APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 3789–3796 Vol. 63, No. 10
0099-2240/97/$04.00+0

Increased Species Diversity and Extended Habitat Range of Sulfur-Oxidizing *Thiomicrospira* spp.

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Received 19 May 1997/Accepted 8 August 1997

We combined traditional cultivation methods and new molecular techniques to study the diversity and habitat range of bacteria of the genus *Thiomicrospira*. Specific primers were designed and used in the PCR to amplify the 16S ribosomal DNA (rDNA) of *Thiomicrospira* spp. and thus detect the presence of these bacteria in environmental samples and enrichment cultures. By using this genus-specific PCR, we were able to amplify 722-bp-long 16S rDNA fragments from different saltwater habitats as well as from a freshwater ecosystem. Furthermore, we were able to isolate most of these bacteria in pure culture by using enrichment cultures for chemolithoautotrophic sulfur-oxidizing bacteria. With denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments followed by hybridization analysis with one of the primers as a genus-specific probe, it was possible to monitor the success of isolation. The combined approach resulted in the isolation of several chemolithoautotrophic bacteria from different habitats: e.g., a coastal sediment along the coast of Chile, a microbial mat of the hypersaline pond Solar Lake (Sinai, Egypt), and the saline spring Artern (Thuringia, Germany). In addition, four different isolates were obtained from sediment and water samples taken at Jadebusen, which is part of the German Waddensea. Comparative analysis of the nearly complete 16S rRNA sequences of these isolates indicated several new species, all grouping with the *Thiomicrospira* species of the gamma subdivision of the class *Proteobacteria*. A freshwater *Thiomicrospira* species could not be isolated, but sequence analysis of the PCR product obtained after amplification of the environmental DNA with the *Thiomicrospira*-specific primers revealed its phylogenetic affiliation. The study indicates an increased species diversity of *Thiomicrospira* and the ubiquity of this sulfur-oxidizing bacterium in habitats with reduced sulfur compounds.

Thiomicrospira species are chemolithoautotrophic bacteria that use reduced sulfur compounds, such as sulfide, thiosulfate, and sulfur, as an energy source and CO_2 as a carbon source (12). These bacteria have been isolated from different marine environments, i.e., coastal mud flats (11, 33, 38) and hydrothermal vent systems (10, 23, 24). However, the diversity and ecological importance of these bacteria in the sulfur cycle are largely unknown. Microscopical identification of these bacteria is difficult, because of their small size (1 to 2 μm by 0.2 μm to 1 to 2 μm by 0.4 μm) (10, 11) and the fact that *Thiomicrospira* can exhibit pleomorphism (11, 39).

Nowadays, the use of molecular techniques, especially those targeting the 16S rRNA or its encoding gene, are more successful in identifying particular bacteria in environmental samples than previously (e.g., reference 20). For this purpose, specific oligonucleotides can be designed and used for hybridization analysis (e.g., reference 14) or PCR amplification (e.g., reference 36).

Recently, the nearly complete 16S rRNA sequences of four *Thiomicrospira* species, i.e., *T. pelophila*, *T. crunogena*, *T. denitrificans*, and *Thiomicrospira* sp. strain MA2-6 were determined (17). These sequences, as well as the 16S rRNA sequences of two other *Thiomicrospira* species, i.e., *T. thyasirae* and *Thiomicrospira* sp. strain L-12, which were determined earlier by Distel and Wood (4) and Ruby and Jannasch (24), respectively, were used to create a phylogenetic framework for characterizing *Thiomicrospira*-related molecular isolates from hydrothermal vent microbial communities (17). Phylogenetic analysis based on these sequences showed that all *Thiomicrospira* species, with the exception of *T. denitrificans*, were forming a coherent group in the gamma subdivision of the class *Proteobacteria*, with their closest relatives being the H_2S-oxidizing bacterial symbionts of different bivalve families. *T. denitrificans* (33), which differs from the other *Thiomicrospira* species in 16S rRNA sequence, as well as in other features, such as motility, oxygen tolerance, percentage G+C content, and the ability to denitrify (10), was grouped with the genus *Thiovulum* (17), another sulfur-oxidizing bacterium, which belongs to the epsilon subdivision of the class *Proteobacteria.*

The work presented in this paper is an extension of a previous study (17). This paper presents the increased species diversity and the extended habitat range of *Thiomicrospira*. A genus-specific PCR was developed to amplify the 16S ribosomal DNA (rDNA) of *Thiomicrospira* bacteria only and thus to detect the presence of these bacteria in different habitats. In parallel with this molecular approach, we attempted to isolate the *Thiomicrospira* bacteria from these habitats. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments followed by hybridization analysis with a *Thiomicrospira*-specific oligonucleotide probe was used to monitor the success of isolation.

Phylogenetic analysis of the 16S rRNA sequences of these bacterial isolates grouped them with the known *Thiomicrospira* species from the gamma subdivision of *Proteobacteria*. This study illustrates the successful combination of microbiology and molecular biology to explore the diversity of bacteria and their presence in different habitats.

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TABLE 1. Environmental samples used in this study

Sample	Habitat	Location
Chimney rock	Hydrothermal vent	Mid-Atlantic Ridge
Sediment and overlying water	Intertidal mud flat	Jadebusen, Germany
Sediment	Continental shelf	Concepción, Chile
Microbial mat	Hypersaline pond	Solar Lake, Sinai, Egypt
Sediment	Saline spring	Artern, Germany
Sediment	Freshwater pond	Duckstein, Germany

MATERIALS AND METHODS

Isolation and cultivation of bacteria. *Thiomicrospira* strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) or from Holger Jannasch (Woods Hole Oceanographic Institution, Woods Hole, Mass.). Bacteria from seawater samples were isolated and cultivated as described by Kuenen and Veldkamp (11) with synthetic seawater supplemented with 0.5% (wt/vol) $Na_2S_2O_3 \cdot 5H_2O$.

