Cah. Biol. Mar. (2002) 43 : 425-428

Expanding the distribution of the *Aquificales* to the deep-sea vents on Mid-Atlantic Ridge and Central Indian Ridge

Anna-Louise REYSENBACH*[1], Dorothee GÖTZ[1], Amy BANTA[1],
Christian JEANTHON[2] and Yves FOUQUET[3]
(1) *Biology Department Portland State University Portland OR 97201 USA*
**corresponding author Fax: (1) 503 725 8570. E-mail: reysenbacha@pdx.edu*
(2) *UMR CNRS 6539 – LEMAR Institut Universitaire Européen de la Mer*
Technopôle Brest-Iroise Place Nicolas Copernic 29280 Plouzané France
(3) *IFREMER BP70 29280 Plouzané Cedex, France*

Introduction

Recently, the use of culture-independent molecular phylogenetic techniques has greatly increased our inventory of archaeal and bacterial diversity at deep-sea vents (e.g. Harmsen et al., 1997; Reysenbach et al., 2000; Takai et al., 2001) and several of the lineages appear to be endemic to deep-sea vents (Reysenbach et al., 2000b; Takai et al., 2000; Longnecker & Reysenbach, 2001). Using environmental data, some of these lineages have now been cultivated (L'Haridon et al., 1998; Campbell et al., 2001). For example, until recently, the chemolithoautotrophic and thermophilic bacterial lineage, the *Aquificales*, was restricted to shallow marine vents and terrestrial thermal springs. However, as part of two independent studies, one in Yellowstone National Park and a second exploring diversity associated with deep-sea hydrothermal vents, two environmental 16S rRNA gene sequences were identified that grouped within the *Aquificales* (pBB and VC bac27, Fig 1) (Reysenbach et al., 2000a; Reysenbach et al., 2000b). These two bacterial sequences were both related to *Aquifex*, yet different enough (>15% difference in 16S rRNA sequence) to comprise a different genus and perhaps even a different family. Using the ecological information gleaned through in situ biogeochemical experiments in Yellowstone (Reysenbach et al., 1999), it was concluded that members of this novel lineage are likely microaerophilic hydrogen-oxidizers. Subsequently, the first isolate representing this lineage was obtained from deep-sea vents at 9° 50'N on the East Pacific Rise (Reysenbach et al., 2000c). This isolate has been named *Persephonella marina* Götz et al., 2002.

In this report we demonstrate that this novel lineage in the *Aquificales* occurs at deep-sea vents in the Mid-Atlantic and Indian Oceans, and together with other deep-sea isolates, forms a distinct lineage from the terrestrial isolates and sequences.

Materials and methods

Collections. In June 2001, sulphide samples were collected from the Rainbow hydrothermal vent field (36°14'N, 32°15'W) and Menez Gwen (37°50'N, 31°32'W) along the Mid-Atlantic Ridge (MAR) using DSV *Nautile*. In April 2001, sulphide samples were collected on the Central Indian Ridge (CIR) using the ROV *Jason* at the Kairei vent field (25°19.23'S, 70°02.42 E, about 2400 m), and the Edmond vent field at about 160 km NNW of Kairei (23°52.68'S, 69°35.80 E, about 3300 m depth) (Van Dover et al., 2001). Upon retrieval shipboard, all sulphides were sectioned and then ground using a mortar and pestle. The slurries were stored anaerobically at 4 °C in serum vials until aliquots were inoculated into enrichment medium.

In order to obtain terrestial representatives, filamentous microbial mats were collected from Calcite Spring, Yellowstone National Park and inoculated into enrichment medium.

