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Spatial patterns of *Aquificales* in deep-sea vents along the Eastern Lau Spreading Center (SW Pacific)

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ABSTRACT

The microbial diversity associated with actively venting deep-sea hydrothermal deposits is tightly connected to the geochemistry of the hydrothermal fluids. Although the dominant members of these deposits drive the structure of the microbial communities, it is less well understood whether the lower abundance groups are as closely connected to the geochemical milieu, or driven perhaps by biotic factors such as microbial community interactions. We used the natural geochemical gradients that exist in the back-arc basin, Eastern Lau Spreading Center and Valu-Fa Ridge (ELSC/VFR) in the Southwestern Pacific, to explore whether the chemolithotrophic *Aquificales* are influenced by geographical location, host-rock of the vent field or deposit type. Using a combination of cloning, DNA fingerprinting (DGGE) and enrichment culturing approaches, all genera of this order previously described at marine vents were detected, i.e., *Desulfurobacterium*, *Thermovibrio*, *Aquifex*, *Hydrogenivirga*, *Persephonella* and *Hydrogenothermus*. The comparison between clone libraries and DGGE showed similar patterns of distribution of different *Aquificales* whereas results differed for the enrichment cultures that were retrieved. However, the use of cultivation-based and -independent methods did provide complementary phylogenetic diversity overview of the *Aquificales* in these systems. Together, this survey revealed that the ELSC/VFR contains some of the largest diversity of *Aquificales* ever reported at a deep-sea vent area, that the diversity patterns are tied to the geography and geochemistry of the system, and that this geochemical diverse back-arc basin may harbor new members of the *Aquificales*.

Introduction

The order *Aquificales* is widespread in terrestrial and deep-sea hydrothermal systems. The lineage is represented by three families: the *Aquificaceae*, *Hydrogenothermaceae* and *Desulfurobacteriaceae* [32]. Genera from all families are found at deep-sea vents, and mostly associated with the hydrothermal deposits that form as the cold oxygenated seawater mixes with the high temperature hydrothermal fluid. Some of the genera described from marine environments are *Aquifex*, *Hydrogenivirga*, *Persephonella*, *Hydrogenothermus* and *Desulfurobacterium* [15,17,21,28,36]. With the exception of *Aquifex* (which grows best over 80 °C), the other marine *Aquificales* genera are moderate thermophiles growing optimally around 65–75 °C [32].

Although the *Aquificales* are not the most prevalent bacterial lineage in hydrothermal deposits, they represent important members of the bacterial communities as they grow at higher temperatures than the dominant *Epsilonproteobacteria*. The *Aquificales* share some of the same metabolic strategies, such as sulfur oxidation and nitrate reduction, with this proteobacterial class [5] but the ability to grow at higher temperatures gives them a specialized niche. However, little is known about the factors that influence their distribution and diversification. Within the genus *Persephonella* there seems to be a large scale (between two major hydrothermal basins) biogeographical distribution [25]. However, whether diversification occurs on smaller scales, like between vent fields within a spreading center, or whether other factors such as deposit type, affect the colonization and whether this pattern is widespread among different genera of the *Aquificales*, remains unknown.

Because hydrothermal vents associated with back-arc basins generally have greater heterogeneity in geology and in geochemistry than those at mid-ocean ridges [14], they provide a unique setting to explore links between environmental variables and microbial diversity in hydrothermal systems. For example, Flores et al. [11] showed that the structure of the archaeal and bacterial communities associated with actively venting hydrothermal

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deposits from several vent fields along the back-arc basin, the Eastern Lau Spreading Center (ELSC) and Valu-Fa Ridge (VFR) in the Southwestern Pacific, was driven in part by the differences in the geochemistry of the hydrothermal vent fields.

Here, we report the diversity of *Aquificales* along the ELSC/VFR and explore the links between their occurrence and the geochemical heterogeneity of this hydrothermal system. The results show that the ELSC/VFR hosts the greatest diversity of *Aquificales* ever reported in a single study at deep-sea vents and that the *Aquificales* community is influenced by geographical location, rock underlying the hydrothermal system (host-rock) and deposit structure. Furthermore, phylogenetic analyses suggest that this system may harbor *Aquificales* genera not yet detected in other deep-sea hydrothermal areas.

Materials and methods

Study site

The ELSC/VFR is located near the Tonga subduction zone in the Southwestern Pacific (between 19°20′ and 22°45′ S) of about 240 km [24,26] (Fig. 1). The ELSC is divided into two segments that have different axial morphologies. The Northern ELSC is the deeper zone (2500–3000 m) and extends from 19°20′ S to 20°32′ S. Two vent fields were sampled in this area: Kilo Moana (KM) and Tow Cam (TC). The Central ELSC (2000–2500 m deep) located between 20°40′ and 21°26′ S contains the ABE site. The shallowest southern part of the ridge is known as the Southern ELSC or the Valu-Fa Ridge (VFR, 1600–2000 m, 21°26′ to 22°45′ S) [24] and three vent fields were sampled: Tu'i Malila (TM), Mariner (MA) and Vai Lili (VL).

