Cah. Biol. Mar. (1998) 39 : 369-372

Microbiology of shallow hydrothermal sites off Palaeochori Bay, Milos (Hellenic Volcanic Arc)

Paul R. DANDO[1], Michael THOMM[2], Hocine ARAB[2], Meike BREHMER[2], Laura E. HOOPER[1], Beate JOCHIMSEN[2], Heinz SCHLESNER[2], Rüdiger STÖHR[1], Juan-Carlos MIQUEL[3] and Scott W. FOWLER[3]

[1] *School of Ocean Sciences, University of Wales, Bangor, Menai Bridge, Anglesey LL59 5EY, U.K.*
Fax: (44) 1248 382620 - e-mail: oss109@sos.bangor.ac.uk

[2] *Institut für Allgemeine Mikrobiologie, Universität Kiel, Am Botanischen Garten 1-9, 24118 Kiel, Germany*
Fax: (49) 431 880 2194 - e-mail: mthomm@ifam.uni-kiel.de

[3] *International Atomic Energy Agency Marine Environment Laboratory BP 800, 98012 Monaco Cedex*
Fax: (33) 9205 7744 - e-mail: fowler@monaco.iaea.org

Introduction

The Hellenic Volcanic Arc, extending from the Turkish coast and the island of Kos in the east to Methana in the west, has been formed by the subduction of the African plate under the Aegean microplate. Extensive gasohydrothermal venting occurs off many of the islands, from the intertidal zone to depths of more than 100 m. The island of Milos has hydrothermal venting over approximately 34 km^2 of seabed. Conspicuous minero-bacterial mats cover extensive brine seep areas, with salinities up to 85 ‰, ΣH_2S to 2.4 mM, NH_4^+ to 0.7 mM and ΣCO_2 to 12 mM (Fitzsimons et al., 1997). The temperatures of venting fluids in only 10 m of water vary between ambient and 123°C. In the highest temperature areas elemental sulphur coats sand grains on the seabed, forming conspicuous yellow patches. The gases released are largely CO_2 but have up to 10% methane, 9% hydrogen and 8% hydrogen sulphide. This mixture of conditions provides microhabitats for a wide variety of Eubacteria and Archaea. Here we describe some of the microbiology of the vent areas, report the isolation of novel prokaryotes and the molecular analyses of archaeal communities in sediments and sinking particulates from Paleochori Bay, in the southeast of Milos.

Materials and methods

The positions from which samples were collected for analysis are shown in Fig. 1. Samples were examined by microscopy and by culture and culture-independent molecular techniques. 1 ml of sediment was used to enrich Archaea in 20 ml of the medium of Stetter et al. (1983), supplemented with $NaHCO_3$, in 100 ml serum bottles containing an H_2/CO_2 atmosphere (80:20, 300 kPa) at 90°C. Positive enrichment cultures were plated onto the same medium solidified with 1% (w/v) phytagel and 2-3% (w/v) sodium alginate and incubated in pressurized stainless steel cylinders. Cultures for mesophilic bacteria were incubated at 20, 30, or 37°C, either in shaken liquid cultures or on solid media.

Microbial community DNA was extracted directly from the sediment or sediment trap samples (Table 1) and then purified. PCR amplification of 16S rDNA: primers for PCR amplification of *Archaea* were Arch 21 F and Arch 958 R (Delong, 1992). For the amplification of *Crenarchaeota* CAF 545 and CAR 952 were used. The PCR products were cloned into the vector pUC57/T which had been pre-cleaved with Eco B21 and treated with terminal deoxynucleotidyl transferase to create ddT overhangs at both 3'ends (T-cloning kit, MBI Fermentas). The PCR products were ligated with the plasmid and circular DNA used for transformation of *E. coli* JM 109.

Achromatium in sediment samples were kept in mixed culture by adding crystals of Na_2S regularly to the bottom of a 5 cm deep sediment layer, in a covered 250 ml pot. The overlying seawater being replaced at approximately 3 day intervals to keep the pH in the range 6.0 to 7.0. In addition, a culture was maintained in a 250 ml glass beaker full of sand, into the base of which 4 mM sodium sulphide solution in seawater was pumped at a rate of 1-4 ml hr^{-1}.

