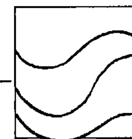

Mesophilic sulfate-reducing bacteria from three deep-sea hydrothermal vent sites

Hydrothermal vents
Bacteria
Sulfate-reducers
Mesophiles
Pleomorphs

Sources hydrothermales
Bactéries
Sulfato-réductrices
Mésophiles
Pléomorphes

Lars ELSGAARD [a]*, Jean GUEZENNEC [b], Nadia BENBOUZID-ROLLET [a] and Daniel PRIEUR [a]

[a] Université Paris 6, Pierre et Marie Curie, and Centre National de la Recherche Scientifique (CNRS), Station Biologique de Roscoff, 29682 Roscoff, France.

[b] Institut Français de Recherche pour l'Exploration de la Mer (IFREMER), Centre de Brest, DRO/EP, Laboratoire de Biotechnologie des Micro-organismes Hydrothermaux, 29280 Plouzané, France.

*Present address:
Lars Elsgaard, Danish Institute of Plant and Soil Science, Department of Soil Science, Research Centre Foulum, P.O. Box 23, DK-8830 Tjele, Denmark.

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ABSTRACT

The present study was conducted to determine the presence and physiology of mesophilic sulfate-reducing bacteria (SRB) at deep-sea hydrothermal vents, which constitute remote ecosystems, largely dependent on their own chemoautotrophic primary production. SRB were enriched and isolated from samples of hydrothermal water, invertebrates, chimneys, and sediment collected at deep-sea (1700 to 2600 m) hydrothermal vent sites in the Lau Basin, in the North Fiji Basin, and at 13°N on the East Pacific Rise. From the hydrothermal fields in the Lau Basin and the North Fiji Basin, SRB were cultured at 30°C from 19 out of 21 samples, including five samples of high-temperature hydrothermal water (> 100°C). Acetate, benzoate, formate, isobutyrate, and lactate were supplied as single electron donors and could all be degraded by SRB. From the site at 13°N, SRB were enriched at 20 or 40°C from seven out of 20 samples. Two *Desulfovibrio* strains, H 2.5 and H 5.3, originated from the tubes of the polychaete *Alvinella* sp. and these strains had optimum temperatures at 37 to 40°C with doubling times of 3.6 and 6.6 hours, respectively. In cultures of strain H 2.5, which had reached the stationary growth phase, almost the entire populations (> 99 %) changed into round pleomorphs (coccoid bodies). However, cultures of vibrio-shaped bacteria were recovered upon transfer of an inoculum to fresh medium. No distinct changes in the composition of the cell membrane phospholipid fatty acids were associated with the morphological transformation. In the deep-sea hydrothermal vent environment, mesophilic SRB occurred frequently and may thrive in anoxic habitats in association with warm vent animal communities.

RÉSUMÉ

Bactéries sulfato-réductrices mésophiles de trois sites d'hydrothermalisme profond.

Cette étude a été entreprise dans le but de déterminer la présence et d'étudier la physiologie de bactéries sulfato-réductrices mésophiles (BSR) dans les sources hydrothermales océaniques, écosystèmes dépendant principalement d'une pro-

duction primaire locale assurée par des micro-organismes chimio-autotrophes. Des BSR ont été cultivées et isolées d'échantillons de fluides hydrothermaux, d'invertébrés, de cheminées et de sédiments collectés sur les sites profonds (1700 à 2600 m) du Bassin de Lau, du Bassin nord-Fidjien et de 13°N sur la ride du Pacifique oriental. Des BSR ont été cultivées à 30°C à partir de 19 échantillons (sur 21), y compris cinq échantillons de fluides hydrothermaux à haute température (> 100°C) récoltés dans le Bassin de Lau et le Bassin nord-Fidjien. Ces BSR peuvent utiliser l'acétate, le benzoate, le formate, l'isobutyrate ou le lactate, fournis individuellement comme donneurs d'électrons. Des cultures de BSR ont été obtenues à 20 et 40°C pour sept des 20 échantillons récoltés sur le site de 13°N. Deux souches utilisant le lactate (*Desulfovibrio* sp.) ont été isolées de tubes du Polychète *Alvinella* sp. Ces souches, H 2.5 and H 5.3, ont un optimum de température à 37 à 40°C avec des temps de doublement de 3,6 et 6,6 heures. Dans les cultures de H 2.5 qui ont atteint la phase stationnaire, la presque totalité de la population (> 99 %) devient pléomorphe (formes coccoïdes). Cependant, des cellules de forme vibrioïde sont à nouveau observées après transfert d'un inoculum dans du milieu frais. Aucun changement significatif dans la composition d'acides gras dérivés des phospholipides membranaires n'a été observé lors des transformations morphologiques. Dans les sources hydrothermales profondes, les BSR mésophiles sont fréquentes et peuvent se développer dans les habitats anoxiques, associés aux communautés animales liées aux sources chaudes.

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INTRODUCTION

Sulfate-reducing bacteria are characterized by their anaerobic energy metabolism based on dissimilatory reduction of SO_4^{2-} to H_2S. Although oxygen tolerance has been reported for some mesophilic SRB (Marschall *et al.*, 1993; Santos *et al.*, 1993), their activity and growth are principally confined to anoxic environments, such as aquatic sediments. In coastal marine sediments, sulfate is abundant and bacterial sulfate reduction may account for up to 50% of the overall mineralization of photosynthetically-derived organic carbon that sinks out from the euphotic water column and deposits at the sea-floor (Jørgensen, 1982).