Enrichment and isolation. Sulphide slurry (0.5 ml) was inoculated into 5 ml of hydrogen oxidizing (HO) medium as described by Götz et al. (2002). All isolates from the CIR vents were enriched with oxygen as the electron acceptor, whereas the MAR isolates were enriched with nitrate (10 mM) as the electron acceptor. Hydrogen was used as the electron donor in all cases. Additional enrichments for nitrate reducers (10 mM nitrate), sulphur reducers (4% w/v sulphur) and methanogens were done on samples from the CIR vents. The filamentous biomass (0.5 ml) from Calcite

Springs was inoculated into 5 ml of the modified HO medium in which the NaCl was omitted. The medium contained S^0 as the electron donor, O_2 as the electron acceptor, the headspace was carbon dioxide. All cultures were incubated at 70 °C, and growth was monitored microscopically for about 2 days. Two enrichments from the CIR (Table 1) were incubated at 90 °C. In order to purify isolates, positive enrichments were used as inocula for end point dilution series. A nitrate enrichment from the CIR which produced some sulphide was subsequently transferred to sulphate (10 mM) reducing medium. All dilution series were repeated 3 times, after which the cultures were considered pure. Attempts to obtain colonies on Gelrite (0.8%) plates were unsuccessful. For long-term storage, pure cultures were stored in two separate collections at -80°C and in liquid nitrogen in the culture medium containing 15 % (v/v) glycerol.

Molecular phylogenetic analysis. One ml of each pure culture was centrifuged, and DNA was extracted from the cell pellets as described previously (Götz et al., 2002). Amplification, cloning, sequencing and phylogenetic analysis of the small subunit (16S) rRNA genes was done as described previously (Götz et al., 2002).

Results

Table 1 depicts the number of positive enrichments obtained, and wherever noted, the primary 16S rRNA sequence affiliation of isolates. We isolated 5 new strains of *Persephonella marina* from both the Kairei and the Edmond vent fields on the CIR, and one from the MAR vent field, Menez Gwen. An additional isolate (H3) was only 97% similar in 16S rRNA sequence to *P. marina*, and may therefore represent a new species (Fig. 1). Another representative of this group was isolated from a sulphide sample from the MAR at the Rainbow vent field. Additionally, two hydrogen oxidizing isolates growing at 90 °C were obtained from sulphides collected at the Edmond site. These isolates were about 98% similar in 16S rRNA sequence to the shallow marine chemolithotrophic hyperthermophile, *Aqulfex pyrophilus* Huber & Stetter, 1992. All strains grew well on nitrate or oxygen, when hydrogen was the electron donor.

Numerous enrichments under nitrate reducing conditions produced cultures identical to those obtained under hydrogen oxidizing conditions. All positive methanogen enrichments resulted in cultures with autofluorescent cocci, initial partial 16S rRNA gene sequence analysis of these cultures confirmed they were members of the Methanocaldococcales. One of the cultures appears to be a new species of *Methanocaldococcus* (Jeanthon & Reysenbach, unpublished data). The sulphur reducing enrichments were not identified further, although based on their morphology may be strains of *Desulfurobacterium* (L'Haridon et al., 1998). One of the nitrate reducing enrichments grew poorly, and sulphide production was noted. This culture was subsequently transferred into

Table 1. Thermophilic isolates and enrichments obtained from the Kairei and Edmond vent fields on the Central Indian Ridge.

