Investigation of the ecological relevance of sulfur-oxidizing bacteria of the genus *Thiomicrospira* in marine habitats with molecular biological and microbiological techniques

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Chapter 1

General introduction

The sulfur-cycle and the role of sulfate-reducing and sulfur-oxidizing bacteria

In 1887 Winogradsky published the results of his investigations on the filamentous bacterium *Beggiatoa*. He postulated chemolithotrophy of these organisms based on growth with hydrogen sulfide and suggested the name "Schwefelbacterien" (sulfur bacteria).

Today we know that the sulfur-cycle is a complex network, in which different sulfur compounds are reduced and oxidized either biologically or chemically. The sulfur transformations are even more complex than those of nitrogen due to the variety of oxidation states of sulfur and the oxidative part of the sulfur cycle itself seems to be more complex than the reductive part (e.g., Jørgensen, 1987).

The most oxidized form of sulfur is sulfate (SO_4^{2-}). A broad range of organisms like higher plants, algae, fungi, and most procaryotes can use sulfate as a sulfur source and carry out assimilatory sulfate-reduction. However, the ability to use sulfate as electron acceptor during the degradation of organic compounds is restricted to the group of sulfate-reducing bacteria. Even though an alternative aerobic metabolism of some sulfate-reducing bacteria was reported (e.g., Dilling and Cypionka, 1990; Krekeler et al., 1997), these bacteria can grow only under anoxic, reduced conditions (Dilling and Cypionka, 1990; Widdel and Bak, 1992).

Dissimilatory sulfate-reduction is the most important process of biomineralization of carbon in anoxic marine sediments (e.g., Jørgensen,

1982; Henrichs and Reeburgh, 1987; Canfield, 1993). Sulfate-reducing bacteria are almost ubiquitous and active in very different marine sediments what might be explained by their enormous metabolical diversity (Widdel and Bak, 1992). During the process of sulfate-reduction hydrogen sulfide (H_2S), the most reduced sulfur compound, is produced as endproduct via sulfite, and seems to be the only extracellular product of significance (Jørgensen, 1987). It is toxic to many organisms because it combines with the iron of cytochromes and other essential iron-containing compounds in the cell. In contrast to the sulfate anion, which is chemically very stable, H_2S oxidizes spontaneously under aerobic conditions. However, H_2S and some other reduced sulfur compounds, like elemental sulfur (S^0), and thiosulfate ($S_2O_3^{2-}$), can also be used by sulfur-oxidizing bacteria as electron donors. The final product of the oxidation of reduced sulfur compounds is generally sulfate and in coastal sediments between 50% and >95% of the produced H_2S becomes reoxidized (Jørgensen, 1987).

Reduced sulfur compounts are used by phototrophic bacteria (purple sulfur bacteria, green sulfur bacteria, green non sulfur bacteria), and by the non phototrophic colorless sulfur bacteria. The phototrophic sulfur bacteria are not able to use H_2O to obtain their reducing power. Therefore they use either an organic compound, H_2 or a reduced sulfur compound. Furthermore they can only grow phototrophically under anaerobic conditions because their pigment synthesis is repressed under oxic conditions.

The colorless sulfur bacteria are a very heterogeneous group that plays an essential role in the oxidative part of the sulfur cycle (e.g., Robertson and Kuenen, 1992). They can be found in almost every environment where reduced sulfur compounds are present, like marine and freshwater habitats, soils, and wastewater treatment plants. There is a wide range of different types with very diverse morphological, physiological, phylogenetical, and ecological properties, and Archea (*Sulfolobus, Acidianus*) are included as well as different types of Bacteria.

The colorless sulfur bacteria are devided into two major groups: (i) organisms that deposit sulfur internally as sulfur globules, which are visible by phase microscopy (e.g., the genera *Beggiatoa, Thiotrix, Thioploca, Achromatium,* and *Thiovulum*), and (ii) bacteria that do not form internal sulfur globules (e.g., the genera *Thiobacillus,* and *Thiomicrospira*) (Kuenen, 1989). The stored sulfur of the former group can serve as an energy reserve.

Within the colorless sulfur bacteria four different physiological types exist: (1) obligate chemolithotrophs, (2) facultative chemolithotrophs (mixotrophs), (3) chemolithoheterotrophs, and (4) chemoorgano-heterotrophs (heterotrophs) (Robertson and Kuenen, 1992). Most require oxygen as their terminal electron acceptor. Exceptions are *Thiobacillus denitrificans* (Taylor and Hoare, 1971; Taylor et al., 1971) and *Thiomicrospira denitrificans* (Timmer-ten Hoor, 1975), which are able to grow anaerobically using nitrate as electron acceptor, and *Thiobacillus ferrooxidans,* which reduces ferric iron under anaerobic conditions (Sugio et al., 1985).

The first step of the aerobic oxidation of H_2S is the formation of elemental sulfur. It is chemically stable in the presence of oxygen, but can be oxidized further by sulfur-oxidizing bacteria to sulfate. During the oxidation of reduced sulfur species H^+ ions are released and the medium is acidified. The acid formed is sulfuric acid (H_2SO_4) and many sulfur-oxidizing bacteria are able to withstand low pH values (e.g. some thiobacilli).

In environments where sulfate-reduction takes place aerobic sulfur-oxidizing bacteria live in a distinct layer in sediments, microbial mats or water columns where hydrogen sulfide and oxygen overlap, to be able to compete with the chemical oxidation of H_2S (e.g., Robertson and Kuenen, 1992). This ecological niche is inhabited, e.g., by *Beggiatoa*, *Thioploca*, and *Thiovulum*, which can form nearly monocultural layers at the sulfide-oxygen interface (Jørgensen and Revsbech, 1983; Jørgensen, 1989). In *Beggiatoa* mats it was found that the biological sulfide oxidation rate was three orders of magnitude higher than the chemical rate, which indicates a very successful competition of these organisms with the chemical process (Jørgensen and Revsbech, 1983).

The genus *Thiomicrospira*

In 1972 Kuenen and Veldkamp isolated a very thin (width of 0.2 - 0.3 μm), comma- or spiral-shaped sulfur-oxidizing bacterium from a Dutch marine mud flat. They found that the organism, due to its size, could be also easily obtained by filtering a mud suspension through a 0.22 μm pore size filter before inoculation of the enrichment culture. The isolate was described as the type species of a new genus: *Thiomicrospira pelophila*. (*thium* sulfur, *micros* small, *spira* spiral: a small sulfur spiral). During the next decades several obligately autotrophic sulfur-oxidizing bacteria were isolated and grouped into this genus, primarily based on their specific morphology, and physiology.

Thiomicrospira species are chemolithoautotrophic bacteria that use reduced sulfur compounds like sulfide, thiosulfate, and sulfur, as electron donor, and CO_2 as carbon source (Kuenen et al., 1992; Kuenen and Robertson, 1989). The physiology and growth characteristics of *Thiomicrospira* species are similar to those of the neutrophilic thiobacilli (Kuenen et al., 1992). A main difference is that *Thiobacillus* species are widespread in the phylogenetic tree, while members of the genus *Thiomicrospira* are present in a monophyletic group in the gamma subdivision of the Proteobacteria, as determined by phylogenetic analysis of their 16S rRNA sequences (Muyzer et al., 1995). Bivalve associated sulfide-oxidizing endosymbionts, e.g., of *Vesicomya chordata*, *Bathymodiolus thermophilus*, and *Calyptogena* spp., were found to be related to *Thiomicrospira* (Muyzer et al., 1995). An exception is

Thiomicrospira denitrificans (Timmer-ten Hoor, 1975), which was assigned as a member of the epsilon subdivision and is affiliated with *Thiovulum*, another sulfur-oxidizing bacterium (Muyzer et al., 1995). The low G + C content of 31.7 mol% separates *Thiomicrospira denitrificans* from other *Thiomicrospira* species, which were described to have G + C contents between 42 and 52 mol% (Kuenen et al., 1992; Wood and Kelly, 1993). In addition, all other *Thiomicrospira* species can grow under aerobic conditions, and do not denitrify (Kuenen and Veldkamp, 1972; Ruby and Jannasch, 1982; Jannasch et al., 1985; Wood and Kelly, 1989; Wood and Kelly, 1993), while *Thiomicrospira denitrificans* is facultatively anaerobic, and is a denitrifier (Timmer-ten Hoor, 1975). Therefore Muyzer et al. (1995) recommended reclassification of this organism as a new genus.

At the beginning of this study five *Thiomicrospira* spp. belonged to the gamma sudivision of the Proteobacteria. These organisms were obtained from two different ecosystems, intertidal mud flats and deep-sea hydrothermal vent systems. At the former habitat reduced sulfur compounds are mainly produced by sulfate-reduction, while at the vent sites they are released by geological processes into the surrounding water (e.g., Jannasch and Mottl, 1985). As mentioned above, *Thiomicrospira pelophila* (Kuenen and Veldkamp, 1972) was isolated from a mud sample of the Dutch Wadden Sea; *Thiomicrospira thyasirae* (Wood and Kelly, 1989; Wood and Kelly, 1993) was isolated from the gills of *Thyasira flexuosa*, which belongs to a genus of the bivalve mollusc family of the *Thyasiridae*. The bivalve was collected off the Plymouth Sound, UK, which is a comparable habitat to the Wadden Sea. *Thiomicrospira*

thyasirae was first described as the putative symbiont of *Thyasira flexuosa*, and named *Thiobacillus thyasiris* (Wood and Kelly, 1989). After sequencing of the 16S rRNA gene it was recognized that this organism was not the endosymbiont (Distel and Wood, 1992) and it was reclassified in a later publication (Wood and Kelly, 1993). The other three organisms were obtained from deep-sea hydrothermal vents: *Thiomicrospira crunogena* (Jannasch et al., 1985) was isolated from the East-Pacific Rise, *Thiomicrospira* sp. str. L-12 (Ruby and Jannasch, 1982) from the Galapagos Rift region, and *Thiomicrospira* sp. str. MA2-6 (Muyzer et al., 1995) was obtained from a vent system of the Mid-Atlantic Ridge.

Kuenen and Veldkamp (1972) already speculated about the ecological role of *Thiomicrospira pelophila* and compared it with *Thiobacillus thioparus*, which was isolated from the same habitat, and which physiological characteristics are similar. They suggested that the curved shape and the extreme thinness of the motile *Thiomicrospira* would facilitate the penetration into very small capillaries between mud particles as compared with the bigger, rod-shaped *Thiobacillus*. Additionally the higher sulfide tolerance of *Thiomicrospira pelophila* might enable this organism to live in another ecological niche than *Thiobacillus*.

The ecological importance of *Thiomicrospira* species in deep-sea hydrothermal vent communities was detected by frequent isolation (Ruby et al., 1981; Jannasch et al., 1985; Ruby and Jannasch, 1982; Wirsen et al., 1993) and by molecular analysis (Muyzer et al., 1995). During the latter study the genetic microbial diversity of two of these habitats at the Mid-

Atlantic Ridge was investigated by using denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16S rDNA fragments. The analysis of the two different hydrothermal vent samples revealed one DGGE band for one sample and three for the other, which probably corresponded to the dominant bacterial populations in these communities. After excision of the bands from the gels, followed by reamplification, sequencing and phylogenetic analysis, two of the bands were assigned to the genus *Thiomicrospira*. In a phylogenetic framework, which was performed for an identification of these ´phylotypes´ in more detail, one sequence was almost identical to the sequence of *Thiomicrospira crunogena*. The other sequence represented an uncultured *Thiomicrospira* species. That indicated that *Thiomicrospira* species are dominant community members at the investigated deep-sea hydrothermal vent sites.

Outline of the dissertation

In a combined approach mainly molecular biological and microbiological methods were used to investigate the ecological role of the genus *Thiomicrospira*. At the beginning of this study little was known about the spatial distribution of this genus, its diversity, number, and activity in different environments. Organisms, which were identified by 16S rDNA sequencing as belonging to the genus *Thiomicrospira* have been isolated from only two ecosystems (Distel and Wood, 1992; Lane et al., 1992; Muyzer et al., 1995).

The first step to study *Thiomicrospira* systematically was the development of a fast and reliable detection method for cells belonging to this genus. In chapter 2 of this dissertation the design of two oligonucleotides is described, which are specific for the 16S rRNA encoding genes of the *Thiomicrospira* spp. present in the gamma subunit of the Proteobacteria. By using these oligonucleotides as primers in a specific PCR, it was tried to elucidate the horizontal distribution of *Thiomicrospira* populations. Enrichment cultures for chemolithoautotrophic sulfur-oxidizing bacteria and DNA extracted from different environments were tested for the presence of *Thiomicrospira*. The results showed a worldwide distribution and an extended habitat range of this genus. Additionally several new isolates were obtained from the enrichment cultures. Phylogenetic analysis of their 16S rRNA genes indicated an increased species diversity.

Chapter 3 also demonstrates a broad distribution of *Thiomicrospira*. It describes the comparison of three *Thiomicrospira* strains based on molecular analysis. These isolates were obtained from deep-sea hydrothermal vents of the Pacific and Atlantic Ocean and were already investigated with microbiological methods during earlier studies (Ruby and Jannasch, 1982; Jannasch et al., 1985; Eberhardt et al., 1995). The obtained results of this study showed that all three strains belong to one species, *Thiomicrospira crunogena*, and gave indications for the worldwide distribution of this species.

During the horizontal survey presented in chapter 2, several new isolates belonging to the genus *Thiomicrospira* were obtained. Sequencing of their 16S rRNA encoding genes already indicated new species. Chapter 4 is the description of two of these new species, introduced as *Thiomicrospira kuenenii* sp. nov. and *Thiomicrospira frisia* sp. nov. Both isolates were obtained from an intertidal mud flat of the German Wadden Sea, and the characterization of these organisms gave indications for an adaptation to this habitat.

Another new species, *Thiomicrospira chilensis* sp. nov., was isolated from a *Thioploca* mat of the continental shelf of Chile. The physiological and biochemical characterization of this organism is described in chapter 5.

The ecological role of *Thiomicrospira* within an intertidal mud flat was investigated because the highest number of species and the greatest diversity was obtained for this habitat as described in chapter 2. The combined approach of molecular biological and microbiological methods

was supplemented with environmental parameters by the use of microsensors for oxygen, pH, and H_2S. The results obtained for this environment are presented in chapter 6.

In parallel a second habitat, a shallow-water hydrothermal vent system, was investigated for two reasons: (i) Muyzer et al. (1995) demonstrated a high significance of *Thiomicrospira* spp. in deep-sea hydrothermal vents. Therefore comparison with results from a shallow-water hydrothermal vent promised to be interesting since several differences between both habitats exist (Tarasov et al., 1990; van den Ende, 1997). (ii) Environmental parameters, which might influence the presence of *Thiomicrospira* populations are much easier to measure at a shallow-water hydrothermal vent. For this environment temperature- and pH-profiles were determined, of which the temperature has probably the strongest influence on the bacterial community (Sievert et al., 1998). The distribution of *Thiomcirospira* spp. in this habitat as well as the influence of the environmental parameters on cells belonging to this genus are presented in chapter 7.

In chapter 8 the results of this dissertation are summarized and discussed in their coherence.

References

Canfield, D. E. 1993. Organic matter oxidation in marine sediments. p. 333-363. *In* R. Wollast et al. (eds.). Interactions of C, N, P and S biogeochemical cycles. Springer.

Dilling, W., and H. Cypionka. 1990. Aerobic respiration in sulfate-reducing bacteria. FEMS Microbiology Letters **71**:123-128.

Distel, D. L., and A. P. Wood. 1992. Characterization of the gill symbiont of *Thyasira flexuosa* (Thyasiridae: Bivalvia) by use of polymerase chain reaction and 16S rRNA sequence analysis. J. Bacteriol. **174**:6317-6320.

Eberhard, C., C. O. Wirsen, and H. W. Jannasch. 1995. Oxidation of polymetal sulfides by chemolithoautotrophic bacteria from deep-sea hydrothermal vents. Geomicrobiol. J. **13**:145-164.

Henrichs, S. M., and W. S. Reeburgh. 1987. Anaerobic mineralization of marine organic matter: Rates and the role of anaerobic processes in the ocean carbon economy. Geomicrobiol. J. **5**:191-237.

Jannasch, H. W., and M. J. Mottl. 1985. Geomicrobiology of deep-sea hydrothermal vents. Science **229**:717-725.

Jannasch, H. W., C. O. Wirsen, D. C. Nelson, and L. A. Robertson. 1985. *Thiomicrospira crunogena* sp. nov., a colorless, sulfur-oxidizing bacterium from a deep-sea hydrothermal vent. Int. J. System. Bacteriol. **35**:422-424.

Jørgensen, B. B. 1982. Mineralization of organic matter in the sea-bed - the role of sulphate reduction. Nature (London) **296**:643-645.

Jørgensen, B. B., and N. P. Revsbech. 1983. Colorless sulfur bacteria, *Beggiatoa* spp. and *Thiovulum* spp., in O_2 and H_2S microgradients. Appl. Environ. Microbiol. **45**:1261-1270.

Jørgensen, B. B. 1987. Ecology of the sulfur cycle: oxidative pathways in sediments. p. 31-63 *In* J. A. Cole, and S. Ferguson (eds.), The nitrogen and sulfur cycles. Cambridge University Press.

Jørgensen, B. B. 1989. Biogeochemistry of chemoautotrophic bacteria. p.117-146 *In* H. G. Schlegel and B Bowien (eds.), Autotrophic Bacteria. Science Tech Publishers, Madison, WI.

Kuenen, J. G., and H. Veldkamp. 1972. *Thiomicrospira pelophila* nov. gen., nov. sp., a new obligately chemolithotrophic colorless sulfur bacterium. Antonie Van Leeuwenhoek **38**:241-256.

Kuenen, J. G. 1989. The colorless sulfur bacteria. *In* Bergey´s manual of systematic bacteriology, Staley, J. T., Bryant, M. P., Pfennig, N., and Holt, J. G. (eds.), Vol. **3**, p. 1834-1837, Williams and Wilkins, Baltimore.

Kuenen, J. G., and L. A. Robertson. 1989. The genus *Thiomicrospira*. *In* Bergey´s manual of systematic bacteriology, Staley, J. T., Bryant, M. P., Pfennig, N., and Holt, J. G. (eds.), Vol. **3**, p. 1858-1861, Williams and Wilkins, Baltimore.

Kuenen, J. G., L. A. Robertson, and O. H. Tuovinen. 1992. The genera *Thiobacillus*, *Thiomicrospira*, and *Thiosphaera*, p. 2638-2657. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (eds.), The prokaryotes, 2nd ed. Springer, Berlin, Germany.

Krekeler, D., P. Sigalevich, A. Teske, H. Cypionka, and Y. Cohen. 1997. A sulfate-reducing bacterium from the oxic layer of a microbial mat from

Solar Lake (Sinai), *Desulfovibrio oxyclinae* sp. nov. Arch. Microbiol. **167**:369-375.

Lane, D. J., A. P. Harrison, D. A. Stahl, B. Pace, S. J. Giovannoni, G. J. Olsen, and N. R. Pace. 1992. Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. J. Bacteriol. **174**:269-278.

Muyzer, G., A. Teske, C.O. Wirsen, H.W. Jannasch. 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. **164**: 165-172.

Robertson, L. A., and J. G. Kuenen. 1992. The colorless sulfur bacteria. p. 385-413. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (eds.), The prokaryotes, 2nd ed. Springer, Berlin, Germany.

Ruby, E. G., C. O. Wirsen, and H. W. Jannasch. 1981. Chemolithotrophic sulfur-oxidizing bacteria from the Galapagos Rift hydrothermal vents. Appl. Environ. Microbiol. **42**:317-324.

Ruby, E. G., and H. W. Jannasch. 1982. Physiological characteristics of *Thiomicrospira* sp. strain L-12 isolated from deep-sea hydrothermal vents. J. Bacteriol. **149**:161-165.

Sievert, S., T. Brinkhoff, G. Muyzer, W. Ziebis, and J. Kuever. 1998. Spatial heterogeneity of bacterial populations at a shallow-water hydrothermal vent in the Aegean Sea (Milos, Greece) as studied by the application of molecular and cultivation-based techniques. Submitted to Appl. Environm. Microbiol.

Sugio, T., C. Domatsu, O. Munaka, T. Tano, and K. Imai. 1985. Role of a ferric iron reducing system in sulfur oxidation of *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol. **49**:1401-1406.

Tarasov, V. G., M. V. Propp, L. N. Propp, A. V. Zhirmunsky, B. B. Namsaraev, V. M. Gorlenko, D. A. Starynin. 1990. Shallow-water gasohydrothermal vents of Ushishir Volcano and the ecosystem of Kraternaya Bight (the Kurile Islands). P. S. Z. N. I. Mar. Ecol. **11**:1-23.

Taylor, B. F., D. S. Hoare, and L. S. Hoare. 1971. *Thiobacillus denitrificans* as an obligate chemolithotroph. Isolation and growth studies. Archiv für Mikrobiologie **78**:193-204.

Taylor, B. F., and D. S. Hoare. 1971. *Thiobacillus denitrificans* as an obligate chemolithotroph. II. Cell suspension and enzymatic studies. Archiv für Mikrobiologie **80**:262-276.

Timmer-ten Hoor, A. 1975. A new type of thiosulphate oxidizing, nitrate-reducing microorganism: *Thiomicrospira denitrificans* sp. nov. Netherlands J. Sea Res. **9**:343-351.

van den Ende, F. P. 1997. Microbial ecology of oxygen-sulfide interfaces in marine benthic ecosystems. PhD thesis, Rijksuniversiteit Groningen, Groningen, Netherlands.

Widdel, F., and F. Bak. 1992. Gram-negative mesophilic sulfate-reducing bacteria. p. 3352-3378. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (eds.), The prokaryotes, 2nd ed. Springer, Berlin, Germany.

Winogradsky, S. 1887. Botanische Zeitung. **45**:489-507, 513-524, 529-540, 545-560, 569-576, 585-609.

Wirsen, C. O., H. W. Jannasch, and S. J. Molyneaux. 1993. Chemosynthetic microbial activity at Mid-Atlantic Ridge hydrothermal vent sites. J. Geophys. Res. **98**:9693-9703.

Wood, A. P., and D. P. Kelly. 1989. Isolation and physiological characterization of *Thiobacillus thyasiris* sp. nov., a novel marine facultative autotroph and the putative symbiont of *Thyasira flexuosa*. Arch. Microbiol. **152**:160-166.

Wood, A. P., and D. P. Kelly. 1993. Reclassification of *Thiobacillus thyasiris* as *Thiomicrospira thyasirae* comb. nov. an organism exhibiting pleomorphism in response to environmental conditions. Arch. Microbiol. **159**:45-47.

Chapter 2

Increased species diversity and extended habitat range of sulfur-oxidizing *Thiomicrospira* spp.

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ABSTRACT

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.

INTRODUCTION

Thiomicrospira species are chemolithoautotrophic bacteria that use reduced sulfur compounds, such as sulfide, thiosulfate and sulfur as energy source and CO_2 as carbon source (12). These bacteria have been isolated from different marine environments, i.e., coastal mud flats (11, 33, 39) and hydrothermal vent systems (10, 23, 24). However, the diversity, and the 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 for PCR amplification (e.g., reference 36).

Recently, the nearly complete 16S rRNA molecules of four *Thiomicrospira* species, i.e., *T. pelophila, T. crunogena, T. denitrificans,* and

Thiomicrospira sp. str. 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. str. 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 *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.

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, 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) x $Na_2S_2O_3$ x $5H_2O$.