Due to the limited nutrient input and the low temperatures, bacterial sulfate reduction and other mineralization processes in deep-sea sediments generally proceed at low rates (Bender and Heggie, 1984; Canfield, 1991). However, the concept of extremely slow process rates in deep-sea organisms has been challenged, notably by the discovery of luxuriant animal communities at deep-sea hydrothermal vents (Jannasch and Taylor, 1984; Gage, 1991). Since their discovery in 1977, hydrothermal vent communities have been found at several sites along the spreading centres of the Earth's mid-ocean ridges and at back-arc spreading centres, which are particularly common in the Western Pacific (Karl, 1987; Hessler and Lonsdale, 1991; Tunnicliffe, 1991). A common feature of the vent communities is dependence on microbial chemoautotrophy based on the oxidation of reduced gases (especially H_2S) in the emitted vent waters (Jannasch and Mottl, 1985). Thus, chemoautotrophic and mixotrophic bacteria occur abundantly in the vent environments, either free-living, in microbial mats or in symbiotic associations with the vent animals (Jannasch and Mottl, 1985; Karl, 1987).

Heterotrophic bacteria have also been found in elevated numbers at the hydrothermal vent sites and may also be associated with the vent animals (Prieur *et al.*, 1989, 1990). However, the role of heterotrophic bacteria in the overall mineralization processes at deep-sea hydrothermal vents has received little attention.

In the course of three deep-sea cruises, samples from hydrothermal vent areas were collected for a microbiological survey of both autotrophs and heterotrophs (Prieur *et al.*, 1989, 1990; Durand, 1992). One of the objectives was to study the presence and diversity of mesophilic SRB in the samples retrieved. We report here the outcome of these enrichment and cultivation studies and discuss the occurrence of mesophilic SRB in hydrothermal vent habitats.

MATERIALS AND METHODS

Study sites and sampling

Samples were retrieved by the manned submersible NAUTILE during the cruises BIOLAU to the Lau back-arc Basin (May, 1989), STARMER II to the North Fiji Basin (June-July, 1989), and HYDRONAUT to the East Pacific Rise at 13°N (November, 1987). Solid samples were collected directly by the mechanical arms of NAUTILE, while water samples were collected with titanium syringes or by a revolving water sampling device (Prieur *et al.*, 1989, 1990). All samples were brought to the surface in an insulated box. During BIOLAU, samples of heated water, invertebrates and chimneys were collected at the hydrothermal fields *Vai Lili* (22°13'S, 176°37'W; ~ 1700 m) and *Hine Hina* (22°32'S, 176°43'W; ~ 1850 m). Warm vents

(2 to 33°C) occurred at both fields and harboured an associated fauna mainly composed of gastropods and bivalves, such as *Alviniconcha hessleri*, *Ifremeria nautilei*, and *Bathymodiolus* spp. (Desbruyères *et al.*, 1994). Active smoker chimneys with exit temperatures of 250 to 400°C were present at *Vai Lili* (Fouquet *et al.*, 1991), but generally no fauna was associated with the walls of the high-temperature chimneys (Desbruyères *et al.*, 1994). During STARMER, samples of chimneys and sediment (*i.e.*, fine and coarse mineral debris) were collected at the *White Lady* hydrothermal vent field (16°59'S, 173°55'W; ~ 2000 m), where warm vents (~ 31°C) and active smoker chimneys (91 to 296°C) were observed, and where the vent fauna was dominated by the same species as these discovered in the Lau Basin (Auzende *et al.*, 1989; Desbruyères *et al.*, 1994). During HYDRONAUT, samples of hydrothermal water, immersed titanium tubes, invertebrates, and chimneys were collected at various hydrothermal vent fields near 12°48'N, 103°57'W (~ 2600 m). In this hydrothermal area, warm vents (~ 20°C) and high-temperature (320°C) smoker chimneys were observed (Desbruyères *et al.*, 1982). The fauna associated with the walls of smoker chimneys included the polychaetes *Alvinella pompejana*, *A. caudata*, and *Paralvinella grasslei* (Desbruyères *et al.*, 1982). The bivalve *Bathymodiolus thermophilus* and the vestimentiferans *Riftia pachyptila* and *Tevnia jerichonana* were found in areas with warm venting (Desbruyères *et al.*, 1982; Prieur *et al.*, 1989).

Enrichment and cultivation

Medium for cultivation of SRB was prepared from a basal salt solution, which contained (in grams per litre of distilled water): $CaCl_2 \cdot 2H_2O$, 0.15; KCl, 0.3; KH_2PO_4, 0.2, $MgCl_2 \cdot 6H_2O$, 3.0; NaCl, 20; Na_2SO_4, 3.0; NH_4Cl, 0.3. Yeast extract was added to 50 mg l^{-1} and resazurin was added in trace amounts as a redox indicator. After autoclaving and cooling under an atmosphere of N_2, the medium was supplemented with trace elements, selenite, vitamins, $NaHCO_3$ (pH buffer) and Na_2S (reductant) according to Pfennig *et al.* (1981). For final reduction of the medium, and to enhance the growth of SRB, a freshly prepared and filter-sterilized (0.2 μm) dithionite solution was added to a concentration of 100 μM $S_2O_4^{2-}$. Carbon sources (acetate, benzoate, formate, isobutyrate, lactate and yeast extract) were added from sterile stock solutions (Widdel and Bak, 1992), and the final pH of the medium was adjusted to 7.4 ± 0.2.