Bacteria from the salt spring Artern (Thuringia, Germany) were isolated and cultivated with the following medium: 4.08 g of $CaSO_4 \cdot 2H_2O$, 0.37 g of $MgSO_4 \cdot 7H_2O$, 0.42 g of $MgCl_2 \cdot 6H_2O$, 0.20 g of KCl, 18.10 g of NaCl, 1.0 g of $(NH_4)_2SO_4$, 0.5 g of K_2HPO_4, 5.0 g of $Na_2S_2O_3 \cdot 5H_2O$, and 1 ml of trace element solution per 1,000 ml. Four milligrams of phenol red was used as a pH indicator. K_2HPO_4 and $Na_2S_2O_3$ were autoclaved separately, each in 10% of the final volume. The pH of the medium was adjusted to 7.0.

Enrichment cultures for freshwater samples have been performed with a medium containing 1.0 g of NaCl, 0.17 g of $MgCl_2 \cdot 6H_2O$, 1.0 g of $(NH_4)_2SO_4$, 0.4 g of $CaCl_2 \cdot 2H_2O$, 0.5 g of K_2HPO_4, 5.0 g of $Na_2S_2O_3 \cdot 5H_2O$, and 1 ml of trace element solution per 1,000 ml. Bromothymol blue (4 mg) was used as a pH indicator. The pH of the medium was adjusted to 7.2. K_2HPO_4 and $Na_2S_2O_3$ were autoclaved separately, each in 10% of the final volume.

For growth of the bacteria on solid plates, the media were supplemented with 1% (wt/vol) agar (DIFCO).

Isolation of the organisms. For enrichment cultures, 10 ml of medium was inoculated with 0.1 g of sample material and incubated at 20°C in the dark to avoid growth of phototrophic bacteria. After growth was obtained, as indicated by a change in the color of the pH indicator, 0.1 ml was transferred to 10 ml of fresh medium. After growth was obtained in these cultures, a PCR with the *Thiomicrospira*-specific primers was performed with isolated DNA or cells taken directly from the cultures. When a PCR product was obtained, indicating the presence of *Thiomicrospira* cells in the culture, 0.1 ml of the cultures was transferred to plates, which were incubated at 20°C in the dark. Different types of the colonies obtained were streaked out on fresh plates. Colonies were then transferred three times to purify them.

Environmental samples. Samples used for the isolation of bacteria and for PCR amplification were obtained from different habitats and geographic locations (Table 1). Samples and partly enriched cultures from Chile, Solar Lake (Sinai, Egypt), and the Duckstein Spring (Germany) were kindly provided by Jan Küver.

DNA isolation. Bacterial genomic DNA was obtained either by direct cell lysis according to a protocol described by Garcia-Pichel et al. (8) or after phenol extraction of bacterial cell pellets and environmental samples. For direct cell lysis, 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 9], 15 mM $MgCl_2$, 500 mM KCl, 0.1% [wt/vol] gelatin, 1% [vol/vol] Triton X-100), 1 μl of 200 mM dithiothreitol, 1 μl of 0.01% (wt/vol) sodium dodecyl sulfate (SDS), and 42 μl of sterile water (Sigma, Deisenhofen, Germany) were added to a bacterial cell pellet in a 500-μl tube. The bacteria were subjected to three freeze-thaw cycles. Thereafter, 1 μl of proteinase K solution (10 μg/ml) was added, and the tubes were incubated at 55°C for 1 h. After incubation, the tubes were briefly centrifuged, and the supernatant (i.e., cell lysate) was transferred to a clean tube and stored at −20°C for further use.

For the phenol extraction protocol, bacterial cells or sediment samples, resuspended in 500 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), were incubated with lysozyme at 37°C for 30 min. Subsequently, SDS and proteinase K were added, and the mixture was then incubated at 55°C for 2 h. An equal volume of Tris-saturated phenol was added and mixed carefully. After centrifugation, the aqueous layer was transferred to a clean tube and extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). This process was repeated until no protein precipitate was observed at the aqueous-organic interphase. Next, 0.1 volume of 5 M NaCl and 2.5 volumes of ice-cold ethanol were added to the aqueous phase, and this mixture was then incubated for 3 h at −80°C. The precipitated DNA was rinsed with 70% (vol/vol) ethanol, dried under a vacuum, and dissolved in TE buffer. The concentration and purity of the DNA preparations were determined by absorption spectrophotometry; the integrity of the DNA was determined by agarose gel electrophoresis (26).

The extracted DNA was used as template DNA in the PCR (25) to amplify the 16S rRNA coding regions (17).

Oligonucleotides used in this study. Figure 1 gives a schematic overview of the different oligonucleotides which were used in this study. Oligonucleotides p1 and p2 were used as primers in a PCR to amplify the nearly complete (1,500-bp) 16S rRNA-encoding gene of eubacteria. The PCR products obtained with these primers were used for dot blot hybridization analysis to test the specificity of the *Thiomicrospira*-specific oligonucleotides (p3 and p4), as well as for sequencing to determine the phylogenetic affiliation of the isolated bacterial strains.

The 16S rRNA sequences of known *Thiomicrospira* species were aligned to each other and to sequences of closely related bacteria by the SEQAPP program developed by Gilbert (9). The specificity of the designed primers p3 and p4 was checked by the Check_Probe program implemented in the Ribosomal Database Project (RDP) (13).

Primer pair p5 and p6 amplify the 16S rDNA of eubacteria and were used to obtain 550-bp-long rDNA fragments for DGGE analysis. The sequences of primers p1 (GM3F), p2 (GM4R), p5 (GM5F), and p6 (907R) have been published by Muyzer et al. (17). The sequences of the oligonucleotides p3 (TMS128F) and p4 (TMS849R) are 5′-GAA TCT RCC CTT TAG TTG-3′ and 5′-CTT TTT AAT AAG RCC AAC AG-3′, respectively.

