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Gerard Muyzer · Andreas Teske · Carl O. Wirsen
Holger W. Jannasch

Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments

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Abstract Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments was used to explore the genetic diversity of hydrothermal vent microbial communities, specifically to determine the importance of sulfur-oxidizing bacteria therein. DGGE analysis of two different hydrothermal vent samples revealed one PCR band for one sample and three PCR bands for the other sample, which probably correspond to the dominant bacterial populations in these communities. Three of the four 16S rDNA fragments were sequenced. By comparison with 16S rRNA sequences of the Ribosomal Database Project, two of the DGGE-separated fragments were assigned to the genus *Thiomicrospira.* To identify these 'phylotypes' in more detail, a phylogenetic framework was created by determining the nearly complete 16S rRNA gene sequence (approx. 1500 nucleotides) from three described *Thiomicrospira* species, viz., *Tms. crunogena, Tms. pelophila, Tms. denitrificans,* and from a new isolate, *Thiomicrospira* sp. strain MA2-6. All *Thiomicrospira* species except *Tms. denitrificans* formed a monophyletic group within the gamma subdivision of the Proteobacteria. *Tms. denitrificans* was assigned as a member of the epsilon subdivision and was distantly affiliated with *Thiovulum,* another sulfur-oxidizing bacterium. Sequences of two dominant 16S rDNA fragments obtained by DGGE analysis fell into the gamma subdivision *Thiomicrospira*. The sequence of one fragment was in all comparable positions identical to the 16S rRNA sequence of *Tms. crunogena.* Identifying a dominant molecular isolate as *Tms. crunogena* indicates that this species is a dominant community member of hydrothermal vent sites. Another 'phylotype' represented a new *Thiomicrospira* species, phylogenetically in an intermediate position between *Tms. crunogena* and *Tms. pelophila.* The third 'phylotype' was identified as a *Desulfovibrio,* indicating that sulfate-reducing bacteria, as sources of sulfide, may complement sulfur- and sulfide-oxidizing bacteria ecologically in these sulfide-producing hydrothermal vents.

Key words *Thiomicrospira* · Hydrothermal vent microbial communities · Ribosomal RNA · Denaturing gradient gel electrophoresis · Polymerase chain reaction · Microbial diversity · Colorless sulfur bacteria · 16S rDNA · Phylogenetic relationships

Abbreviations *PCR* Polymerase chain reaction · *DGGE* Denaturing gradient gel electrophoresis

Introduction

The microbial communities of the hydrothermal vent ecosystems have been known for more than a decade (Jannasch 1985) and continue to inspire searches for new bacterial and archeal species, undertakings of sometimes adventurous and unique character. Although several different bacteria have been isolated from these communities, little is known about the microbial diversity of these communities and about the relative abundance of their individual inhabitants. Sulfur-oxidizing bacteria of the genus *Thiomicrospira* have been isolated frequently from deep-sea hydrothermal vent samples (Ruby et al. 1981; Jannasch et al. 1985), which indicates their ecological importance in this habitat. In this study, we complemented cultivation-based studies of hydrothermal vent communities with a new molecular approach, denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments, and focused on the congruences of the strategies.

DGGE of enzymatically amplified 16S rDNA has recently been introduced into molecular microbial ecology to determine the genetic diversity of natural microbial

G. Muyzer (✉) · A. Teske
Molecular Ecology Unit,
Max-Planck-Institute for Marine Microbiology,
Fahrenheitstrasse 1, D-28359 Bremen, Germany
Tel. +49-421-2208123; Fax +49-421-2208130
e-mail: gerard@postgate.mpi-mm.uni-bremen.de

C. O. Wirsen · H. W. Jannasch
Department of Biology, Woods Hole Oceanographic Institution,
Woods Hole, Mass. 02543, USA

communities (Muyzer et al. 1993) and to identify the phylogenetic position of the community members (Muyzer and De Waal 1994). PCR amplifications from 16S rRNA genes of natural bacterial populations, using gene-specific primers, yield mixtures of products of identical length, but different in nucleotide sequence. DGGE separates these PCR products on the basis of differences in melting behavior in polyacrylamide gels containing a linear gradient of DNA denaturants, thereby giving direct visualization of the dominant participants in the microbial populations. Subsequently, separated bands can be excised, re-amplified, and sequenced directly (Muyzer and De Waal 1994). Cloning of PCR products is circumvented, and cloning biases in the analysis of PCR products are avoided.

Here we applied the DGGE approach to determine the genetic complexity of hydrothermal vent microbial communities and to assess the abundance of sulfur-oxidizing bacteria. 16S rDNA fragments obtained after enzymatic amplification of genomic DNA isolated from two deep-sea hydrothermal vent samples were analyzed by DGGE. The most dominant PCR fragments were sequenced and, after searching the Ribosomal Database Project for related sequences, identified as *Thiomicrospira* species. For a closer identification of our molecular isolates, we constructed a phylogenetic framework for all known *Thiomicrospira* species.