Bacteria from the salt spring Artern (Thuringia, Germany) were isolated and cultivated using the following medium: 4.08 g $CaSO_4$ x 2 H_2O, 0.37g $MgSO_4$ x 7 H_2O, 0.42g $MgCl_2$ x 6 H_2O, 0.20 g KCl, 18.10 g NaCl, 1.0 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 5.0 g $Na_2S_2O_3$ x 5 H_2O, 1 ml trace element solution per 1,000 ml. Four milligrams 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 NaCl, 0.17g, $MgCl_2$ x 6 H_2O, 1.0 g $(NH_4)_2SO_4$,

0.4 g $CaCl_2 \times 2H_2O$, 0.5 g K_2HPO_4, 5.0 g $Na_2S_2O_3 \times 5H_2O$, and 1 ml of trace element solution per 1,000 ml. Bromothymolblue (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 a 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 colonies obtained were streaked out on fresh plates. Colonies were then transfered 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.

TABLE 1. Environmental samples used in this study

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

DNA isolation. Bacterial genomic DNA was obtained either by direct cell lysis following 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 10x 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 incubated at 55°C for 2 h. An equal volume of Tris-saturated phenol was added and mixed carefully. After centrifugation the aquaeous layer was transfered 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 aquaeous-organic interphase. Next, 0.1 volume of 5 M NaCl and 2.5 volumes of ice-cold ethanol were added to the aquaeous 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 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 a 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 using the

SEQAPP program developed by Gilbert (9). The specificity of the designed primers p3 and p4 was checked by using 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 for 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.

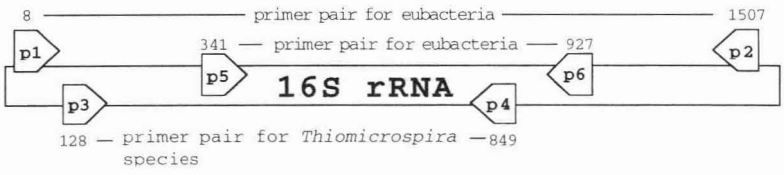

FIG. 1. Schematic diagram of the oligonucleotides used in this study. Primers p1 and p2 are specific for the 16S rDNA of eubacteria and amplify the nearly complete gene. These PCR products have been used for dot-blot hybridization analysis to test the specificity of primers p3 and p4, and for phylogenetic 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.

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°C 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, digoxygenin (Dig)-labelled 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 using the D-Gene™ system (Bio-Rad Laboratories, Inc.). Two different DGGE protocols were used. The first protocol (18) used: 1.5 mm thick, 6% (w/v) polyacrylamide gels, 0.5x 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, 1x 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 pre-hybridized for 4 h at 52°C with 50 ml of a solution containing 2% (wt/vol) blocking reagent (Boehringer Mannheim Biochemicals, Germany) in 5x SSC (1x 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 pre-hybridization solution. The blot was incubated in this mixture overnight at 52°C. After hybridization, the membrane was washed for 30 min at hybridization temperature, first with a solution containing 2x SSC-0.1% (wt/vol) SDS and then twice with a solution containing 0.1x 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 enzym-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, Forster City, Calif.) was used to sequence the 16S rDNA fragments. Sequencing primers were the same as described previously by Buchholz-Cleven et al. (3). The sequence reactions 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 using 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 numbers. The sequences obtained in this study are available from GenBank under accession numbers AF013971 to AF013978, and under 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 position 128 to 145 (TMS128F, primer p3) and for position 830 to 849 (TMS849R, primer p4) (*E. 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 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 on 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	Andreas Teske	-
Nitrobacter sp.	alpha	Andreas Teske	-
Rhodopseudomonas palustris	alpha	DSM 123	-
Nitrospira briensis C-128	beta	John Waterbury	-
Nitrosolobus multiformis	beta	ATCC 25196	-
Leptothrix discophora	beta	Liesbeth de Vrind	-
Beggiatoa alba	gamma	DSM 1416	-
Escherichia coli (strain W3110)	gamma	Sigma D-0421	-
Nitrococcus mobilis	gamma	ATCC 25380	-
Thiomicrospira crunogena	gamma	Holger Jannasch	+
Thiomicrospira pelophila	gamma	DSM 1534	+
Thiomicrospira sp. str. L-12	gamma	Holger Jannasch	+
Thiomicrospira sp. str. MA2-6	gamma	Holger Jannasch	+
Thiomicrospira thyasirae	gamma	DSM 5322	+
symb. of *Calyptogena magnifica*	gamma	East Pacific Ocean	-
symb. of *Bathymodiolus*	gamma	East Pacific Ocean	-
symb. 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 separated 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 enrichement 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 Duckstein.

The relative abundance of *Thiomicrospira* in the habitat was determined by hybridisation 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.

TABLE 3. Habitat range of *Thiomicrospira*

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

[a] PCR with eubacterial primer pair p5 and p6 followed by DGGE and hybridization analysis using the *Thiomicrospira*-specific oligonucleotide p4.

[b] - = not detected, + = detected, n.d. = not determined

[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.

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 environmental 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 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. str. MA2-6, and *Thiomicrospira* sp. str. L-12) were used as markers in the electrophoresis (Fig. 2, lanes 1 to 5 and 14 to 18).

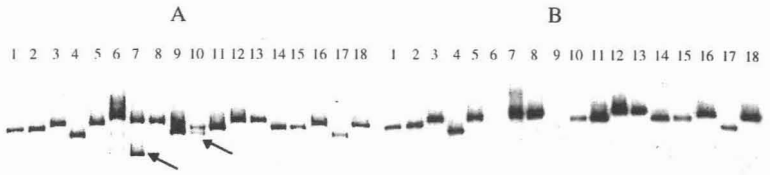

FIG. 2. Hybridization analysis of DGGE profiles to monitor the success of isolation of *Thiomicrospira* bacteria from different habitats. (A) DGGE separation pattern of rDNA fragments obtained after enzymatic amplification of known *Thiomicrospira* strains, enrichment cultures, and bacterial isolates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

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 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, lane 1 and 8). Lane 4 shows a profile of an enrichment culture obtained after inculation 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).

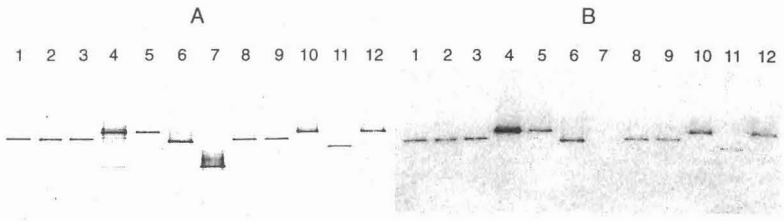

Fig. 3. Hybridization analysis and DGGE profiles of PCR products obtained after enzymatic amplification of enrichment cultures, and bacterial isolates from Jadebusen and of known *Thiomicrospira* strains with primer pair p5 and p6, which is specific for eubacteria in general. (A) DGGE separation pattern of rDNA fragments. Lane 1 and 8, *T. pelophila;* lane 2, enrichment culture obtained after inoculation of a 0.2-µm-pore-size filtered sediment sample; lane 3, isolate JB-A1F; lane 4, unfiltered enrichment JB-A1; lane 5, isolate JB-A2; lane 6, isolate JB-A1; lane 7, *'Rhodobacter'*-like isolate; lane 9, *T. thyasirae;* lane 10, *T. crunogena;* lane 11, *Thiomicrospira* sp. strain MA2-6; lane 12, *Thiomicrospira* sp. strain L-12. (B) Results after hybridization analysis using 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.

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 for one or more of the known *Thiomicrospira* species (see Fig. 2A and Fig. 3A, lanes 1 and 3) indicating identical sequences. To substantiate this assumption, as well as to determine the phylogenetic relationship of 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. str. L-12, and *Thiomicrospira* sp. str. 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*).

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 with 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 an oil field microbial communities (35). Phylogenetic analysis indicated a close relationship of the Duckstein sequence with the sequence of the isolate CH-1, 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.

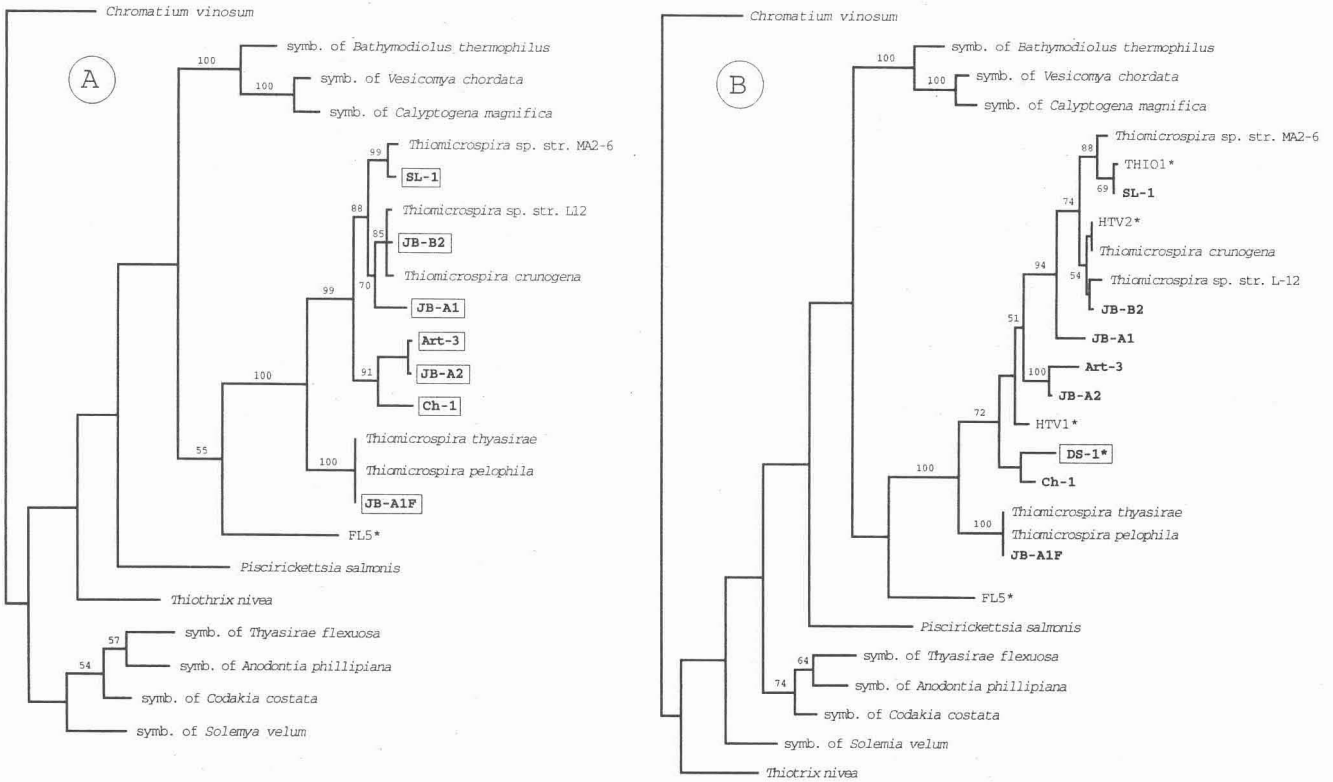

FIG 4: Phylogenetic trees showing the relationships of bacterial and molecular isolates with sequences from RDP and GenBank. (A) Tree based on nearly complete (1,500 nucleotides) 16S rRNA sequences. (B) Tree inferred from partial (700 nucleotides) 16S rRNA sequences giving the affiliation of the freshwater 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.

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 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 for 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 of 44 mol% for *T. pelophila*, while a G+C content of (51.9 $\pm$ 1.6) mol% (10 determinations) was found for *T. thyasirae* (39). According to Stackebrandt and Liesack (28), this big difference in G+C 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 contradicting result has also 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 as 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 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 to determine 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 new species.

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

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REFERENCES

1. Benson, D.A., Boguski, M.S., Lipman, D.J., and Ostell J. 1997 Genbank. Nucleic Acids Res. 25: 1-6.

2. Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller. 1981. Gene organisation and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. 148: 107-127.

3. Buchholz-Cleven, B. E. E., B. Rattunde, and K. L. Straub. 1996. Screening for genetic diversity of isolates of anaerobic Fe(II)-oxidizing bacteria using DGGE and whole-cell hybridization. Syst. Appl. Microbiol. In press.

4. Distel, D. L., and A. P. Wood. 1992. Characterization of the gill symbiont of *Thyasira flexuosa* (Thyasiridae: Bivalvia) by use of polymerase chain reaction and 16S rRNA sequence analysis. J. Bact. 174: 6317-6320.

5. Don, R.H., P.T. Cox, B. Wainwright, K. Baker, and J.S. Mattick. 1991. "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. 19: 4008.

6. Ferris, M. J., G. Muyzer, and D. M. Ward. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. 62: 340-346.

7. Fox, G.E., J.D. Wisotzkey, and P. Jurtshuk, jr. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42: 166-170.

8. Garcia-Pichel, F., L. Prufert-Bebout, and G. Muyzer. 1996. *Microcoleus chthonoplastes*: phenotypical and phylogenetic analyses reveal a coherent, cosmopolitan cyanobacterial taxon. Appl. Environ. Microbiol. 62: 3284-3291.

9. Gilbert, D.G. 1992. SeqApp - a biosequence analysis application. Indiana University, Bloomington.

10. Jannasch, H. W., C. O. Wirsen, D. C. Nelson, L. A. Robertson. 1985. *Thiomicrospira crunogena* sp. nov., a colorless sulfur bacterium from a deep-sea hydrothermal vent. Int. J. Syst. Bacteriol. 35: 422-424.

11. Kuenen, J. G. and H. Veldkamp. 1972. *Thiomicrospira pelophila,* gen. n., sp. n., a new obligately chemolithotrophic colourless sulfur bacterium. Antonie van Leeuwenhoek 38: 241-256.

12. Kuenen, J. G., Robertson, L. A., and O. H. Tuovinen. 1991. The genera *Thiobacillus, Thiomicrospira,* and *Thiosphaera.* In: Balows A, Trüper, H. G., Dworkin, M., Harder, W., Schleifer, K. H. (eds.) The Prokaryotes, 2nd edn. Springer, Berlin Heidelberg New York, pp. 2638-2657.

13. Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek R., McCaughey, M.J. and Woese, C.R. 1997. The RDP (Ribosomal Database Project). Nucleic Acids Res. 25: 109-110.

14. McSweeney, C.S., R.I. Mackie, A.A. Odenyo, and D.A. Stahl. 1993. Development of an oligonucleotide probe targeting 16S rRNA and its application for detection and quantification of the ruminal bacterium *Synergistes jonesii* in a mixed-population chemostat. Appl. Environ. Microbiol. 59: 1607-1612.

15. Murray, A.E., J.T. Hollibaugh, and C. Orrego. 1996. Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl. Environ. Microbiol. 62: 2676-2680.

16. Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59: 695-700.

17. Muyzer, G., A. Teske, C.O. Wirsen, H.W. Jannasch. 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. 164: 165-172.

18. Muyzer, G., S. Hottenträger, A. Teske, and C. Wawer. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA - A new molecular approach to analyse the genetic diversity of mixed microbial communities. In: A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (eds.). Molecular

microbial ecology manual. 2. edition. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 3.4.4.1-3.4.4.22.

19. Muyzer, G., T. Brinkhoff, U. Nubel, C. Santegoeds, H. Schafer, and C. Wawer. 1997. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (eds.). Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands. In press.

20. Muyzer, G. and N. Ramsing. 1996. Molecular methods to study the organization of microbial communities. Water Science Techn. 32: 1-9.

21. Olsen, G.J., C.R. Woese, and R. Overbeek. 1994. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. 176: 1-6.

22. Romanowski, G., Lorenz, M.G., Wackernagel,W. 1993. Use of polymerase chain reaction and electroporation of *Escherichia coli* to monitor the persistence of extracellular plasmid DNA introduced into natural soils. Appl. Environ. Microbiol. 59: 3438-3446.

23. Ruby, E.G., C.O. Wirsen, and H.W. Jannasch. 1981. Chemolithotrophic sulfur-oxidizing bacteria from the Galapagos Rift hydrothermal vents. Appl. Envrion. Microbiol. 42: 317-324.

24. Ruby, E.G., and H.W. Jannasch. 1982. Physiological characteristics of *Thiomicrospira* sp. strain L-12 isolated from deep-sea hydrothermal vents. J. Bacteriol. 149: 161-165.

25. Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.

26. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

27. Santegoedts, C. M., S. C. Nold, and D. M. Ward. 1996. Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat. Appl. Environ. Microbiol. 62: 3922 - 3928.

28. Stackebrandt, E., and W. Liesack. 1993. Nucleic acids and classification, p. 151-194. In: M. Goodfellow and A. G. O'Donnell (ed.) Handbook of new bacterial systematics, Academic Press Ltd, London.

29. Stackebrandt, E., and B.M. Goebel. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44: 846-849.

30. Swofford, D. L. 1991. PAUP: Phylogenetic Analysis Using Parsimony. Version 3.1. Computer program distributed by the Illinois Natural History Survey.

31. Teske, A., C. Wawer, G. Muyzer, and N. B. Ramsing. 1996. Distribution of sulfate-reducing bacteria in a stratified Fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl. Environ. Microbiol. 62: 1405-1415.

32. Teske, A., P. Sigalevich, Y. Cohen, and G. Muyzer. 1996. Molecular identification of bacteria from a co-culture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. Appl. Environ. Microbiol. 62: 4210 - 4215.

33. Timmer-ten Hoor, A. 1975. A new type of thiosulphate oxidizing, nitrate-reducing microorganism: *Thiomicrospira denitrificans* sp. nov. Netherlands J. Sea Res. 9: 343-351.

34. VanDamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters, and J. Swings. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60: 407-438.

35. Voordouw, G., S.M., Armstrong, M.F. Reimer, B. Fouts, A.J. Telang, Y. Shen, and D. Gevertz. 1996. Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fementative, and sulfide-oxidizing bacteria. Appl. Environ. Microbiol. 62: 1623-1629.

36. Voytek, M.A., and B.B. Ward. 1995. Detection of ammonium-oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with the PCR. Appl. Environ. Microbiol. 61: 1444-1450.

37. Ward D.M., C. M. Santegoeds, S.C. Nold, N.B. Ramsing, M.J. Ferris, and M.M. Bateson. 1996 Biodiversity within hot spring microbial mat communities: molecular monitoring of enrichment cultures. Antonie van Leeuwenhoek 71: 143-150.

38. Wood, A. P. and D. P. Kelly. 1989. Isolation and physiological characterization of *Thiobacillus thyasiris* sp. nov., a novel marine facultative autotroph and the putative symbiont of *Thyasira flexuosa.* Arch. Microbiol. 152: 160-166.

39. Wood, A. P. and D. P. Kelly. 1993. Reclassification of *Thiobacillus thyasiris* as *Thiomicrospira thyasirae* comb. nov. an organism exhibiting pleomorphism in response to environmental conditions. Arch. Microbiol. 159: 45-47.

Chapter 3

A new *Thiomicrospira* strain from the Mid-Atlantic Ridge compared to known hydrothermal vent isolates

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ABSTRACT

A new autotrophic *Thiomicrospira* strain, MA-3, was isolated from the surface of a polymetal sulfide deposit collected at a Mid-Atlantic Ridge hydrothermal vent site. The DNA homology among three vent isolates, *T. crunogena*, *T.* strain L-12 and *T.* strain MA-3, was 99.3% or higher, grouping them as the same species, *T. crunogena*^{T} (type strain, ATCC no. 35932). The fact that strains *T. crunogena* and *T.* L-12 were isolated from Pacific vent sites demonstrates a cosmopolitan distribution of this species.

Sulfur-oxidizing chemolithotrophs isolated from hydrothermal environments are described as mesophiles and facultative or obligate autotrophs (6,7,10,14,19) with diverse oxidative capabilities (12,13,15). Twelve of ninety five sulfur-oxidizing isolates from the Galapagos hydrothermal vent site were physiologically described as *Thiomicrospira* strains (19), and one Mid-Atlantic Ridge (MAR) isolate was phylogenetically placed in the same genus (17). Combined molecular and cultivation approaches have greatly furthered the concept of *Thiomicrospira* diversity in both marine as well as a freshwater habitat (2). Presently sulfur-oxidizing chemolithoautrotrophs isolated and fully described from deep sea hydrothermal vents are limited to two species of *Thiomicrospira* (14,18) and one species of *Thiobacillus* (7), none of which is acidophilic. Furthermore, natural microbial populations as well as certain MAR isolates and the two previously described *Thiomicrospira*

species from Pacific vent sites (*T. crunogena* and *T.* L-12) have been shown to oxidize sulfidic minerals at near neutral pH (8,23). The most active of these Atlantic vent isolates (MAR strain MA-3) is physiologically and phylogenetically characterized and compared to the two different Pacific vent site *Thiomicrospira* isolates.

Strain MA-3 was isolated from enrichments of surface scrapings of a polymetal sulfide rock collected from a depth of 3620 m at the MAR Trans-Atlantic Geotransverse (TAG) hydrothermal vent site. *Thiomicrospira crunogena* and *T.* strain L-12 were regrown from our culture collection. The medium used for enrichment and laboratory experimentation was artificial seawater (ASW) containing vitamins, trace elements and phenol red indicator (8). The pH of filter-sterilized (0.2 μm pore size) medium was set at 7.4 with HCl. Reduced sulfur sources for autotrophic growth were sodium thiosulfate (10 mM; T-ASW), elemental sulfur (1% w/v), sodium sulfide (500 μM) (5), sodium sulfite (1 and 5 mM), and natural and commercial metal sulfides, e.g., pyrite and chalcopyrite, as ground and sterilized slurries (4% w/v) (8). Organic substrates were glucose, lactate, galactose, peptone, yeast extract and casamino acids, tested individually at 0.1% in ASW medium. Agar (1.5%) medium made with T-ASW was used for the initial isolation, counting of colony forming units (CFU), and stock culture maintenance. Growth was determined by acridine orange epifluorescent cell counts (11), pH indicator change, elemental sulfur deposition, and incorporation of radiolabeled $^{14}CO_2$. The latter was done in T-ASW or metal sulfide amended ASW medium using radiolabeled

$NaH^{14}CO_3$ (8,21,23). Testing for the possible excretion of acid stable products during CO_2 incorporation in growing cultures at 24°C, the procedure of Ruby and Jannasch (18) was modified by acidifying the filtrate to pH 3.0 and then sparging with air to remove residual radiolabeled bicarbonate. Maximum growth rates were determined at 24°C in 100% and 10% air saturated medium by direct cell counts and by $^{14}CO_2$ incorporation. Growth temperature range was determined in liquid cultures (1°C increments above 38°C). The pH range for growth was tested in T-ASW set at initial pH values in the range of 4.5 to 9.0 at 0.5 pH intervals. Iron oxidation supporting CO_2 incorporation was studied using ASW medium with 10 mM ferrous sulfate at pH 7.0 with a gaseous headspace of 20%, 2% or 1% oxygen (balanced by N_2). T-ASW with 1% oxygen in the gas phase served as the positive control. Iron toxicity was tested in oxygen-free (N_2 gassed) ASW medium containing from 0 to 90 mM ferrous sulfate at near-neutral pH and inoculated with pregrown, centrifuged and washed cells. Viability, as CFU's, was assessed by plating subsamples at various time points on T-ASW agar medium. Nitrate respiration was tested by counting cell number increase in anoxic T-ASW medium supplemented with 2 mM KNO_3. The requirement for sodium ions and the possible substitution by potassium was tested in NaCl-free T-ASW medium (18). Effects of ambient versus in situ pressure (1 and 350 bar) on growth were measured by CO_2 incorporation at 24°C (14,18). The 16S rRNA encoding gene from isolate MA-3 was amplified using primers and PCR conditions as described by Muyzer et al. (17). The 16S rRNA sequence of strain MA-3 was aligned manually, using the sequence editor SEQAPP (9), to sequences of other

bacteria obtained from the Ribosomal Database Project (RDP; 16), and from Genbank (1). The sequence is available from Genbank under accession number AF069959. The DNA base composition for G + C, the ubiquinone analysis and the DNA-DNA hybridizations were all carried out as described in detail by Brinkhoff et al. (3).