Cells of *Achromatium volutans* Hinze, 1903 were obtained free from diatoms, cyanobacteria and other contaminants by first collecting the cells from a suspension by filtering on a mesh of 5 µm pore size, washing with 0.2 µm filtered sea water and further purified by differential centrifugation until no visible contaminants could be observed by microscopy. The cells were homogenized in a 100 µl glass Potter homogenizer in cold distilled water. Enzyme assays for ribulose*bis*phosphate carboxylase (EC 4.1.1.39), sulphate adenylyltransferase (EC 2.7.7.4) and adenylylphosphosulphate reductase (EC 1.8.99.2), were carried out by previously described procedures (Dando et al., 1985) at a temperature of 20°C.

Thioploca sp. were kept in a microcosm in a 50x350x400 mm tank of sediment from Palaeochori Bay, enriched with Na_2S and S°, with 7 cm of sea water of 37 ‰ salinity overlying. The pH of the water was again kept within the range of 6.0 to 7.0 by periodically replacing some of the water. Time lapse video was used to observe movements of the bacteria within the microcosms as well as within capillary slides of 400 µm depth.

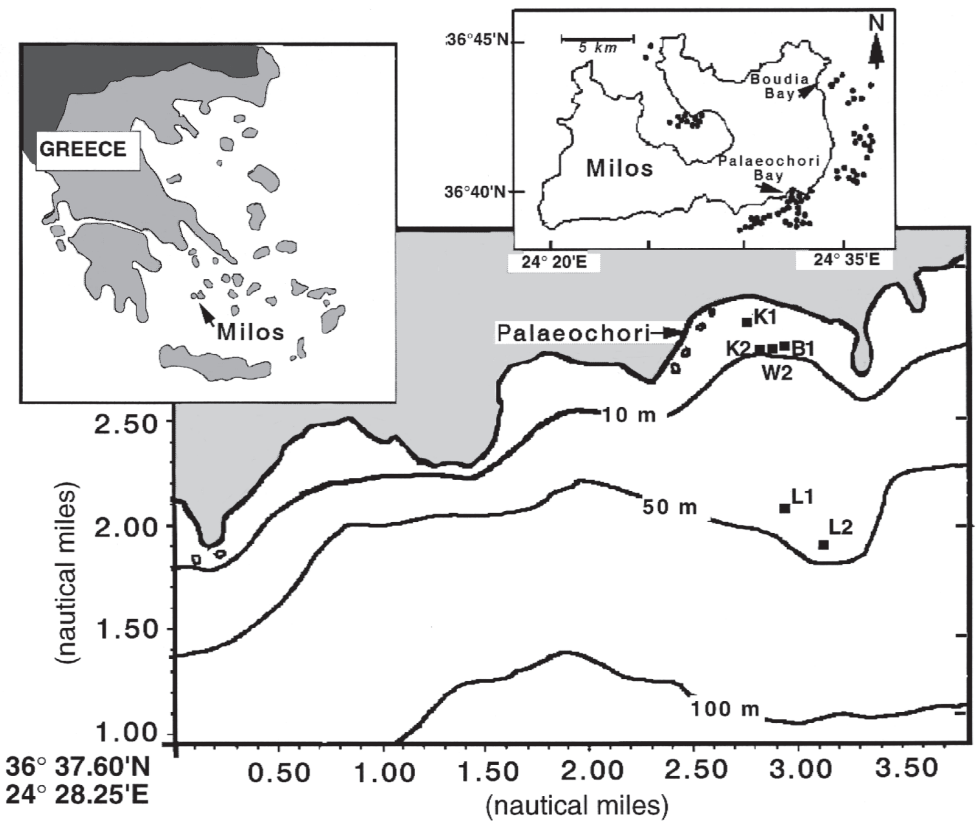

Figure 1. Location of the sampling sites. The positions of the hydrothermal vent areas are indicated, by closed squares, on the inset map of Milos.

