group 2A.

The cultured members of group 2A, *H. aquamarina*, *H. axialensis* and *H. meridiana*, were, respectively, isolated from an unreported location and depth off the coast of California, a 27 °C diffuse flow vent on Axial Seamount in 1998 and from an Antarctic hypersaline lake (ZoBell & Upham, 1944; James *et al.*, 1990; Kaye *et al.*, 2004). Characterized *H. meridiana* strains Slthf1 and Eplume2 were, respectively, cultured from a 9 °C diffuse flow site on the Southern EPR and from a 2 °C hydrothermal plume above the MEF on the Endeavour Segment of the Juan de Fuca Ridge (Kaye *et al.*, 2004). Hydrostatic-pressure–temperature growth experiments suggest that *H. axialensis* and *H. meridiana* strain Slthf1 can thrive in cool to warm pressurized habitats (13–30 °C, > 25 MPa), but grow only very slowly or not at all under cold (2 °C) deep-sea conditions (Kaye & Baross, 2004). This observation serves as another indicator of the hypothesized endemism of this group in cool to warm subseafloor habitats.

Ninety-six percent of the clones from library D, a deep seawater sample from the eastern flank of the Juan de Fuca Ridge located 4.6 km from the basaltic outcrop at Baby Bare, fell into the *H. glaciei–H. neptunia–H. variabilis* group 2B. This clade was also moderately well represented in the marker 33 library, but not detected in any other sample. *Halomonas neptunia* and *H. glaciei* were, respectively, isolated from a 2 °C hydrothermal plume above the MEF and from Antarctic fast ice (Reddy *et al.*, 2003; Kaye *et al.*, 2004). *Halomonas variabilis*, more distantly related to the other members of this group, was isolated from the Great Salt Lake (Fendrich, 1988). The remaining isolates in group 2B were obtained from a variety of cold environments: a 2 °C deep-sea hydrothermal plume, and Arctic and Antarctic seawater. One strain, isolated from 194 °C hydrothermal fluid from a vent on the Trans-Atlantic Geotraverse (TAG) hydrothermal mound on the Mid-Atlantic Ridge, very likely did not derive from that high-temperature environment (Okamoto *et al.*, 2001). The clones in group 2B may represent a cold-adapted, deep-sea *Halomonas* lineage, given the predominance of this group in a cold deep seawater sample and given that many cultured strains were isolated from permanently cold environments.

Library E, which derived from a deep seawater sample procured in 2000 from within the axial valley of the MEF without a detectable hydrothermal plume signal, was comprised almost exclusively of clones belonging to group 2C with *H. sulfidaeris* as its only characterized member. *Halomonas sulfidaeris* was isolated from a sample of sulfide rock collected from the MEF in 1995 (Kaye *et al.*, 2004). An incompletely characterized isolate in this group was isolated from sediment in the Sea of Okhotsk. As with libraries D and H, the low clone diversity implicates a resident population that is locally enriched. In this case, the enriched population may be linked directly to the widespread massive metal sulfide deposits for which the MEF and the encompassing 15 km stretch of the Endeavour Segment are known (Kelley *et al.*, 2001).

The deep seawater sample taken near Axial Seamount was the only sample from which *Marinobacter* 16S rRNA gene sequences were amplified (library F; Fig. 2). Like *Halomonas*, the genus *Marinobacter* is nearly ubiquitous, contains moderately halophilic and euryhaline species and includes strains that have been isolated frequently from hydrothermal vent environments (Kaye & Baross, 2000; Edwards *et al.*, 2003). Partial characterization of *Marinobacter* spp. from samples of oxidized sulfide rock and metalliferous sediments collected from the Endeavour Segment and Middle Valley revealed that some species are psychrophilic Fe^{2+} oxidizers, a phenotype consistent with success in cold, oxic hydrothermal vent environments (Edwards *et al.*, 2003). Moreover, these and other Fe^{2+}-oxidizing bacteria likely play a significant role in seafloor weathering of hydrothermal deposits (Edwards, 2004). The Axial Seamount samples without detectable *Marinobacter* sequences contained higher concentrations of hydrogen sulfide, suggesting a possible antagonistic effect on this group.