On board ship, the BIOLAU samples were transferred to reduced basal medium supplemented with acetate and lactate (20 mM each), while the STARMER samples were transferred to basal medium amended with either acetate, benzoate, formate, isobutyrate, lactate (all at 20 mM) or yeast extract (0.2 %) as single carbon sources. During both cruises, enrichments were incubated at 28 to 30°C (a few also at 55 to 60°C) under a headspace of N_2. Upon indication of bacterial growth (turbidity increase), the samples were kept at 5°C until cultivation and isolation of SRB. During HYDRONAUT (Prieur *et al.*, 1989; 1990), samples were transferred to a medium chemically reduced by ascorbic acid and thioglycolate and supplemented with ferrous iron (Fe^{2+}), yeast extract, acetate, and lactate (Pfennig *et al.*, 1981). The enrichments for the present study were incubated at 20 or 40°C under atmospheric pressure. The activity of SRB (*i.e.*, H_2S production) was detected by blackening of the medium due to the precipitation of FeS.

BIOLAU enrichments were transferred (~ 0.5 ml) to lactate-acetate-amended medium (20 mM each) in 10 ml screw-cap tubes and incubated at 30°C. Cultures showing both H_2S production (Cord-Ruwisch, 1985) and growth (microscopic examination) were scored as positive SRB enrichments and were transferred to media with acetate or lactate as single carbon sources. Upon regrowth of SRB, these cultures were used as inocula for agar shake purifications (Pfennig *et al.*, 1981). Golden, brown colonies with a slight shine that developed in the deep agar were typically found to represent SRB. Such colonies were picked and transferred to 10 ml medium in screw-cap tubes. About 20 μl of $S_2O_4^{2-}$ solution (30 mM) was added before the tubes were capped and incubated. Sulfide-producing cultures that grew from single agar colonies were scored as positive for SRB cultivation.

STARMER enrichments were transferred to 10 ml medium with one of the following final substrate concentrations: acetate 20 mM; benzoate 7 to 10 mM; formate 20 mM; lactate 20 mM; or isobutyrate 7 to 10 mM. Cultures showing both H_2S production and growth at 30°C were scored as positive for SRB enrichment. Similarly, cultures that could be grown from single colonies after use of the agar shake procedure (Pfennig *et al.*, 1981) were scored as positive for SRB cultivation. Isolation of SRB into pure cultures was done by repeated application of the agar shake procedure. Purity checks for aerobic contaminants were made by streaking and incubating (30 and 55°C) the cultures on plates with YPG agar (basal salt solution supplemented with 0.2 % yeast extract, 0.2 % peptone, 0.1 % glucose, and 1.5 % agar). The absence of anaerobic contaminants was verified by microscopic examination of cultures grown for two to three weeks in reduced medium supplemented with yeast extract (0.5 %), pyruvate (5 mM), and fumarate (5 mM) as substrates. Also, agar shake dilution series were made with YPG medium that was supplemented with ferrous iron and reduced by ascorbic acid rather than H_2S and $S_2O_4^{2-}$. Due to the precipitation of black FeS, the H_2S-producing SRB colonies could be distinguished from fermentative contaminants.

HYDRONAUT enrichments, originally handled by Prieur *et al.* (1989, 1990), were cultivated at 30°C on a basal medium supplemented with 20 mM lactate as single electron donor. Two strains (H 2.5 and H 5.3) were isolated by the agar shake method and their physiology and morphology were studied in detail.

Microbiological and chemical analyses

Bacterial growth was quantified spectrophotometrically (Milton Roy, Spectronic 20D) as the increase in optical density at 578 nm (ΔOD_{578}). Specific growth rates (μ) and

doubling times ($T_d = \mu^{-1} \times \ln 2$) were calculated from linear regressions of ln ΔOD_{578} versus the incubation time (three to five data points from the exponential growth phase were used). Sulfide production was assayed either qualitatively by the precipitation of CuS (Cord-Ruwisch, 1985) or quantitatively by the methylene blue method (Cline, 1969). Presence of the sulfite reductase desulfoviridin was detected by a red fluorescence when one drop of NaOH (5 N) was added to a dense cell suspension under UV-light at 365 nm (Postgate, 1984). Direct cell counts were made by epifluorescence microscopy after staining with acridine orange (Hobbie *et al.*, 1977). Relative numbers of round pleomorphs and normal cells were determined by phase contrast microscopy of samples fixed on agar-coated slides. The agar-slide method was also used for preparation of photomicrographs (Pfennig and Wagener, 1986). The molar ratio of guanine and cytosine in the DNA bases (GC %) was determined spectrophotometrically (Ulitzur, 1972) and by the thermal denaturation procedure (Marmur, 1961; Marmur and Doty, 1962). For analyses of phospholipid-linked fatty acids, SRB were grown at 34°C in 1-litre batches of 20 mM lactate medium. The cells were harvested by centrifugation (10.000 *g*, 20 min) and the cell pellets were washed in 50 mM K-phosphate buffer (pH 6.4) and lyophilized. The yield of SRB was about 180 mg dry weight per litre. Phospholipid fatty acids were analysed as previously described (White *et al.*, 1979). Briefly, lipids were extracted using a modified Bligh-Dyer method (Bligh and Dyer, 1959) and separated into three classes using silicic column chromatography. The polar lipids were esterified by a mild methanolysis and fatty acid methyl esters were analysed by gas chromatography and coupled gas chromatography-mass spectrometry. The location of double bonds was determined as described by Nichols *et al.* (1986).