Strain MA-3 is an autotrophic, obligately aerobic, gram negative, motile (by phase microscopy) and slightly vibroid bacterium measuring 0.5 to 0.7 by 1.3 to 2.0 μm. The colonies on T-ASW agar are white, smooth and entire, produce elemental sulfur and sulfate (i.e. sulfuric acid). It grows aerobically on reduced sulfur compounds (H_2S, sulfur and thiosulfate) and sulfidic minerals, all of which may be available in its natural vent habitat, but not on sulfite. It did not demonstrate nitrate respiration. The growth rate on thiosulfate at 24°C is 0.8 h^{-1} (doubling time 51 min.) in the presence of either 100% or 10% air saturated medium. It did not grow heterotrophically on any of the tested organic compounds. Strain MA-3 of the species *T. crunogena* has been deposited as ATCC no. 700270 in the American Type Culture Collection.

At near neutral pH, strain MA-3 will utilize commercial pyrite or natural metal sulfides (primarily chalcopyrite) collected from MAR vent sites. Growth on these minerals is optimal at pH 6.5 to 7.0, with doubling times averaging from 10 to 50 h depending on the mineral, and ceases when the pH drops below 6.0 to 5.5. On thiosulfate, strain MA-3 grows optimally at pH 7.5 and ceases at comparably low pH's. Strain MA-3 did not grow autotrophically on ferrous iron as a substrate. In fact, ferrous iron

was significantly toxic at increasing concentrations (Fig. 1). In the iron free control, cells remained almost completely viable (as CFU's) over the experimental time period.

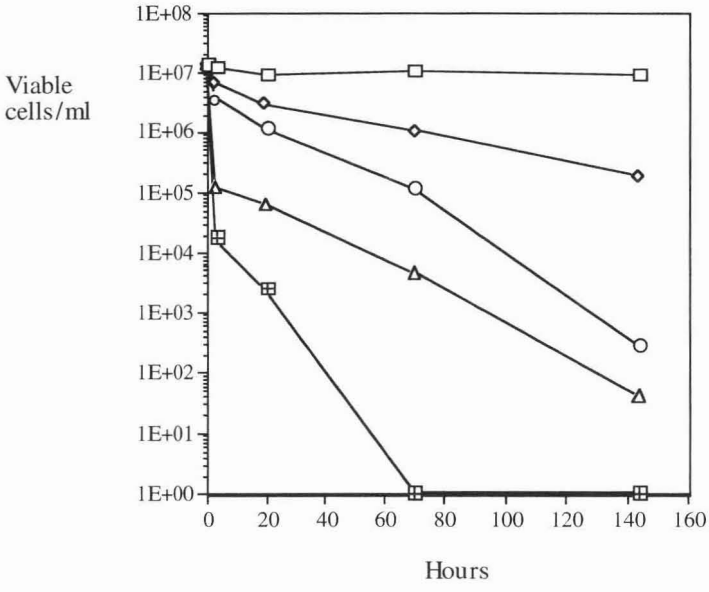

Figure 1. Survival, as colony forming units, of pre-grown cells of strain MA-3 exposed to varied concentrations of Fe^{++} at near neutral pH under anoxic conditions at 23°C. Symbols: iron in mM, □ none, ◇ 10, ○ 30, △ 60, ⊞ 90.

Growth of this isolate at a hydrostatic pressure of its normal habitat in the deep-sea (ca. 350 bar) did not show a barophilic adaptation, however it is quite barotolerant demonstrating a rate of CO_2 fixation at 350 bar approximately 83% of the rate measured at 1 bar. The reduction in growth rate is similar to that reported for *T. crunogena* and *T.* strain L-12 at their in situ pressures of 250 bar (14,18). Strain MA-3 grows over a temperature range of 4° to 41°C (optimum 28° to 32°C). This maximum growth temperature of 41°C is the highest reported for the three species. Cells did not grow at 42°C, but survived 4 days at this temperature and then grew at a lower temperature (24°C), while cells incubated at 44°C for an equivalent time period did not grow upon transfer. Sodium is required for growth of strain MA-3, as no growth occurred at less than 50 mM NaCl concentrations over 1 week. Optimum growth occurred at 200 - 400 m M NaCl. Potassium, as KCl, was unable to substitute for sodium. In T-ASW medium, strain MA-3 excreted approximately 4 to 5% of its fixed carbon as acid stable organic compounds, of unknown composition, during exponential growth and a maximum of 12% in the stationary phase.

Most physiological characteristics of strain MA-3 compared to those of the two earlier described *Thiomicrospira* isolates, *T. crunogena* and *T.* strain L-12, are similar (e.g. motility, utilization of the same reduced sulfur sources) including the presence of ubiquinone Q-8 with traces of Q-7. Differences among the three isolates are listed in Table 1.

Table 1. Growth characteristics of three *Thiomicrospira* isolates from deep sea hydrothermal vents.

ORGANISM	***T. crunogena****	***T.* L-12****	**Strain MA-3**
Source	Vestimentiferan tube worm casing 21°N EPR	Mussel periostracum Galapagos Rift	Polymetal sulfide rock TAG site - MAR
Maximum growth rate (h^{-1})	0.8	0.32	0.8
Temp. range (°C)	4 - 38.5	10 - 35	4 - 41
(optimum)	(28 - 32)	(25)	(28 - 32)
pH range	5.0 - 8.5	5.5 - 8.5	5.5 - 8.5
(optimum)	(7.75)	(8.0)	(7.5)
% Fixed carbon excreted (24 h)	8.5	9.0	12
% Growth in situ bar / 1 bar	80	75	83
Minimum NaCl requirement	45 mM	80 mM	50 mM
G + C content	44.2 ± 0.2***	44.4 ± 0.2***	44.6 ± 0.3

*Data from reference 14, **data from reference 18, ***data from this study.

In its maximum rate of growth on thiosulfate and its growth temperature range, strain MA-3 resembles *T. crunogena* more than *T.* strain L-12. Similarly, slight differences hold for the excretion of fixed carbon, pH optima, minimum salinities, and G+C contents. The 16S rRNA encoding gene of strain MA-3 was nearly completely sequenced to determine its phylogenetic affiliation. By parsimony analysis of the 16S rRNA sequences, the new isolate was grouped with other *Thiomicrospira* species isolated from hydrothermal vents communities, i.e., *T. crunogena*, and *T.* strain L-12. The closest relative is *T. crunogena* (14) to which it has a sequence similarity value higher than 99%. Only two base pair differences could be found between the sequences of *T. crunogena* and strain MA-3: at *E. coli* positions (4) 978 and 1015 there was a G in the sequence of *T. crunogena* versus an A in the sequence of strain MA-3. The DNA-DNA homology among the 3 strains is presented in Table 2. Strain MA-3 has a 99.3% homology with the other two strains. The two Pacific vent strains, *T. crunogena* and *T.* strain L-12, share a 100% homology to each other. The type species of the genus *Thiomicrospira*, *T. pelophila*, shows only a 27-33.5% homology with all three vent strains. This value, being below the homology threshold of 60-70%, defines (20) *T. pelophila* as a separate species.

Table 2. Percentage DNA-DNA homology for three *Thiomicrospira* isolates from deep-sea hydrothermal vents and *T. pelophila*, the type species of the genus.

	T. pelophila	**T. strain L-12**	***T. crunogena***
T. strain L-12	27		
T. crunogena	33.5	100	
T. strain MA-3	27	99.3	99.3

The study results in two new findings: (i) despite physiological differences among the three compared isolates, their almost identical 16S rRNA sequences and DNA-DNA hybridization values define them as one species (20,22), *T. crunogena*^{T} (type strain, ATCC no. 35932), (ii) the identification of these isolates from Mid-Atlantic Ridge and East Pacific Rise deep-sea hydrothermal vent sites indicates a highly cosmopolitan occurrence of this species. In view of the 16S RNA sequence similarity of the three isolates compared in this paper, a further strain isolated from the Mid-Atlantic Ridge, MA2-6 (17), has a similarity value of about 97% with *T. crunogena* indicating it as a separate species (20). On the other hand, a new intertidal *Thiomicrospira* isolate, strain JB-B2, shows a 99% similarity to both, *T. crunogena* and *T.* strain L-12 (2) and, therefore, may be yet another strain of the species *T. crunogena.*

In view of the greater than 99.3% DNA homology of the three compared *T. crunogena* strains, the observed differences of a physiological

nature under equal growth conditions (Table 1) indicate a certain range of phenotypic adaptations to environmentally different vent sites. The metabolic capability of metal sulfide oxidation affords these chemolithotrophs an additional and stable source of energy in environments where massive polymetal sulfide deposits exist, namely at deep sea hydrothermal vent sites. This versatility may contribute to the fact that the genus *Thiomicrospira* appears to dominate, by molecular analysis (17), the sulfur-oxidizing bacterial communities at deep-sea hydrothermal vents. The present work presents a striking case of wide geographic distribution of a single sulfur-oxidizing bacterial species at Atlantic and Pacific deep-sea vent sites. If this can indeed be extended toward intertidal zones, as suggested by the genetic relatedness of strain JB-B2 (2), a truly worldwide distribution of the species *T. crunogena* would be apparent.

The ubiquinones were identified by B. Tindall, and the G + C determination and DNA:DNA hybridizations were done by J. Burghardt, both of the DSMZ identification service (Braunschweig, Germany). This work was supported by grants OCE 92-000458, OCE 9615830 and OCE 9714195 from the National Science Foundation, and with financial support from the Max-Planck-Society, Munich, Germany.

REFERENCES

1. **Benson, D. A., M.S. Boguski, D. J. Lipman, and J. Ostell.** 1997. Genbank. Nucleic Acids Res. **25:** 1-6.

2. **Brinkhoff, T. and G. Muyzer.** 1997. Increased species diversity and extended habitat range of sulfur-oxidizing *Thiomicrospira* spp. Appl. Environ. Microbiol. **63**: 3789-3796.

3. **Brinkhoff, T., G. Muyzer, C. O. Wirsen and J. Kuever.** 1998. Characterization of *Thiomicrospira kuenenii* sp. nov. and *Thiomicrospira frisia* sp. nov., two mesophilic obligately chemolithoautrophic sulfur-oxidizing bacteria from an intertidal mud flat. Int. J. Syst. Bacteriol. in press.

4. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148**: 107-127.

5. **Cline, J. D.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanog. **14:** 454-458.

6. **Durand, P., A. Benyagoub, and D. Prieur.** 1994. Numerical taxonomy of heterotrophic sulfur-oxidizing bacteria isolated from southwestern Pacific hydrothermal vents. Can. J. Microbiol. **40:** 690-697.

7. **Durand, P., A. Reysenbach, D. Prieur, and N. Pace.** 1993. Isolation and characterization of *Thiobacillus hydrothermalis* sp. nov., a mesophilic obligately chemolithotrophic bacterium isolated from a deep-sea hydrothermal vent in Fiji Basin. Arch. Microbiol. **159:** 764-766.

8. **Eberhard, C., C. O. Wirsen, and H. W. Jannasch.** 1995. Oxidation of polymetal sulfides by chemolithoautotrophic bacteria from deep-sea hydrothermal vents. Geomicrobiol. J., **13:** 145-164.

9. **Gilbert, D.G.** 1992. SeqApp - a bio-sequence analysis application. Indiana University, Bloomington, USA.

10. **Gugliandolo, C., and , T. L. Maugeri.** 1993. Chemolithotrophic, sulfur-oxidizing bacteria from a marine, shallow hydrothermal vent of Vulcano (Italy). Geomicrobiol. J. **11:** 109-120.

11. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:** 1225-1228.

12. **Huber, H., and K. O. Stetter.** 1989. *Thiobacillus prosperus* sp. nov., represents a new group of halotolerant metal-mobilizing bacteria isolated from a marine geothermal field. Arch. Microbiol. **151:** 479-485.

13. **Huber, H., and K. O. Stetter.** 1990. *Thiobacillus cuprinus* sp. nov., a novel facultatively organotrophic metal-mobilizing bacterium. Appl. Environ. Microbiol. **56:** 315-322.

14. **Jannasch, H. W., C. O. Wirsen, D. C. Nelson, and L. A. Robertson.** 1985. *Thiomicrospira crunogena* sp. nov., a colorless sulfur-oxidizing bacterium from a deep-sea hydrothermal vent. Int. J. Syst. Bacteriol. **35:** 422-424.

15. **Javor, B. J., D. B. Wilmot, and R. D. Vetter.** 1990. pH-Dependent metabolism of thiosulfate and sulfur globules in the chemolithotrophic marine bacterium *Thiomicrospira crunogena*. Arch. Microbiol. **154:** 231-238.

16. **Maidak, B.L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese.** 1997. The RDP (Ribosomal Database Project). Nucleic Acids Res. **25**: 109-110.

17. **Muyzer, G., A. Teske, C. O. Wirsen, and H. W. Jannasch.** 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. **164**:165-172.

18. **Ruby, E. G., and H. W. Jannasch.** 1982. Physiological characteristics of *Thiomicrospira* sp. strain L-12 isolated from deep-sea hydrothermal vents. J. Bacteriol. **149:** 161-165.

19. **Ruby, E. G., C. O. Wirsen, and H. W. Jannasch.** 1981. Chemolithotrophic sulfur-oxidizing bacteria from the Galapagos Rift hydrothermal vents. Appl. Environ. Microbiol. **42:** 317-342.

20. **Stackebrandt, E. , and B. M. Goebel.** 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44**: 846-849.

21. **Tuttle, J. H., and H. W. Jannasch.** 1977. Thiosulfate stimulation of microbial dark assimilation of carbon dioxide in shallow marine environments. Microb. Ecol. **4:** 9-25.

22. **Wayne, L.G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. J. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Truper.** 1987. Report of the Ad Hoc Committee on reconcilation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37**: 463-464.

23. **Wirsen, C. O., H. W. Jannasch, and S. J. Molyneaux.** 1993. Chemosynthetic microbial activity at Mid-Atlantic Ridge hydrothermal vent sites. J. Geophys. Res. **98:** 9693-9703.

Chapter 4

Characterization of *Thiomicrospira kuenenii* sp. nov. and *Thiomicrospira frisia* sp. nov., two mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacteria isolated from an intertidal mud flat

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SUMMARY

Two new members of the genus *Thiomicrospira* were isolated from an intertidal mud flat sample with thiosulfate as the electron donor and CO_2 as carbon source. On the basis of differences in genotypic and phenotypic characteristics, we propose that strain JB-A1^T and strain JB-A2^T are members of two new species, *Thiomicrospira kuenenii* and *Thiomicrospira frisia*, respectively. The cells were Gram-negative, vibrios or slightly bent rods. Strain JB-A1^T was highly motile, whereas strain JB-A2^T showed a much lower degree of motility combined with a strong tendency to form aggregates. Both organisms were obligately autotrophic and strictly aerobic. Nitrate was not used as electron acceptor. Chemolithoautotrophic growth was observed with thiosulfate, tetrathionate, sulfur, and sulfide. Neither isolate was able to grow heterotrophically. For strain JB-A1^T, growth was observed between pH values of 4.0 and 7.5 with an optimum at pH 6.0; whereas for strain JB-A2^T growth was observed between pH 4.2 and 8.5 with an optimum at 6.5. The temperature limits for growth were between 3.5 and 42°C, and 3.5 and 39°C, respectively. The optimum growth temperature for strain JB-A1^T was between 29 and 33.5°C; whereas strain JB-A2^T showed optimal growth between 32-35°C. The average maximum growth rate was 0.35 h^{-1} for strain JB-A1^T and 0.45 h^{-1} for strain JB-A2^T.

INTRODUCTION

The genus *Thiomicrospira* was first described by Kuenen & Veldkamp in 1972, who isolated the type species *Thiomicrospira pelophila* from the intertidal mud flats of the Dutch Wadden Sea. Since then, several other species have been isolated from different marine environments with a specific focus on deep-sea hydrothermal vents (Ruby & Jannasch, 1982; Ruby et al., 1981; Jannasch et al., 1985; Wood & Kelly, 1989; Eberhard et al., 1995). All members of this genus are obligately chemolithoautotrophic sulfur-oxidizing bacteria. Heterotrophic growth of *Thiomicrospira thyasirae* (DSM 5322), as described by Wood & Kelly (1989; 1993), was not reproducible during our studies (J. Kuever, unpublished results). *Thiomicrospira* spp. seem to play an important role in the re-oxidation of reduced sulfur compounds in marine habitats, but were also found in a freshwater habitat with high sulfide concentrations (Brinkhoff & Muyzer, 1997). Except for *Thiomicrospira denitrificans*, all *Thiomicrospira* species isolated so far form a monophyletic group within the gamma subdivision of the Proteobacteria (Muyzer et al., 1995; Brinkhoff & Muyzer, 1997).

MATERIALS AND METHODS

Culture media. The medium used for enrichments, isolation and routine culture work was liquid medium based on the medium for *Thiomicrospira pelophila* (TP) (Kuenen & Veldkamp, 1972), with the exception

that the trace element solution described by Widdel & Bak (1992) was used. The medium was supplied with 20 mM thiosulfate and bromothymol blue as a pH-indicator. The initial pH of the medium was 7.2. Growth on organic compounds and other reduced sulfur sources was tested in TP medium without thiosulfate. For solid media 1.2% (w/v) Difco Noble agar was added. A complex organic medium was used to determine general heterotrophic growth potential (Marine Broth 2216, Difco) and to detect possible heterotrophic contamination. Heterotrophic growth was tested for eight weeks.

Isolation of bacteria. Enrichment cultures were obtained by inoculating 10 ml medium with 0.1 g sediment from the Jadebusen area close to Sehestedt, Northern Germany, which belongs to the Wadden Sea of the North Sea. The cultures were incubated at 22°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 and by sulfur deposition, 0.1 ml was transferred to 10 ml fresh medium. For isolation of pure cultures 0.1 ml of the enrichment cultures were transferred onto thiosulfate agar plates and repeatedly streaked out. Colonies were transferred at least three times to be considered pure. The isolates were differentiated by partial sequencing of the 16S rRNA gene obtained from several pure cultures, because morphological features of the colonies were identical. *T. pelophila* (DSM 1534) and *T. thyasirae* (DSM 5322) were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSMZ) (Braunschweig, Germany) and *T. crunogena* was kindly provided by H. Jannasch.

Growth experiments. Batch cultures were grown in 500 ml flasks containing 100 ml medium on a rotary shaker at 22°C in the dark. Routine cultivation of the isolates and utilization of different substrates were investigated in 15 ml tubes containing 10 ml TP medium at 22°C and 30°C. Large scale cultivation was done at 22°C in 3 l and 20 l glass carboys supplied with 40 mM thiosulfate, in which the pH was monitored by a sterilized pH-electrode (Ingold, Germany) and readjusted by titration with Na_2CO_3 (1 M) through a personal computer program controlling a peristaltic pump. The program was developed by Volker Meyer at the Max-Planck-Institute for Marine Microbiology. The fermenter were aerated with sterile filtered pressurized air through sparkling devices.

The maximum growth rate in TP-medium was determined at 30°C by direct counts (acridine orange staining and epifluorescence microscopy) (Hobbie et al., 1977), increase in optical density or protein concentration in pH controlled fermenters, and from the rate of CO_2-incorporation, using $NaH^{14}CO_3$ (Wirsen et al., 1993; Eberhard et al., 1995; Tuttle & Jannasch, 1977). A rough estimate of the optimal pH value and the lowest and highest values tolerated by the isolates was determined by using TP medium adjusted to different initial pH values and supplied with pH indicators covering different pH ranges (bromocresol purple (5.2-6.8), phenol red (6.8-8.4), and thymol blue (8.0-9.6)). Screening for acidification by a color change of the pH indicator indicated growth. The optimal pH values for both isolates were determined by direct counts as indicated above. The CO_2-incorporation rate was measured at the optimal pH and 0.5 pH units above and below the optimum. The determination of the maximum growth rate under these conditions

(Tuttle & Jannasch, 1977; Wirsen et al., 1993) confirmed the optimal pH for growth. The lowest pH for growth was determined in a pH-controlled 3 l fermenter system.

The optimal growth temperature of the new isolates was determined in a thermally insulated aluminum block which was heated electrically to +52.5°C at one end and cooled to +3.5°C with a refrigerated circulation thermostat at the other end. The block contained thirty rows of four holes, so that samples could be incubated simultaneously at temperature intervals of 1.5°C with a maximum of four replicates. The temperature limits of growth were established by screening for acidification for ten days. The optimal growth temperature was determined within 24 hours after inoculation.

The Na^+-requirement of the isolates was tested in TP medium supplied with 20 mM thiosulfate containing varying Na^+-concentrations (40, 57, 83, 100, 140, 290, 470, 640, 1240, 1580 mM) and incubated at 30°C. All Na^+-concentrations included the sodium thiosulfate; increased concentrations were provided by addition of NaCl.

Utilization of inorganic electron donors. The ability to oxidize and grow on different reduced sulfur compounds and formate was tested by using TP medium supplemented with one of the following compounds: thiosulfate (20 mM), tetrathionate (10 mM), sulfite (3, 5 or 20 mM), thiocyanate (3, 5 or 20 mM), elemental sulfur (0.1% (w/v)), formate (20 or 40 mM). Growth on sulfide was determined by using 0.2% (w/v) slush agar gradients over 8 mM sulfide-containing agar plugs (Nelson & Jannasch, 1983). Uninoculated controls showed no pH change and no turbidity.

Autotrophic growth on hydrogen was tested on solid mineral medium supplied with bicarbonate (30 mM) incubated in a jar containing a gas mixture of 80% hydrogen and 20% air (v/v) and in liquid media (Drobner et al., 1990).

Formation of intermediate products. Formation of intermediates during growth on thiosulfate was monitored by cyanolysis (Kelly et al., 1969) and high performance liquid chromatography (HPLC) (Rethmeier et al., 1997).

Anaerobic growth. Whether nitrate was used as an electron acceptor in the absence of oxygen was tested under autotrophic and heterotrophic conditions by using bicarbonate buffered (30 mM) TP medium supplied with 20 mM KNO_3 and prepared anaerobically. Hungate tubes contained 10 ml liquid medium under a 90%/10% (v/v) N_2/CO_2 gas phase and the varied electron donors (see above). In addition, anaerobically prepared agar dilution series using the same electron donors as noted above were monitored for colony formation.

Utilization of organic electron donors. Heterotrophic growth was checked in TP medium without pH-indicator supplied with one of the following compounds: fructose (5 mM), glucose (5 mM), acetate (20 mM), pyruvate (10 mM), lactate (10 mM), casamino acids (0.1 and 0.01% (w/v)), peptone (0.1 and 0.01% (w/v)), and yeast extract (0.1 and 0.01% (w/v)). The same additions in TP medium with bromothymol blue containing 20 mM thiosulfate were used for testing for an inhibitory effect of organic compounds on thiosulfate oxidation.

Ubiquinone analysis. The ubiquinone fraction was isolated, purified, and identified by B. Tindall (DSMZ Identification Service, Braunschweig, Germany) from cells harvested from large scale incubations. Respiratory lipoquinones were extracted from 100 mg freeze dried cell material using the two stage method described by Tindall (1990a, b). The lipoquinones were separated into their different classes by thin layer chromatography (Macherey-Nagel Art. No. 805 203), using hexane: *tert*-butylmethylether as solvent (9:1), UV absorbing bands corresponding to ubiquinones were removed from the plate and further analyzed by HPLC. The latter was carried out on a LDC Analytical (Thermoseparation products) HPLC fitted with a reverse phase column (Macherey-Nagel, 2 mm x 125 mm, 3 µm, RP18). Methanol served as eluant. Respiratory lipoquinones were detected at 269 nm.