PCR amplification of 16S rDNA fragments. PCR amplifications were performed as described by Muyzer et al. (17). When humic acids were expected to be present in the DNA extracts, bovine serum albumin (Sigma, St. Louis, Mo.) was added to the PCR solution to a final concentration of 3 mg/ml (22). A so-called "touchdown" PCR (5) was performed for primer pair p5 and p6 (annealing temperature from 50 to 40°C in 20 cycles). For the primer pairs p1 and p2 (annealing temperature of 40°C) and p3 and p4 (annealing temperature of 44°C), no touchdown PCR was used.

Amplification products were first analyzed by electrophoresis in 2% (wt/vol) Nusieve agarose (FMC, Vallensbaek Strand, Denmark) gels containing ethidium bromide (0.5 μg/ml [26]) before further characterization by dot blot hybridization, DGGE analysis, or DNA sequencing.

Dot blot hybridization. PCR-amplified 16S rDNA fragments obtained with primer pair p1 and p2 were used to test the specificity of the *Thiomicrospira*-specific oligonucleotides. The PCR products were spotted onto a nylon membrane (Hybond-N^+; Amersham, Buckinghamshire, United Kingdom) with the Bio-Rad dot blot apparatus (Bio-Rad Laboratories, Inc.). High-performance liquid chromatography-purified, digoxigenin (Dig)-labeled oligonucleotides were ordered directly from Biometra (Göttingen, Germany). The hybridization protocol for the dot blot analysis is the same as the one described below under "Hybridization analysis of blotted DGGE patterns," with the exception that the hybridization temperatures were varied to obtain a specific positive hybridization signal.

DGGE analysis of PCR products. DGGE was performed with the D-Gene system (Bio-Rad Laboratories, Inc.). Two different DGGE protocols were used. The first protocol (18) used 1.5-mm-thick, 6% (wt/vol) polyacrylamide gels, 0.5× TAE electrophoresis buffer (20 mM Tris-acetate, 10 mM acetate, 0.5 mM Na_2EDTA [pH 7.4]), and an electrophoresis time of 4 h at a constant voltage of 200 V. A new protocol (19), which resulted in much sharper bands (compare Fig. 2 and 3) used 1-mm-thick, 6% (wt/vol) polyacrylamide gels, 1× TAE electrophoresis buffer (pH 8.3), and an electrophoresis time of 20 h at a constant voltage of 100 V. After electrophoresis, the gels were stained with ethidium bromide and photographed as described before (18).

Hybridization analysis of blotted DGGE patterns. Denaturing gradient gel patterns were transferred to nylon membranes (Hybond-N^+) by electroblotting and hybridized as described by Muyzer et al. (18). The membrane was prehybridized for 4 h at 52°C with 50 ml of a solution containing 2% (wt/vol) blocking reagent (Boehringer Mannheim Biochemicals, Mannheim, Germany) in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% (wt/vol) *N*-lauroylsarcosine–0.02% (wt/vol) SDS. One hundred picomoles of the Dig-labeled *Thiomicrospira*-specific probe p4 (TMS849R) was added to 6 ml of the prehybridization solution. The blot was incubated in this mixture overnight at 52°C. After hybridization, the membrane was washed for 30 min at hybridization tempera-

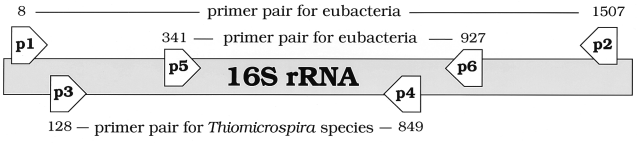

FIG. 1. Schematic diagram of the oligonucleotides used in this study. Primers p1 and p2 are specific for the 16S rDNA of eubacteria and amplify the nearly complete gene. These PCR products have been used for dot blot hybridization analysis to test the specificity of primers p3 and p4 and for phylogenetic analysis. Primers p3 and p4 are specific for *Thiomicrospira* bacteria only. These primers were used to detect the presence of *Thiomicrospira* species in environmental samples and enrichment cultures and for phylogenetic analysis of *Thiomicrospira* species which could not be isolated in pure cultures. Primer p4 has also been used as a probe in hybridization experiments to identify amplified *Thiomicrospira* sequences in DGGE profiles. Primers p5 and p6 amplify the 16S rDNA of eubacteria and were used to obtain 550-bp-long rDNA fragments for DGGE analysis.

ture, first with a solution containing 2× SSC–0.1% (wt/vol) SDS and then twice with a solution containing 0.1× SSC–0.1% (wt/vol) SDS. Subsequently, the membrane was rinsed with a solution of 0.1 M malic acid (pH 7.5)–0.15 M NaCl. The bound probe was detected by an enzyme-linked immunoassay with an anti-Dig alkaline phosphatase conjugate (Boehringer Mannheim Biochemicals). After 30 min of incubation, the membrane was washed twice for 15 min with a solution of 0.1 M malic acid (pH 7.5)–0.15 M NaCl and equilibrated for 5 min with a solution containing 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 50 mM $MgCl_2$. An enzyme-catalyzed reaction with the chemiluminescent reagent CSPD (Tropix, Inc., Bedford, Mass.) allows the detection of the hybrids with X-ray film (Kodak, Inc., Rochester, N.Y.).

Sequencing of PCR products. PCR products were purified by using the Qiaquick Spin PCR purification kit (Qiagen, Inc., Chatsworth, Calif.). The *Taq* Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) was used to sequence the 16S rDNA fragments. Sequencing primers were the same as those described previously by Buchholz-Cleven et al. (3). The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer.

Comparative analysis of 16S rRNA sequences. The 16S rRNA sequences were aligned to those obtained from the RDP (13) and GenBank (1). Sequence alignments were prepared with the sequence editor SEQAPP (9). Phylogenetic trees were created with the parsimony algorithm (heuristic search with random addition of sequences and TBR (tree bisection-reconnection) branch swapping; all characters in the alignment were used with equal weight) implemented in the software program PAUP (30). Bootstrap analysis (100 replicates) was used to validate the reproducibility of the branching pattern of the trees.