Fatty acid nomenclature

Fatty acids are designated as total number of carbon atoms:number of double bonds, with the position of the double bond closest to the aliphatic (ω) end of the molecule indicated with the geometry "c" for *cis* and "t" for *trans.* The prefixes "i" and "a" refer to iso and anteiso branching, respectively. For example, i-C17:1ω7c is *cis*-15-methyl-9-hexadecenoic acid and a-C15:0 is 12-methyltetradecanoic acid.

RESULTS

During BIOLAU, SRB could be enriched at 30°C from 14 samples while only two were devoid of culturable SRB (Tab. 1). The SRB were present in samples of warm water taken around animal assemblages as well as samples of high-temperature hydrothermal water (100 to 400°C). However, no thermophilic SRB could be enriched from two subsamples (B04 E02 and B06 E24) originally incubated at 60°C. Similarly, 1 ml subsamples of the enrichments at 30°C gave no H_2S production when transferred (5 % inoculum) to acetate-lactate-amended medium and incubated at 55°C for two weeks. Thus, by this tentative procedure no thermophilic growth of vegetative cells or spores

Table 1

Enrichment and cultivation of mesophilic SRB from BIOLAU samples.

Hydrothermal field	Sample No.	Sample description	SRB enrichment [a]	SRB cultivation [b]
Hine Hina	B02 E01	Water 17°C, inside a mussel	+	A,L
	B03 E01	Water 18°C, inside a mussel	+	L
Vai Lili	B04 E01	Water 20°C, inside a mussel	+	A,L
	B04 E02	Water > 100°C, ~ 15 cm above black smoker exit	+	–
	B04 E03	Water 20°C, base of chimney, near gastropods	+	A,L
	B04 E04	Water 20°C, base of chimney, near gastropods	–	–
	B04 E05	Water < 240°C, ~ 15 cm above white smoker exit	+	L
	B04 E06	Water < 100°C, ~ 15 cm above black smoker exit	–	–
	B04 E07	Water 340°C, at black smoker exit	+	L
	B04 E08	Water < 240°C, at black smoker exit	+	L
	B04 E20	Piece of hydrothermal chimney	+	L
	B06 E02	*Alviniconcha*, - shell surface	+	A,L
	B06 E04	*Alviniconcha*, - gill	+	L
	B06 E07	*Alviniconcha*, - foot	+	–
	B06 E24	Piece of hydrothermal chimney	+	L
	B09 E01	Water > 400°C, at black smoker exit	+	L

[a] Positive cultures (+) showed growth and sulfide production at 30°C with acetate and lactate as electron donors.

[b] Cultures grown from single agar colonies with acetate (A) or lactate (L) as sole electron donor.

from the original sample could be demonstrated. Of the 14 enrichment cultures obtained, 12 contained desulfoviridin, and lactate was the substrate used by most of the cultures (Tab. 1). This indicated a predominance of *Desulfovibrio* spp. in the enrichments. Four cultures from warm vent areas, however, showed a substantial sulfide production and growth when cultivated with acetate as sole electron donor (Tab. 1). These cultures appeared as round to oval cells with a diameter of about 1 μm (Fig. 1*a*).

The STARMER samples showed a broad metabolic diversity of mesophilic SRB (Tab. 2). Thus, all five substrates tested supported H_2S production, and SRB from single agar colonies could be cultivated on all substrates except formate. Pure cultures of SRB were obtained and morphologically examined. The benzoate-grown bacteria were small, slightly curved rods (2 to 3 μm), some of which were sluggishly motile. Cells grown on lactate appeared as vibrio-shaped (2 to 4 μm), while isobutyrate selected for non-motile oval-shaped cells (3 to 4 μm). Acetate-grown cultures were oval to rod-shaped (2 to 3 μm) or thin, rod to vibrio-shaped cells (2 to 3 μm) with no apparent motility (Fig. 1*b*, *c*). No thermophilic SRB grew from three subsamples (S05 E01, S07 E02, and S07 E03) incubated at 55°C on a medium supplemented with lactate, acetate and yeast extract. Similarly, the enrichments grown at 30°C showed no sulfide production or growth when transferred (5 % inoculum) to media with their respective electron donors and incubated at 55°C for up to six weeks.