DNA base composition. For determining the G+C content, cells were disrupted and the DNA purified on hydroxyapatite (Cashion et al., 1977). The DNA was hydrolyzed with P1 nuclease and the nucleotides dephosphorylized with bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleosides were analyzed by HPLC according to Tamaoka & Komagata (1984). The instrument was calibrated with non-methylated LAMBDA-DNA (Sigma), G+C content 49.86 mol%. The G+C content was calculated from the ratio of deoxyguanosine (dG) and deoxyadenosine (dA) (Mesbah et al., 1989).

DNA-DNA hybridization. The DNA-DNA hybridizations were carried out by J. Burghardt (DSMZ, Braunschweig, Germany). The DNA was isolated as described above and the hybridization was carried out as

described by De Levy et al. (1970) with the modifications described by Huß et al. (1983) using a Gilford System 2600 spectrophotometer equipped with a Gilford 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992).

Preparation of cell free extracts. Cells were harvested from late exponential growth phase and passed three times through a French press (Extrusion pressure 49 MPa). Breakage of the cells was monitored by phase contrast microscopy. Cell debris was removed by centrifugation and the supernatant was used for further experiments.

Enzyme assays. The ribulose bisphosphate carboxylase (RuBisCO) activity in the cell free extracts was measured at 30°C according to the procedure of Beudeker et al. (1980) as modified by Nelson & Jannasch (1983). An extract from spinach was used as a reference. Protein was determined by the Comassie brilliant blue dye binding technique (Bradford, 1976) using a Biorad protein assay kit.

Phylogenetic analysis. The 16S rRNA sequences of strain JB-A1^T (*T. kuenenii*), and JB-A2^T (*T. frisia*) and the phylogenetic position was already published previously (Brinkhoff & Muyzer, 1997). In this paper we only show a similarity matrix of the 16S rRNA sequences of all described *Thiomicrospira* spp. and closely related organisms.

Nucleotide sequence accession number. The nearly complete 16S rRNA sequences of strain JB-A1^T (*T. kuenenii)*, and strain JB-A2^T (*T. frisia*) are available from GenBank under accession no. AF013978 and AF013974, respectively.

RESULTS

Isolation of strain JB-A1^T and JB-A2^T. Subsequent to enrichment, pure cultures were obtained by subculturing single colonies and the two strains JB-A1^T and JB-A2^T were chosen for continued study. After the 16S rRNA genes had been sequenced and showed less than 96% similarity to all described *Thiomicrospira* spp. and to each other (see Table 1) these cultures were used for further characterization.

Table 1: 16S rDNA similarity values between strain JB-A1^T, strain JB-A2^T and related taxa of the gamma subclass of the Proteobacteria[a]

	Chr. vinosum	*Ttx. nivea*	*P. salmonis*	*B. thermo-philus* sym.	*Tms. pelophila*	*Tms. thyasirae*	*Tms. crunogena*	*Tms.* sp. L-12	*Tms.* sp. MA2-6	*Tms.* sp. JB-A1^T
Chr. vinosum										
Ttx. nivea	88.5									
P. salmonis	85.2	87.1								
B. thermophilus sym.	85.5	86.9	84.6							
Tms. pelophila	84.9	84.3	83.9	86.0						
Tms. thyasirae	85.2	84.7	84.0	85.9	99.9					
Tms. crunogena	84.5	84.2	85.1	85.6	92.3	92.2				
Tms. sp. L-12	84.6	84.3	84.8	85.5	91.7	91.7	99.1			
Tms. sp. MA2-6	84.7	84.8	84.5	86.1	92.5	92.5	97.2	97.1		
Tms. sp. JB-A1^T	83.9	84.4	84.9	85.4	92.1	92.1	95.6	95.7	95.0	
Tms. sp. JB-A2^T	83.9	84.1	84.6	85.4	91.1	91.1	94.5	94.3	93.4	94.1

[a]: The following accession numbers were used: *Chromatium vinosum* (M26629), *Thiothrix nivea* (M79435), *Piscirickettsia salmonis* (U36915), *Bathymodiolus thermophilus* gill symbiont (M99445), *Thiomicrospira pelophila* (L40809), *Thiomicrospira thyasirae* (AF016046), *Thiomicrospira crunogena* (L40810), *Thiomicrospira* sp. L-12 (L01576), *Thiomicrospira* sp. MA2-6 (L40811), *Thiomicrospira* sp. JB-A1^T (AF013978), *Thiomicrospira* sp. JB-A2^T (AF013974).

Morphology. Cells of strain JB-A1^T appeared as single motile vibrios of 0.3-0.4 x 1-2.5 µm in size (Fig. 1A), whereas cells of JB-A2^T were slightly bent rods of 0.3-0.5 x 1-2.7 µm (Fig. 1B). Compared to JB-A1^T which was highly motile, strain JB-A2^T showed a reduced level of motility. In the late exponential growth phase, cells of strain JB-A2^T showed a tendency to clump, resulting in the formation of aggregates. Both isolates were Gram negative and spore-formation was absent.

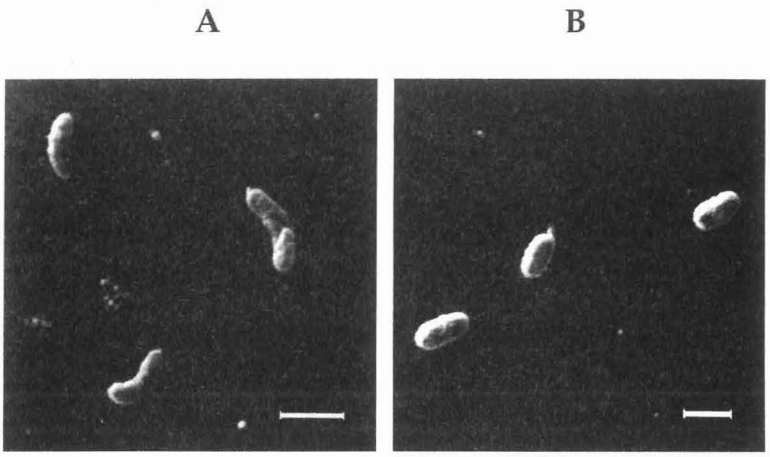

Figure 1: Electron micrographs of both strains. Picture A shows the typical morphology of strain JB-A1^T, very similar to all other described *Thiomicrospira* spp.; picture B shows the typical rod like morphology of strain JB-A2^T, which is different to other *Thiomicrospira* spp. For both pictures the bar represents 1 µm.

Growth conditions. Both isolates are strictly aerobic and grow autotrophically on thiosulfate, tetrathionate, sulfur, and sulfide, but not on sulfite and thiocyanate. Growth on thiosulfate lowered the pH to 3.5. Thiosulfate was completely oxidized to sulfate, with a recovery of 95% to 99%. Formation of elemental sulfur was only observed on solid media, but not in liquid media. No growth occurred in TP medium supplemented with any of the organic compounds tested. The oxidation of thiosulfate was not inhibited by any of the organic substrates. Nitrate was not used as a terminal electron acceptor. Hydrogen was not used as an electron donor for autotrophic growth. Vitamin B_{12} was not essential for growth. Maximum growth rates on thiosulfate at 30°C and optimal pH were obtained from the average of four different procedural determinations, i.e., CO_2-incorporation, direct counts, optical density and protein production. The rate for strain JB-A1^T was 0.35 h^{-1} (range 0.3 h^{-1} to 0.4 h^{-1}), whereas strain JB-A2^T showed a slightly higher rate of 0.45 h^{-1} (range 0.4 h^{-1} to 0.5 h^{-1}). The rates were nearly the same for 100% and 20% air saturated medium.

When the pH was readjusted to the optimal pH during growth on thiosulfate the new isolates showed no sulfur precipitation at all, whereas *T. crunogena* showed a strong precipitation. In cultures of strain JB-A1^T and strain JB-A2^T sulfur was detectable in very low concentrations of 200 µM or below, and only at the end of the growth phase. Sulfite was only found at concentrations below 10 µM.

The pH range for growth on thiosulfate for strain JB-A1^T was between 4.0 and 7.5. Above a pH of 7.5 no growth was observed. At a pH of 7.5 the lag phase was significantly extended. CO_2-incorporation was found to be optimum at pH 6.0, but at pH of 5.5 and 6.5 the maximum growth rate was only slightly lower. Strain JB-A2^T was able to grow between pH 4.2 and 8.5 and showed an optimum CO_2-incorporation at 6.5. At a pH of 6.0 the maximum growth rate was slightly lower; whereas at a pH of 7.0 it showed a significant decrease.

For comparison we used *T. pelophila* and *T. crunogena* as reference strains. Both showed good agreement with previously published results (Kuenen & Veldkamp, 1972; Jannasch et al., 1985). *T. pelophila* was even able to grow in medium with an initial pH of 9.0. Growth was monitored over a time period of three weeks.

The temperature ranges for growth were between 3.5 and 42°C, and 3.5 and 39°C for strain JB-A1^T and for strain JB-A2^T respectively, although growth below 3.5°C may proceed very slowly. The optimum growth temperature for strain JB-A1^T was between 29 and 33.5°C; whereas strain JB-A2^T showed optimal growth between 32-35°C. Growth was determined by acidification of the medium over a period of 10 days.

Both isolates showed a requirement for Na^+ -ions which could not be satisfied by a substitution of K^+. Strain JB-A1^T was able to grow at a Na^+-concentration between 100 and 640 mM, whereas strain JB-A2^T showed growth between 100 and 1240 mM. For both isolates a Na^+-concentration of 470 mM resulted in the best growth, although strain JB-A1^T showed similar

rates between 140 and 470 mM. For *T. pelophila* which was used as reference strain in this experiment, reproducible growth was detectable at a Na^+-concentration as low as 40 mM, although the cells were very long and showed pleomorphism under these conditions as previously described (Kuenen & Veldkamp, 1972; Wood & Kelly, 1993). For *T. crunogena* growth was obtained in the same range as previously published (Jannasch et al., 1985).

RuBisCO activity. Ribulose bisphosphate carboxylase was found in cell free extracts of both isolates. The specific activity for strain JB-A1^T was 9.75 nmol C fixed x mg protein^{-1} and min^{-1}, and strain JB-A2^T showed a specific activity of 12.65 nmol C fixed x mg protein^{-1} and min^{-1}. Values for *T. pelophila* and *T. crunogena* were in a similar range and agreed well to previously published data (Kuenen & Veldkamp, 1973).

DNA base ratio and ubiquinone content. The G+C content for strain JB-A1^T was 42.4 $\pm$ 0.2 mol% and 39.6 $\pm$ 0.4 mol% for strain JB-A2^T. Both isolates contained Q-8 as the major ubiquinone and small traces of ubiquinone Q-7.

Phylogenetic analysis and DNA-DNA hybridization. The similarity matrix values and the results of the DNA-DNA-hybridization are shown in Table 1 and 2, respectively. Comparison of the nearly complete 16S rRNA-genes shows that strain JB-A1^T has at least 4% difference and JB-A2^T has at least 5% difference to all other described *Thiomicrospira* spp. The level of DNA-DNA-hybridization between the different *Thiomicrospira* strains shown in Table 2 gives values of less then 30% for JB-A1^T and JB-A2^T.

Table 2: Levels of DNA-DNA similarity for *Thiomicrospira* spp.

	% Homology		
Organism	***T. pelophila***	***T. crunogena***	***T. kuenenii* (JB-A1^T)**
T. pelophila	100		
T. crunogena	33.5	100	
***T. kuenenii* (JB-A1^T)**	25	29.3	100
***T. frisia* (JB-A2^T)**	27	27	25

DISCUSSION

The isolates, strain JB-A1^T and strain JB-A2^T are both chemolithoautotrophic sulfur-oxidizing bacteria belonging to the genus *Thiomicrospira*. The similarity values of the 16S rRNA sequences for the new isolates as listed in Table 1 are below 97%. According to the definition of Stackebrandt and Goebel (1994) this already indicates that strain JB-A1^T and strain JB-A2^T do not belong to presently described species.

The level of DNA-DNA-hybridization shown in Table 2 is far below the critical value of 70% (Wayne et al., 1987). In addition, as Table 3 shows, there are also significant physiological differences between the new isolates and the previously described species (for example: pH minimum, sulfur formation). Therefore, we propose that the isolates JB-A1^T and JB-A2^T be considered as two new species and be given the names *Thiomicrospira kuenenii* and *Thiomicrospira frisia*.

Table 3 also extends the description of this genus by adding some important biochemical data about the already described *Thiomicrospira* spp., which were so far not documented. An analysis of which ubiquinones are present in the respiratory chain of these organisms was rather incomplete.

When *T. crunogena* was cultivated with thiosulfate in liquid medium a strong precipitation of sulfur was observed, which later was completely consumed and oxidized to sulfate, as previously described (Javor et al., 1990). At a pH below 7.0 this precipitation was even more obvious and sulfur oxidation was inhibited after the thiosulfate was completely consumed. We observed the same in our experiments with *T. crunogena*, but not for the new isolates. The latter formed no sulfur precipitation during growth on thiosulfate, even if the pH was not readjusted to the optimal value. In batch cultures without pH-control very little sulfur was observed, but only at very low pH values. Therefore, we conclude that the metabolism of thiosulfate in our strains might be slightly different than in *T. crunogena*.

Another very interesting feature of both new isolates is the adaptation to lower pH values. This is also the case for *T. pelophila*, which could also grow well at a pH lower than 6.5 (Kuenen & Veldkamp, 1972; J. Kuever, unpublished results). This might be typical for sulfur-oxidizing bacteria of tidal mud flats. In marsh muds, tidal flats, or coastal sediments microenvironments of low pH are likely to exist (Jørgensen, 1977), whereas in deep-sea habitats the overlying water column brings a steady supply of well-buffered sea water with a more alkaline pH. The higher optimal pH for growth of the *Thiomicrospira* spp. isolated from these habitats would imply such an adaptation.

Table 3: Morphological and physiological characteristics among *Thiomicrospira* spp.[a]

	Organism			
	T. pelophila	*T. crunogena*	*T. kuenenii* (JB-A1^T)	*T. frisia* (JB-A2^T)
Shape	vibrio	vibrio	vibrio	rod
Width (μm)	0.2-0.3	0.4	0.3-0.4	0.3-0.5
Length (μm)	1-2	1.5	1-2.5	1.3-2.7
Motility	+	+	+	+[b]
G+C content (mol%)	45.7[c] (45)	44.4[c](44)	42.4[c]	39.6[c]
Ubiquinone	Q-8	Q-8	Q-8	Q-8
Maximum growth rate (h^{-1})	0.3	0.8	0.35	0.45
Optimal pH	7.0[d]	7.5-8.0	6.0	6.5
pH range	5.6-9.0	5.0-8.5	4.0-7.5	4.2-8.5
Optimal temperature (°C)	28-30	28-32	29-33.5	32-35
Temperature range (°C)	3.6-42[e]	4-38.5	3.6-42[e]	3.6-39[e]
Optimal Na^+ concentration (mM)	470	n.d.	470	470
Na^+ concentration range (mM)	40-1240[f]	at least 50	100-640	100-1240
Vitamin B_{12}-dependent	+	-	-	-
Ribulose 1,5-bisphosphate carboxylase	+	+	+	+
Formation of sulfur from thiosulfate at pH 7.0 in liquid medium	+	+	-	-

[a]: data from Kuenen and Veldkamp, 1972; 1973; Kuenen and Robertson, 1989; Jannasch et al. 1985; and own data.
[b]: motility can rapidly decrease during growth.
[c]: determined by HPLC; values in brackets were determined previously by thermal denaturation.
[d]: data from present study, determined by CO_2-incorporation.
[e]: Growth would be likely to occur below 3.6°C.
[f]: in medium without NaCl there was at least 20 mM $Na_2S_2O_3$ present; cells of *T. pelophila* showed pleomorphism at low Na^+-concentrations.

If microzonations of low pH are present at tidal mud flats, H_2S would be released from the FeS as one dominant reduced sulfur compound in these habitats (Canfield & Thamdrup, 1996). Because *Thiomicrospira* spp. and probably other sulfur-oxidizing bacteria acidify their environment, they might take direct advantage of this process by using the H_2S or its chemical oxidation products as electron donors, thus favoring this reaction by establishing a more acidic environment. If the sulfide is not directly consumed, other reduced sulfur compounds such as thiosulfate, polythionates, sulfur, sulfite and sulfate will be produced through chemical oxidation (Canfield & Thamdrup, 1996; Zhang & Millero, 1993). Except sulfite and sulfate, all these compounds are excellent substrates for growth of *Thiomicrospira*.

It has been demonstrated that deep sea hydrothermal vent *Thiomicrospira* spp. can grow autotrophically while oxidizing various mineral sulfides at circumneutral pH (Eberhard et al., 1995). The optimal pH for growth on these mineral sulfides was found to be 6.5 to 7.0 compared to an optimal pH of 7.5 for growth on thiosulfate. This might indicate a slightly acidic pH optimum for growth on mineral sulfides. In terrestrial systems the maximum rate of dissolution for pyrite occurs between a pH of 1 to 5 (Schüring et al., 1997). An adaptation to lower pH values as it was found for the new *Thiomicrospira* spp. described in this paper may be an advantage in habitats rich in FeS and FeS_2. These minerals should be considered as likely electron donors for *Thiomicrospira* spp. or other sulfur-oxidizing bacteria in sediments from the Wadden Sea. We were able to get very low, but

reproducible growth of both isolates on FeS, but not on synthetic pyrite (J. Kuever, unpublished results).

Description of *Thiomicrospira kuenenii* sp. nov. *Thiomicrospira kuenenii* (kue. nen´i.i M. L. gen. n. kuenenii of Kuenen; named after J.G. Kuenen, a Dutch microbiologist of the Delft School of Microbiology, who proposed the genus *Thiomicrospira*).

Cells are Gram-negative, motile, and vibrio shaped (0.3–0.4 x 1.-2.5 µm). *Thiomicrospira kuenenii* is strictly aerobic and grows autotrophically on thiosulfate, tetrathionate, sulfur, and sulfide, but not on sulfite, and thiocyanate. The organism does not grow heterotrophically. When thiosulfate is used as the primary energy source very small amounts of sulfur and sulfite are produced. Compared to other *Thiomicrospira* spp. sulfur precipitation in liquid medium is less obvious. During growth on reduced sulfur compounds the pH decreases from neutrality to around 3.5. Thiosulfate is completely oxidized to sulfate. Autotrophic growth on thiosulfate occurs between pH. 4.0 and 7.5 and at a temperature of 3.5 to 42°C; optimum growth occurs at pH 6.0 and at 29 to 33°C. The optimal Na^+-concentration for growth is 470 mM; growth is possible between Na^+-ion concentrations of 100 and 640 mM. Carbon dioxide is fixed by means of ribulose bisphosphate carboxylase. Nitrate is not used as terminal electron acceptor. On thiosulfate agar, cells produce white to yellowish, smooth, entire colonies (diameter on 1.2% (w/v) agar is 2-5 mm) in which sulfur is deposited and acid is produced. Ubiquinone Q-8 is present in the respiratory chain. The G+C content of the DNA is 42.4 mol%. As determined by a 16S rRNA gene sequence analysis, *Thiomicrospira kuenenii* belongs to the gamma subdivision of the

Proteobacteria and is closely related to previously described members of the genus *Thiomicrospira*. The strain JB-A1 is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under number DSM 12350, which is the type strain of the species. The GenBank accession number for the nearly complete 16S rRNA gene sequence of *T. kuenenii* is AF013978

Description of *Thiomicrospira frisia* sp. nov. *Thiomicrospira frisia* (fri´si.a M. L. fem. adj. frisia pertaining to Frisia, a coastal region in the northwest of Germany and the Netherlands).

Cells are Gram-negative, motile, and rod shaped (0.3-0.5 x 1.3–2.7 μm). During late exponential growth cells have a strong tendency to clump and to form aggregates. *Thiomicrospira frisia* is strictly aerobic and grows autotrophically on thiosulfate, tetrathionate, sulfur, and sulfide, but not on sulfite, and thiocyanate. The organism does not grow heterotrophically. When thiosulfate is used as the primary energy source small amounts of sulfur and sulfite are produced. During growth on reduced sulfur compounds the pH decreases from neutrality to around 3.5. Thiosulfate is completely oxidized to sulfate. Autotrophic growth on thiosulfate occurs between pH 4.2 and 8.5 and at a temperature of 3.5 to 39°C; optimum growth occurs at pH 6.5 and at 32 to 35°C. The optimal Na^+-concentration for growth is 470 mM; growth is possible between a Na^+-ion concentration of 100 to 1240 mM. Carbon dioxide is fixed by means of ribulose bisphosphate carboxylase. Nitrate is not used as terminal electron acceptor. On thiosulfate agar, cells produce white to yellowish, smooth, entire colonies (diameter on 1.2% (w/v) agar is 2-5 mm) in which sulfur is deposited and acid is produced. Ubiquinone Q-8 is present in

the respiratory chain. The G+C content of the DNA is 39.6 mol%. As determined by a 16S rRNA gene sequence analysis, *Thiomicrospira frisia* belongs to the gamma subdivision of the Proteobacteria and is closely related to previously described members of the genus *Thiomicrospira*. The strain JB-A2 is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under number DSM 12351, which is the type strain of the species. The GenBank accession number for the nearly complete 16S rRNA gene sequence of *T. frisia* is AF013974.

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REFERENCES

Beudeker, R.F., G.C. Cannon, J.G. Kuenen, & J.M. Shively. (1980). Relations between D-ribulose-1,5-biphosphate carboxylase, carboxysomes and CO_2 fixing capacity in the obligate chemolithotroph *Thiobacillus neapolitanus* grown under different limitations in the chemostat. Arch. Microbiol. **124**: 185-189.

Bradford, M.M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Brinkhoff, T. & G. Muyzer. (1997). Increased species diversity and extended habitat range of sulfur-oxidizing *Thiomicrospira* spp. Appl. Environ. Microbiol. **63**: 3789-3796.

Canfield, D.E., & B. Thamdrup. (1996). Fate of elemental sulfur in an intertidal sediment. FEMS Microbiol. Ecol. **19**: 95-103

Cashion, P., M.A. Holder-Franklin, J. McCully, & M. Franklin. (1977). A rapid method for the base ratio determination of bacterial DNA. Anal. Biochem. **81:** 461-466.

De Levy, J., H. Cattior, and A. Reynaerts. (1970). The quantitative measurement of DNA hybridization from renaturation rates. Eur. J. Biochem. **12:** 133-142.

Drobner E., H. Huber, & K.O. Stetter. (1990). *Thiobacillus ferrooxidans*, a facultative hydrogen oxidizer. Appl. Environ. Microbiol. **56:** 2922-2923.

Eberhard, C., C.O. Wirsen, & H.W. Jannasch. (1995). Oxidation of polymetal sulfides by chemolithoautotrophic bacteria from deep-sea hydrothermal vents. Geomicrobiol. J. **13**: 145-164.

Hobbie, J.E., R.J. Daley, & S. Jasper. (1977). Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**: 1225-1228.

Huß, V.A.R., H. Festl, & K.H. Schleifer. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. Syst. Appl. Microbiol. **4**: 184-192.

Jahnke, K.-D. (1992). BASIC computer program for evaluation of spectroscopic DNA renaturation data from GILFORD SYSTEM 2000 spectrophotometer on a PC/XT/AT type personal computer. J. Microbiol. Methods **15**: 61-73.

Jannasch, H.W., C.O. Wirsen, D.C. Nelson, & L.A. Robertson. (1985). *Thiomicrospira crunogena* sp. nov., a colorless sulfur-oxidizing bacterium from a deep-sea hydrothermal vent. Int. J. Syst. Bacteriol. **35**: 422-424.