Nearly complete 16S rRNA sequences analyses have been published for *Thiomicrospira* sp. strain L-12 (Ruby and Jannasch 1982) and *Tms. thyasirae* (Wood and Kelly 1993). These species are grouped into the gamma subdivision of the Proteobacteria (Distel and Wood 1992; Lane et al. 1992). Based on 5S rRNA sequences, *Thiomicrospira* sp. strain L-12 and *Tms. pelophila* were assigned to the gamma subdivision of the Proteobacteria (Lane et al. 1985). The phylogenetic relationships of *Tms. pelophila* (Kuenen and Veldkamp 1972), *Tms. denitrificans* (Timmer-ten Hoor 1975), and *Tms. crunogena* (Jannasch et al. 1985) based on 16S rRNA sequences have not yet been elucidated. We, therefore, determined nearly complete sequences of 16S rRNA genes (comprising approximately 1500 nucleotides) of these three species and of a new isolate *Thiomicrospira* sp. strain MA2-6. Here we present the refined *Thiomicrospira* phylogeny, integrated with our analysis of the dominant DGGE fragments. We illustrate the microbiological and ecological relevance of these molecular data and argue for closer connections between molecular-based and cultivation-based microbial ecology.

Materials and methods

Bacteria and hydrothermal vent samples

Table 1 gives a summary of the different bacteria and environmental samples used in this study.

DNA extraction

Genomic DNA from bacterial cells and natural samples was obtained by using a modification of the method described by Rochelle et al. (1992). One gram of hydrothermal vent sample was resuspended in 2 ml of 0.15 M NaCl, 0.1 M EDTA (pH 8). After the addition of 250 µl of lysozyme solution (80 mg/ml), the samples were incubated for 60 min at 37°C and were mixed every 10 min. The samples were placed on ice, and 2 ml of a solution containing 0.5 M Tris-HCl (pH 8), 0.1 M NaCl, and 10% (w/v) SDS was added. Subsequently, the samples were incubated on a mixture of ice and NaCl. Proteinase K (20 mg/ml; 100 µl) was added, and the sample was incubated for 10 min at 55°C. The samples were placed on ice again, and 4 ml of Tris-buffered phenol was added to the sample. After centrifugation at 6,000 × *g* for 10 min at 4°C, the aqueous phase was removed, placed in a clean tube, carefully mixed with an equal volume of phenol:chloroform:isoamyl alcohol (50:49:1, by vol.), and centrifuged at 6,000 × *g* for 10 min at 4°C. This step was repeated until no protein precipitate was visible at the organic/inorganic interface. NaCl (0.1 vol. of 5 M) and 100% (v/v) ethanol (2.5 vol.) were added to the aqueous phase, mixed, and incubated overnight at –20°C. The genomic DNA was precipitated by centrifugation at 6,000 × *g* for 10 min at 4°C, dried under vacuum, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

PCR amplification of the 16S rRNA gene

The extracted DNA was used as target DNA in the polymerase chain reaction (Saiki et al. 1988) to amplify the 16S ribosomal RNA coding regions (Medlin et al. 1988). Two primers, GM3F and GM4R (Table 2), were used to amplify the nearly complete 16S rRNA gene. Two other primers, GM5F and 907R, were used to amplify a 550-bp rDNA fragment, which was used for DGGE

Table 1 Bacteria and hydrothermal vent samples used in this study

Bacterium/sample	Isolated/collected from	Location	Source
Thiomicrospira crunogena[a]	Scrapings from the outer side of a tubeworm	East Pacific Rise	H. W. Jannasch
Thiomicrospira denitrificans[b]	Intertidal mud flat	Dutch Wadden Sea	DSM 1251
Thiomicrospira pelophila[c]	Intertidal mud flat	Dutch Wadden Sea	DSM 1534
Thiomicrospira sp. strain MA2-6[d]	Slurp sample of whitish material from the base of a beehive smoker	Mid-Atlantic Ridge	H. W. Jannasch
Sample no. 2613[e]	Outer side of a chimney rock sample	Mid-Atlantic Ridge	H. W. Jannasch
Sample no. 2609[f]	Outer side of a chimney rock sample	Mid-Atlantic Ridge	H. W. Jannasch

[a] Jannasch et al. 1985
[b] Timmer ten Hoor 1975
[c] Kuenen and Veldkamp 1972
[d] Collected from Snake Pit site, Mid-Atlantic Ridge, 23° 20´´ N, 44° 57´´ W; temperature during sampling: 17–35°C
[e] Collected from active site 1 at Moose site, Snake Pit, Mid-Atlantic Ridge, 23° 20´´ N, 44° 57´´ W, at a depth of 3523 m
[f] Collected from TAG site at a depth of 3648 m