Nucleotide sequence accession number. The sequences obtained in this study are available from GenBank under accession no. AF013971 to AF013978 and AF016046.

RESULTS

Primer design. The *Thiomicrospira*-specific primers p3 and p4 were obtained after comparative analysis of aligned 16S rRNA sequences from known *Thiomicrospira* species and from several other bacteria belonging to the different subgroups of the class *Proteobacteria*. *Thiomicrospira*-specific sequences were found for positions 128 to 145 (TMS128F [primer p3]) and positions 830 to 849 (TMS849R [primer p4]) (*Escherichia coli* numbering) (2). Both primers include one wobble base, i.e., an R (A or G), to be complementary to the 16S rRNA sequences of all of the *Thiomicrospira* species belonging to the gamma subdivision of *Proteobacteria*. A specificity check with the Check_Probe option in the RDP showed no mismatches with the two *Thiomicrospira* sequences present in the RDP. At least two mismatches were found for oligonucleotide p3, and three mismatches were found for p4 with 16S rRNA sequences from other bacteria.

Specificity of the *Thiomicrospira*-specific oligonucleotides. The specificity of the *Thiomicrospira*-specific oligonucleotides was first tested in a dot blot hybridization assay with nearly complete 16S rDNA fragments obtained after enzymatic amplification of target DNAs from a variety of bacteria, including close relatives of *Thiomicrospira*, i.e., the endosymbiotic bacteria of *Riftia pachyptila*, *Calyptogena magnifica*, and *Bathymodiolus thermophilus* (Table 2). Different hybridization temperatures were used to obtain a specific signal with *Thiomicrospira* bacteria only. We found that for both oligonucleotide probes (i.e., p3 and p4), a hybridization temperature of 52°C gave a specific signal with good intensity (results not shown).

***Thiomicrospira*-specific PCR.** The two oligonucleotides p3 and p4 were also used as primers in the PCR with an annealing temperature of 44°C to amplify 16S rRNA encoding gene fragments from *Thiomicrospira* bacteria in natural samples and enrichment cultures. PCR products of about 722 bp were obtained with the five *Thiomicrospira* species, but not with target DNAs of all other bacteria tested, including the three endosymbionts tested and *T. denitrificans.*

Sensitivity of the *Thiomicrospira*-specific PCR. To determine the detection limit of the *Thiomicrospira*-specific PCR, we made a serial dilution of genomic DNA of *Thiomicrospira* sp. strain MA2-6 in DNA of other bacteria. Using a PCR with 35 cycles, we could still obtain a specific PCR product with 2.4 pg of added *Thiomicrospira* DNA.

TABLE 2. Specificity analysis of *Thiomicrospira*-specific oligonucleotides

Bacterium	Affiliation[a]	Source[b]	Result[c]
Nitrobacter agilis	Alpha	ATCC 14128	−
Nitrobacter sp.	Alpha	Andreas Teske	−
Rhodopseudomonas palustris	Alpha	DSM 123	−
Nitrosospira briensis C-128	Beta	John Waterbury	−
Nitrosolobus multiformis	Beta	ATCC 25196	−
Leptothrix discophora	Beta	Liesbeth de Vrind	−
Beggiatoa alba	Gamma	DSM 1416	−
Escherichia coli (W3110)	Gamma	Sigma D-0421	−
Nitrococcus mobilis	Gamma	ATCC 25380	−
Thiomicrospira crunogena	Gamma	Holger Jannasch	+
Thiomicrospira pelophila	Gamma	DSM 1534	+
Thiomicrospira sp. strain L-12	Gamma	Holger Jannasch	+
Thiomicrospira sp. strain MA2-6	Gamma	Holger Jannasch	+
Thiomicrospira thyasirae	Gamma	DSM 5322	+
Endosymbiont of *Calyptogena magnifica*	Gamma	East Pacific Ocean	−
Endosymbiont of *Bathymodiolus*	Gamma	East Pacific Ocean	−
Endosymbiont of *Riftia pachyptila*	Gamma	East Pacific Ocean	−
Desulfobacter curvatus	Delta	DSM 3379	−
Desulfobotulus sapovorans	Delta	DSM 2055	−
Desulfovibrio baculatus	Delta	DSM 2555	−
Desulfovibrio desulfuricans	Delta	DSM 1926	−
Desulfovibrio vulgaris	Delta	DSM 644	−
Thiomicrospira denitrificans	Epsilon	DSM 1251	−
Wolinella succinogenes	Epsilon	DSM 1740	−
Campylobacter jejuni	Epsilon	DSM 4688	−

[a] Phylogenetic affiliation based on 16S rRNA sequence comparison. Different subdivisions of the class *Proteobacteria* are shown (21).
[b] DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; ATCC, American Type Culture Collection, Rockville, Md.
[c] −, no reaction; +, positive reaction.

Survey of different habitats for *Thiomicrospira*. Several samples from different habitats and separate geographic locations (Table 1) were tested for the presence of *Thiomicrospira* species as well as for the relative abundance of these bacteria among others (Table 3). The presence of *Thiomicrospira* bacteria in environmental samples as well as enrichment cultures was determined with the *Thiomicrospira*-specific PCR. By using the specific primer pair, we obtained a PCR product of the expected size of about 722 bp with environmental DNA from nearly all habitats tested, as well as with DNA obtained from all enrichment cultures (Table 3). For the first time, we detected the presence of *Thiomicrospira* species in enrichment cultures obtained from sediment samples of the freshwater pond at Duckstein.

The relative abundance of *Thiomicrospira* in the habitat was determined by hybridization analysis of DGGE patterns of PCR products obtained with the eubacterial primers p5 and p6 with the *Thiomicrospira*-specific oligonucleotide. By this approach, positive results were found only with enrichment cultures. No positive results were obtained when environmental DNA was tested, indicating the low abundance of *Thiomicrospira* in these habitats.