During the HYDRONAUT cruise, enrichment cultures of mesophilic SRB were obtained from seven (out of twenty) samples, and five of these were from invertebrates or their tubes (Prieur *et al.*, 1989, 1990). At 20°C, SRB enrichments were obtained from tubes of *Riftia* sp. and two *Alvinella* sp.; from a hydrothermal chimney sample; and from the digestive gland of *Bathymodiolus thermophilus*. At 40°C, enrichments were obtained from the tubes of two *Alvinella* sp. (the same that gave enrichments at 20°C); from a sample of *Paralvinella grasslei*; and from a hydrothermal water sample. The two SRB strains H 2.5 and H 5.3 were isolated from the tubes of *Alvinella* sp. and the physiology of these strains was studied during growth on lactate-amended basal medium.

HYDRONAUT strains H 2.5 and H 5.3

Cells of strain H 2.5 contained desulfoviridin and had a GC content of 55 to 57 %, as determined by the methods of thermal denaturation and spectroscopy, respectively. Sulfide production and growth took place concurrently, and the molar ratio of sulfide production to lactate consumption was about 1 to 2.2 (Fig. 2 and 3). When cultivated on a 20 mM lactate medium, the cell number increased to 7.0×10^8 cells ml^{-1} and growth was accompanied by a pH decrease from 7.3 to 6.9. The optimum temperature for growth was about 37°C (Fig. 4), where a doubling time of 3.6 h was reached. No growth occurred at 5 or 44°C (two weeks of incubation). Cells in the exponential growth phase were vibrio-shaped, 3 to 5 μm long (Fig. 1*d*), but turned into round pleomorphs with a fainted contrast during the stationary phase (Fig. 1*e*). However, upon transfer of an

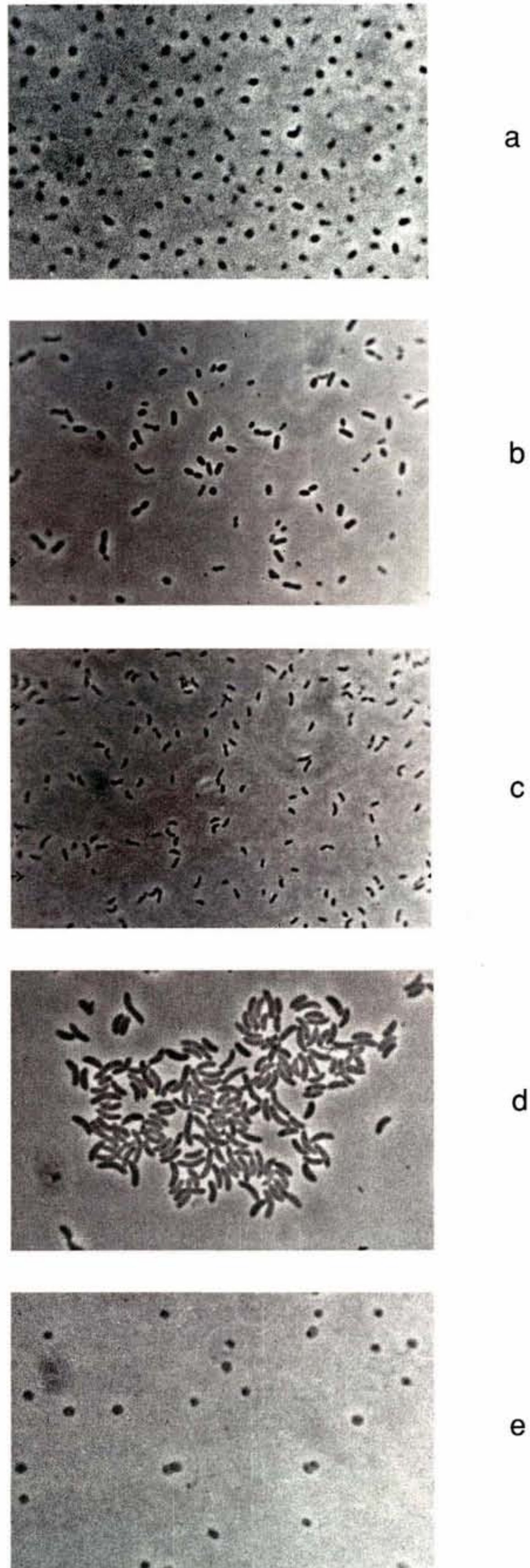

Figure 1

Sulfate-reducing bacteria from deep-sea hydrothermal vent samples. (a): Acetate oxidizers from BIOLAU. (b,c): Acetate oxidizers from STARMER. (d): Desulfovibrio *strain H 2.5 from HYDRONAUT. (e): Round pleomorphs in old cultures of strain H 2.5. Phase-contrast micrographs, × 1000.*

Table 2

Enrichment and cultivation of mesophilic SRB from STARMER samples.

Hydrothermal field	Sample No.	Sample description	SRB enrichment [a]	SRB cultivation [b]
White Lady	S05 E01	Piece of hydrothermal chimney	+	A,B,I,L
	S07 E02	Black sediment (mineral debris)	+	B,L
	S07 E03	Piece of hydrothermal chimney	+	B,L
	S12 E04	Black sediment (coarse mineral debris)	+	A,B,I,L
	S12 E05	Black sediment (fine grained mineral debris)	+	A,I,L

[a] Positive cultures (+) showed growth and sulfide production at 30°C with either acetate, benzoate, formate, isobutyrate or lactate as electron donors.