Table 2 Primer sequences and positions (*F* forward primer, *R* reverse primer). The numbering of the positions is according to that of the16S rRNA of *Escherichia coli* (Brosius et al. 1981)

Primer	Position	Sequence
GM3F	8–24	5′-AGAGTTTGATCMTGGC-3′
GM4R	1492–1507	5′-TACCTTGTTACGACTT-3′
GM5F	341–357	5′-CCTACGGGAGGCAGCAG-3′
907R	907–927	5′-CCGTCAATTCCTTTRAGTTT-3′
GC-clamp[a]		5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′

[a] The GC-clamp was attached to the 5′-end of the GM5F primer

analysis. At the 5′-end of the GM5F primer, an additional 40-nucleotide GC-rich sequence (GC-clamp) was added to obtain a stable melting behavior of the DNA fragments in the DGGE (Muyzer et al. 1993). The sequences and the exact position of the primers are given in Table 2.

PCR amplifications were performed with a Techne PHC-3 Temperature Cycler (Techne, Cambridge, UK) as follows: 10–100 ng of target DNA, 25 pmol of each of the appropriate primers, 10 µmol of each deoxyribonucleoside triphosphate, and 10 µl of 10 × PCR buffer [100 mM Tris-HCl (pH 9), 15 mM $MgCl_2$, 500 mM KCl, 0.1% (w/v) gelatin, 1% (v/v) Triton X-100] were adjusted to a final volume of 50 µl with sterile water (Sigma, Deisenhofen, Germany) and overlaid with two drops of mineral oil (Sigma, Deisenhofen, Germany). SuperTaq DNA polymerase (HT Biotechnology, Cambridge, UK) was added to the reaction mixture after the initial denaturing step (94°C, 5 min), at a temperature of 80°C. In addition, to increase the specificity of the amplification and to reduce the formation of spurious by-products, a "touchdown" PCR (Don et al. 1991) was performed, meaning that the annealing temperature was set 10°C above the expected annealing temperature (50°C for GM3F/GM4R and 70°C for GM5F/907R) and decreased by 1°C every second cycle until a touchdown of 40 or 60°C, at which temperature ten additional cycles were carried out. Primer extension was carried out at 72°C for 3 min. The total number of cycles was 30.

Aliquots (5 µl) of the amplification products were analyzed by electrophoresis in 2% (w/v) Nusieve agarose (FMC, Vallensbaek Strand, Denmark) gels containing ethidium bromide (0.5 µg/ml; Sambrook et al. 1989).

DGGE analysis

DGGE was performed using a Bio-Rad Protean II system, as described previously (Muyzer et al. 1993, 1995). PCR samples were applied directly onto 6% (w/v) polyacrylamide gels in 0.5 × TAE [20 mM Tris-acetate (pH 7.4), 10 mM acetate, 0.5 mM Na_2EDTA], with gradients formed with 6% (w/v) acrylamide stock solutions (acrylamide/*N, N*′-methylene bisacrylamide, 37:1, w/w) that contained 20% and 60% denaturant [100% denaturant is 7 M urea and 40% (v/v) formamide, deionized with AG501-X8 mixed bed resin (Bio-Rad, Munich, Germany)]. Electrophoresis was performed at a constant voltage of 200 V and a temperature of 60°C. After electrophoresis, the gels were incubated for 15 min in Milli-Q water containing ethidium bromide (0.5 mg/l), rinsed for 10 min in Milli-Q water, and photographed with UV transillumination (302 nm) with Cybertech CS1 (Cybertech, Berlin, Germany) equipment.

Sequencing of PCR products

Amplified DNA fragments were separated from free PCR primers by electrophoresis in 2% (w/v) agarose gels using the Biometra E91 Electrophoresis Unit (Biometra, Göttingen, Germany). Electrophoresis was continued until the PCR product migrated into the buffer-filled well, from where it was taken up with a pipette and transferred into a clean 1.5-ml tube. After addition of 0.1 vol. of 5 M NaCl and 2.5 vol. of 100% (v/v) ethanol and incubation for 1 h at –80°C, the PCR product was pelleted by centrifugation. The pellet was dried under vacuum and redissolved in 50 µl of sterile water.