Javor, B.J., D.B. Wilmot, & R.D. Vetter. (1990). pH-dependent metabolism of thiosulfate and sulfur globules in the chemolithotrophic marine bacterium *Thiomicrospira crunogena*. Arch. Microbiol. **154**:231-238.

Jørgensen, B.B. (1977). The sulfur cycle of a coastal marine sediment (Limfjorden, Denmark). Limnol. Oceanogr. **22**:814-832.

Kelly, D.P., L.A. Chambers, & P.A. Trudinger. (1969). Cyanolysis spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. Anal. Chem. **41**:898-901.

Kuenen, J.G., & H. Veldkamp. (1972). *Thiomicrospira pelophila*, gen. n., sp. n., a new obligately chemolithotrophic colourless sulfur bacterium. Ant. Leeuwenhoek **38**: 241-256.

Kuenen, J.G., & H. Veldkamp. (1973). Effects of organic compounds on growth of chemostat cultures of *Thiomicrospira pelophila, Thiobacillus thioparus* and *Thiobacillus neapolitanus.* Arch. Microbiol. **94:** 173-190.

Kuenen, J.G., & L.A. Robertson. (1989). Genus *Thiomicrospira.* Kuenen and Veldkamp. In: Staley J.T., Bryant, M.P., Pfennig, N., Holt, J.G. (eds.) Bergey`s Manual of Systematic Bacteriology, vol. 3. Williams and Wilkins, Baltimore, pp. 1858-1861.

Mesbah, M., U. Premachandran, & W. Whitman. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. Int. J. Syst. Bacteriol. **39**: 159-167.

Muyzer, G., A. Teske, C.O. Wirsen, & H.W. Jannasch. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. **164**: 165-172.

Nelson, D.C., & H.W. Jannasch. (1983). Chemoautotrophic growth of a marine *Beggiatoa* in sulfide-gradient cultures. Arch. Microbiol. **136**:262-269.

Rethmeier, J., A. Rabenstein, M. Langer, & U. Fischer. (1997). Detection of traces of oxidized and reduced sulfur compounds in small samples by combination of different high-performance liquid chromatography methods. J. Chromat. A **760**: 295-302.

Ruby, E.G., & H.W. Jannasch. (1982). Physiological characteristics of *Thiomicrospira* sp. strain L-12 isolated from deep-sea hydrothermal vents. J. Bacteriol. **149**: 161-165.

Ruby, E.G., C.O. Wirsen, & H.W. Jannasch. (1981). Chemolithoautotrophic sulfur-oxidizing bacteria from the Galapagos Rift hydrothermal vents. Appl. Environ. Microbiol. **42**:317-342.

Schüring, J. Kölling, & H.D. Schulz. (1997). The potential formation of acid mine drainage in pyrite-bearing hard-coal tailings under water-saturated conditions: an experimental approach. Environ. Geol. **31**: 59-65.

Stackebrandt, E. & B.M. Goebel. (1994). Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44**: 846-849.

Tamaoka, J., & K. Komagata. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol. Lett. **25**: 125-128.

Tindall, B.J. (1990a). A comparative study of the lipid composition of *Halobacterium saccaravorum* from various sources. System. Appl. Microbiol. **13:** 128-130.

Tindall, B.J. (1990b). Lipid composition of *Halobacterium lacusprofundi*. FEMS Microbiol. Lett. **66:** 199-202.

Tuttle, J.H., & H.W. Jannasch. (1977). Thiosulfate stimulation of microbial dark assimilation of carbon dioxide in shallow marine environments. Microb. Ecol. **4**:9-25.

Wayne, L.G., D.J. Brenner, R.R. Colwell, P.A.D. Grimont, O.Kandler, M.I. Krichevsky, L.H. Moore, W.E.C. Moore, R.G.E. Murray, E. Stackebrandt, M. P. Starr & H.G. Trüper. (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37**: 463-464.

Widdel, F., & F. Bak. (1992). Gram-negative mesophilic sulfate-reducing bacteria. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds.), The Procaryotes. Sec. Edition Volume IV. Springer Verlag, New York, pp. 3352-3378.

Wirsen, C.O., H.W. Jannasch, & S.J. Molyneaux. (1993). Chemosynthetic microbial activity at Mid-Atlantic Ridge hydrothermal vent sites. J. Geophys. Res. **98:** 9693-9703.

Wood, A.P., & D.P. Kelly. (1989). Isolation and characterization of *Thiobacillus thyasiris* sp. nov., a novel marine facultative autotroph and the putative symbiont of *Thyasira flexuosa.* Arch. Microbiol. **152**: 160-166.

Wood, A.P., & D.P. Kelly. (1993). Reclassification of *Thiobacillus thyasiris* as *Thiomicrospira thyasirae* comb. nov. An organism exhibiting pleomorphism in response to environmental conditions. Arch. Microbiol. **159**: 45-47.

Zhang, J.-Z., & F.J. Millero. (1993). The products from the oxidation of H_2S in seawater. Geochim. Cosmochim. Acta **57**: 1705-1718.

Chapter 5

Characterization of *Thiomicrospira chilensis* sp. nov., a mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacterium isolated from a *Thioploca* mat

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SUMMARY

A new member of the genus *Thiomicrospira* utilizing thiosulfate as the electron donor and CO_2 as carbon source was isolated from a sediment sample dominated by the filamentous sulfur bacterium *Thioploca*. Although the investigated physiological properties are nearly identical to other described species of the genus, we propose that strain Ch-1^T is a member of a new species, *Thiomicrospira chilensis*, on the basis of differences in genotypic characteristics (16S rRNA sequence, DNA-homology, G+C content). Strain Ch-1^T was highly motile combined with a slight tendency to form aggregates in the stationary growth phase. The organism was obligately autotrophic and strictly aerobic. Nitrate was not used as electron acceptor. Chemolithoautotrophic growth was observed with thiosulfate, tetrathionate, sulfur, and sulfide. The isolate was not able to grow heterotrophically. For strain Ch-1^T, growth was observed between pH values of 5.3 and 8.5 with an optimum at pH 7.0. The temperature range for growth was between 3.5 and 42°C. The optimum growth temperature was between 32 and 37°C. The average maximum growth rate on thiosulfate was 0.4 h^{-1}. This is the second described *Thiomicrospira* spp. which has a rod-shaped morphology. Therefore discrimination between vibrio-shaped *Thiomicrospira* and rod-shaped *Thiobacilli* is no longer valid.

INTRODUCTION

Most *Thiomicrospira* spp. described so far were isolated from deep-sea hydrothermal vents and intertidal mud flats (Kuenen & Veldkamp, 1972; Ruby et al., 1981; Ruby & Jannasch, 1982; Jannasch et al., 1985; Wood & Kelly, 1989; Eberhard et al., 1995; Brinkhoff et al., 1998). All members of this genus are obligately chemolithoautotrophic sulfur-oxidizing bacteria. The ecological importance of *Thiomicrospira* spp. in marine habitats rich in reduced sulfur compounds has been already demonstrated (Muyzer et al., 1995; Brinkhoff & Muyzer, 1997), but very little is known about their role in sulfide influenced habitats which have a mat-like community structure. In these habitats they have to compete with other very specialized bacteria for the electron donor sulfide or other reduced sulfur compounds. In light influenced mat systems they have to compete with phototrophic bacteria. Nevertheless, a *Thiomicrospira* sp. was isolated out of a sediment sample from the Solar Lake, Egypt, which can be regarded as an model system for such an ecosystem (Brinkhoff and Muyzer, 1997). In this paper we used samples from a different mat-like community, the *Thioploca* mats on the Chilean coastal shelf, to obtain *Thiomicrospira* isolates.

MATERIALS AND METHODS

Culture media and isolation of bacteria. The medium (TP) used and the procedure for isolation was as described previously (Brinkhoff et al., 1998).

Growth experiments and utilization of electron donors. All growth experiments and tests for electron donors were carried out as described previously (Brinkhoff et al., 1998); the results are summarized in table 3.

Biochemical and molecular biological analysis. The formation of intermediates during growth on thiosulfate was monitored by cyanolysis (Kelly et al., 1969) and high performance liquid chromatography (HPLC) (Rethmeier et al., 1997). The ubiquinones were analyzed by B. Tindall (DSMZ Identification Service, Braunschweig, Germany) and the DNA base composition together with the DNA-DNA hybridizations were carried out by J. Burghardt (DSMZ, Braunschweig, Germany) as described previously (Brinkhoff et al., 1998).

The ribulose bisphosphate carboxylase (RuBisCO) activity in the cell free extracts was measured at 30°C as described before (Brinkhoff et al., 1998). The 16S rRNA sequence of strain Ch-1^T and its phylogenetic position have been reported (Brinkhoff & Muyzer, 1997). In this paper we show a similarity matrix of the 16S rRNA sequences of all described *Thiomicrospira* spp. and closely related organisms.

Nucleotide sequence accession number. The sequence of strain Ch-1^T (*T. chilensis*) is available from Genbank under accession no. AF013975.

RESULTS

Isolation of strain Ch-1^T. Enrichment cultures were inoculated with mud from the continental shelf of Chile at the Bay of Concepcion at 36°32´S (Fossing et al., 1995). Using sulfide gradients we observed a high number of spirillum like organisms containing mainly two sulfur globules and forming "Bakterienplatten" located in the regions of optimal H_2S and O_2 concentrations. These organism looked very similar to *Thiospira bipunctata*, which was first described by Molisch (1912). A newer publication revised the taxonomic position and described them as *Aquaspirillum bipunctata* (Dubinina et al., 1993). After several attempts to enrich and isolate these organisms we only got a good enrichment culture using thiosulfate as the electron donor. Differing from the original enrichment on sulfide, this culture was dominated by short vibrios and rods containing no sulfur globules. Using solid modified medium TP, as described recently (Brinkhoff et al., 1998), we were able to isolate a vibrio-shaped organism (Ch-2). After filtration of the enrichment culture through a 0.45 µm filter a rod-shaped sulfur oxidizing bacterium (Ch-1) was obtained in pure culture. Both isolates were identified as *Thiomicrospira* sp. using the specific PCR approach (Brinkhoff & Muyzer, 1997). Based on nearly complete sequencing of the 16S rRNA encoding genes we could distinguish both isolates. The vibrio shaped *Thiomicrospira* sp. isolate was nearly identical (99.5%) to *Thiomicrospira*

crunogena, whereas the rod shaped isolate showed less than 96% similarity to all described *Thiomicrospira* spp. (see Table 1). Therefore only *Thiomicrospira* sp. Ch-1^T. was further characterized.

Table 1: 16S rDNA similarity values between strain Ch-1^T and related taxa of the gamma subclass of the Proteobacteria[a]

	Chr. vinosum	*Cal. magnifica* gill symbiont	*Tms. pelophila*	*Tms. thyasirae*	*Tms. crunogena*	*Tms.* sp. L-12	*Tms.* sp. MA2-6	*"Tms. kuenenii"*	*"Tms. frisia"*
Chr. vinosum									
Cal. magnifica gill symbiont	84								
Tms. pelophila	85.0	84.7							
Tms. thyasirae	85.6	85.1	99.9						
Tms. crunogena	84.9	84.2	92.5	92.5					
Tms. sp. L-12	86.0	84.6	92.4	92.4	99.2				
Tms. sp. MA2-6	84.9	84.7	92.6	92.7	97.3	97.4			
"Tms. kuenenii"	84.1	83.1	94.1	92.3	95.7	96.1	95.1		
"Tms. frisia"	84.1	84.0	91.1	91.3	94.7	94.8	93.5	92.2	
Tms. sp. Ch-1^T	83.8	84.8	92.1	92.3	94.9	94.9	93.9	93.1	95.3

[a]: The following accession numbers were used: *Chromatium vinosum* (M26629), *Calyptogena magnifica* gill symbiont (M99446), *Thiomicrospira pelophila* (L40809), *Thiomicrospira thyasirae* (AF016046), *Thiomicrospira crunogena* (L40810), *Thiomicrospira* sp. L-12 (L01576), *Thiomicrospira* sp. MA2-6 (L40811), *"Thiomicrospira kuenenii"* (AF013978), *"Thiomicrospira frisia"* (AF013974), *Thiomicrospira* sp. Ch-1^T (AF013975).

Morphology. Cells of strain Ch-1^T appeared as single motile rods of 0.3-0.5 x 0.8-2 µm in size (Fig. 1). In the late exponential growth phase, cells of strain Ch-1^T showed a tendency to clump, resulting in the formation of aggregates, as described previously for *T. frisia* (Brinkhoff et al., 1998). Spore formation was absent and the Gram reaction was negative.

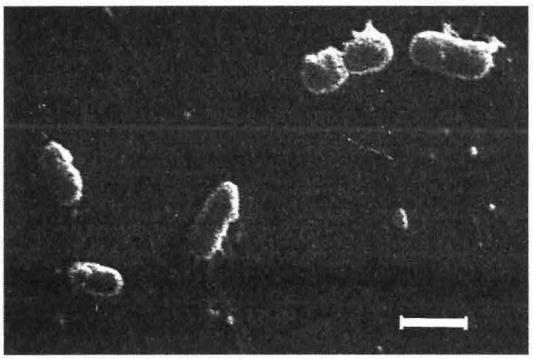

Figure 1: Electron micrograph of strain Ch-1^T (bar represents 1 µm) showing the typical rod-shaped cell form.

Growth conditions. The isolate is strictly aerobic and grows autotrophically on thiosulfate, tetrathionate, sulfur, and sulfide, but not on sulfite and thiocyanate. Growth on thiosulfate lowered the pH from 7.2 to 4.5. Thiosulfate was completely oxidized to sulfate, with a recovery of 92% to 99%. Intermediate formation of elemental sulfur was observed on solid media and in liquid media. No growth occurred in TP medium

supplemented with any of the organic compounds tested. The oxidation of thiosulfate was not inhibited by any of the organic substrates. Nitrate (10 mM) was not used as a terminal electron acceptor. Hydrogen was not used as an electron donor for autotrophic growth. Vitamin B_{12} was not essential for growth. For strain Ch-1^T, growth was observed between pH values of 5.3 and 8.5 with an optimum at pH 7.0. At 30°C and optimal pH the maximum growth rate on thiosulfate is 0.4 h^{-1}.

When the pH was readjusted to the optimal pH during growth on thiosulfate the new isolate showed a strong sulfur precipitation only at the beginning of the growth phase, similar to *T. crunogena*. The only intermediate found was sulfite at a concentration varying from 0.1 to 2.1 mM, whereas tetrathionate was not detected.

RuBisCO activity. Ribulose bisphosphate carboxylase was found in cell free extracts of the new isolate. The specific activity for strain Ch-1^T was 8.2 nmol C fixed x mg protein^{-1} and min^{-1}.

DNA base ratio and ubiquinone content. The G+C content for strain Ch-1^T was 49.9 ± 0.2 mol%; the isolate contained Q-8 as the major ubiquinone and small traces of ubiquinone Q-7.

Phylogenetic analysis and DNA-DNA hybridization. A 16S rRNA sequence similarity matrix and the results of the DNA-DNA-hybridizations are shown in Table 1 and 2, respectively. These results show clearly that strain Ch-1^T can be considered as a new *Thiomicrospira* species.

Table 2: Levels of DNA-DNA similarity for *Thiomicrospira* spp.

Organism	% Homology			
	T. pelophila	*T. crunogena*	*"T. kuenenii"* (JB-A1^T)	*"T. frisia"* (JB-A2^T)
T. pelophila	100			
T. crunogena	33.5	100		
"T. kuenenii" (JB-A1^T)	25	29.3	100	
"T. frisia" (JB-A2^T)	27	27	25	100
"T. chilensis" (Ch-1^T)	31	27	16	18

DISCUSSION

In contrast to *Thiomicrospira* spp. which are dependent on oxygen, *Thioploca* spp. are well adapted to use nitrate as a terminal electron acceptor. Using extracted DNA from these *Thioploca* mats the specific PCR-approach for *Thiomicrospira* spp. was negative (Brinkhoff & Muyzer, 1997), suggesting a neglectable role for this genus in this habitat compared to hydrothermal vent systems. Nevertheless, we were able to isolate two *Thiomicrospira* spp. from this sampling site which was dominated by filamentous sulfur oxidizing bacteria. The additional finding of another isolate of *T. crunogena* supports the thesis of Wirsen and coworkers (1998)

that this species can be found worldwide and seems to be favored by using enrichment techniques.

Comparison of the nearly complete 16S rRNA-gene sequence shows that strain Ch-1^T has at least a 4% difference to all other described *Thiomicrospira* spp. According to the definition of Stackebrandt & Goebel (1994) this already indicates that strain Ch-1^T does not belong to a presently described species. The level of DNA-DNA-hybridization shown in Table 2 is far below the critical value of 70% (Wayne et al., 1987). In addition, as Table 3 shows, there are also some physiological differences between the new isolate and other previously described species. The DNA base ratio of 49.9% for strain Ch-1^T is much higher than for the other species of the genus. Based on the great genotypic differences it can be speculated that *T. chilensis* also has some metabolic properties which separates it from other *Thiomicrospira* spp., but which were not detected using the general description methods. Together with *T. frisia*, strain Ch-1^T shares a rod-like cell form, which is not found for the other *Thiomicrospira* spp. Differentiation of *Thiomicrospira* spp. against *Thiobacillus* spp. based on morphology is no longer valid. Together with *T. frisia* and *Thiomicrospira* sp. strain Art-3, strain Ch-1^T falls into a subcluster of rod-shaped organisms (Kuever, unpublished results) within the *Thiomicrospira* cluster, which was notable in the phylogenetic tree published by Brinkhoff & Muyzer (1998). Therefore, we propose that the isolate Ch-1^T be considered as a new species of this genus and be given the name *Thiomicrospira chilensis*.

Table 3: Morphological and physiological characteristics among *Thiomicrospira* spp.*

	Organism				
	T. pelophila	*T. crunogena*	*"T. kuenenii"* (JB-A1T)	*"T. frisia"* (JB-A2T)	*"T. chilensis"* (Ch-1T)
Shape	vibrio	vibrio	vibrio	rod	rod
Width (μm)	0.2-0.3	0.4	0.3-0.4	0.3-0.5	0.3-0.5
Length (μm)	1-2	1.5	1-2.5	1-2.7	0.8-2
Motility	+	+	+	+†	+
G+C content (mol%)	45.7 ‡ (45)	44.2 ‡(44)	42.4 ‡	39.6 ‡	49.9
Ubiquinone	Q-8	Q-8	Q-8	Q-8	Q-8
Maximum growth rate (h-1)	0.3	0.8	0.35	0.45	0.4
optimal pH	7.0 $	7.5-8.0	6.0	6.5	7.0
pH range	5.6-9.0	5.0-8.5	4.0-7.5	4.2-8.5	5.3-8.5
optimal temperature (°C)	28-30	28-32	29-33.5	32-35	32-37
temperature range (°C)	3.5-42 »	4-38.5	3.5-42 »	3.5-39 »	3.5-42 »
optimal Na+ concentration (mM)	470	n.d.	470	470	470
Na+ concentration range (mM)	40-1240¶	at least 45	100-640	100-1240	100-1240
Vitamin B12-dependent	+	-	-	-	-
Ribulose 1,5-bisphosphate carboxylase	+	+	+	+	+
Formation of sulfur from thiosulfate at pH 7.0 in liquid medium	+	+	-	-	+

*: data from Kuenen & Veldkamp, 1972; 1973; Kuenen & Robertson, 1989; Jannasch et al. 1985; Brinkhoff et al., 1998; and own data.
†: motility can rapidly decrease during growth.
‡: determined by HPLC; values in brackets were determined previously by thermal denaturation.
$: data from present study, determined by CO_2-incorporation.
»: Growth would be likely to occur below 3.5°C.
¶: in medium without NaCl there was at least 20 mM $Na_2S_2O_3$ present; cells of *T. pelophila* showed pleomorphism at low Na+ concentrations.

Description of *Thiomicrospira chilensis* sp. nov. *Thiomicrospira chilensis* (chi.len´sis M. L. adj. n. chilensis from Chile, a country of South America). Cells are Gram-negative, motile, and rod shaped (0.3–0.5 x 0.8-2 μm). *Thiomicrospira chilensis* is strictly aerobic and grows autotrophically on thiosulfate, tetrathionate, sulfur, and sulfide, but not on sulfite, and thiocyanate. The organism does not grow heterotrophically. When thiosulfate is used as the primary energy source, sulfur and very small amounts of sulfite are produced. During growth on reduced sulfur compounds the pH decreases from neutrality to around 4.8. Thiosulfate is completely oxidized to sulfate. Autotrophic growth on thiosulfate occurs between pH 5.3 and 8.5 and at a temperature of 3.5 to 42°C; optimum growth occurs at pH 7.0 and at 32 to 37°C. The optimal Na^+-concentration for growth is 470 mM; growth is possible between Na^+-ion concentrations of 100 and 1240 mM. Carbon dioxide is fixed by means of ribulose bisphosphate carboxylase. Nitrate is not used as terminal electron acceptor. On thiosulfate agar, cells produce white to yellowish, smooth, entire colonies (diameter on 1.2% (wt/vol) agar is 2-5 mm) in which sulfur is deposited and acid is produced. Ubiquinone Q-8 is present in the respiratory chain. The G+C content of the DNA is 49.9 mol%. As determined by a 16S rRNA gene sequence analysis, *Thiomicrospira chilensis* belongs to the gamma subdivision of the Proteobacteria and is closely related to previously described members of the genus *Thiomicrospira*. The strain Ch-1 is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under number DSM

12352, which is the type strain of the species. The GenBank accession number for the nearly complete 16S rRNA gene sequence of *T. chilensis* is AF013975.

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REFERENCES

Brinkhoff, T. & Muyzer, G. (1997). Increased species diversity and extended habitat range of sulfur-oxidizing *Thiomicrospira* spp. *Appl Environ Microbiol* **63**, 3789-3796.

Brinkhoff, T., Muyzer, G., Wirsen, C. O. & Kuever, J. (1998) Characterization of *Thiomicrospira kuenenii* sp. nov. and *Thiomicrospira frisia* sp. nov., two mesophilic obligately chemolithotrophic sulfur-oxidizing bacteria isolated from an intertidal mud flat. *I J Syst Bacteriol*; in press.

Dubinina, G. A., Grabovich, M. Y., Lysenko, A. M., Chernykh, N. A. & Churikova, V. V. (1993) Revision of the taxonomic position of colorless sulfur spirilla of the genus *Thiospira* and description of a new species *Aquaspirillum bipunctata* comb. nov. *Microbiologya (Engl. Transl.)* **62**, 638-644.

Eberhard, C., Wirsen, C. O. & Jannasch, H. W. (1995). Oxidation of polymetal sulfides by chemolithoautotrophic bacteria from deep-sea hydrothermal vents. *Geomicrobiol J* **13**, 145-164.

Fossing, H., Gallardo, V. A., Jørgensen, B. B., Hüttel, M., Nielsen, L. P., Schulz, H., Canfield, C. E., Forster, S., Glud, R., Gundersen, J., Küver, J., Ramsing, N. B., Teske, A., Thamdrup, B., Ulloa, O. (1995). Concentration and transport of nitrate by the mat-forming sulphur bacterium *Thioploca*. *Nature* **374**, 713-715.

Jannasch, H. W., Wirsen, C. O., Nelson, D. C. & Robertson, L. A. (1985). *Thiomicrospira crunogena* sp. nov., a colorless sulfur-oxidizing bacterium from a deep-sea hydrothermal vent. *Int J Syst Bacteriol* **35**, 422-424.

Kelly, D. P., Chambers, L. A. & Trudinger, P. A. (1969). Cyanolysis spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. *Anal Chem* **41**, 898-901.