Isolation of bacteria in pure cultures. The presence of *Thiomicrospira* species in different habitats for which *Thiomicrospira* bacteria have not been described before prompted us to obtain these bacteria in pure cultures. The culture medium for chemolithotrophic microorganisms was inoculated with envi-

TABLE 3. Habitat range of *Thiomicrospira*

Habitat	PCR result for[a]:				*Thiomicrospira* strain(s) isolated from habitat
	Environmental samples		Enrichment cultures		
	Eubacterial[b]	*Thiomicrospira* specific[c]	Eubacterial	*Thiomicrospira* specific	
Hydrothermal vents	ND	+	ND	ND	HTV1, HTV2[d]
Intertidal flat (water)	−	+	+	+	JB-B2
Intertidal flat (sediment)	−	+	+	+	JB-A1, JB-A1F, JB-A2
Continental shelf	ND	−	+	+	Ch-1
Hypersaline pond	−	+	+	+	SL-1
Saline spring	−	+	+	+	Art-3
Freshwater pond	ND	+	ND	+	DS-1[e]

[a] −, not detected; +, detected; ND, not determined.
[b] PCR with eubacterial primer pair p5 and p6 followed by DGGE and hybridization analysis with the *Thiomicrospira*-specific oligonucleotide p4.
[c] PCR with the *Thiomicrospira*-specific primer pair p3 and p4.
[d] Molecular isolates from the same hydrothermal vent samples obtained in an earlier study (17).
[e] Molecular isolate obtained after sequencing of the PCR product obtained with the *Thiomicrospira*-specific primer pair p3 and p4.

ronmental material and incubated at 20°C in the dark. After growth was visible, cells were transferred from the enrichment cultures onto agar plates containing the same medium. Between 500 and 2,000 bacterial colonies were obtained. All known *Thiomicrospira* strains form colonies with an intensive yellow color because of sulfur precipitation. Those colonies were further identified with the PCR specific for *Thiomicrospira*. Every colony selected was transferred three times onto new agar plates before it was considered to be a pure isolate.

DGGE and hybridization analysis to monitor the success of isolation. DGGE of 16S rDNA fragments obtained after enzymatic amplification with general primers for eubacteria and followed by hybridization analysis with the Dig-labeled *Thiomicrospira*-specific oligonucleotide p4 was used to monitor the success of isolation. Figure 2 shows the results of such an analysis. DGGE analysis of a mixture of PCR products from an enrichment culture containing acetate, fumerate, and sulfide showed bands with different mobilities (Fig. 2A, lane 6), indicating the presence of several different bacteria. However, none of the bands was reacting with the Dig-labeled *Thiomicrospira*-specific probe (Fig. 2B, lane 6). Enrichments for chemolithoautotrophic sulfur-oxidizing bacteria, such as *Thiomicrospira*, with the general enrichment used as an inoculum resulted in the presence of only two bands (Fig. 2A, lane 7). Hybridization analysis of this sample with the *Thiomicrospira*-specific probe showed a positive signal with only one band, indicating the enrichment of *Thiomicrospira* (Fig. 2B, lane 7). The other band (Fig. 2A, lane 7, indicated by an arrow) is probably a PCR product amplified from another bacterium which can also grow chemolithoautotrophically. Filtration of this enrichment culture through a sterile 0.45-μm-pore-size filter resulted in the purification of the *Thiomicrospira* isolate (Fig. 2A and B, lane 8). No growth was obtained after filtration through a 0.2-μm-pore-size filter. The same approach was followed for the isolation of *Thiomicrospira* bacteria from the microbial mat of the hypersaline pond Solar Lake. Lane 9 (Fig. 2A) shows the DGGE profile of PCR products obtained from a general enrichment on acetate, fumarate, and sulfide, in which no *Thiomicrospira* bacteria could be detected (Fig. 2B, lane 9). However, lane 10 in Fig. 2A shows the enrichment for chemolithotrophic bacteria in which two bands are present, which are close to each other, but from which only the upper band belongs to a *Thiomicrospira* bacterium (Fig. 2B, lane 10). Lane 11 (Fig. 2A and B) demonstrates the isolation of a *Thiomicrospira* strain in pure culture obtained after 0.45-μm-pore-size filtration. Selective enrichment of a sediment sample from Artern, Germany, showed only one band (Fig. 2A, lanes 12 and 13), which gave a positive reaction with the *Thiomicrospira*-specific oligonucleotide probe (Fig. 2B, lanes 12 and 13). The *Thiomicrospira* bacteria (i.e., *T. pelophila*, *T. thyasirae*, *T. crunogena*, *Thiomicrospira* sp. strain MA2-6, and *Thiomicrospira* sp. strain L-12) were used as markers in the electrophoresis (Fig. 2, lanes 1 to 5 and 14 to 18).

DGGE and hybridization analysis of the isolates from Jadebusen. Figure 3A shows the DGGE profiles of PCR products of different enrichment cultures and isolates obtained from