The double-stranded PCR products were sequenced directly. Purified PCR product (1 µl; 20–50 ng) was added to a 0.5-ml tube containing 2 µl 5 × sequencing buffer [200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl], 1 µl of a 5% (v/v) Nonidet P-40 solution, and 1 µl of 'nested' sequencing primer (5 pmol/µl). The volume was adjusted to 10 µl with sterile water. The mixture was incubated for 10 min at 95°C, with a short centrifugation step after 5 and 10 min to re-collect the sample in the bottom of the tube. After denaturation, the sequencing reaction was started by subsequently adding: 1.0 µl of 0.1 M DTT, 2.0 µl of a dNTP solution (200 nM of each dGTP, dATP, dTTP), 0.5 µl of (α-^{33}P)-dCTP (Amersham, Braunschweig, Germany; 10 µCi/µl; 3,000 Ci/mmol), and 1 µl (1 unit) of Sequenase (version 2.0; USB, Bad Homburg, Germany). The samples were incubated for 5 min at 37°C. Thereafter, 3.5-µl aliquots were added to 2.5 µl of the dideoxynucleotide termination solutions (80 µM each of dGTP, dATP, cTTP, and dCTP, and 8 µM of each of the dideoxynucleotides). After 5 min of incubation at 37°C, the reaction was stopped by adding 4 µl of 'stop solution' [96% (v/v) formamide, 20 mM EDTA). The samples were heated for 5 min at 95°C, and 2.5 µl of the samples was loaded onto a 0.25–0.40 mm wedge sequencing gel [6% (v/v) acryl/bisacrylamide (30:1, w/w) and 7 M urea]. After electrophoresis at a constant power of 60 W for 4 h, the gel was fixed, dried, and exposed to Kodak XAR5 X-ray film.

Phylogenetic analysis of the 16S rRNA sequences

The 16S rRNA sequences were aligned to those of other bacteria obtained from the Ribosomal Database Project (RDP; Larsen et al. 1993). The Similarity_Rank program of the Ribosomal Database Project was used to search for close evolutionary relatives. Sequence alignments were prepared with the sequence alignment editor Seqapp (Gilbert 1992). Matrices of evolutionary distances were computed from the sequence alignment using the program Dnadist implemented in the software package Phylip (version 3.5) developed by Felsenstein (1989). Dnadist calculates distance matrices according to the Jukes-Cantor model, which assumes independent mutations at all sites with equal probability (Jukes and Cantor 1969). From these distance matrices, phylogenetic trees were inferred with the program Fitch, which uses the least-squares algorithm of Fitch and Margoliash (1967) to match evolutionary distances with branch lengths of the phylogenetic tree. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed using the program Seqboot, also implemented in the software package Phylip 3.5c. Nucleotides of the sequence dataset are deleted and replaced, to produce random variation typical of the variation to be obtained by introducing new sequences. The sequence dataset is varied and analysed multiple times. The percentages of the resamplings that support the branching pattern of the phylogenetic tree, the bootstrap values, are drawn into the phylogenetic trees (Figs. 1, 3). A bootstrap value of 100 indicates that a branching pattern was confirmed in all resamplings, whereas a bootstrap value of 50 indicates that the branching pattern was reproduced only in 50% of the resamplings. Bootstrap values drawn into a phylogenetic tree always refer to the distal branchings, i.e., those leading to the branch tips, not to the roots. The sequences are available from Genbank under accession numbers L40808–L40814.