Results and Discussion

I. Isolation of anaerobic hyperthermophilic Archaea

Sediment samples were taken from four sites in Palaeochori Bay (K1, K2, B1 and W2), water depth 5-10 m (Fig. 1). A new hyperthermophilic Crenarchaeote, *Stetteria hydrogenophila* Jochimsen et al., 1997 representing a new genus within the family of *Desulfurococcacae,* was isolated from a depth of 10 m (Jochimsen et al., 1997). In addition, four novel coccoid isolates, P3, P5, P6 and P8, were obtained which grew in culture at 90°C. 16S rRNA sequencing revealed that two strains showed a sequence similarity to the described species *Thermococcus chitonophagus* Huber et al., 1995 and *Pyrococcus horikoshii* Gonzalez et al., 1998 ≤95%. The 16S rRNA of strains P3 and P8 showed 98% sequence similarity to that of *Pyrococcus abyssi* Erauso et al., 1993 and *Staphylothermus marinus* Stetter et Fiala, 1986 respectively. In DNA/DNA hybridization experiments under relaxed conditions, strains P6 and P5 did not show any homology to *Pyrodictium occultum* Stetter et al., 1984 *Staphylothermus marinus, Thermococcus celer* Zillig, 1983 and *Pyrococcus woesei* Zillig et al., 1986, suggesting that strains P5 and P6 represent new species or genera.

II. Occurrence of aerobic micro-organisms

Measurements in Palaeochori Bay sediments showed oxygen penetration varying from a depth of 4 mm outside the vent to 8 mm in the recharge area 2 m from the vent centre. Closer to the vent, in the higher temperature area < 1.8 m from the centre, hydrogen sulphide emerged from the sediment and oxygen was already depleted at the sediment surface (W. Ziebis, pers. comm.). The high H_2S content was due to the hydrothermal fluids and to the activity of sulphur reducing Archaea. In accordance with this finding no aerobes could be enriched from sediment samples at temperatures between 60-85°C. However, a variety of aerobic mesophilic Bacteria were isolated. These probably thrive in ambient seawater. 39 of the 80 strains obtained were endospore forming gram-positive Bacteria phenotypically resembling *Bacillus* sp. The presence of so many endospore-forming bacilli may indicate that spore formation is used as a survival mechanism in a situation where the rapidly changing conditions can frequently become lethal for growing cells. Endospore forms could not be isolated from the water column or a non-hydrothermal site on the same coast.

Samples	BK 16S6-6 (sediment core) site B1	Sediment trap site C	Sediment trap site C
Site	Paleochori bay	Particles collected	Particles collected
Date	16/09/96	12-24 /09/96	25/09/96
Water depth	7 m Temp.: 80°C	60 m Bottom depth: 90 m	60 m Bottom depth: 90 m
Position	36°40.391'N 24°31.220'E	36°39.11'N 24°31.81'E	36°39.11'N 24°31.81'E
PCR primers	CAF 545/CAR 952 (crenarchaeal specific primers)	ARCH 21F/ ARCH 958R (universal archaeal primers)	ARCH 21F/ ARCH 958R CAF 545/ CAR 952
Sequence similarity after cloning; numbers of identical nucleotides (n/n) in %.	*Staphylothermus marinus* (452/456; 99%) 12 of 39 clones = 31%	*Thermosphaera aggregans* (671/744; 90%) 5 of 27 clones = 18%	*Staphylothermus marinus* (593/606; 97%) 16 of 32 clones = 50%
Frequency (%) of each of the clones on total clone numbers.	*Desulfurococcus mobilis* (440/456; 96%) 7 of 39 clones = 18%	*Stetteria hydrogenophila* (835/898; 92%) 1 of 27 clones = 41%	*Sulfophobococcus zilligii* (520/573; 90%) 2 of 32 clones = 6%
	Thermodiscus maritimus (448/457; 98%) 15 of 39 clones=38%	*Sulfophobococcus zilligii* (859/924; 92%) 7 of 27 clones = 26%	14 sequences represent new archaeal lineages (44%)
	5 sequences represent new archaeal lineages (13%)	4 sequences represent new archaeal lineages (15%)	

Table 1. Sequence analysis of environmental samples

16S rRNA analyses revealed that a halophilic spore-forming isolate differed from previously described *Bacillus* species. This isolate grew at NaCl-concentrations between 2 and 23% and had a L-ornithine, D-glutamate type of cell wall. The latter property discriminates this strain from all hitherto described halophilic bacilli.