Vent Field	Sample#	Sulphide Description	Temp °C	H_2-Oxidation	NO_3^--Reduction	Methanogen	S^0-Reduction	SO_4^{2-}-Reduction
KAIRAI FIELD	**295-1**	Fe-oxyhydroxide, distinct inner and outer band of anhydrite, pyrite, little chalcopyrite	365	*P. marinus* strain	Short rods; *P. marinus* strain	NG	NG	NG
	296-6	Fe-oxyhydroxide, pyrite with pyrrhotite, no visible anhydrite or barite		NG	NG	NG	short broad rods	NG
	297-1	Anhydrite, some Fe-oxyhydroxide; inside pyrite, chalcopyrite	355	*P. marinus* strain	Short rods	Large, AF cocci (80°C)	motile short rods	NG
	298-1	Chalcopyrite, pyrite, black shiny surface outside	335	NG	Rods, sulphide noted	NG	NG	*Thermodesulfo-bacterium* sp., nov.; 90% similar to *Thdb*;
EDMOND FIELD	**300-1**	Fe-oxyhydroxide, white precipitate, patches of reddish-brown/black	371	*P. marinus* strain	Short rods	NG	NG	NG
	301-2	Fe-oxyhydroxide coating, tubes with pyrite, some white material		*Persephonella sp. nov.* strain H3	Short rods	AF cocci	Rods	NG
	301-7	Fe-oxyhydroxide, some white precipitate at base		*A. pyrophilus*-strain 1(90°C); *P. marinus* strain	NG	NG	NG	NG
	301-8	Some anhydrite, pyrite, black outer surface		*P. marinus* strain; *A. pyrophilus* strain 2 (90°C)	NG	NG	Rods	NG
	301-11	Fe-oxyhydroxide, some white precipitate, pyrite		*P. marinus* strain	NG	AF cocci	NG	NG

AF = Autofluorescent, NG = no growth

Unless a temperature is given all isolates or enrichments were incubated at 70°C

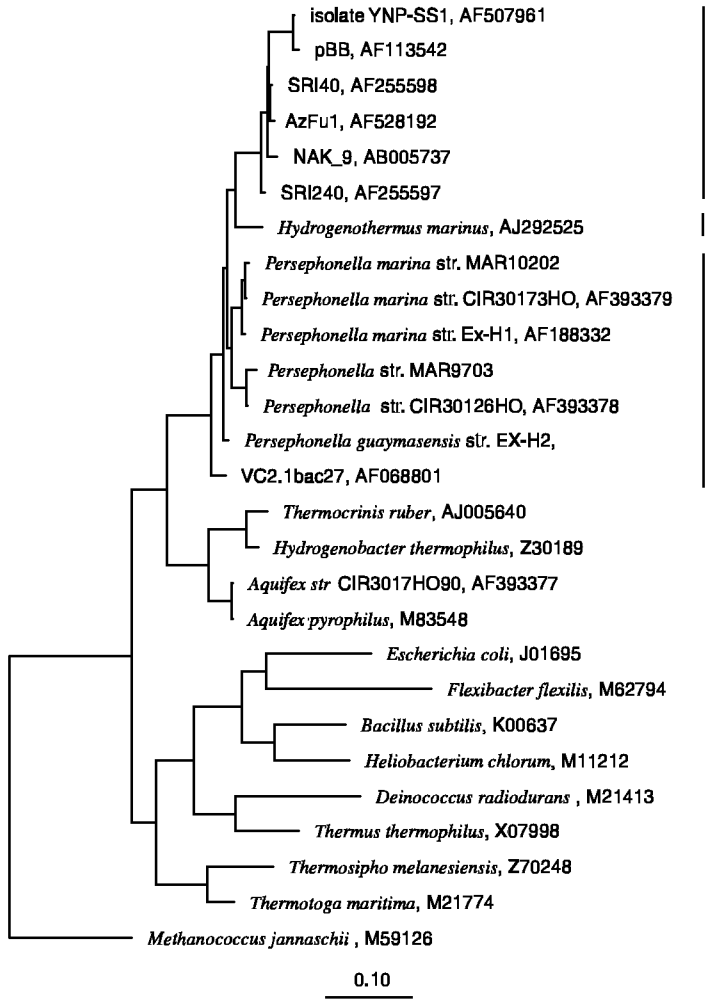

Figure 1. Maximum likelihood phylogenetic tree using16S rRNA sequences. The two lineages of the *Aquificales* are shown, the GenBank numbers of the sequences used for the analysis follow the species name.

sulphate reducing media, and growth was significantly enhanced. Based on 16S rRNA sequence analysis, the isolate is a new species of *Thermodesulfobacterium* (or a new genus).

The Genbank accession numbers for the new isolates first described here are AF507959- AF507961.