[b] Cultures grown from single agar colonies with acetate (A), benzoate (B), isobutyrate (I), or lactate (L) as sole electron donor.

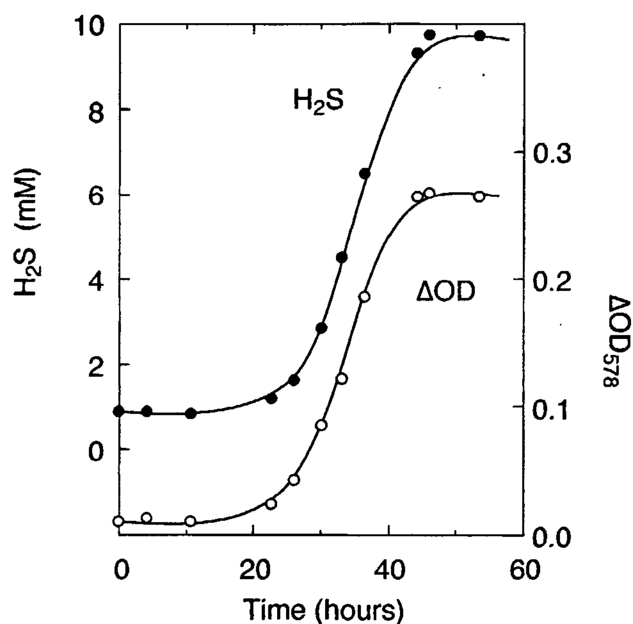

Figure 2

Sulfide production (•) and growth (o) of strain H 2.5. The culture was grown at 34°C on a basal medium amended with 20 mM lactate.

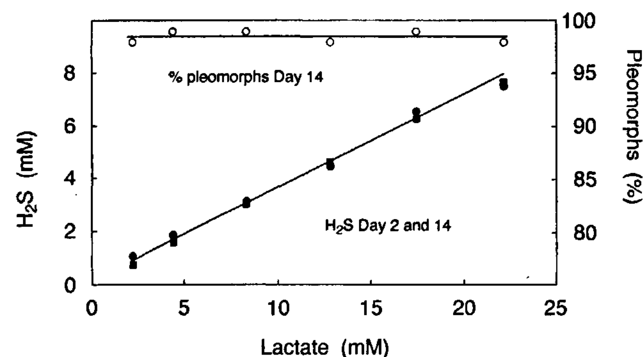

Figure 3

Sulfide production (•) and frequency of round pleomorphs (o) in strain H 2.5 grown at different lactate concentrations for 14 days. The sulfide production after two days of incubation (▪) is also shown.

Table 3

*Fatty acid distribution in the membrane phospholipids of strain H 2.5 (*Desulfovibrio sp.*) occurring as vibrio-shaped cells or round pleomorphs [a].*

Fatty acids	% of total fatty acids	
	vibrios	pleomorphs
Straight chain saturates		
14:0	0.28	0.32
15:0	0.51	0.50
16:0	18.37	17.35
17:0	3.91	3.28
18:0	4.71	3.29
Straight chain unsaturates		
16:1ω7c	5.06	4.77
16:1ω7t	0.17	0.18
17:1ω6c	0.26	0.21
18:1ω9	0.13	0.10
18:1ω7c	2.75	1.82
Branched chain saturates		
i-14:0	0.16	0.24
i-15:0	6.95	8.72
a-15:0	5.80	7.43
i-16:0	4.47	5.05
i-17:0	9.52	9.31
a-17:0	7.21	7.03
i-18:0	0.31	0.23
Branched chain unsaturates		
i-15:1ω7c	0.37	0.57
a-15:1ω7	0.15	0.22
i-16:1ω7c	3.70	4.23
i-16:1ω7t	0.20	0.16
i-17:1ω7c	16.33	16.13
i-17:1ω7t	0.78	1.00
a-17:1ω7c	5.94	6.16
a-17:1ω7t	0.22	0.26
i-18:1ω7c	1.10	0.92
Total branched	63.21	67.66
Total unsaturates	37.16	36.73
Total branched unsaturates	28.79	29.65

[a] Strain H 2.5 was grown at 34°C with 20 mM lactate as electon donor. Cultures harvested after 2 days occurred as vibrio-shaped cells, while cultures harvested after 5 days occurred as round pleomorphs.

inoculum to fresh medium, a culture of vibrio-shaped cells was recovered. A decrease in the ΔOD_{578} from 0.28 to 0.16 was associated with the morphological transformation of the culture during a stationary phase of three days. When cultures were grown at lactate concentrations of 2 to 18 mM, the resulting H_2S concentrations attained from 2 to 10 mM and more than 99 % of the cells turned into round pleomorphs within two weeks of incubation (Fig. 3). No significant changes in the phospholipid fatty acid composition of the cell membrane were observed during the morphological transformation (Tab. 3). Thus, in cultures of both vibrio-shaped and pleomorphic cells, the fatty acid profiles were characterized by high proportions (63 to 68 %) of branched (including mono-unsaturated) fatty acids with i-C17:1ω7c predominating.