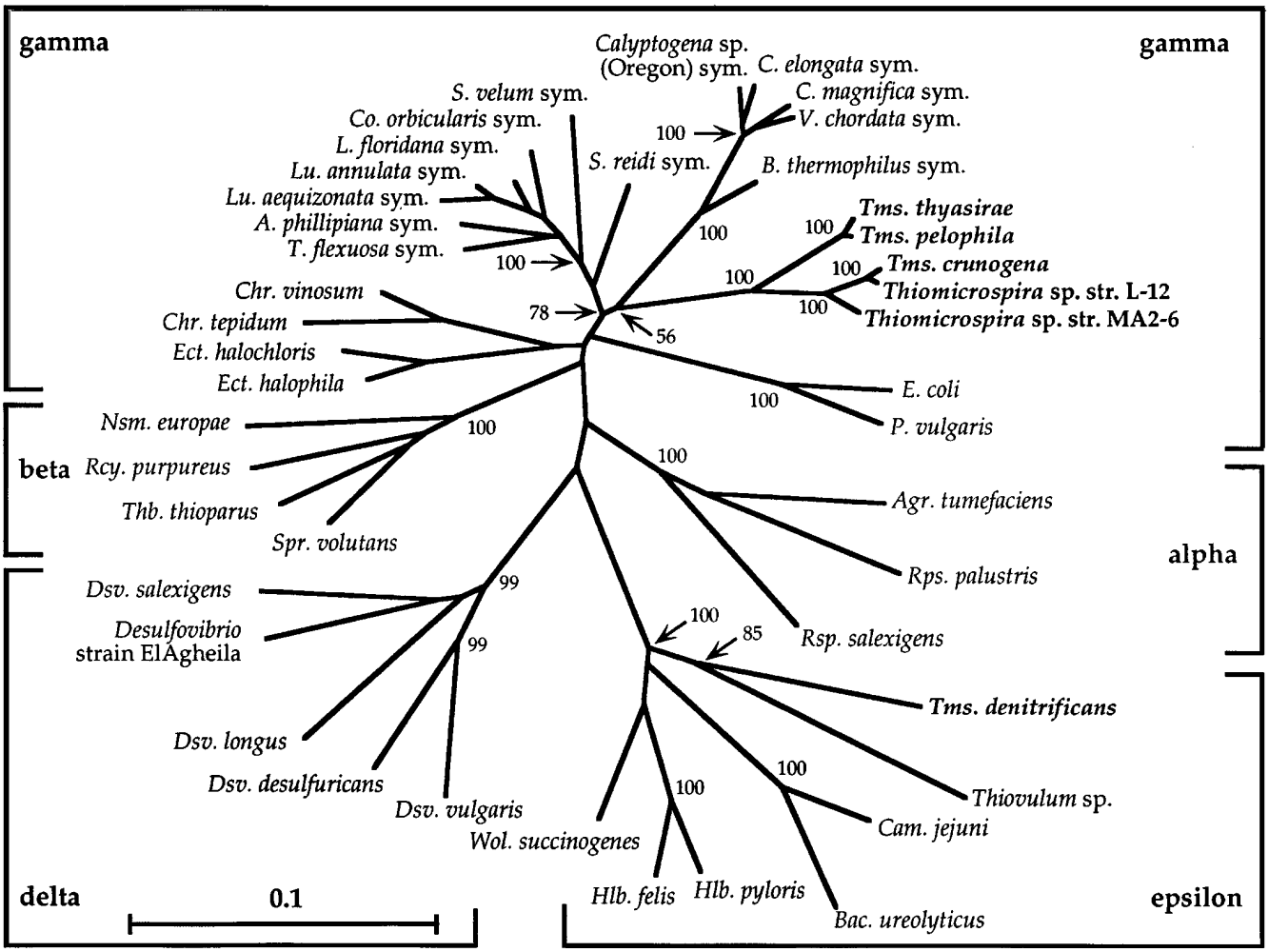

Fig. 1 Unrooted evolutionary distance tree based on nearly complete 16S rRNA sequences, showing the phylogenetic relationships of *Thiomicrospira thyasirae, Tms. pelophila, Tms. crunogena, Thiomicrospira* strains L-12 and MA2-6, and *Tms. denitrificans.* Sequences used in preparing this figure were derived from the Ribosomal Database Project (RDP; Larsen et al. 1993). Bootstrap values (see text for explanation) from distance analyses (100 replicates) refer to species distal to the associated node. The scale bar represents 0.1 estimated nucleotide change per sequence position. The five different subdivisions of the Proteobacteria, i.e., alpha, beta, gamma, delta, and epsilon (Woese 1987; Olsen et al. 1994), are indicated. Abbreviations of bacterial genera: *Agr. Agrobacterium, Bac. Bacteroides, Cam. Campylobacter, Chr. Chromatium, Dsv. Desulfovibrio, E. Escherichia, Ect. Ectothiorhodospira, Hlb. Helicobacter, Nsm. Nitrosomonas, P. Proteus, Rcy. Rhodocyclus, Rps. Rhodopseudomonas, Rsp. Rhodospirillum, Spr. Spirillum, Thb. Thiobacillus, Tms. Thiomicrospira, Wol. Wolinella.* Abbreviations of bivalve genera: *A. Anodontia, B. Bathymodiolus, C. Calyptogena, Co. Codakia, L. Lucina, Lc. Lucinoma, S. Solemya, T. Thyasira, V. Vesicomya.* Genbank accession numbers of the new *Thiomicrospira* 16S rRNA sequences: *Tms. denitrificans* (DSM 1251), L40808; *Tms. pelophila* (DSM 1534), L40809; *Tms. crunogena* (ATCC 35932), L40810; *Thiomicrospira* sp. strain MA2-6, L40811

Results

Phylogeny

The phylogeny of *Thiomicrospira* (Fig. 1) is based on analysis of nearly complete 16S rDNA sequences, comprising approximately 1500 positions. All *Thiomicrospira* species and strains, with the exception of *Tms. denitrificans*, belong to the gamma subdivision of the Proteobacteria (Fig. 1). Within the gamma subdivision, *Thiomicrospira* species form a distinct, monophyletic lineage. Their next relatives are obligately host-associated, symbiotic H_2S-oxidizing bacteria. Affiliated with the gamma *Thiomicrospira* cluster are the H_2S-oxidizing symbionts of the bivalva families Lucinidae, Thyasiridae, Solemyidae, Vesicomyidae, and Mytilidae (bootstrap value of 78% for the bivalve symbiont lineages and *Thiomicrospira*). These bivalve symbionts are divided into two distinct, monophyletic lineages, which correspond to the traditional systematic division of their respective hosts (Distel et al. 1994). A specific affiliation of the *Thiomicrospira* group with one of these lineages is uncertain. The phylogenetic tree (Fig. 1) indicates that the gamma *Thiomicrospira* species share, as a deep-branching lineage, their root with the Vesicomyidae and Mytilidae symbionts, but this is not significantly supported by bootstrap analysis (bootstrap value of 56%). *Thiomicrospira* shows no specific affilia-