Discussion

This report expands the known distribution of one of the two primary lineages of the *Aquificales*, the *Persephonella*-lineage. The presence of members of the *Aquificales* at deep-sea vents was first detected from molecular phylogenetic analysis of the contents of an in situ growth chamber experiment deployed on a hydrothermal vent at Snake Pit, on the MAR (Reysenbach et al., 2000b). Subsequently, the first isolate of this lineage was obtained from 9°N on the EPR (Reysenbach et al., 2000c). A second isolate was obtained from vents at Guaymas Basin (Götz et al., 2002). The presence of this group in Indian Ocean and Atlantic deep-sea vent sulphides, confirms their potential global distribution at deep-sea vents. Interestingly, only strains of *Persephonella marina* (Type strain HI) and *Persephonella* sp. strain H3, have been obtained from more than one deep-sea vent biogeographic province. *P. guaymasensis* has only been obtained from Guaymas Basin. This may be a reflection of the very different chemistry (high pH fluids rich in ammonia) and mineralogy (calcite precipitation) of this vent area, or the small sample size that we have screened for these chemolithotrophic thermophiles.

Although the *Persephonella marina* strains from the Pacific, Mid-Atlantic and Indian Ocean deep-sea vents are so closely related (about 99% similar to each other) based on 16S rRNA sequence, it is likely that analysis of less-conserved genes will provide higher genetic and functional resolution of the different strains. It is generally accepted that 16S rRNA provides very limited resolution particularly regarding endemism (Beja et al., 2002; Staley & Gosink, 1999). It is interesting to note that all terrestrial isolates and sequences form a separate lineage from the shallow and deep marine isolates. Additional analyses using less conserved genes are needed to confirm that the deep-sea, shallow marine and terrestrial isolates form different phylogeographic clades. Recently, close relatives to the terrestrial isolates were obtained from a gold mine in Japan, suggesting that this group is ubiquitous in hot subsurface environments (Takai et al., 2002).

Porous sulphide structures provide a relatively stable niche for thermophiles, where the nutrients of growth are supplied by the hydrothermal fluid and seawater. Hydrogen is often measured in millimolar concentrations in end member hydrothermal fluids, and provides one of the most abundant electron donors for chemolithoautotrophic thermophiles such as sulphate-, sulphur-, iron-reducers and methanogens (McCollom & Shock, 1997). It is therefore not surprising that (Harmsen et al., 1997) showed, using fluorescent in situ hybridization (FISH), that members of the hydrogen-utilizing Aquificales and sulphur-reducing genus *Desulfurobacterium* may account for up to 40% of the microbial community associated with vent chimney's. The

sulphide structure is also an ideal environment for microaerophiles, as oxygen availability will be sporadic and limited. The ability of *Persephonella* spp. to utilize additional electron acceptors such as nitrate, sulphur, thiosulphate, gives the organism the metabolic plasticity to have a competitive advantage over other thermophiles that are able to utilize only a limited suite of substrates (Götz et al., 2002).

The closely related terrestrial members (e.g. pBB, strains YNP-SS1 and Az-Ful) of the *Persephonella* lineage are often associated with significant iron and sulphide biomineralization (Reysenbach et al., 1999). Therefore, the potential role of *Persephonella* spp. in biomineralization within sulphide structures cannot be ruled out. Although our current understanding of the diversity associated with sulphide chimneys is still rudimentary, some patterns of diversity of are emerging. As we design novel approaches to culturing these organisms in the laboratory, the role of microorganisms in sulphides will become more apparent.

Acknowledgements

The authors thank the shipboard members of the CIR cruise, pilots of the ROV *Jason* and *Nautile*, crew of the RV *Knorr* and *Atalante*. This work was supported by grant NSF OCE 0083134 to Anna-Louise Reysenbach.