Strain H 5.3 was morphologically characterized as small, curved rods (2 to 3 μm). The strain was enriched on a medium with formate as sole electron donor, but grew better with lactate. Growth and sulfide production occurred with an optimum at 40°C (Fig. 4) where a doubling time of 6.6 hours was reached. No growth was observed at 5 or 44°C. The strain contained desulfoviridin and had a broad pH optimum around 7.2 (data not shown). Round pleomorphs also occurred in the stationary phase of cultures of strain H 5.3, but to a lesser extent than observed for strain H 2.5.

DISCUSSION

The high number of sulfate-reducing enrichments indicated that mesophilic SRB occurred frequently at the deep-sea hydrothermal vent sites. Moreover, the present enrichments represent only the fraction of culturable SRB, which

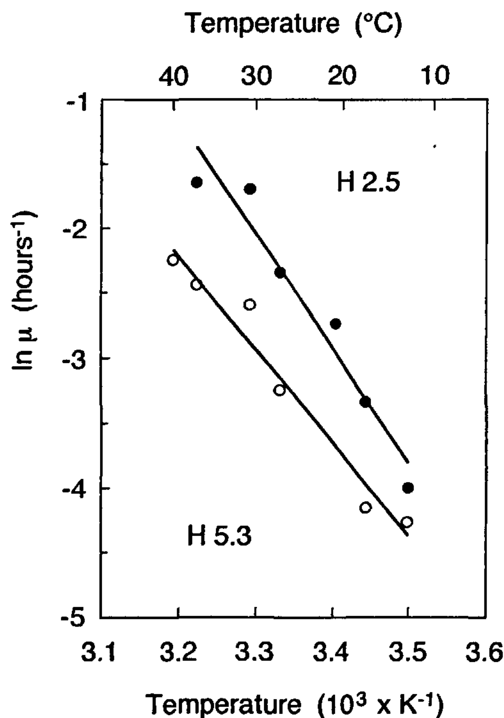

Figure 4

Arrhenius plots of the effect of temperature on the specific growth rates (ln μ) for strain H 2.5 (•) and H 5.3 (o).

proliferated at 30°C and atmospheric pressure. Mesophilic SRB could be cultivated even from samples of hydrothermal water with measured *in situ* temperatures of 100 to 400°C (Tab. 1). This raises the question of their origin in the hydrothermal system. Activity and growth of mesophilic microorganisms have previously been detected in high-temperature water samples collected at deep-sea hydrothermal vents (Bianchi, 1986, 1988 ; Winn *et al.*, 1986 ; Karl *et al.*, 1988). Possible origins include the leakage of seawater through fissures in the walls of hydrothermal chimneys. However, prolonged survival of bacteria in the high-temperature hydrothermal fluids (> 200°C) is unlikely as they do not seem to possess any special mechanisms of protection against such thermal injury (Karl *et al.*, 1988). Thus, the presence of mesophilic SRB in the hydrothermal water samples may have been due to entrapment of ambient seawater during sampling. In this case, the SRB presently cultivated from hydrothermal water samples originated from oxic bottom sea water at the hydrothermal vent sites. This conclusion would be consistent with earlier reports of mesophilic SRB in bottom sea water at deep-sea hydrothermal vents (Lilley *et al.*, 1983 ; Bianchi, 1986, 1988). Hence, previous enumerations ranged from 10^2 to 3.6×10^4 SRB ml^{-1} in warm vent waters of the Galapagos Spreading Centre (Lilley *et al.*, 1983), while 5.6×10^3 SRB ml^{-1} were found in warm water (20°C) above an *Alvinella* colony at 13°N (Bianchi, 1986, 1988). The latter number was > 300-fold higher than found outside the hydrothermal area, while mesophilic SRB were found to be virtually absent from normal, cold deep-sea bottom waters (Bianchi, 1988). The presence of anaerobic SRB in the oxic bottom water may be explained most readily if the bacteria are regularly introduced to the sea water from anoxic habitats in the hydrothermal environment (*cf.* Gottschal, 1986). However, anoxic sediments, where the activity of SRB normally occurs, are virtually absent at the active mid-ocean and back-arc spreading centres, which are characterized by their continuous formation of new ocean crust (Karl, 1987). Based on laboratory experiments with oxic sediment, the occurrence of sulfate reduction in reduced microniches was suggested by Jørgensen (1977). In the hydrothermal environment, such microniches could be envisaged to occur within or around the dense animal communities, where high nutrient concentrations and favourable temperatures for mesophilic growth occur.

Sulfate-reducing bacteria similar to *Desulfovibrio* were isolated from all three vent sites with lactate as electron donor in the enrichment medium. These bacteria were vibrio-shaped and contained the sulfite reductase desulfoviridin. Also, several HYDRONAUT isolates, including strain H 2.5, were found to contain the phospholipid fatty acid i-C17:1ω7c, that represents a biomarker fatty acid for the genus *Desulfovibrio* (Elsgaard *et al.*, 1991; Vainshtein *et al.*, 1992). During BIOLAU and STARMER, enrichment with other short-chain fatty acids and benzoate resulted in different cultures of SRB. Based on the morphological characteristics and the utilization of selective electron donors, the taxonomic affiliation of these isolates was tentatively examined. The morphology of the acetate-oxidizers was similar to *Desulfobacter*, which comprise

the mesophilic SRB with the most effective acetate utilization (Widdel, 1987; Widdel and Bak, 1992). The benzoate-oxidizers were morphologically different from most described species (Widdel and Bak, 1992), but resembled a newly isolated SRB affiliated to *Desulfoarculus* (Drzyzga *et al.*, 1993). Lastly, isobutyrate selected for SRB which showed resemblance to the nutritionally versatile *Desulfobacterium* (Widdel and Bak, 1992).