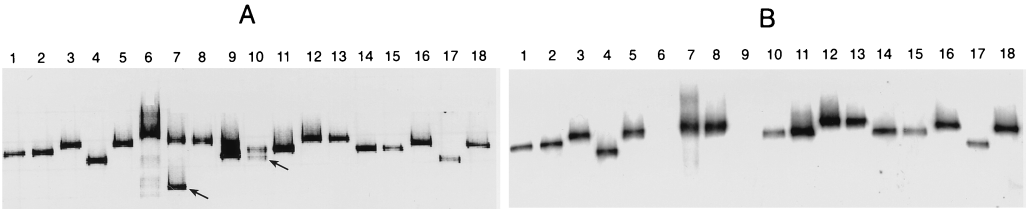

FIG. 2. Hybridization analysis of DGGE profiles to monitor the success of isolation of *Thiomicrospira* bacteria from different habitats. (A) DGGE separation pattern of rDNA fragments obtained after enzymatic amplification of known *Thiomicrospira* strains, enrichment cultures, and bacterial isolates with primer pair p5 and p6, which are specific for all eubacteria. Lanes: 1 and 14, *T. pelophila*; 2 and 15, *T. thyasirae*; 3 and 16, *T. crunogena*; 4 and 17, *Thiomicrospira* sp. strain MA2-6; 5 and 18, *Thiomicrospira* sp. strain L-12; 6, general enrichment of bacteria from a sediment sample from the coast of Chile (C1); 7, enrichment for chemolithoautotrophic bacteria (C2) with the general enrichment C1 as an inoculum; 8, bacterial isolate (CH-1) after filtration of the enrichment C2 through a 0.45-μm-pore-size filter; 9, general enrichment of bacteria from a microbial mat sample from Solar Lake (S1); 10, enrichment for chemolithoautotrophic bacteria (S2) with the general enrichment S1 as an inoculum; 11, bacterial isolate (SL-1) after filtration of the enrichment S2 through a 0.45-μm-pore-size filter; 12, enrichment for sulfur-oxidizing bacteria from Artern; 13, bacterial isolate from Artern. (B) Results after hybridization analysis with the *Thiomicrospira*-specific, Dig-labeled oligonucleotide p4, whose target sequence is located within the rDNA amplified with primer pair p5 and p6. Note that comparison of both panels shows the presence of sulfur-oxidizing bacteria other than *Thiomicrospira* in the enrichments C2 and S2 (see arrows in panel A, lanes 7 and 10, respectively).

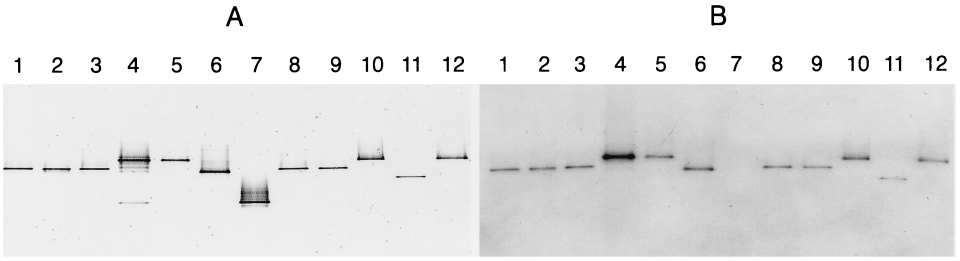

FIG. 3. Hybridization analysis and DGGE profiles of PCR products obtained after enzymatic amplification of enrichment cultures and bacterial isolates from Jadebusen and of known *Thiomicrospira* strains with primer pair p5 and p6, which are specific for eubacteria in general. (A) DGGE separation pattern of rDNA fragments. Lanes: 1 and 8, *T. pelophila*; 2, enrichment culture obtained after inoculation of a 0.2-μm-pore-size-filtered sediment sample; 3, isolate JB-A1F; 4, unfiltered enrichment JB-A1; 5, isolate JB-A2; 6, isolate JB-A1; 7, "*Rhodobacter*"-like isolate; 9, *T. thyasirae*; 10, *T. crunogena*; 11, *Thiomicrospira* sp. strain MA2-6; 12, *Thiomicrospira* sp. strain L-12. (B) Results after hybridization analysis with the *Thiomicrospira*-specific, Dig-labeled oligonucleotide p4, whose target sequence is located within the rDNA amplified with primer pair p5 and p6. Note the absence of a hybridization reaction with the PCR product of the "*Rhodobacter*"-like isolate in lane 7.

Jadebusen, Germany. Lane 2 (Fig. 3A) shows the DGGE profile from an enrichment culture inoculated with a mud suspension which was passed through a 0.2-μm-pore-size filter before inoculation, as described by Kuenen and Veldkamp (11) for the isolation of *T. pelophila*. Lane 3 (Fig. 3A) shows the band from the isolate from this culture, which halts at the same position in the gel as the band in the original enrichment culture (Fig. 3A, lane 2) and as the band of *T. pelophila* (Fig. 3A, lanes 1 and 8). Lane 4 shows a profile of an enrichment culture obtained after inoculation with an unfiltered mud sample from the same sediment. Here we obtained several bands halting at different positions in the gel, indicating several different bacteria. Lanes 5, 6, and 7 show bands from the purified isolates of this enrichment culture. In Fig. 3A, lanes 1 and 8 to 12, we have applied the PCR products of the known *Thiomicrospira* species as markers. Hybridization analysis of these DGGE patterns with the *Thiomicrospira*-specific probe p4 gave positive signals with the bands obtained from the enrichment (Fig. 3B, lanes 2 and 4) and isolates (Fig. 3B, lanes 3, 5, and 6), as well as with the known *Thiomicrospira* species (Fig. 3B, lanes 1 and 8 to 12). No hybridization signal was obtained with the lower band in lane 4 or with the band in lane 7, which halts at the same position in the gel (compare Fig. 3A and B).

Phylogenetic analysis of bacterial isolates. Some of the bands in the DGGE profiles of our new isolates were at the same position in the gel as those of one or more of the known *Thiomicrospira* species (Fig. 2A and 3A, lanes 1 and 3), indicating identical sequences. To substantiate this assumption, as well as to determine the phylogenetic relationship among our new bacterial isolates, we sequenced their 16S rRNA encoding genes.

Phylogenetic analysis of the 16S rRNA sequences showed that all bacterial isolates cluster together with the sequences of the five known *Thiomicrospira* species, forming a monophyletic group with a bootstrap value of 100% (Fig. 4A). Addition of several closely and distantly related sequences, as well as the choice of different outgroup sequences, did not change this grouping (results not shown).

So far, the *Thiomicrospira* species obtained from hydrothermal vent communities (i.e., *T. crunogena*, *Thiomicrospira* sp. strain L-12, and *Thiomicrospira* sp. strain MA2-6) have been grouped together, but have been separated from those species (i.e., *T. pelophila* and *T. thyasirae*) which came from intertidal mud flats (17). However, the results presented in this paper demonstrate that this split is no longer valid. Two of the four isolates from Jadenbusen (JB-A1 and JB-B2), as well as the isolate from the microbial mat of the hypersaline pond Solar Lake (SL-1), are all grouped together with the *Thiomicrospira* species from hydrothermal vent environments.