Kuenen, J.G. & Veldkamp, H. (1972). *Thiomicrospira pelophila*, gen. n., sp. n., a new obligately chemolithotrophic colourless sulfur bacterium. *Ant Leeuwenhoek* **38**, 241-256.

Kuenen, J.G. & Veldkamp, H. (1973). Effects of organic compounds on growth of chemostat cultures of *Thiomicrospira pelophila, Thiobacillus thioparus* and *Thiobacillus neapolitanus. Arch Microbiol* **94,** 173-190.

Kuenen, J. G. & Robertson, L. A.. (1989). Genus *Thiomicrospira*. Kuenen and Veldkamp. In: *Bergey`s Manual of Systematic Bacteriology, vol. 3*, pp. 1858-1861. Edited by J. T. Staley, M. P. Bryant, N. Pfennig, , J. G. Holt. Baltimore, ML, Williams and Wilkins.

Molisch, H. (1912) Neue farblose Schwefelbakterien. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 2* **33**, 55-62.

Muyzer, G., Teske, A., Wirsen, C. O. & Jannasch, H.W. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**, 165-172.

Rethmeier, J., Rabenstein, A., Langer, M. & Fischer, U. (1997). Detection of traces of oxidized and reduced sulfur compounds in small samples by combination of different high-performance liquid chromatography methods. *J Chromat* A **760**, 295-302.

Ruby, E. G. & Jannasch, H. W. (1982). Physiological characteristics of *Thiomicrospira* sp. strain L-12 isolated from deep-sea hydrothermal vents. *J Bacteriol* **149**, 161-165.

Ruby, E. G., Wirsen, C. O. & Jannasch, H. W. (1981). Chemolithoautotrophic sulfur-oxidizing bacteria from the Galapagos Rift hydrothermal vents. *Appl Environ Microbiol* **42**,317-342.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846-849.

Swofford, D. L. (1991) PAUP: phylogenetic analysis using parsimony. Version 3.1 Illionois Natural History survey, Champaign, IL, U.S.A.

Tuttle, J. H., & Jannasch, H. W. (1977). Thiosulfate stimulation of microbial dark assimilation of carbon dioxide in shallow marine environments. *Microb Ecol* **4**, 9-25.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P. & Trüper, H. G. (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463-464.

Wirsen, C. O., Brinkhoff, T., Kuever, J., Muyzer, G., Molyneaux, S., & Jannasch, H. W. (1998) A new *Thiomicrospira* strain from the Mid-Atlantic Ridge compared to known hydrothermal vent isolates. *Appl Environ Microbiol* **64**, 4057-4059.

Wirsen, C. O., Jannasch, H. W. & Molyneaux, S. J. (1993). Chemosynthetic microbial activity at Mid-Atlantic Ridge hydrothermal vent sites. *J Geophys Res* **98,** 9693-9703.

Wood, A. P. & Kelly, D. P. (1989). Isolation and characterization of *Thiobacillus thyasiris* sp. nov., a novel marine facultative autotroph and the putative symbiont of *Thyasira flexuosa*. *Arch Microbiol* **152**, 160-166.

Chapter 6

A polyphasic approach to study the diversity and vertical distribution of sulfur-oxidizing *Thiomicrospira* species in coastal sediments of the German Wadden Sea

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ABSTRACT

Recently, four *Thiomicrospira* strains were isolated from a coastal mud flat of the German Wadden Sea (T. Brinkhoff and G. Muyzer. Appl. Environ. Microbiol. 63:3789-3796, 1997). Here we describe the application of a polyphasic approach to investigate the functional role of these closely related bacteria. Microsensor measurements showed an oxygen penetration in the sediment to a depth of about 2.0 mm. The pH decreased from 8.15 in the overlaying water to a minimum pH of 7.3 at a depth of 1.2 mm. Further down in the sediment the pH increased to about 7.8, and remained constant. Most probable number (MPN) counts of chemolithoautotrophic sulfur-oxidizing bacteria revealed nearly constant numbers along the vertical profile, ranging from 0.93 to 9.3 x 10^5 cells per gram sediment. A specific PCR was used to detect the presence of *Thiomicrospira* cells in these MPN counts, and to obtain their 16S rRNA sequences. The number of *Thiomicrospira* cells did not decrease with depth. It was found that *Thiomicrospira* was not a dominant sulfur-oxidizing bacterium in this habitat. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments followed by hybridization analysis with a genus specific oligonucleotide probe showed the diversity of *Thiomicrospira* strains in the MPN cultures. Sequence analysis of the highest MPN dilutions in which *Thiomicrospira* was detected, revealed the presence of four clusters of several closely related sequences. Only one out of the ten retrieved *Thiomicrospira* sequences was related to known isolates from the same habitat. Slot-blot

hybridization of rRNA isolated from different sediment layers showed that, in contrast to the number of *Thiomicrospira* cells, the *Thiomicrospira*-specific rRNA decreased rapidly below the oxic layer of the sediment. The study indicates the enormous sequence diversity of closely related microorganisms present in one habitat, which so far has only been found by sequencing of molecular isolates. In addition, it gives indication that most of the *Thiomicrospira* populations in the sediment were quiescent.

INTRODUCTION

The rRNA approach (25), the use of ribosomal RNA as molecular marker to detect and identify particular bacteria in their natural habitat (e.g., reference 3), and to explore microbial diversity without the need of cultivation (e.g., references 6, 7) is nowadays routinely used in microbial ecological studies. In some of these studies, the molecular approach was combined with microbiological methods in an attempt to isolate the relevant microorganisms (e.g., reference 13). In other studies molecular biological techniques were used in combination with geochemical techniques, or with the application of microsensors (e.g., see reference 4 for an overview) to characterize environmental parameters. However, the combination of molecular biological techniques, microbiological methods, and geochemical techniques or microsensors has only been used in a few studies (e.g., see the combined papers of Ramsing et al. [28] and Teske et al.

[37]). Yet this combination of techniques and concepts of different disciplines is necessary to obtain a better understanding of the interactions between microorganisms and their natural environment, which is the aim of microbial ecology. Here we describe the use of a comprehensive approach to study the functional role of different closely related *Thiomicrospira* strains in one habitat, the intertidal mud flat.

Thiomicrospira species are chemolithoautotrophic bacteria, that use reduced sulfur compounds as energy source, and CO_2 as carbon source; they are obligate aerobes (18). 16S rRNA sequence comparison showed that they form a monophyletic group within the gamma subdivision of the class Proteobacteria (8, 22). In a recent study we demonstrated the ubiquity of *Thiomicrospira* in environments with reduced sulfur compounds (8). In addition, we were able to isolate *Thiomicrospira* species from most of these habitats, and demonstrated a high species diversity within this genus. Four isolates, i.e., JB-A1, JB-A1F, JB-A2, and JB-B2, were obtained from a sample taken from an intertidal coastal mud flat of the Jadebusen Bay which is part of the German Wadden Sea. Comparative sequence analysis of their 16S rRNA genes demonstrated that these isolates were phylogenetically affiliated to different members of the genus *Thiomicrospira*. Two of the isolates from the Jadebusen sediment, i.e., JB-A1 and JB-A2, were biochemically and physiologically characterized, and were recently described by Brinkhoff and coworkers (9) as new species of the genus *Thiomicrospira* (17), i.e., *Thiomicrospira kuenenii* (JB-A1), and *Thiomicrospira frisia* (JB-A2). The third isolate, strain JB-A1F, had a 16S rRNA sequence identical to *Tms. pelophila* (17). The fourth isolate, JB-B2,

was phylogenetically related to *Tms. crunogena*. Apart from our isolates, 3 other *Thiomicrospira* species, i.e., *Tms. pelophila* (17), *Tms. denitrificans* (39), and *Tms. thyasirae* (46,47) were isolated from this habitat. The presence of several *Thiomicrospira* isolates in one habitat with only minor variations in their genotypical and phenotypical features prompted us to study their abundance and vertical distribution in the sediment with the aim to reveal their niche differentiation. For this purpose, we used tools and techniques from different disciplines. Microsensor measurements were performed on sediment cores to profile environmental parameters, such as oxygen, sulfide and pH (e.g., references 19, 31). In parallel, 2 additional cores were sliced, and the slices were used for subsequent molecular biological and microbiological analysis. The most-probable-number (MPN) technique was used to determine the relative abundance of chemolithoautotrophic sulfur-oxidizing bacteria. A genus specific PCR (8) allowed us to detect the presence of *Thiomicrospira* species in the cultures. Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rDNA fragments (see reference 24 for an overview) obtained after enzymatic amplification with primers for Bacteria, followed by hybridization analysis with a *Thiomicrospira*-specific oligonucleotide probe (8) was applied to identify different *Thiomicrospira* strains in the MPN tubes. In addition, the specific primers for the 16S rDNA of *Thiomicrospira* (8) were used to obtain DNA fragments for sequence analysis. Ribosomal RNA slot-blot hybridization (e.g., references 29, 36) was performed to determine the abundance of 16S rRNA in different

sediment layers, and so to infer the physiological status of present *Thiomicrospira* populations.

The results of this polyphasic approach showed the presence of many different *Thiomicrospira* populations down to a depth of 40 mm. As a significant amount of *Thiomicrospira*-specific rRNA was only present in the oxic layer of the sediment we conclude that only those *Thiomicrospira* populations living in the oxic part of the sediment were metabollically active.

MATERIALS AND METHODS

Sampling. Three cores (Ø 5 cm and 20 cm long) were taken from an intertidal coastal sediment of the Jadebusen Bay, which is part of the German Wadden Sea, on September 25, 1996. Sediment cores were taken during low tide. During high tide the water depth was ca. 35 cm at the sampling side. Air, water and sediment temperature was 8°C, when the samples were taken. The salinity was 35‰. The cores were brought directly into the laboratory for further analysis. In addition, seawater from the same location was also taken.

Microsensor measurements. Microsensor measurements on a sediment core were performed at 15°C in a flow cell with aeration and circulation of filtered Jadebusen water. The oxygen concentration in the flow cell was kept at air saturation. Measurements were done at a light intensity of 830 $\mu E/m^2s$. Microsensors mounted on micro-manipulators

were positioned at the sediment surface under guidance of a dissection microscope. Profiles were recorded by penetrating the sediment in steps of 100 µm using the micromanipulator.

Microsensors were used to measure: oxygen (32), hydrogen sulfide (16, 20), and pH (31). The oxygen and pH electrodes were calibrated as described before (32). The H_2S microsensor was calibrated for total dissolved sulfide by measuring the signal in dilution series of a standard solution (sulfide dissolved in Jadebusen water, flushed with nitrogen to avoid oxidation of sulfide). The concentration of total dissolved sulfide (H_2S, HS^-, S^{2-}) in the dilution series was measured by chemical analysis (11).

Slicing of sediment cores. Cores taken for MPN counts and RNA extraction were sliced by pushing up the sediment in the plexiglas cylinder, which was used for sampling. At every two mm a layer was scraped off with a sharp spatula. The sediment of each layer was stirred before using it for MPN counts to obtain an even distribution of the cells. The sediment samples for RNA isolation were frozen directly in liquid nitrogen and stored at -80°C until further use.

MPN counts of sulfur-oxidizing bacteria. MPN counts were performed for the first four layers of the sediment, i.e., 0-2 mm, 2-4 mm, 4-6 mm, and 6-8 mm depth, and then for every second layer, i.e., 10-12 mm, 14-16 mm, 18-20 mm, etc. Altogether twelve layers, spanning a total sediment depth of about 4 cm were investigated. Tubes with 9.9 ml *Tms. pelophila* (TP) medium (17) were inoculated with 0.1 g of sediment and vigorously mixed. From these tubes dilution series were made in 1 : 10 steps. The cultures were incubated at 20°C in the dark to avoid growth of

phototrophic bacteria. Sulfur-oxidizing bacteria from the different layers were counted in a threefold MPN dilution series. Cultures were incubated for four weeks. Growth was monitored by a color change of the pH indicator. The presence of *Thiomicrospira* cells in the MPN cultures was determined by using a *Thiomicrospira* specific primer set (8) in a PCR with whole cells taken directly from the cultures as sources of template DNA. The numbers of sulfur-oxidizing bacteria and *Thiomicrospira* cells were determined by using the MPN index of the American Public Health Association (5) for three parallels.

Oligonucleotides used in this study. Details of the different oligonucleotides used in this study are presented in Table 1. The oligonucleotides TMS128F and TMS849R are specific for the 16S rDNA of bacteria belonging to the genus *Thiomicrospira* (8) and have been used for the PCR detection of *Thiomicrospira* species in the MPN cultures and for sequencing of obtained PCR products. The primer pair GM5F and 907R amplifies the 16S rDNA of members of the domain Bacteria and was used to obtain 550 bp long fragments for DGGE analysis (22). The probes used in the rRNA slot-blot hybridization were S-*-Univ-1392-a-A-15 (27), which targets the 16S and 18S rRNA of all known forms of life, and S-D-Bact-0338-a-A-18 (2), which is specific for members of the domain Bacteria. Probes TMS849A and TMS849G target the 16S rRNA of members of the genus *Thiomicrospira*; they are modifications of oligonucleotide TMS849R (see Table 1).

TABLE 1. Oligonucleotides used in this study

Name[a]	Sequence (5'to 3')	Target site[b]	T_d/T_a[c]	assay[d]
GM5F[e]	CCT ACG GGA GGC AGC AG	341-357	-/55°C	1
907R[f]	CCG TCA ATT CCT TTR AGT TT	907-926	-/55°C	1
TMS128F[f]	GAA TCT RCC CTT TAG TTG	128-145	-/44°C	1/2
TMS849R[f]	CTT TTT AAT AAG RCC AAC AG	830-849	52°C/44°C	1/2/3
TMS849A	CTT TTT AAT AAG ACC AAC A	831-849	41°C/-	4
TMS849G	CTT TTT AAT AAG GCC AAC A	831-849	41°C/-	4
S-*-Univ-1392-a-A-15[f, g]	ACG GGC GGT GTG TRC	1392-1406	44°C/-	4
S-D-Bact-0338-a-A-18[g]	GCT GCC TCC CGT AGG AGT	338-355	54°C/-	4

[a] F (forward) and R (reverse) designations refer to primer orientation in relation to the rRNA gene.

[b] *E. coli* numbering of the 16S rRNA nucleotides (10).

[c] T_d, dissociation temperature in the hybridization assays; T_a, annealing temperature in the PCR

[d] 1, PCR; 2, sequencing; 3, DNA hybridisation; 4, rRNA slot-blot hybridization.

[e] This primer has a GC-clamp attached to its 5'-end (23)

[f] R, adenine (A)/guanine (G) degeneracy.

[g] Names according to the Oligonucleotide Probe Database (OPD; http://www.cme.msu.edu./OPD/) (1).

T_d determinations of *Thiomicrospira*-specific probes. The temperature of dissociation (T_d), defined as the temperature at which half of the bound probe is released from the hybrid, was determined by a graded-temperature wash series as previously described (29). RNAs isolated from *Tms. frisia* (9) and *Tms. crunogena* (15) were used for the T_d determinations of the *Thiomicrospira* specific oligonucleotides TMS849A and TMS849G. Probe TMS849A has no mismatch to the 16S rRNA of *Tms. frisia,* and only one mismatch to the 16S rRNA of *Tms. crunogena.* The opposite is true for probe TMS849G; no mismatch with the 16S rRNA of *Tms. crunogena,* and one mismatch with the 16S rRNA of *Tms. frisia.* Both probes had a T_d of 41°C and were pooled for general detection of *Thiomicrospira*-rRNA in slot-blot hybridization. The combination of both probes matches the 16S rRNA of all known *Thiomicrospira* species.

rRNA extraction and slot-blot hybridization. Nucleic acids were isolated by bead beating, phenol extraction and isopropanol precipitation based on the method described by Stahl et al. (36), and MacGregor et al. (21) with slight modifications. Sediment samples were resuspended in $NaPO_4$-buffer (120 mM, pH 8) with 1% (wt/vol) polyvinylpolypyrrolidone (PVPP, acid washed according to Holben et al. [14]), and subsequently mixed with phenol-chloroform-isoamylalcohol (PCI) equilibrated with TE-buffer (100 mM Tris, pH 8, 10 mM EDTA) to extract the nucleic acids. After precipitation, the nucleic acids were resuspended in DNAse-buffer (6 m M $MgCl_2$, 40 mM Tris, pH 7.5), treated with DNAse for 30 min, extracted again with PCI, and precipitated with ethanol. RNA was blotted onto nylon membranes (Magna Charge, Micron Separations, Mass.) in triplicates and

probed with radioactively labeled oligonucleotides as described previously (36). For the general probes, the membranes were prehybridized, and hybridized at 40°C, and washed at 44°C for probe S-*-Univ-1392-a-A-15, and at 54°C for probe S-D-Bact-0338-a-A-18. For the *Thiomicrospira*-specific probes prehybridization and hybridization were done at 30°C, and washing at 41°C. Intensity of the hybridization signal was measured with a Phosphor Imager (Molecular Dynamics, Sunnyvale, Ca.) and quantified according to an *Escherichia coli* rRNA standard (Boehringer, Mannheim, Germany).

PCR amplification of 16S rDNA fragments. PCR amplifications were performed as described by Muyzer et al. (22). A touchdown PCR was performed for primer pair GM5F and 907R. For primer pair TMS128F and TMS849R (annealing temperature of 44°C) no touchdown PCR was used.

DGGE analysis of PCR products. DGGE was performed using the D-Code™ system (Bio-Rad Laboratories, Inc.). The following conditions were used: 1 mm thick, 6% (w/v) polyacrylamide gels, 1x TAE electrophoresis buffer (pH 8.3), 20-70% denaturant, and an electrophoresis time of 20 h at a constant voltage of 100 V (see reference 23 for details). After electrophoresis, the gels were stained with ethidium bromide and photographed as described before (23).

Hybridization analysis of blotted DGGE patterns. Denaturing gradient gel patterns were transferred to nylon membranes (Hybond-N^+) by electroblotting and hybridized with the Dig-labeled *Thiomicrospira* specific probe TMS849R, as described before (8).

Sequencing of PCR products. PCR products were purified by using the Qiaquick PCR purification kit (Qiagen Inc., Chatsworth, Calif.). The *Taq* Dideoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) was used to sequence the 16S rDNA fragments. Sequencing primers were the *Thiomicrospira* specific primer TMS128F and TMS849R (8). The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer.

Comparative analysis of 16S rRNA sequences. Alignments of the 16S rRNA sequences were prepared as described before (8). A phylogenetic tree was created using the neighbor-joining algorithm with maximum likelihood as a model of sequence evolution, as implemented in the test version of the program PAUP 4 developed by David Swofford. Bootstrap analysis (1000 replicates) was used to validate the reproducibility of the branching pattern of the trees.

Nucleotide sequence accession numbers. The sequences obtained in this study are available from EMBL Nucleotide Sequence Database under accession numbers AJ011066 - AJ011075.

RESULTS

Microsensor measurements. Oxygen measurements in the sediment core showed a concentration of 250 µM O_2 in the water overlying the sediment, and a decrease from 225 µM O_2 at the sediment surface to 0 µM O_2 at a depth of 2.0 mm (Fig. 1). The pH measurements showed pH values

of about 8.15 in the overlying water. From the sediment surface down to a depth of 1.2 mm the pH decreased to values of about 7.3. From 1.2 mm to 6.5 mm depth the pH increased to about pH 7.8, and remained constant at lower depth (Fig. 1). H_2S was not detectable in the first 4 cm of the sediment.

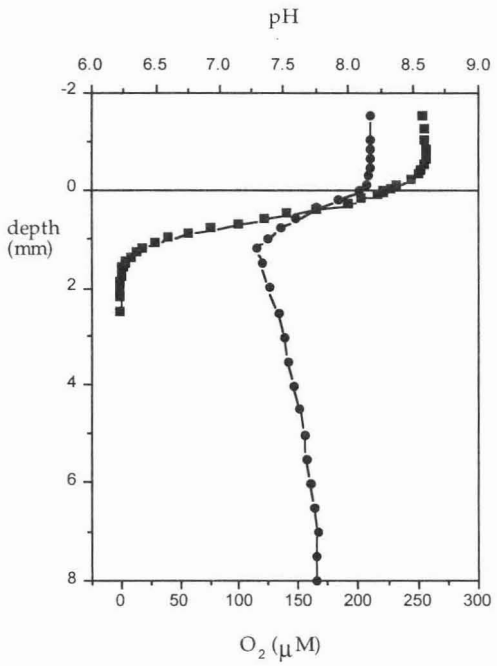

FIG. 1. Oxygen (■) and pH (●) microprofiles in a sediment core from the Jadebusen Bay. Note that oxygen was only found in the top 2.0 mm of the sediment. Sulfide was also measured, but could not be detected. The sediment surface is at depth 0 mm. Negative values of depth indicate the measurements in the water phase overlaying the sediment; positive values indicate the depth in the sediment.

MPN counts of sulfur-oxidizing bacteria. Cultivable chemolithoautotrophic sulfur-oxidizing bacteria from different layers of the Jadebusen sediment were counted by MPN serial dilution. The results of growth in the MPN counts are shown in Figure 2. Numbers between 0.93 to 9.3 x 10^5 cells of sulfur-oxidizing bacteria per gram sediment were found in nearly all layers. The only exception is the second layer (2-4 m m depth) which showed a slightly higher value of 4.3 x 10^6 cells per gram sediment. However, there is no significant decrease of sulfur-oxidizing bacteria with depth.

Screening of the MPN cultures for the presence of *Thiomicrospira*. To obtain cell numbers of *Thiomicrospira* present in the sediment layers, the MPN cultures were tested with a *Thiomicrospira* specific PCR. The results are given in Figure 2. They indicate that *Thiomicrospira* cells are present in all sediment layers which have been investigated, down to 4 cm depth. Furthermore, *Thiomicrospira* cells were found in every layer in relatively constant numbers, between 0.93 and 9.3 x 10^3 cells per gram sediment. Comparison of the cell numbers of sulfur-oxidizing bacteria and *Thiomicrospira* showed that the latter is only a minor part (ca. 1%) of the sulfur-oxidizing bacterial populations.

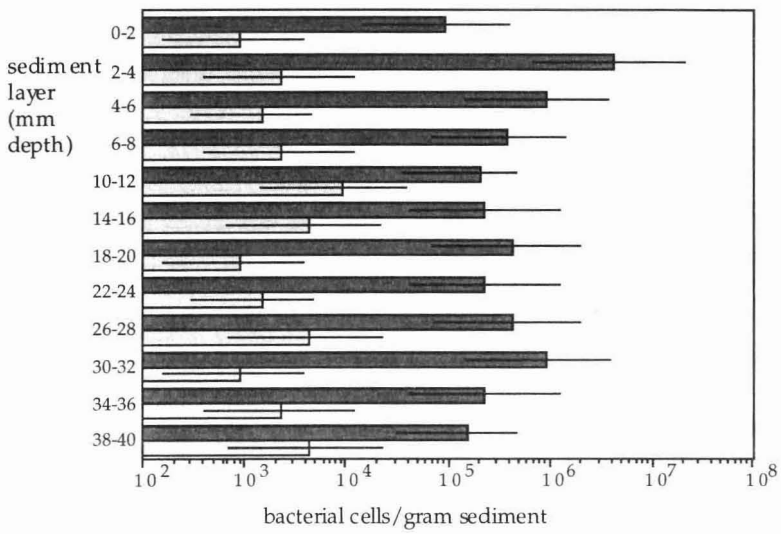

FIG. 2. Most-Probable-Number (MPN) counts of chemolithoautotrophic sulfur-oxidizing bacteria (dark bars) in sediment samples from the Jadebusen Bay, an intertidal coastal mud flat area of the German Wadden Sea. The *Thiomicrospira* cell numbers (light bars) were determined by enzymatic amplification of the 16S rDNA using genus-specific primers. The errors bars indicate 95% confidence intervals.