During the present study, we attempted the enrichment of moderately thermophilic SRB at 55 to 60°C. This group includes the only forms (*Desulfotomaculum* spp.) known to develop heat resistant endospores (Widdel, 1992), that might be a selective advantage in the fluctuating environment of the deep-sea hydrothermal vents. Although not conclusive, our results indicated that spore-forming, thermophilic SRB were absent from the BIOLAU and STARMER samples. In general, desulfotomacula are more frequent in freshwater than in marine environments, although most species may readily adapt to saline conditions (Postgate, 1984). Certain thermophilic species, however, such as *D. geothermicum* and *D. australicum*, have been isolated from saline environments and are even dependent on NaCl for optimal growth (Daumas *et al.*, 1988; Love *et al.*, 1993).

HYDRONAUT strains H 2.5 and H 5.3

The previous assignment of strain H 2.5 to the genus *Desulfovibrio* (Elsgaard *et al.*, 1991) was confirmed by the presence of desulfoviridin and by the stoichiometry of H_2S production to lactate consumption (1 to 2.2), which indicated an incomplete lactate oxidation to acetate (Widdel and Bak, 1992). Strain H 5.3 equally could be assigned to *Desulfovibrio*, as the cells were vibrio-shaped, contained desulfoviridin, and grew with lactate according to the same stoichiometry as strain H 2.5 (data not shown). The two strains differed clearly, however, in regard to morphology, specific growth rates, and the extent of pleomorph formation.

Strain H 2.5 and H 5.3 both had optimum temperatures at 37 to 40°C, but did not grow (within two weeks of incubation) at 5°C, which is well above the average temperature in deep-sea bottom waters (2 to 3°C). Thus, the growth of these SRB in the deep-sea environment may either be extremely slow or be confined to warm areas in the hydrothermal vent habitats. The temperature optima of the two strains were in accordance with their isolation from tubes of the polychaete *Alvinella* sp., which has been found to thrive at 20 to 40°C on the walls of hydrothermal smoker chimneys (Desbruyères *et al.*, 1982; Chevaldonné *et al.*, 1991). As calculated from Arrhenius plots (Fig. 4), the temperature coefficients Q_{10} (*i.e.*, the ratio of rates at temperatures 10°C apart) for strain H 2.5 and H 5.3, respectively, were 2.3 and 2.8 for the temperature range from 15 to 25°C. Similar temperature coefficients were previously reported for pure cultures of SRB from coastal and freshwater environments (Bak, 1988). Thus, no unusual temperature adaptations characterized the two deep-sea vent isolates.

The spherical morphology of the pleomorphs in strain H 2.5 and H 5.3 indicated that their bacterial cell walls were weakened and at least partly lost. This occurred without significant changes in the phospholipid fatty acid composition of the cell membrane. Thus, the fatty acid profiles of the two morphologies in strain H 2.5 were almost identical and dominated by i-C17:1ω7c (Tab. 3). Indeed, the lipid analyses clearly showed that the two morphologies represented the same bacteria. Aberrant forms, also described as coccoid bodies, previously have been reported from old cultures of *Desulfovibrio* (Skyring *et al.*, 1977), where a high H_2S concentration has been suggested as favouring their appearance (Postgate, 1984). The results presented in Fig. 3 showed, however, that the occurrence of pleomorphs was probably not related to the H_2S concentration *per se*. Thus, at least it could be concluded that H_2S concentrations in the range of 2 to 10 mM had no different effect on pleomorph formation. In other vibrio-shaped bacteria, coccoid bodies also have been observed and have been interpreted as a stage in their life cycle (Williams and Rittenberg, 1956; Felter *et al.*, 1969; Krieg, 1984). However, the mechanism of formation and the viability of such pleomorphs differ among bacterial groups (Krieg, 1984). Levin and Vaughn (1968) studied the spontaneous formation of spheroplasts in cultures of *Desulfovibrio aestuarii* strain 30 and found that the cells lost their viability prior to spheroplast formation. Thus, the spheroplast formation in *Desulfovibrio* could merely be related to the rapid growth rate in laboratory cultures, which necessitate a high level of lysozyme-like enzymes during cell-wall synthesis. Upon cessation of growth the same enzymes could catalyse the rupture of the cell walls.

CONCLUSIONS

Twenty-six of 41 samples collected at three deep-sea hydrothermal vent sites gave rise to enrichments of mesophilic SRB that grew well at 30°C. The samples represented both warm-vent animals and high-temperature hydrothermal features and different species of SRB were obtained that were able to degrade the electron donors acetate, benzoate, formate, isobutyrate and lactate. This indicated an important role of mesophilic SRB in the anaerobic mineralization processes in basaltic deep-sea vent environments from which anoxic sediments are virtually absent. The habitats of the mesophilic SRB might be associated with the dense animal communities that thrive in the warm vent areas.

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