By DGGE analysis, we were not able to separate *T. pelophila*, *T. thyasirae*, and the bacterial isolate JB-A1F, indicating identical or very similar sequences. However, because of missing sequence data for the 16S rRNA sequence of *T. thyasirae*, we resequenced this molecule. Comparison of the old and the new 16S rRNA sequences demonstrated eight differences. For further phylogenetic analysis, we used the corrected sequence for *T. thyasirae*. Comparison of this sequence with the sequence of *T. pelophila* gave only one mismatch (i.e., an A at position 1456 in the *T. thyasirae* sequence versus a G in the sequence of *T. pelophila* (insert).

The sequence of one of the four isolates from Jadebusen (i.e., JB-A1F) was 100% identical to the sequence of *T. pelophila*, which might indicate the isolation of the same species after more than 25 years. The other three sequences from strains isolated from Jadebusen were grouped with sequences from other *Thiomicrospira* species.

We also obtained a sequence of the isolate from an enrichment culture inoculated with an unfiltered mud suspension from Jadebusen (Fig. 3A, lane 7). A similarity search with sequences stored in the GenBank sequence database (1) indicated 95% similarity to the 16S rRNA sequence of *Rhodobacter sphaeroides* and gave similarity (S_{ab}) values of about 0.8 with sequences of *Rhodobacter* species stored in the RDP database (13). *Rhodobacter* species are phototrophic bacteria, which can also grow in the dark heterotrophically and in some cases with inorganic electron donors; they belong to the alpha subdivision of the class *Proteobacteria.*

Phylogenetic analysis of the freshwater *Thiomicrospira* spp. Although it was not possible to obtain a pure culture of the chemolithoautotrophic bacterial isolate from the freshwater ecosystem Duckstein, we were still able to retrieve phylogenetic information from this system. By using the *Thiomicrospira*-specific primer pair, we obtained a PCR product from an enrichment culture for chemolithoautotrophic bacteria. Sequencing of this product resulted in one sequence, indicating the presence of one *Thiomicrospira* species. In the comparative analysis of this sequence, we included other partial sequences which were shown before to be related to *Thiomicrospira*, such as the HTV1 and HTV2 sequences, which were obtained from hydrothermal vent microbial communities (17), and the THIO1 sequence, which was obtained after amplification of DNA extracted from oil field microbial communities (35). Phylogenetic analysis indicated a close relationship of the Duckstein sequence with the sequence of the isolate CH-1,

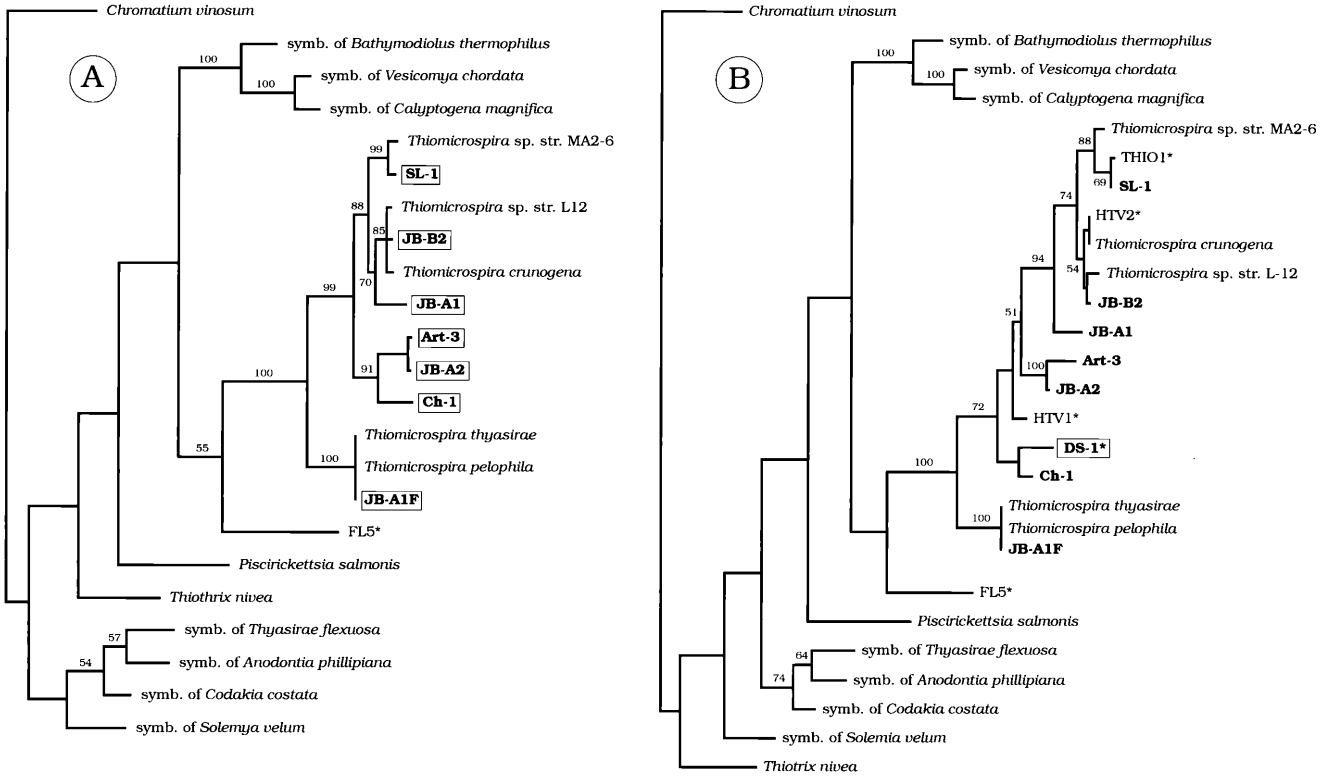

FIG. 4. Phylogenetic trees showing the relationships of bacterial and molecular isolates with sequences from the RDP and GenBank. (A) Tree based on nearly complete (1,500 nucleotides) 16S rRNA sequences. (B) Tree inferred from partial (700 nucleotides) 16S rRNA sequences giving the affiliation of the freshwater molecular isolate DS-1. The sequences determined in this study are framed. Molecular isolates other than the endosymbiotic (symb.) bacteria are marked with an asterisk. The sequence of *Chromatium vinosum* was used as an outgroup. The numbers on the branches refer to bootstrap values; only values above 50% are shown.