DGGE and hybridization analysis of MPN cultures. Figures 3A and 3C show the DGGE results of 16S rDNA fragments obtained from the lowest and highest MPN dilution in which *Thiomicrospira* was detected. PCR products from known *Thiomicrospira* strains isolated from the Wadden Sea sediment (8, 9, 18) were applied to both sides of the gels as

standards (Fig. 3A and 3C, lanes 1 and 2, and lanes 15 and 16). The bacterial diversity in the MPN cultures, indicated by the number of bands, ranged from one band (see Fig. 3A, lanes 3, 4, 6, 9, and Fig. 3C, lanes 8, 10, 12, 14) to six bands (Fig. 3A, lane 8). Bands halting at the same position in the gels were rare, indicating a high diversity of chemolithoautotrophic sulfur-oxidizing bacteria in these cultures.

Hybridization analysis of these DGGE patterns with the *Thiomicrospira*-specific probe TMS849R gave positive signals with bands from all *Thiomicrospira* species used as standards (Fig. 3B and 3D, lanes 1, 2, 15 and 16) as well as with one or more bands of every tested MPN culture (compare lanes 3 to 14 in Fig. 3A, with the same lanes in Fig. 3B, and lanes 3 to 14 in Fig. 3C, with the same lanes in Fig. 3D). Some of the bands in the DGGE patterns did not hybridize with the *Thiomicrospira* specific probe (compare lanes 8 and 14 in Fig. 3A, with the same lanes in Fig. 3B, and lanes 4 and 6 in Fig. 3C, with the same lanes in Fig. 3D) indicating the growth of other bacteria under these culture conditions.

Two or more positive hybridization signals in one lane indicated the presence of two or more *Thiomicrospira* strains in the respective culture. This was mainly the case for MPN cultures at the lowest dilution (compare lane 11 in Fig. 3A with the same lane in Fig. 3B, and lanes 5, 7, 11, and 13 in Fig. 3C, with the same lanes in Fig. 3D). However, also in one of the highest dilutions hybridization signals from two bands were obtained (compare lane 4 in Fig. 3C with lane 4 in Fig. 3D).

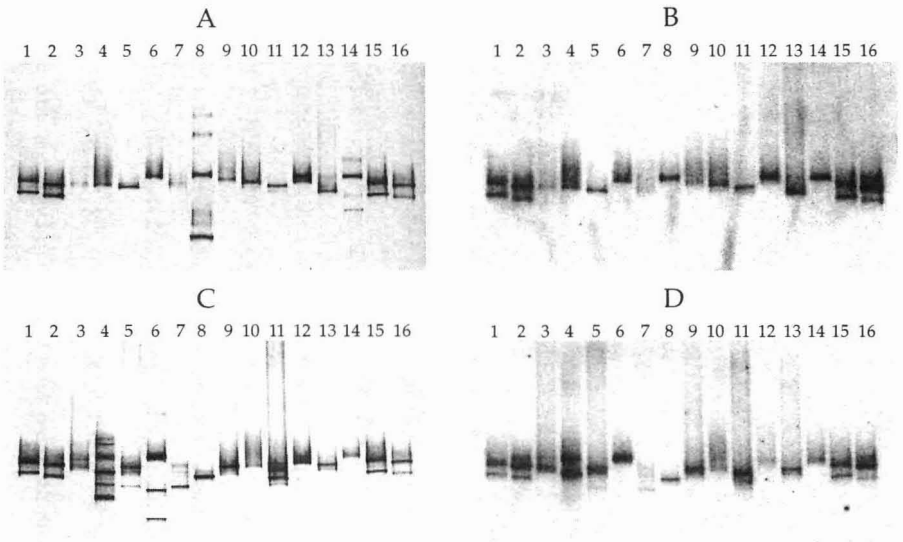

FIG. 3. Hybridization analysis of DGGE profiles of 16 rDNA fragments obtained with primers specific for Bacteria, and template DNA from MPN cultures and *Thiomicrospira* isolates from coastal sediments of the Wadden Sea. (A) DGGE patterns. Lanes: 1 and 15; *T. pelophila* (lower band) and *Thiomicrospira* sp. str. JB-B2 (upper band); 2 and 16, *Thiomicrospira kuenenii* (str. JB-A1) (lower band) and *Thiomicrospira frisia* (str. JB-A2)(upper band); 3, lowest dilution MPN culture of the sediment layer 0-2 mm dept; 4, highest dilution MPN culture of sediment layer 0-2 mm depth in which *Thiomicrospira* could still be detected; 5 and 6, equivalent MPN cultures of 2-4 mm depth; 7 and 8, equivalent MPN cultures of 4-6 mm depth; 9 and 10, equivalent MPN cultures of 6-8 mm depth; 11 and 12, equivalent MPN cultures of 10-12 mm depth; 13 and 14, equivalent MPN cultures of 14-16 mm depth. (B) Hybridization analysis of DGGE pattern A with the *Thiomicrospira* specific, Dig-labeled oligonucleotide, whose target sequence is located within the rDNA amplified.
(C) DGGE patterns. Lanes: 1, 2 , 15, and 16, *Thiomicrospira* standards (see panel A); 3 and 4, equivalent MPN cultures of 18-20 mm depth; 5 and 6, equivalent MPN cultures of 22-24 mm depth; 7 and 8, equivalent MPN cultures of 26-28 mm depth; 9 and 10, equivalent MPN cultures of 30-32 mm depth; 11 and 12, equivalent MPN cultures of 34-36 mm depth; 13 and 14, equivalent MPN cultures of 38-40 mm depth. (D) Hybridization analysis of DGGE pattern C. Comparison of panels A and B, and C and D, also shows the presence of chemolithoautotrophic bacteria other than *Thiomicrospira* in some of the MPN cultures.

Phylogenetic analysis of *Thiomicrospira* sequences. Although the *Thiomicrospira* strains from the highest dilutions in which *Thiomicrospira* was present were not isolated in pure cultures, partial sequences of the 16S rDNA from nearly all of these MPN cultures could be obtained by using the *Thiomicrospira*-specific primer pair for amplification and sequencing. Both DNA strands of approximately 700 bp long fragments were sequenced. Only for the MPN cultures of two sediment layers (18-20 mm and 34-36 mm depth) mixed sequences were obtained. These sequences showed ambiguities at positions where differences in *Thiomicrospira* 16S rDNA occur, indicating the presence of more then one *Thiomicrospira* strain in these cultures. This is in agreement with the hybridization result of the DGGE pattern of the respective culture from 18-20 mm depth where two positive signals were obtained (Fig. 3D, lane 4), but for the culture from 34-36 mm depth only one signal was obtained (Fig. 3D, lane 12).

Phylogenetic analysis of the obtained sequences showed that all MPN isolates group with the known *Thiomicrospira* sequences (Fig. 4). The sequences obtained in this study are grouped in four clusters in the phylogenetic tree. No correlation was found between this phylogenetic grouping and sediment depth; cluster 1 contains sequences from 0-2 mm, 6-8 mm, and 30-32 mm depth, cluster 2 from 2-4 mm, and 38-40 mm depth, cluster 3 from 4-6 mm, 10-12 mm, 14-16 mm, and 22-24 mm depth, and the sequence from a depth of 26-28 mm is grouping with the sequences of *Tms. pelophila*, *Tms. thyasirae*, and *Thiomicrospira* sp. str. JB-A1F (cluster 4). Within each cluster less then 1.8% sequence difference was found,

while between each cluster, or with other *Thiomicrospira* sequences more than 3% sequence difference was obtained.

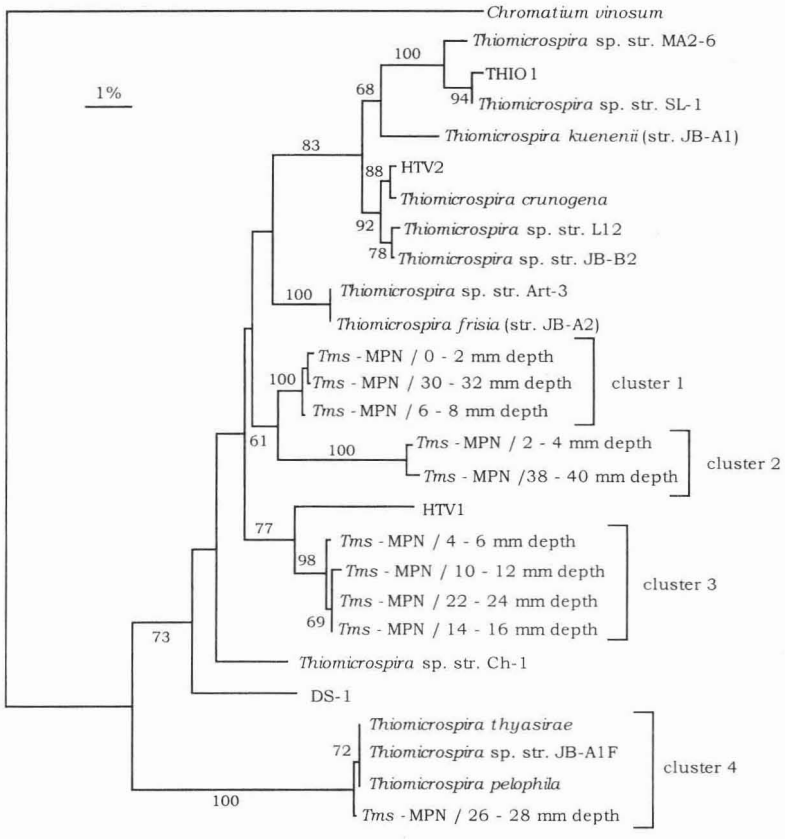

FIG. 4. Unrooted tree showing the phylogenetic relationships of *Thiomicrospira* strains present in the highest MPN dilutions for *Thiomicrospira*. The tree is based on partial 16S rRNA sequences and was produced by using the neighbor joining algorithm with maximum likelihood correction. The sequences determined in this study are indicated as *Tms-MPN/x-y mm depth*. The sequence of *Chromatium vinosum* was used as an outgroup. The numbers on the branches refer to bootstrap values (1000 replicates); only values above 50% are shown.

rRNA slot-blot hybridization. The unexpected presence of relatively high numbers of *Thiomicrospira* cells in the anoxic part of the sediment prompted us to determine the metabolic state of these bacteria. Ribosomal RNA was extracted from sliced sediment samples, and used in rRNA slot-blot hybridization. The samples were hybridized with probes specific for all known forms of life (universal probe), specific for members of the domain Bacteria, and with a probe specific for *Thiomicrospira* (see Table 1). The following sediment layers were investigated: 0-2 mm, 2-4 mm, 4-6 mm, 6-8 mm, 10-12 mm, 14-16 mm, 26-28 mm, and 38-40 mm depth. The results are summarized in Figure 5. While the concentration of bacterial rRNA was relatively constant, varying with depth from 0.95 to 1.27 μg per gram sediment (data not shown), the vertical profile of *Thiomicrospira*-specific rRNA showed a steep decrease within the first 6 mm of the sediment; within the layers measured between the 7 and 40 mm depth the *Thiomicrospira* rRNA never exceeded more than 25% of the specific rRNA concentration reached in the first 2 mm of the sediment.

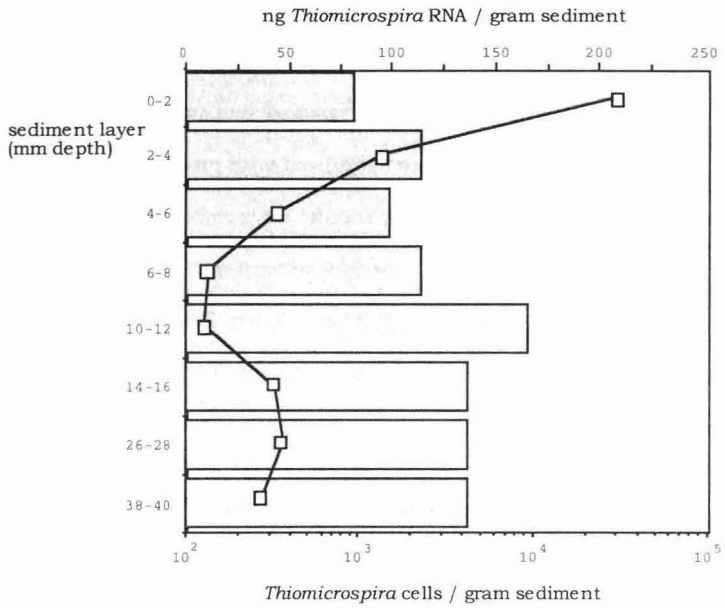

FIG. 5. Amount of *Thiomicrospira*-specific rRNA in sediment samples from different depths and MPN counts of *Thiomicrospira* cells for these layers. The results indicate the presence of a small, but active *Thiomicrospira* population in the oxic surface layer of the sediment.

DISCUSSION

Microsensor measurements for oxygen indicated an oxygen penetration in the sediment to a depth of 2.0 mm. However, it must be stated that the Wadden Sea sediment is a very dynamic habitat with diel and seasonal fluctuations in environmental conditions, which have a great influence on the oxygen sulfide and pH gradients in the sediments. For instance, Rasmussen and Jørgensen (30) found that oxygen could penetrate to a depth of 1.3 mm in summer, and to a depth of 5.1 mm during winter in a coastal sediment in Aarhus Bay, Denmark. Also Visscher and coworkers (40, 41, 42) found diel and seasonal fluctuations in oxygen and sulfide profiles for microbial ecosystems from coastal mud flats.

Free sulfide could not be detected in this sediment core, which might be the result of the low in situ water temperature of 8°C at the time that the core was taken. It has been demonstrated by Vosjan (43) that the sulfate reduction rate in the Wadden Sea sediment is temperature dependent, and that it was 10 times lower at 4°C than at 18°C in an experiment with lactate addition to the sediment to stimulate sulfate reduction. Thamdrup et al. (38) reported that H_2S was not detectable in coastal marine sediment of Aarhus Bay, Denmark, although sulfate reduction proceeded at significant levels. This rapid removal of H_2S was attributed to reactions with Fe and Mn oxides. This process might also occur in the Wadden Sea sediment.

The pH profile is consistent with the oxygen profile. There is no net photosynthesis, and the initial decrease of the pH is caused by oxidation of organic material, whereby the minimum in the pH profile corresponds with the depletion of oxygen. The increase in pH is probably caused by denitrification or by reduction of iron or manganese.

Comparison of the numbers of chemolithoautotrophic sulfur-oxidizing bacteria with the numbers of *Thiomicrospira* cells (see Fig. 2) indicates that *Thiomicrospira* is not a dominant sulfur-oxidizer in the Wadden Sea sediments. The *Thiomicrospira* populations are about 1% of the total number of sulfur-oxidizing bacteria detected in this study. This was confirmed by the absence of hybridization signals in DGGE profiles of 16S rDNA fragments obtained after PCR amplification of total community DNA from sediment samples (data not shown). However as mentioned before, the results described are just of one moment in time; the abundance of *Thiomicrospira* might differ at different seasons. Visscher and van Gemerden (41) found different MPN values for different sulfur bacteria, including the colorless sulfur bacteria to which *Thiomicrospira* is associated in spring and fall, and a decrease in these values with depth. Furthermore, *Thiomicrospira* appears to be more abundant in other habitats, such as in hydrothermal vent systems (22).

DGGE-hybridization analysis of the MPN cultures suggest that some *Thiomicrospira* strains are opportunistic microorganisms, rapidly growing under the culture conditions used (see Fig. 3). Growth of sulfur-oxidizing bacteria was determined by a color change of the pH indicator present in the culture medium. The presence of *Thiomicrospira* in the cultures

corresponded with the cultures where fastest growth was obtained for (data not shown). Growth in other tubes was generally observed at least one week later. This indicates that *Thiomicrospira* spp. belong to the fastest growing sulfur-oxidizing bacteria under these culture conditions.

Comparison of the hybridized DGGE patterns shows that different *Thiomicrospira* spp. were present in the MPN cultures of the lowest and the highest dilution from most sediment layers. Only in the cultures from 0-2 mm depth (Fig. 3A and B, lanes 3 and 4) and from 30-32 mm depth (Fig. 3C and D, lanes 9 and 10), the *Thiomicrospira* spp. in both cultures might have been identical, because the hybridized bands of the lowest and the highest dilutions ran at the same position in the gel. It has been shown before that numerically lower populations may overgrow numerically superior populations that are less adapted to the enrichment conditions employed (34, 44). This causes problems if only enrichment cultures are used to study bacterial populations, because then only the fast-growing bacteria are obtained. To avoid this problem dilution of the inoculum to extinction may favor the recovery of numerically superior populations that are less adaptive (44). The use of the MPN technique allows not only the determination of the number of bacteria which are able to grow with a particular substrate, it also makes the detection of the most abundant of these bacteria possible. However, it should be mentioned that there might be *Thiomicrospira* populations present which might not be able to grow under the cultivation conditions used.

Comparison of the sequences presented in this paper with those published before from the same habitat (8, 22) showed, with one exception,

the absence of previously isolated strains in the highest MPN dilutions for *Thiomicrospira*. This indicates that most of these isolates, which were all obtained with enrichment techniques (8, 17, 46, 47) do not belong to the most abundant *Thiomicrospira* spp. Only the sequence of 'Tms-MPN/26-28 mm depth' showed a high similarity (greater than 99,8%) to the sequence of *Tms. pelophila*, and related species. The other new sequences formed 3 monophyletic clusters with no close known relative. Differences between sequences within one cluster were always less than 1.8%, which by the definition of Stackebrandt and Goebel (35) might be regarded as sequences coming from different strains of the same species. However, the differences of more than 3% between sequences of the different clusters, and other *Thiomicrospira* strains, might indicate the presence of additional new species in this habitat.

Slot-blot hybridization of rRNA extracted from the different sediment layers was used to infer the metabolic activity of the *Thiomicrospira* populations. The total amount of rRNA depends on the number of cells and the amount of rRNA per cell. Cells which are well growing have a higher number of ribosomes, and thus a higher amount of rRNA, than cells which are not growing (27). While cell numbers for *Thiomicrospira* were relatively constant along the whole vertical profile of the sediment, *Thiomicrospira*-specific rRNA decreased sharply from the oxic to suboxic zone of the sediment (Fig. 5). By combining the MPN and rRNA slot-blot hybridization results we conclude that *Thiomicrospira* is a metabolically active member of the bacterial community within the first 4 mm of the sediment, from the oxic to suboxic region, while their relative

activity is low in the anoxic zone of the sediment. This finding is in agreement with physiological characteristics of *Thiomicrospira* species, which, with the exception of *Tms. denitrificans*, are all obligate aerobes (9, 15, 17). *Tms. denitrificans* is able to grow anaerobically with nitrate as electron acceptor (39). However, 16S rRNA sequence analysis showed that *Tms. denitrificans* is phylogenetically affiliated to the genus *Thiovulum* belonging to the epsilon subdivision of the class Proteobacteria (22). The oligonucleotides designed for the detection of *Thiomicrospira* do not hybridize with *Tms. denitrificans* (8).

In recent years many investigations on the exploration of microbial diversity in natural samples have demonstrated a high diversity of closely related sequences within one environment (e.g., reference 6, 12). However, no information in these studies was obtained about the metabolic state of the microorganisms. Here we also found a high diversity of closely related sequences, all belonging to different populations of *Thiomicrospira*. However, by the polyphasic approach performed in this study, we have indications that these populations show decreasing growth rates depending on their localization in the sediment. This leads to the conclusion that *Thiomicrospira* is only metabolically active in the oxic part of the sediment and that most of the *Thiomicrospira* populations, found in the anoxic part of the sediment, are probably dormant. The strategy of how these populations survive under anaerobic conditions is unknow, and might be the aim of future studies.

Similar results have also been described by Wawer and coworkers (45), who demonstrated, by comparative DGGE analysis of [NiFe]

hydrogenase gene fragments obtained by enzymatic amplification of bacterial genomic DNA and total RNA from microbial communities, that only one *Desulfovibrio* population out of the 5 which were present, expressed the [NiFe] hydrogenase gene after addition of hydrogen as substrate, indicating niche differentiation.

The question remains why the number of *Thiomicrospira* cells in the anoxic layers of the sediment is relatively constant. It might partly be explained by mixing of the sediment by physical turbation, as caused by waves, what can have a potential impact on the upper few centimeters of the sediment. Furthermore, bioturbation by benthic animals is known to influence particle transport (33, 48) and to have an effect on the distribution of microorganisms in the sediment.

Comparison of the physiological features of three *Thiomicrospira* species, *Tms. pelophila, Tms. kuenenii,* and *Tms. frisia,* isolated from the Wadden Sea sediment showed differences in pH range, and salt tolerance (9). Changes in pH values and salt concentrations might occur in an intertidal coastal mud flat. For this reason it is likely that different *Thiomicrospira* populations are adapted to different environmental conditions, and that populations which are inactive at one moment, might be active at another, when specific environmental conditions favor their growth.

In summary, we demonstrated the existence of many different *Thiomicrospira* populations in the intertidal mud flat habitat. However, these populations are not a dominant proportion (ca. 1%) of the sulfur-oxidizing bacterial community, and most of them are quiescent. Only

those populations present in the oxic sediment layers seem to be metabolically active. From these results we conclude that the functional role of *Thiomicrospira* in intertidal mud flats is minor, and only limited to the oxic part of the sediment, and that other sulfur-oxidizing bacteria might be mainly responsible for the oxidation of reduced sulfur compounds produced by sulfate reduction.

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REFERENCES

1. **Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin.** 1996. The Oligonucleotide Probe Database. Appl. Eviron. Microbiol. **62:**3557-3559.

2. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide

probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919-1925.

3. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59**:143-169.

4. **Amann, R. I., and M. Kühl.** 1998. In situ methods for assesment of microorganisms and their activities. Current Opinion in Microbiology**1:** 352-358.

5. **American Public Health Association.** 1969. Standard methods for the examination of water and wastewater, including bottom sediments and sludge, p. 604-609. American Public Health Association, Washington, D. C.

6. **Barns S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace.** 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc. Natl. Acad. Sci. USA **91**:1609-1613.

7. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62**:1935-1943.

8. **Brinkhoff, T. and G. Muyzer**. 1997. Increased species diversity and extended habitat range of sulfur-oxidizing *Thiomicrospira* spp. Appl. Environ. Microbiol. **63**:3789-3796.

9. **Brinkhoff, T., G. Muyzer, C. O. Wirsen, and J. Kuever.** 1998. Characterization of *Thiomicrospira kuenenii* sp. nov. and *Thiomicrospira frisia* sp. nov., two mesophilic obligately chemolithoautotrophic sulfur-

oxidizing bacteria isolated from an intertidal mud flat. Int. J. Syst. Bacteriol, in press.

10. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148**:107-127.

11. **Cline, J. D.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. **14**:454-458.

12. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) **345**:60-63.

13. **Großkopf, R., P. H. Janssen, and W. Liesack.** 1998. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl. Environ. Microbiol. **64**:960-969.

14. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**:703-711.

15. **Jannasch, H. W., C. O. Wirsen, D. C. Nelson, and L. A. Robertson.** 1985. *Thiomicrospira crunogena* sp. nov., a colorless sulfur-oxidizing bacterium from a deep-sea hydrothermal vent. Int. J. Syst. Bacteriol. **35**:422-424.

16. **Jeroschewski, P., C. Steuchart, and M. Kühl.** 1996. An amperometric microsensor for the determination of H_2S in aquatic environments. Anal. Chem. **68**:4351-4357.

17. **Kuenen, J. G. and H. Veldkamp.** 1972. *Thiomicrospira pelophila,* gen. n., sp. n., a new obligately chemolithotrophic colourless sulfur bacterium. Antonie van Leeuwenhoek **38**:241-256.