References

Beja O., Koonin E. V., Aravind L., Taylor L. T., Seitz H., Stein J. L., Bensen D. C., Feldman R. A., Swanson R. V. & DeLong E. F. 2002. Comparative genomic analysis of archaeal genotypic variants in a single population and in two different oceanic provinces. *Applied and Environmental Microbiology*, **68**: 335-345.

Campbell B. J., Jeanthon C., Kostka J. E., Luther G. W. & Cary S. C. 2001. Growth and phylogenetic properties of novel bacteria belonging to the epsilon subdivision of the Proteobacteria enriched from *Alvinella pompejana* and deep-sea hydrothermal vents. *Applied and Environmental Microbiology*, **67**: 4566-4572

Götz D., Beveridge T., Rushdi A., Simoneit B., & Reysenbach A.-L. 2002. *Persephonella marina*, gen. nov., sp. nov. and *Persephonella guaymasensis* sp. nov, two novel thermophilic hydrogen-oxidizing microaerophiles from deep-sea hydrothermal vents. *International Journal of Systematic and Evolutionary Microbiology*, **52**: 1349-1359.

Harmsen H. J. M., Prieur D. & Jeanthon C. 1997. Distribution of microorganisms in deep-sea hydrothermal vent chimneys investigated by whole-cell hybridization and enrichment culture of thermophiles. *Applied Environmental Microbiology*, **63**: 2876.

L'Haridon S., Cilia V., Messner P., Raguenes G., Gambacorta A., Sleytr U. B., Prieur D. & Jeanthon C. 1998. *Desulfurobacterium thermolithotrophum* gen. nov., sp. nov., a novel autotrophic, sulphur-reducing bacterium isolated from a deep-sea hydrothermal vent. *International Journal of Systematic Bacteriology*, **48**: 701-11.

Longnecker K. & Reysenbach A. L. 2001. Expansion of the geographic distribution of a novel lineage of *epsilon-Proteobacteria* to a hydrothermal vent site on the Southern East Pacific Rise. *Fems Microbiology Ecology*, **35**: 287-293.

McCollom T. M. & Shock E. L. 1997. Geochemical constraints on chemolithoautotrophic metabolism by microorganisms in seafloor hydrothermal systems. *Geochemica et Cosmochimica Acta*, **61**: 4375-4391.

Reysenbach A. L., Ehringer M. & Hershberger K. 2000a. Microbial diversity at 83°C in Calcite Springs, Yellowstone National Park: another environment where the *Aquificales* and "Korarchaeota" coexist. *Extremophiles*, **4**: 61-67.

Reysenbach A. L., Longnecker K. & Kirshtein J. 2000b. Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. *Applied Environmental and Microbiology*, **66**: 3798-806.

Reysenbach A.-L., Banta A., Boone D. R., Cary C. & Luther G. 2000c. Microbial essentials at hydrothermal vents. *Nature*, **404:** 835.

Reysenbach A.-L., Seitzinger S., Kirshstein J. & McLaughlin E. 1999. Molecular constraints on a high temperature evolution of early life. *Biological Bulletin*, **196**: 367-372.

Staley J. T. & Gosink J. J. 1999. Poles apart: biodiversity and biogeography. *Annual Review of Microbiology*, **53**: 189-215.

Takai T., Komatsu T., Inagki F. & Horikoshi K. 2001. Distribution of Archaea in a black smoker chimney structure. *Applied Environmental Microbiology*, **67**: 3618-3629.

Takai K., Hirayama H., Sakihama Y., Inagaki F., Yamato Y. & Horikoshi K. 2002. Isolation and metabolic characteristics of previously uncultured members of the order *Aquificales* in a subsurface gold mine. *Applied Environmental Microbiology*, **68**: 3046-3054.

Van Dover C. L., Humphris S. E., Fornari D., Cavanaugh C., Collier R., Goffredi S. K., Hashimoto J., Lilley M. D., Reysenbach A. L. et al. 2001. Biogeography and ecological setting of Indian Ocean hydrothermal vents. *Science*, **294**: 818-823.