which was obtained from a marine sediment sample from the coast of Chile (Fig. 4B). The THIO1 sequence was found to be closely related to the sequence of a *Thiomicrospira* strain (SL-1) isolated from the microbial mats of Solar Lake.

DISCUSSION

rRNA sequencing of chemolithoautotrophic isolates indicated an increased diversity belonging to the genus *Thiomicrospira*. However, we also found that the 16S rRNA sequence of one isolate from Jadebusen, i.e., JB-A1F was 100% identical to the sequence of *T. pelophila*, an organism isolated more than 25 years ago by Kuenen and Veldkamp (11). Resequencing of the 16S rRNA of *T. thyasirae* indicated eight differences between the old sequence described by Distel and Wood (4) and the new sequence presented in this study. These sequence differences are probably sequence errors in the old sequence, since these aberrant nucleotides were not found in any of the other *Thiomicrospira* sequences. For further analysis, we used the new sequence of *T. thyasirae*. This sequence is 99.9% identical (one mismatch in 1,424 bp) to the sequence of *T. pelophila*. Such a high sequence identity indicates that *T. pelophila* and *T. thyasirae* are two strains of the same species (29). However, Kuenen and Veldkamp (11) described a G+C content of 44 mol% for *T. pelophila*, while a G+C content of (51.9 ± 1.6) mol% (10 determinations) was found for *T. thyasirae* (39). According to Stackebrandt and Liesack (28), this big difference in G+C content between the two bacteria, however, argues against one species. They described that strains should not differ by more than 3% within a well-defined species and not more than 10% within a well-defined genus. A similar contradictory result has been described by Fox et al. (7) for three psychrophilic *Bacillus* strains. 16S rRNA sequence comparison of the *Bacillus* strains showed more than 99.5% sequence similarity. However, DNA-DNA hybridization between the strains was too low to consider them members of the same species. The authors concluded that 16S rRNA sequences might not resolve between recently diverged species. So, although *T. pelophila* and *T. thyasirae* have similar phenotypical features and nearly identical 16S rRNA sequences, they could still be two different species. A polyphasic approach (34) should be employed to characterize in more detail the taxonomic relationships among the different *Thiomicrospira* strains, especially those between *T. pelophila*, *T. thyasirae*, and isolate JB-A1F.

DGGE analysis of PCR-amplified 16S rDNA fragments was originally introduced into microbial ecology as a means of determining the genetic diversity of mixed microbial populations (6, 15–17, 31). However, recently Teske et al. (32) demonstrated the power of this approach in testing the purity of bacterial strains. Furthermore, Buchholz-Cleven et al. (3) used DGGE analysis for the rapid screening of bacterial isolates as a first step for further characterization. Ward and coworkers (27, 37) used DGGE of PCR-amplified 16S rDNA fragments to characterize enrichment cultures. Here, we have demonstrated the combined use of DGGE of PCR-amplified rDNA products and hybridization analysis with a genus-specific oligonucleotide probe to monitor the successful isolation of bacteria in pure cultures. This strategy facilitates the isolation and characterization of new species.

In this study, we were able to detect *Thiomicrospira* in different habitats. However, detection of *Thiomicrospira* in envi-

ronmental samples was only possible after PCR amplification with the *Thiomicrospira*-specific primer pair p3 and p4. *Thiomicrospira* bacteria could not be detected in natural samples after amplification with the eubacterial primers p5 and p6, followed by hybridization analysis with the *Thiomicrospira*-specific Dig-labeled oligonucleotide probe p4. The reason for this might be the low number of *Thiomicrospira* bacteria in the samples in relation to the number of other bacteria present. Muyzer et al. (16) showed that the sensitivity of DGGE analysis of PCR products obtained with general eubacterial primers was about 1%, meaning that a particular bacterial population representing 1% of the total community could still be detected in the DGGE profile. Recently, Murray et al. (15) found a similar result.

With our *Thiomicrospira*-specific oligonucleotide probes, we could detect a specific signal with PCR-amplified 16S rDNA fragments obtained from 2.4 pg of *Thiomicrospira* genomic DNA. Unfortunately, it was not possible to relate this amount of target DNA to a cell number, because neither the size of the *Thiomicrospira* genome nor the rRNA operon copy number is known.

It might be possible that *Thiomicrospira* bacteria are only dominant in a distinct, relatively small zone of the sediment, in which oxygen and sulfide overlap. We are therefore now using the following strategy: first (i), a horizontal survey using the group-specific PCR to determine the presence of *Thiomicrospira* bacteria in different habitats as presented in this paper and then (ii) a vertical study of one environment to determine the presence of different *Thiomicrospira* species in microhabitats in order to unravel the niche differentiation of these bacteria. For the latter purpose, molecular methods will be combined with microsensor measurements to characterize environmental parameters, such as oxygen concentration, sulfide concentration, and pH, and with classical microbiological techniques.

ACKNOWLEDGMENTS

We are grateful to Jan Küver for advice and help and Bridget Laue and Gijs Kuenen for helpful discussions. We thank Holger Jannasch for providing us with *Thiomicrospira* cultures and Andreas Teske and Cathrin Wawer for providing us with DNA from several bacteria.

This research was financially supported by the Max-Planck-Society, Munich, Germany.

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