Sampling and DNA extraction

Rock samples were collected with the ROV Jason II during the cruise TUIM05MV on the R/V Melville in 2005 and placed in an insulated box in the ROV basket. Once shipboard, rocks were imaged, then sectioned into subsamples for molecular analysis and culture isolation. Subsamples for DNA analysis were ground and immediately extracted shipboard using the UltraClean™ Soil DNA Isolation Kit (MO BIO). Rock slurries were ground and kept under a N_2 atmosphere for culture enrichments.

Culturing

Rock slurries from chimneys were used to inoculate serum vials containing modified MSH mineral medium [15]. This medium is routinely used for the isolation and growth of *Aquificales*. All experiments were done with CO_2 as the only carbon source but different electron donors and acceptors were used (H_2/nitrate, H_2/O_2, S°/H_2), as in Götz et al. [15]. Enrichments were incubated at 70 °C without agitation. Enrichment cultures were purified by multiple (5–8) dilution-to-extinction series. Purity of isolates was checked by sequencing the 16S rRNA gene as described below.

PCR-DGGE and cloning

For DGGE analysis, *Aquificales* partial 16S rRNA genes were amplified by PCR using the set of primers 338F-GC (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGGCAGCAG) and AQFX540R (TCGCGCAACGTTCGGGACC) [35]. Each reaction contained: 1× PCR Buffer (Promega), 2 mM $MgCl_2$, each dNTP (200 μM), 0.5 μM of each forward and reverse primer, 1 U of *Taq* polymerase (Promega) and 10 ng of gDNA. PCRs were as follows: 1 cycle of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C, followed by a final extension of 7 min at 72 °C. PCR products were run on a 6% polyacrylamide gel with a denaturant gradient ranging from 30% to 70% during 4 h at 200 V as described previously [27]. In addition to the PCR products, a custom genomic DNA ladder was run to allow comparison between different gels. Gels were stained with SYBR Green and visualized under a UV lamp and recorded using the Kodak 1D Image Software. DGGE bands from the gel were stabbed with sterile pipette tips and reamplified by PCR using the same sets of primers without the GC-clamp.

The diversity of *Aquificales* was also studied by cloning and sequencing. About 500 bp of the 16S rRNA gene were amplified by PCR using the bacterial-specific primer 8F (AGAGTTTGATCCTGGCTCAG) and the *Aquificales*-specific primer AQFX540R using the same PCR conditions above mentioned. PCR products were cloned using the TOPO TA cloning kit (Invitrogen). Putative positive colonies were picked, grown in TYGPN broth and their plasmids extracted as described by Ng et al. [29]. Inserts were amplified by using primers M13F and M13R as described previously [30].

Sequencing and phylogenetic analysis

16S rRNA gene products obtained from DGGE bands, plasmid inserts and isolates were purified with a PCR clean-up kit (MO BIO) and sequenced using the ABI PRISM Big Dye Terminator kit and an ABI sequencer according to the manufacture's protocol. Sequences were subjected to a BLAST search [3] to obtain an indication of their phylogenetic affiliation. Sequences were aligned in the ARB software [23] according to secondary structure constraints of the 16S rRNA molecule. Similarity matrices of aligned sequences were constructed in ARB. Sequences sharing > 97% similarity in 16S rRNA sequences were grouped as the same operational taxonomic unit (OTU). A maximum-likelihood tree was constructed as described in Ferrera et al. [10] using only nearly full 16S rRNA gene sequences (>1400 nt). Clone sequences (~500 nt) were then added by using the 'quick add by parsimony' tool of ARB.

Nucleotide sequences for the 16S rRNA gene were deposited in GenBank database under accession numbers AM989515–AM989552 for clone sequences, FM955251–FM955253 for new isolates and AM993050–AM993094 for DGGE sequences.

Statistical analysis

DGGE gel images were analyzed using the software Gel 2K created by Svein Norland (University of Bergen) and freely available at http://folk.uib.no/nimsn/gel2k. The software detects bands and creates a density profile for each lane. Bands occupying the same position within and between gels were identified and two similarity matrices were constructed based on (i) presence or absence of bands and (ii) the relative contribution of each band to the total intensity of the lane. Matrices were imported into the software PRIMER-E [7] to calculate similarities in community structure by using the Bray–Curtis coefficient. For the intensity matrix, values were transformed with square root normalization and cluster analysis was performed on these matrices. Differences in microbial community structure were visualized using non-metric multidimensional scaling (MDS). To test whether differences in community structure between vent fields, rock type or sulfide deposit type were significant, a one-way analysis of similarity (ANOSIM) was performed.

BIOENV in Primer-E was used to explore whether biotic data (OTUs) correlated to a range of environmental variables (Na, Ca, Sr, Si, Mn, Fe, B, F, H_2S, H_2, CO_2, pH and temperature). BIOENV calculates the measure of agreement between the two similarity matrices, the biotic (Bray–Curtis) and the abiotic (Euclidean) matrices with Spearman rank correlation [6].