Surface sediments collected from a low-temperature (18.5±1.2°C), pH 5.8, diffuse vent area, at 46 m depth (L1) contained white 'threads' embedded in a mucopolysacchraride matrix. They were identified as sheaths, of diameters up to 175 µm, containing trichomes of the giant sulphur-oxidizing bacteria *Thioploca* sp. The mean trichome width was 6.7 ± 0.56 µm with a cell length of 4.0 - 12.5 µm. A few trichomes were also isolated from sediment collected at the W2 site. In laboratory microcosms the sheaths penetrated up to 40 - 60 mm into the sediment, often being completely buried, but formed in the water column under low oxygen conditions. At times some of the trichomes projected from the sheath, or left the sheath entirely. Individual trichomes moved synchronously up and down within the sheaths on a 20 min cycle at speeds of 8.4±3.1 mm h^{-1}. The reason for this short periodicity is unknown but may serve to circulate water within the sheath. *Thioploca* is common in reducing sediments, particularly off the western coast of the Americas, but has not previously been reported from hydrothermal areas.

Achromatium volutans, another giant sulphur-oxidizing bacteria was found at 5-10 m sites (K1, K2, W2) and at 46 m depth (L2). The cells had a diameter of 5-90 µm and contained sulphur globules of 1-2 µm diameter. They were found in the upper 1-15 mm of the sediment, forming "white sand" when numerous, due to the reflective sulphur globules in the cells coating the sand grains. *A. volutans* also colonized slate settlement plates placed 1-5 cm above the sediment. Cells frequently accumulated in high density in the surface 1-2 mm. These mats of *A. volutans* were less abundant in 1996-7 than when first described at the sites in 1992-4, possibly due to grazing by increased numbers of the gastropod, *Cyclope neritea* (Linné, 1758). In the laboratory cultures, it was observed that *A. volutans* cells moved to the surface as sediment sulphide concentrations increased and moved deeper into the sediment as they fell. The cells were motile in NaCl concentrations of 2-5 %, with highest mobility at 3-3.5 %, but were inactive in 1 and 6 % NaCl and at dissolved sulphide concentrations > 1 mM. In capillary microslides with an oxygen input from air microbubbles the cells aligned in a band less than 200 µm wide in the microaerobic zone parallel to the air interface (Figure 2). *A. volutans* did not survive well in sea water at normal oxygen saturation, but did so, and continued to divide, in anoxic seawater, suggesting that it may respire using nitrate.

Enzyme activity measurements on *Achromatium* extracts showed activity for ribulosebisphosphate carboxylase (0.22 nmol CO_2 fixed mg $protein^{-1}$ min^{-1}), adenylylphosphosulphate reductase (0.15 µmol mg^{-1} min^{-1}) and sulphate adenylyltransferase (0.17 µmol mg^{-1} min^{-1}). These enzymes are indicative of autotrophic sulphur oxidizing activity and this is the first occasion that autotrophic activity has been shown for *Achromatium* sp. The assumption that it was chemo-autotrophic was based solely on its habitat and upon the accumulation of elemental sulphur in the cells. When a bell jar was placed on top of one of the microbial mats containing *A. volutans,* the pH in the overlying water fell to

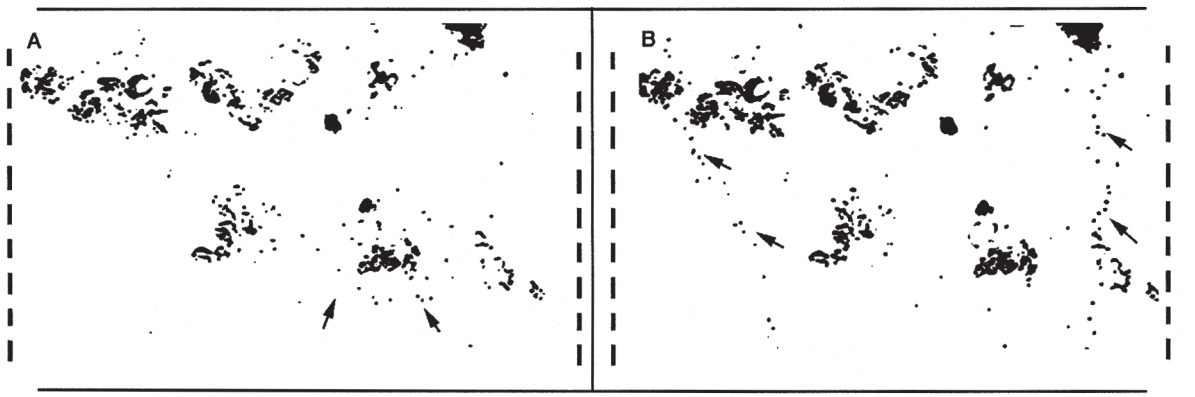

Figure 2. Directed movement of *Achromatium volutans,* the cells are indicated by arrows, the larger objects present are sand and detrital particles: A) position of the cells 18 min after start of experiment with cells in de-oxygenated seawater and air bubbles and B) 71 minutes later. Thick dotted lines indicate tube ends.