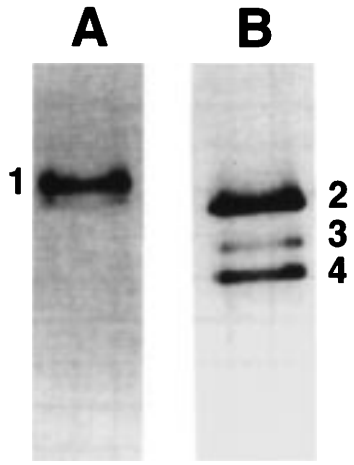

Fig. 2 Negative image of an ethidium-bromide-stained DGGE separation pattern of PCR-amplified 16S rDNA fragments (positions 357–907) from two hydrothermal vent samples. *Lane A* No. 2609, one dominant band (*1*); *lane B* no. 2613, three dominant bands (*2,3,4*). The denaturant gradient from gel top to bottom was 20–60%

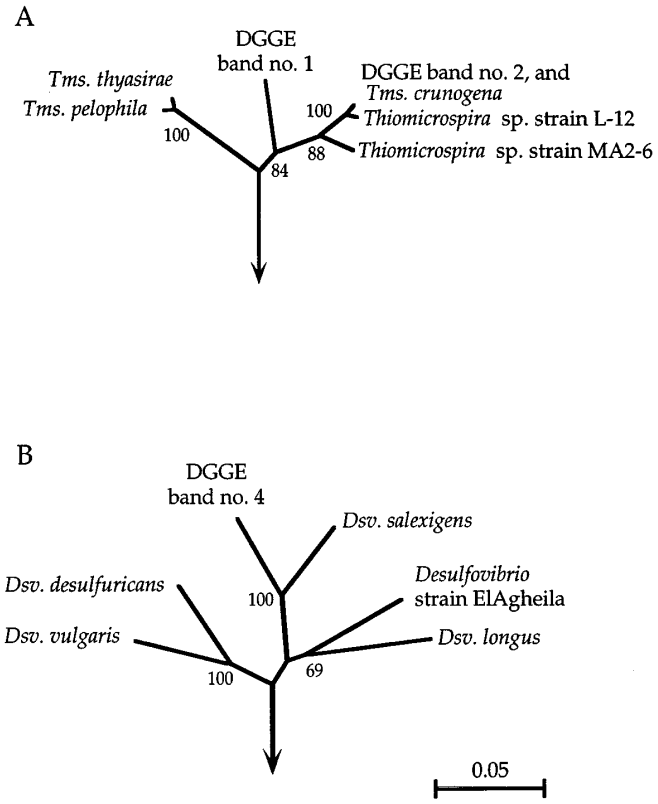

Fig. 3 A, B Phylogenetic relationships of the sequenced 16S rRNA DGGE fragments. **A** *Thiomicrospira* cluster of the gamma subdivision of the Proteobacteria, with sequences from DGGE fragments no. 1 and 2 (see Fig. 2). **B** *Desulfovibrio* species of the delta subdivision of the Proteobacteria, with the sequence of DGGE fragment no. 4 (see Fig. 2B). The trees are based on the evolutionary distance estimates obtained after comparision of *E. coli* positions 357–907 of aligned 16S rDNA sequences. The *scale bar* represents 0.05 estimated nucleotide change per sequence position. Bootstrap values (see text for explanation) indicate how many of 100 resamplings and reiterations of the phylogenetic analysis support the branching pattern. They always refer to the distal branchings, i.e., those leading to the branch tips, not to the roots. Genbank accession numbers: DGGE band no. 1, L40812; DGGE band no. 2, L40813; DGGE band no. 4, L40814

tion to any of the free-living H_2S- or sulfur-oxidizing bacteria of the gamma and beta subdivisions, such as *Thiobacillus*, *Chromatium*, or *Ectothiorhodospira*.

The gamma subdivision *Thiomicrospira* species can be divided into two pairs of very closely related species, which share very similar 16S rRNA sequences: (1) *Tms. thyasirae* and *Tms. pelophila* (6 nucleotide differences out of 1500 positions, i.e., less than 1% difference) and (2) *Tms. crunogena* and *Thiomicrospira* sp. strain. L-12 (12 nucleotide differences out of 1500 positions, i.e., less than 1% difference). *Thiomicrospira* sp. strain MA2-6 is related to the latter pair. This clustering might reflect the different habitats from which the bacteria were isolated. *Tms. thyasira* was isolated from the gills of the bivalve *Thyasira flexuosa* collected from a marine sediment off Plymouth (Wood and Kelly 1989), while *Tms. pelophila* was isolated from an intertidal mud flat in the Dutch Wadden Sea (Kuenen and Veldkamp 1972). *Thiomicrospira* sp. strain L-12, *Tms. crunogena*, and *Thiomicrospira* sp. strain MA2-6 were all isolated from hydrothermal vent areas (Ruby and Jannasch 1982; Jannasch et al. 1985).