It can be difficult to infer specific phenotypic attributes from phylogeny, especially for the genus *Halomonas*, within which 16S rRNA gene sequences of different species are typically $> 97\%$ identical (Arahal *et al.*, 2002; de la Haba *et al.*, 2010). Although it is not yet possible to demonstrate the precise phenotype of the bacteria represented in the clone libraries, the results suggest that certain subpopulations of *Halomonas* dominate over other *Halomonas* groups in different hydrothermal vent, subseafloor and deep-sea habitats. In addition, only members of *Halomonas* 16S rRNA group 2 were shown to be present in deep-sea and hydrothermal vent ecosystems, bolstering a broad ecotype division within the genus between marine isolates and strains collected from hypersaline habitats, saline soils and food-curing brines, although this distinction belies several exceptions. The groups analyzed here appear to comprise functional intraspecific ecotypes with $> 99\%$ sequence identity in the 16S rRNA gene among members of a given clade. Further physiological experiments are required to decipher the phenotypic diversity among the phylogenetically closely related *Halomonas* and *Marinobacter* groups. We assume that all of the clones represent euryhaline species, but the exact temperature ranges for growth (including possible psychrophily), especially under *in situ* hydrostatic pressures, and possible mixotrophic or autotrophic growth on Fe^{2+}, have yet to be determined. It would be instructive to attempt to isolate psychrophilic *Halomonas* and *Marinobacter* spp. using cold temperatures, elevated salinity and *in situ* hydrostatic pressure during enrichment.

Recent work has revealed that multiple species of *Halomonas* and *Marinobacter* produce novel, highly efficient Fe^{3+} siderophores (Martinez *et al.*, 2000; Hickford *et al.*, 2004; Homann *et al.*, 2009a, b). The presence of mechanisms to specifically obtain metals emanating from vents may help explain the success of these two genera in iron-rich environments. Interestingly, at least one of the siderophore-producing *Halomonas* species is also a Mn^{2+} oxidizer, indicating that this species may be involved in both the iron and the manganese cycles in hydrothermal vent environments (Homann *et al.*, 2009b). While these are not the only microorganisms important in iron cycling, their presence in hydrothermal fields where iron concentrations are high suggests an active role in the iron cycle for *Halomonas* and *Marinobacter*.

To determine the global distribution of *Halomonas* and *Marinobacter* species, we mapped the origins of sequences deposited in GenBank (Fig. 1b). Clones and isolates have been retrieved from every ocean, and many of the

sampling sites were either influenced by hydrothermal processes or highly saline (Table S2). The occurrence of these lineages is extremely widespread in hydrothermal settings, including the Juan de Fuca Ridge, EPR, Mid-Atlantic Ridge, Kumano Nada mud volcano, Nankai Trough, Murray Seamount, Loihi Seamount, Suiyo Seamount and North Fiji Basin.

Conclusions

The application of taxonomically focused PCR primers designed to detect natural populations of *Halomonas* and *Marinobacter* revealed niche partitioning among *Halomonas* groups 2A, 2B and 2C. Complementary phylogenetic and physiological data suggest that *Halomonas* group 2A represents a subseafloor clade, whereas *Halomonas* groups 2B and 2C represent cold-adapted lineages, the latter possibly associated with metal sulfide deposits. A monophyletic *Marinobacter* clade detected may represent Fe^{2+}-oxidizing facultative chemoautotrophs. Both *Halomonas* and *Marinobacter* are detected in cultures and clone libraries globally, revealing that they are cosmopolitan genera with localized enrichments in deep-sea, hydrothermal vent and subseafloor settings. Their widespread distribution and ease of culturing suggest that *Halomonas* and *Marinobacter* are excellent candidates as model hydrothermal vent microorganisms for laboratory-based studies of deep-sea hydrothermal vent metal cycling.

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Authors' contributions

J.Z.K. helped to conceive the study, participated in the sequencing of the 16S rRNA genes, performed the molecular alignments and analyses and drafted the manuscript. J.B.S. helped with phylogenetic analyses and biogeography and helped draft the discussion. K.J.E. helped with editing and manuscript conception. J.A.B. helped to conceive the study and edited the manuscript. All authors read and approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. *Halomonas* and *Marinobacter* ecotypes from hydrothermal vent, subseafloor and deep-sea environments.

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