2.05, compared with 8.5 in the seawater and >6.9 in the underlying sediment, due to bacterial oxidation of sulphide.

Filamentous gliding bacteria, currently of an unknown genus, also reached high numbers on the surface of reducing sediment from sites W2 and K1 in mesocosms. The filaments, 12 µm to 1050 µm in length, with a width of 3 µm, often formed a single network covering the surface of the sediment and aggregated into foci of 200-300 filaments. Time-lapse video observations showed that the cluster of filaments could move together as a single organism. *Achromatium* cells often gathered at the nuclei of some of these foci of the gliding bacteria. However these concentrations of *Achromatium* and filaments were not observed in newly collected sediments.

The hydrothermal brine has been shown to rise and fall within the sediment, following changes in sea level (Fitzsimons et al., 1997). The motility of the different bacteria living at the oxic-anoxic interface will therefore be important in maintaining an optimum position in a frequently changing chemical gradient.

III. Molecular composition of hot vent communities in Palaeochori Bay

Primers specific for 16S rRNA of Crenarchaeota (R. Hellwig, pers. comm.) and Archaea (DeLong, 1992) were used to amplify the 16S rRNA encoding genes from DNA extracted from sediments and a sediment trap (Table 1). At the B1 site (Fig. 1) close relatives of *"Thermodiscus maritimus"* Stetter, 1986, *Staphylothermus marinus* and *Desulfurococcus mobilis* Zillig & Stetter, 1983 were identified as the predominant archaeal species. 5 sequences represent hitherto unknown archaeal lineages. At the sediment trap C, at 60 m depth in 90 m deep water, a variety of Archaea were detected in material collected over a period of 12 days. The dominant species in this sample were relatives of *Stetteria hydrogenophila, Sulfophobococcus zilligii* Hensel et al., 1997 and of *Thermosphaera aggregans* Huber et al., 1998 (Table 1). 4 sequences belong to thus far not cultivated archaeal lineages. In the material collected at the same site on 25 September, relatives of *Staphylothermus marinus* and *Sulfophobococcus zilligii* were detected, but 44% of the 16S rRNA PCR amplificates belong to thus far unknown archaeal phyla.

The data accumulating from studies of the Milos vents are demonstrating a high degree of both archaeal and eubacterial diversity, reflecting the very varied physical and chemical conditions present around the vents.

Acknowledgements

The IAEA Marine Environment Laboratory operates under an agreement between the International Atomic Energy Agency and the Government of the Principality of Monaco. We thank Jan Kuever for confirmation of the identity of *Thioploca,* Stefie Schwabe and the late Rob Palmer for the collection of samples from the deeper sites and Prof. Varnavas for arranging the fieldtrips. This work was supported by EC MAST contracts MAS2-93-0058 and MAS3-95-0021, the Fonds der Chemischen Industrie and the Royal Society.

References

Dando P. R., Southward A. J., Southward E. C., Terwilliger N.B. & Terwilliger R. C., 1985. Sulphur-oxidising bacteria and haemoglobin in gills of the bivalve mollusc *Myrtea spinifera. Marine Ecology Progress Series,* **23**: 85-98.

De Long E. 1992. Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences,* **89:** 5685-5689.

Fitzsimons M. F., Dando P. R., Hughes J. A., Thiermann F., Akoumianaki I. & Pratt S. M. 1997. Submarine hydrothermal brine seeps off Milos, Greece: observations and geochemistry. *Marine Chemistry,* **57:** 325-340.

Jochimsen B., Peinemann-Simon S., Völker H., Stüben D., Botz R., Stoffers P., Dando P. R. & Thomm M. 1997. *Stetteria hydrogenophila, gen. nov.* and *sp. nov.,* a novel obligate mixotrophic sulphur-dependent Crenarchaeon isolated from Milos, Greece. *Extremophiles,* **1:** 67-73.

Stetter K. O., König H., & Stackebrandt E. 1983. *Pyrodictium* gen. nov., a new genus of submarine disc-shaped sulphur reducing archaebactera growing optimally at 105°C. *Systematic and Applied Microbiology,* **4:** 535-551.