Tms. denitrificans is a member of the epsilon subdivision of the Proteobacteria. The epsilon subdivision contains the genera *Campylobacter*, *Bacteroides*, *Wolinella*, *Thiovulum*, *Helicobacter*, and *Arcobacter* (Paster and Dewhirst 1988; Vandamme et al. 1991). Although sulfur oxidation is a widespread feature in this subdivision, *Tms. denitrificans* is not closely related to any of these genera. The least distant relative of this organism is the sulfur-oxidizing bacterium *Thiovulum* (La Riviere and Schmidt 1991). The genetic distance of their 16S rRNA sequences (13% sequence difference) and the ecophysiological differences argue against a close connection of *Tms. denitrificans* and *Thiovulum*. Considering its isolated phylogenetic position, *Tms. denitrificans* deserves reclassification as a new genus. This will require a more detailed physiological comparison of *Tms. denitrificans* with other epsilon subdivision sulfide or sulfur oxidizers.

DGGE analysis

DGGE analysis of PCR products, comprising approximately 550 nucleotides from position 357 to 907, revealed one dominant band for the hydrothermal vent sample no. 2609, and three bands for sample no. 2613 (Fig. 2). Three of the four bands were sequenced. The sequencing of a DGGE fragment requires elution of the fragment from the denaturant gel and subsequent re-amplification (Muyzer et al. 1995). This was unsuccessful with DGGE band no. 3, probably because of its low intensity as compared to DGGE band nos. 1, 2, and 4.

For sequence comparisons and phylogenetic analysis, alignments of DGGE fragment sequences and corre-

sponding parts from complete 16S rRNA sequences were prepared (positions 357–907, *Escherichia coli* numbering). The phylogenetic affiliations of the DGGE fragments are shown in Fig. 3.

The sequence of DGGE band no. 1 was phylogenetically affiliated with the gamma *Thiomicrospira* cluster (Fig. 3 A). Here it held a phylogenetic position between the two branches of the gamma subdivision *Thiomicrospira* species, with *Tms. thyasirae* and *Tms. pelophila* on one side and *Tms. crunogena*, *Thiomicrospira* sp. strain L-12, and *Thiomicrospira* sp. strain MA2-6 on the other. The phylogenetic position of this DGGE fragment strongly suggests that it is derived from a new *Thiomicrospira* species. The sequence of DGGE fragment no. 2 was in all comparable positions (i.e., readable in the sequence gel; unreadable and, therefore, unknown nucleotides were not considered) identical to the corresponding fragment from *Tms. crunogena* (Fig. 3A). This DGGE fragment is, therefore, derived from *Tms. crunogena* or at least from a phylogenetically very closely related bacterium that shows no 16S rRNA sequence differences from *Tms. crunogena* in the area covered by the DGGE fragment. DGGE fragment no. 4 was not derived from *Thiomicrospira* species; it was a *Desulfovibrio* fragment, most closely related to *Desulfovibrio salexigens* (Fig. 3B).

In former studies (Muyzer et al. 1993; Muyzer and de Waal 1994), two bacterial primers were used that amplified 16S rDNA fragments of about 200 bp, encompassing positions 341 to 534. However, the small size of these fragments limits an optimal phylogenetic analysis (Muyzer and de Waal 1994). Here, we used two primers that amplify 16S rDNA fragments of about 550 bp. These fragments are short enough to be separated well by DGGE (Lessa and Applebaum 1993) and are long enough to give a reliable phylogenetic inference. We compared phylogenetic trees based on nearly complete 16S rRNA sequences and on DGGE fragment sequences and found ambiguities only at the root level of deeply branching groups, which are also difficult to resolve by analysis of complete 16S rRNA sequences (Evers et al 1993).

Discussion

In this study, the phylogeny of *Thiomicrospira* was originally established as a framework for the identification of the molecular isolates, but also showed more general implications. First, the phylogeny of *Thiomicrospira* is relevant for the evolution of "autotrophic animals," in most cases bivalves associated with symbiotic sulfide-oxidizing bacteria that provide the animal with autotrophically fixed carbon (Cavanaugh et al. 1981; Felbeck et al. 1981). Two monophyletic lineages of obligately bivalve-associated symbiotic sulfide-oxidizers are phylogenetically affiliated with the gamma-subdivision *Thiomicrospira* species. The lineages of these free-living and host-associated sulfide-oxidizers are affiliated at their roots only. This supports an early origin of the host-symbiont relationship within each lineage and a subsequent associated evolution of host bivalvia and bacterial symbionts (Distel et al. 1994) independent from the evolution of free-living *Thiomicrospira* species. Consequently, these *Thiomicrospira* species are viewed as modern descendants of the last free-living ancestor of the obligate bivalve symbionts. This scenario allows a prediction: free-living bacteria, closely related to obligately host-associated bacteria of an ancient symbiosis, do not exist.

The phylogeny of *Tms. denitrificans* illustrates the limitation of the use of metabolic and morphological features as reliable taxonomic markers. This organism is assigned to the genus *Thiomicrospira* based on its morphology and the ability to grow as an obligate chemolithotroph by oxidation of sulfide and thiosulfate, although differences in G+C content have been noted (Timmer-ten Hoor 1975). The low G+C content of *Tms. denitrificans* (36 mol%) separates this organism from other *Thiomicrospira* species with G+C contents between 42 and 52 mol% (Kuenen et al. 1991; Wood and Kelly 1993). In addition, physiological differences between the different *Thiomicrospira* species did not support the unity of the genus. *Tms. pelophila* and *Tms. crunogena* are motile, can grow under oxic conditions, and do not denitrify, while *Tms. denitrificans* is not motile, grows strictly anaerobically, and is a denitrifier (Timmer-ten Hoor 1975; Jannasch et al. 1985; Kuenen et al. 1991). Considering these distinctive traits of *Tms. denitrificans* together with its isolated phylogenetic position, distantly related to *Thiovulum*, reclassification of this organism as a new genus is recommended. Its morphology, size, and lack of motility distinguish *Tms. denitrificans* significantly from *Thiovulum*. According to Garcia Pichel (1989), *Thiovulum* is the fastest swimmer among all bacteria. By this ability, *Thiovulum* forms free-floating veils to create and maintain steep gradients of oxygen and hydrogen sulfide.

The low number of bands in the DGGE patterns for these two hydrothermal vent samples argues for a comparatively small number of dominant bacterial species, although this does not exclude the possibility of numerous other bacteria occurring in smaller numbers in these hydrothermal vent microbial communities. This finding is supported by the results of a recent study on the microbial diversity and community structure of an active, hydrothermal vent system located at Loihi Seamount, Hawaii (Moyer et al. 1994, 1995). By using a different molecular approach, restriction fragment length polymorphism (RFLP) analysis of cloned PCR-amplified 16S rRNA genes, the authors found two dominant operational taxonomic units (OTUs) that accounted for 72.9% of 48 16S rDNA clones. In a subsequent paper, Moyer et al. (1995) has shown that one of the dominant OTU clusters, OTU 2, is closely affiliated with the sulfur-oxidizing bacterium *Thiovulum* sp., which belongs to the epsilon subdivision of the Proteobacteria, and that the other cluster, OTU 1, is affiliated to *Xanthomonas* sp., which belongs to the gamma subdivision of the Proteobacteria. Bacterial populations more complex than those of hydrothermal vent sites were found by DGGE analysis of a permanently stratified marine watercolumn; these highly differentiated

DGGE band patterns indicated a bacterial ecosystem with more diverse dominating bacterial populations (A. Teske et al., unpublished results).

After sequencing the re-amplified DGGE bands, we were able to determine the phylogenetic relationships of the dominant hydrothermal vent microbial community members. A phylogenetic framework of *Thiomicrospira* was created to allow a detailed identification of two of the separated PCR-amplified 16S rRNA gene fragments. DGGE fragment no. 2 from sample no. 2613 was derived from *Tms. crunogena* or a very closely related organism of identical 16S rRNA DGGE fragment sequence. DGGE fragment no. 1 from sample no. 2609 was related to the gamma *Thiomicrospira* sequences. The phylogenetic position of this DGGE fragment, in the center of the gamma *Thiomicrospira* cluster, strongly suggests that it is derived from a new *Thiomicrospira* species. If the physiological properties of this species are not fundamentally different from those of other *Thiomicrospira* species, it should be possible to isolate this organism. This finding indicates that more *Thiomicrospira* species are waiting to be discovered in nature.

DGGE fragment no. 4 from sample no. 2613 was not derived from *Thiomicrospira* species, but was identified as a *Desulfovibrio* fragment, most closely related to *Desulfovibrio salexigens*. Sulfate-reducing bacteria have been isolated on various occasions from deep-sea hydrothermal vent sites (Elsgaard et al. 1991), but have so far not been considered as a potentially dominant part of the hydrothermal vent bacterial community. A *Desulfovibrio* phylotype among the dominant DGGE fragments argues for a more important role of these sulfate reducers in the hydrothermal vent community.

Although molecular approaches avoid the bias that is inevitable in studies based on selective cultivation (Ward et al. 1992), it has been observed in many cases that the phylogenetic analysis of microbial communities using rRNA-based and PCR-based methods yield a variety of unknown sequences that cannot be ascribed to known bacterial genera or species (Liesack and Stackebrandt 1992). From this point of view, it appears impractical to forgo the use of pure cultures in microbial ecology altogether, as is sometimes suggested. The present study was meant to exemplify how molecular approaches can be used jointly with those of classical microbiology to acquire new capabilities, e.g., those of the DGGE approach, with the store of physiological and biochemical information that is available and still arises from studies of pure cultures.

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