18. **Kuenen, J. G., L. A. Robertson, and O. H. Tuovinen.** 1992. The genera *Thiobacillus, Thiomicrospira,* and *Thiosphaera,* p. 2638-2657. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer, Berlin, Germany.

19. **Kühl, M., and N. P. Revsbech.** 1998. Microsensors for the study of interfacial biogeochemical processes. *In* B. P. Boudreau, and B. B. Jørgensen (ed.), The benthic boundary layer, Oxford Univ. Press, Oxford. In press.

20. **Kühl, M., C. Steuckart, G. Eickert, and P. Jeroschewski.** 1998. A H_2S microsensor for profiling biofilms and sediments: Application in an acidic lake sediment. Aquat. Microb. Ecol. In press.

21. **MacGregor, B. J., D. P. Moser, E. W. Alm, K. H. Nealson, and D. A. Stahl.** 1997. Crenarchaeota in Lake Michigan sediment. Appl. Environ. Microbiol. **63:**1178-1181.

22. **Muyzer, G., A. Teske, C. O. Wirsen, H. W. Jannasch.** 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. **164**:165-172.

23. **Muyzer, G., T. Brinkhoff, U. Nübel, C. Santegoeds, H. Schäfer, and C. Wawer.** 1998. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, 3.4.4:1-27. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.). Molecular microbial ecology manual,3 rd ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.

24. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek, **73**: 127-141.

25. **Olsen, G. J., D. J. Lane, S. J. Giovannoni, and N. R. Pace.** 1986. Microbial ecology and evolution: a ribosomal RNA approach. Ann. Rev. Microbiol. **40**:337-365.

26. **Pace, N. R., D. A. Stahl, D. J. Lane, and G. J. Olsen.** 1986. The analysis of natural microbial populations by ribosomal RNA sequences. Adv. Microb. Ecol. **9**:1-55.

27. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59**:1354-1360.

28. **Ramsing, N. B., H. Fossing, T. G. Ferdelman, F. Andersen, and B. Thamdrup.** 1996. Distribution of bacterial populations in a stratified fjord (Mariager Fjord, Denmark) quantified by in situ hybridization and related to chemical gradients in the water column. Appl. Environ. Microbiol. **62**:1391-1404.

29. **Raskin, L., J. M. Stromley, B. E. Rittmann, and D. A. Stahl.** 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl. Environ. Microbiol. **60**:1232-1240.

30. **Rasmussen, H., and B. B. Jørgensen.** 1992. Microelectrode studies of seasonal oxygen uptake in a coastal sediment: role of molecular diffussion. Mar. Ecol. Prog. Ser. **81**: 289-303.

31. **Revsbech, N. P., and B. B. Jørgensen.** 1986. Microelectrodes: their use in microbial ecology. Adv. Microb. Ecol. **9:**293-352.

32. **Revsbech, N. P.** 1989. An oxygen microelectrode with a guard cathode. Limnol. Oceanogr. **55:**1907-1910.

33. **Rhoads, D. C.** 1974. Organism sediment relations on the muddy sea floor. Oceanogr. Mar. Biol. Ann. Rev. **12**:263-300.

34. **Santegoeds, C. M., S. C. Nold, and D. M. Ward.** 1996. Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from hot spring cyanobacterial mat. Appl. Environ. Microbiol. **62**:3922-3928.

35. **Stackebrandt, E. and B. M. Goebel.** 1994. Taxonomic Note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44**:846-849.

36. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54:**1079-1084.

37. **Teske, A., C. Wawer, G. Muyzer, and N. B. Ramsing.** 1996. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl. Environ. Microbiol. **62**:1405-1415.

38. **Thamdrup, B., H. Fossing, and B. B. Jørgensen.** 1994. Manganese, iron, and sulfur cycling in a coastal marine sediment, Aarhus Bay, Denmark. Geochim. Cosmochim. Acta **58**:5115-5129.

39. **Timmer-ten Hoor, A.** 1975. A new type of thiosulfate oxidizing, nitrate-reducing microorganism: *Thiomicrospira denitrificans* sp. nov. Neth. J. Sea Res. **9**:343-351.

40. **Visscher P. T., J. Beukema, and H. van Gemerden.** 1991. In situ characterization of sediments: measurements of oxygen and sulfide profiles with a novel combined needle electrode. Limnol. Oceanogr. **36**: 1476-1480.

41. **Visscher P. T., and H. van Gemerden.** 1993. Sulfur cycling in laminated marine ecosystems. *In* Oremland, R.S. (ed.) Biogeochemistry of Global Change: Radiatively active trace gases. Chapman and Hal, New York, pp. 672-690.

42. **Visscher P. T., and F. P. van den Ende.** 1994. Diel and spatial fluctuations of sulfur transformations. *In* Stal L.J., Caumette P. (eds.) Microbial mats. Structure, development and environmental significance. NATO ASI Ser. Vol. G35. Springer, Berlin, pp. 353-359.

43. **Vosjan, J. H.** 1975. Ecologische en fysiologische aspecten van bacteriële sulfaatreductie in het waddengebied. Ph.D.-thesis Rijksuniversiteit Groningen, Groningen, The Netherlands.

44. **Ward, D. M., C. M. Santegoeds, S. C. Nold, N. B. Ramsing, M. J. Ferris and M. M. Bateson.** 1997. Biodiversity within hot spring microbial mat communities: molecular monitoring of enrichment cultures. Antonie van Leeuwenhoek **71**:143-150.

45. **Wawer, C., M. S. M. Jetten, and G. Muyzer.** 1997. Genetic diversity and expression of the [NiFe] hydrogenase large-subunit gene of *Desulfovibrio* spp. in environmental samples. Appl. Environ. Microbiol. **63**:4360-4369.

46. **Wood, A. P., and D. P. Kelly.** 1989. Isolation and physiological characterization of *Thiobacillus thyasiris* sp. nov., a novel marine

facultative autotroph and the putative symbiont of *Thyasira flexuosa*. Arch. Microbiol. **152**:160-166.

47. **Wood, A. P., and D. P. Kelly.** 1993. Reclassification of *Thiobacillus thyasiris* as *Thiomicrospira thyasirae* comb. nov. an organism exhibiting pleomorphism in response to environmental conditions. Arch. Microbiol. **159**:45-47.

48. **Yingst, J. Y., and D. C. Rhoads.** 1980. The role of bioturbation in the enhancement of bacterial growth rates in marine sediments. *In* K. L. Tenore, and B. C. Coull (ed.), Marine Benthic Dynamics, p. 407-421. University of South Carolina Press, Columbia.

Chapter 7

Distribution and diversity of sulfur-oxidizing *Thiomicrospira* spp. at a shallow water hydrothermal vent in the Aegean Sea (Milos, Greece)

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ABSTRACT

A shallow-water hydrothermal vent system in the Aegean Sea close to the island of Milos (Greece) was chosen to study the distribution and diversity of the chemolithoautotrophic sulfur-oxidizing bacterium *Thiomicrospira*. Different regions around a solitary vent were investigated with a combination of microbiological and molecular biological methods. Physicochemical parameters from an accompanying study, investigating the general bacterial community structure of the same vent (Sievert et al., 1998) completed our work. The results showed an inverse relationship between the abundance of *Thiomicrospira* spp. and the environmental parameters. Cell numbers decreased towards the center of the vent (horizontal distribution), as well as with depth (vertical distribution), corresponding with an increase in temperature and a decrease in pH. *Thiomicrospira* was found to be one of the most abundant sulfur-oxidizers and was even dominant in one region. The predominance of one particular *Thiomicrospira* population was indicated by the isolation of this organism from a high dilution enrichment, its identification in environmental samples by hybridization analysis of DGGE patterns, and sequencing of the excised DGGE band. These results support earlier indications that *Thiomicrospira* spp. are dominant members of hydrothermal vent communities.

INTRODUCTION

Members belonging to the genus *Thiomicrospira* are chemolithoautotrophic bacteria, obtaining energy from reduced sulfur compounds, and carbon from CO_2 (Kuenen et al., 1992; Brinkhoff et al., 1998d). Although *Thiomicrospira* spp. were detected in and isolated from several different environments (e.g., Brinkhoff and Muyzer, 1997; Wirsen et al., 1998), their ecological importance remained obscure for most of these habitats. Several isolates were obtained from intertidal mud flats (Kuenen and Veldkamp, 1972; Wood and Kelly 1989, 1993; Brinkhoff and Muyzer, 1997), but in a recent study it was found that only low numbers of *Thiomicrospira* cells were present in this habitat, pointing to a minor role of these microorganisms (Brinkhoff et al., 1998b). On the other hand studies on bacterial species composition in deep-sea hydrothermal vent systems indicated a more important role of *Thiomicrospira*. By using molecular biological methods, i.e., DGGE analysis and sequencing of PCR amplified 16S rDNA fragments, it was demonstrated that *Thiomicrospira* spp. were dominant community members of hydrothermal vent sites at the Mid-Atlantic Ridge (Muyzer et al., 1995). One of the ecological functions of *Thiomicrospira* in these systems, where light and photosynthesis are absent, is primary production (Jannasch and Wirsen, 1979). The main chemosynthetic usable chemical energy at these vents are reduced sulfur compounds. This and the biochemical versatility of

Thiomicrospira spp. seems to be the key to their predominance at the vents (Jannasch and Mottl, 1985).

Hydrothermal vent systems are not only located in the deep-sea. They have also been described for several shallow-water areas (e.g., Trager and DeNiro, 1990; Vidal et al., 1978; Sorokin, 1991; Tarasov et al., 1990; Hashimoto et al., 1993; Olafsson et al., 1991). These shallow water vents differ from their deep-sea counterparts by the presence of light (Tarasov et al., 1990), and can be influenced by wave action (van den Ende, 1997). Furthermore, the biology of these ecosystem is different, too; e.g., at shallow-water vents photosynthetic organisms, such as benthic micro-algae, can be present (Tarasov et al., 1990; Dando et al., 1995a).

Offshore the Greek island Milos in the Aegean Sea shallow-water vents were found at depths varying from the littoral zone to 115 m (Dando et al., 1995a). A wealthy macrofauna depending on endosymbiotic bacteria is absent at the Milos vents (Dando et al., 1995a; Thiermann et al., 1997). Until now the only sulfur-oxidizing bacteria (SOB) described for the hydrothermal vents around Milos were thiobacilli and *Achromatium volutans* (Dando et al., 1995a; van den Ende, 1997). These identifications were only based on microscopical observations. In case of *Achromatium volutans* this approach is well suited because of the particular morphological features of this organism. But for thiobacilli microscopical identification is more problematic, because of a lack of conspicous morphological features. The rod-shaped morphology might easily be confused with, e.g., the recently described rod-shaped isolates of *Thiomicrospira* (Brinkhoff et al., 1998a, c).

Nowadays detection and identification of microorganisms is performed indirectly by sequencing or probing of their 16S rRNA or its encoding gene. For our purpose, we have developed specific primers and probes, which are used in PCR or hybridization analysis to identify and characterize *Thiomicrospira* spp. in environmental samples, enrichments, and most-probable-number (MPN) cultures (Brinkhoff and Muyzer, 1997; Brinkhoff et al., 1998b). Here we present the results of an investigation to determine the distribution and diversity of *Thiomicrospira* populations in the shallow-water hydrothermal vent system of Milos, in an attempt to understand the ecological importance of these microorganisms.

MATERIALS AND METHODS

Sampling. Samples were taken in June and September 1996, and in June 1997 at a solitary vent site, located at a water depth of 8 m in the Palaeochori Bay of the Greek island Milos in the Aegean Sea. For a detailed site description see Sievert et al. (1998). Sediment cores and water samples from the sediment surface were taken with polycarbonate cylinders (∅ 36 mm) by SCUBA diving, along a transect, at several distances from the vent center. In June 1996 samples were taken at 10, 123, 165, and 235 cm distance from the vent center, and in September 1996 at 30, 117, and 200 cm distance. For the cores taken at 123 and 117 cm the surface consisted of a white precipitate, which had a thickness of 2 to 5 mm. In September 1996 a second core was taken at 117 cm distance, one week later than the other

samples, at which time no white precipitate was present. The distances were chosen according to different physicochemical parameters found at these places, as described in the accompanying paper by Sievert et al. (1998). The cores were brought to the shore, sliced immediately and further processed for MPN counts and DNA extraction.

Subsampling of sediment cores. For all samples taken in June 1996 the following layers were investigated: water directly from above the sediment surface, and sediment samples from 0 - 10, 10 - 20, and 20 - 30 mm depth. For samples taken in September 1996 cores were sliced with a finer resolution as follows: water directly from above the sediment surface, and sediment from 0 - 5, 8 - 13, and 16 - 26 mm depth. Dilutions for the MPN series were performed directly after slicing and subsamples of the same layers for DNA extraction were frozen immediately in liquid N_2 until further processing.

MPN counts of sulfur-oxidizing bacteria. MPN counts for samples taken in September 1996 were performed in three paralleles as described by Sievert et al. (1998). For each core also a MPN count with water directly from the sediment surface was performed. With the second core taken at a distance of 117 cm a MPN series, in which the salinity was increased to 55‰ by adding NaCl, was performed. This salinity was almost the same as from the outflowing brine (Thierman et al., 1997). This MPN series was carried out to investigate whether there are SOB specifically adapted to the higher salt concentration. All other cultures had a salinity of 30‰. The cultures were incubated at their approximate in situ temperatures as shown in Figure 1. Growth was monitored by a color change of the pH

indicator and checked microscopically. The presence of *Thiomicrospira* cells in the MPN cultures was determined by using a *Thiomicrospira* specific primer set in a PCR with cells taken directly from the cultures (Brinkhoff and Muyzer, 1997). The numbers of SOB and *Thiomicrospira* cells were determined by using the MPN index of the American Public Health Association (1969) for three parallels.

Isolation and cultivation of bacteria. High dilution enrichments were performed with samples taken in June 1996 and June 1997, with the intention to obtain the most abundant *Thiomicrospira* species. The medium used and the culture conditions were the same as for the MPN cultures and are described by Sievert et al. (1998). Aliquots from the tubes were transferred on solid agar plates, containing the same culture medium and 1% (wt/vol) agar (DIFCO). All known *Thiomicrospira* species and strains isolated so far form intensively yellow colored colonies, because of sulfur precipitation. Additionally they all produce acid. Colonies with these characteristics were chosen for identification with a *Thiomicrospira* specific PCR (Brinkhoff and Muyzer, 1997). The obtained positive colonies were transferred at least three times to be considered as pure.

Nucleic acid extraction. DNA was extracted from sediment and water samples by the method described by Zhou et al. (1996), modified by Sievert et al. (1998). Two g of the frozen samples were first subjected 5 cycles of thawing at 30°C and freezing in liquid nitrogen. Then 5.4 ml extraction buffer (100 mM TRIS [pH 8], 100 mM EDTA [pH 8], 100 mM Na-phosphate [pH 8], 1.5 M NaCl, and 1% (wt/vol) hexa-decyl-trimethyl-

ammonium bromide [CTAB]) was added, together with 40 µl proteinase K (Boehringer Mannheim, 10 mg/ml) and incubated at 37°C for 30 min on a horizontal shaker. Thereafter 0.6 ml 20% (wt/vol) SDS was added and incubated at 65°C for 2 h in a water-bath with gentle end-over-end inversions every 25 min. After centrifugation at 6000x *g* for 10 min the supernatant was collected. The pellet was again extracted with extraction buffer and 20% (wt/vol) SDS at 65°C for 10 min and the supernatants were combined. It followed the addition of an equal volume of chloroform:isoamylalcohol and centrifugation at 6000x *g* for 10 min. The aqueous phase was collected and the genomic DNA was precipitated by addition of 0.6 volumes of isopropanol. Following an incubation for 1 h at room temperature the precipitate was centrifuged at 14,000x *g* for 25 min, the supernatant decanted and the pellet washed with icecold 80% (wt/vol) ethanol, by centrifuging at 14,000x *g* for 15 min. After drying the pellet under vacuum it was resuspended in 100 µl of TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA [pH 8]), and stored at -20°C until further analysis.

Oligonucleotides used for PCR. The oligonucleotides TMS128F and TMS849R are specific for the 16S rDNA of bacteria belonging to the genus *Thiomicrospira* (Brinkhoff and Muyzer, 1997). With these primers about 700 bp long 16S rDNA fragments are obtained. The primers have been used for the identification of *Thiomicrospira* isolates and for the detection of bacteria belonging to this genus in the MPN cultures. The PCR products obtained from the highest MPN dilutions *Thiomicrospira* could be detected in were sequenced, to determine the phylogenetic affiliation of the respective organisms.

Oligonucleotides GM3F and GM4R are specific for the 16S rDNA of Bacteria and were used as primers in a PCR to amplify the nearly complete (1,500 bp) encoding gene. The PCR products obtained with these primers were used for sequencing and to determine the phylogenetic affiliation of the isolated bacterial strains.

The primer pair GM5F and 907R amplifies the 16S rDNA of Bacteria and was used to obtain 550 bp long fragments for DGGE analysis. The sequences of both primer pairs (GM3F and GM4R, GM5F and 907R) have been published before by Muyzer et al. (1995).

PCR amplification of 16S rDNA fragments. PCR amplifications were performed as described by Muyzer et al. (1995). A touchdown PCR (Don et al., 1991) was performed for primer pair GM5F and 907R (annealing temperature from 65 to 55°C in 20 cycles). For primer pair TMS128F and TMS849R (annealing temperature of 44°C), and GM3F and GM4R (annealing temperature of 40°C) no touchdown PCR was used. Amplification products were analyzed as described by Muyzer et al. (1995), before further characterization by DGGE analysis or DNA sequencing.

DGGE analysis of PCR products. DGGE was performed using the D-Gene™ system (Bio-Rad Laboratories, Inc.). The protocol as described by Brinkhoff and Muyzer (1997) was used: 1 mm thick, 6% (wt/vol) polyacrylamide gels, 1 x TAE electrophoresis buffer (pH 8.3), 20-70% denaturant, 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 (Muyzer et al., 1998).

Hybridization analysis of blotted DGGE patterns. Denaturing gradient gel patterns were transferred to nylon membranes (Hybond-N$^+$, Amersham, UK) by electroblotting and hybridized with the Dig-labeled *Thiomicrospira* specific probe TMS849R, as previously described (Brinkhoff and Muyzer, 1997).

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 for the nearly complete 16S rDNA of bacterial isolates were GM3F, GM1F, and GM4R (Buchholz-Cleven et al., 1997). Sequencing primers for *Thiomicrospira* 16S rDNA fragments obtained from MPN cultures with the specific primer pair were the same *Thiomicrospira* specific oligonucleotides TMS128F and TMS849R (Brinkhoff and Muyzer, 1997). One band in the DGGE gel, which hybridized with the *Thiomicrospira* specific probe TMS849R, was excised from the gel, reamplified with the primer pair GM5F and 907R, and sequenced with the *Thiomicrospira* specific primer TMS849R. 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 Ribosomal Database Project (RDP; Maidak et al., 1997) and GenBank (Benson et al., 1997). Sequence alignments were prepared with the sequence editor SEQAPP (Gilbert, 1992). Phylogenetic trees were created using the neighbor joining

algorithm with maximum likelihood correction as implemented in the test version of PAUP 4 developed by Swofford.

Nucleotide sequence accession numbers. The sequences obtained in this study are available from EMBL Nucleotide Sequence Database under accession numbers xxxx.

RESULTS

MPN counts of sulfur-oxidizing bacteria and *Thiomicrospira* cells. Cultivable aerobic chemolithoautotrophic SOB and *Thiomicrospira* cells of samples taken in September 1996 from different zones and layers of the sediment, as well as from the overlaying water, were counted by MPN serial dilution. A *Thiomicrospira* specific PCR (Brinkhoff and Muyzer, 1997) was used to screen the MPN cultures for the presence of *Thiomicrospira* cells (Brinkhoff et al., 1998b). The results are shown in Figure 1. Numbers of SOB and *Thiomicrospira* cells were variable for the different zones and layers. Generally the numbers of SOB varied between not detectable and 1.4 x 10^6 cells/g sediment (wet weight). For *Thiomicrospira* cells the numbers were between not detectable and 2.7 x 10^5.

In the first zone, with 30 cm distance from the vent center, no or only low numbers of SOB up to 2.4 x 10^2 cells/g sediment could be obtained. *Thiomicrospira* cells were not detectable in the MPN cultures of this zone (Fig. 1A).

In the second zone, at a distance of 117 cm from the vent center, the numbers of SOB and *Thiomicrospira* cells were different for the two investigated cores (Fig. 1B and 1C). The surface of the first core (Core I, Fig. 1B) contained the white preciptate mentioned above, which was absent at the surface of the second core (Core II, Fig. 1C). Core II was used for the MPN counts with the increased salinity. For Core II the cell numbers obtained for the three sediment layers were slightly lower than those obtained for Core I. However, cell numbers, which were obtained for the surface of Core II were significant lower (Fig. 1C). The highest numbers for SOB and *Thiomicrospira* in this zone were obtained with the sample from the sediment surface of Core I, which contained the white precipitate (4.1×10^4 and 1.9×10^4, respectively [see Fig. 1B]).

For the third zone, at 200 cm distance of the vent center, the lowest numbers of SOB and *Thiomicrospira* cells were obtained for the sediment surface (Fig. 1D). Highest numbers of SOB (1.4×10^6) were found in the second sediment layer (8-13 mm depth), while highest numbers of *Thiomicrospira* cells (2.7×10^5) were found in the first sediment layer (0-5 mm depth). Here *Thiomicrospira* species were also the dominant SOB. Comparison of the different zones revealed generally the highest numbers for SOB and *Thiomicrospira* cells in the sediment layers of the third zone.

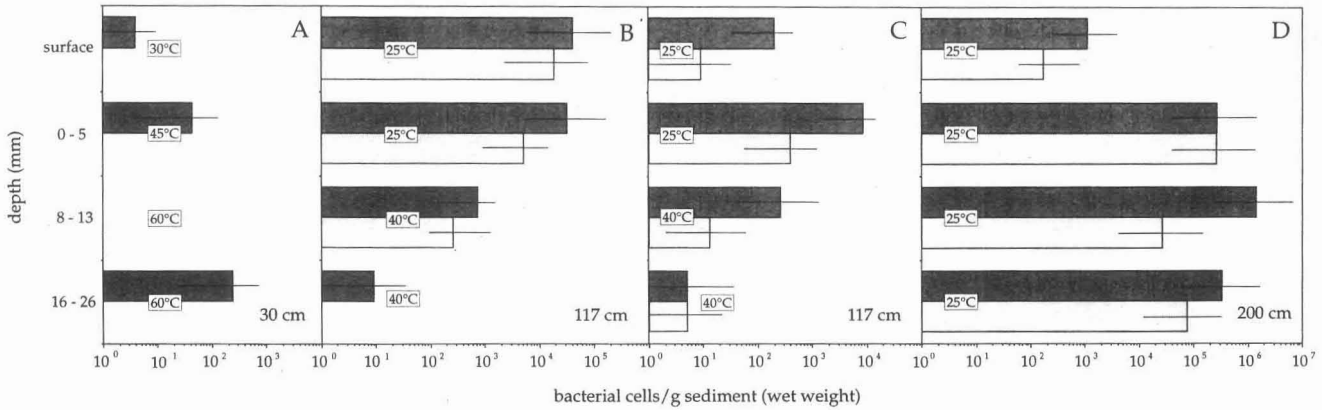

Fig. 1: Most probable number (MPN) counts of chemolithoautotrophic sulfur-oxidizing bacteria (dark bars) in sediment samples from the shallow-water hydrothermal vent system of Milos. The *Thiomicrospira* cell number (light bars) were determined by enzymatic amplification of the 16S rDNA using genus-specific primers. The error bars indicate 95% confidence intervalls. The temperature values shown are the incubation temperatures of the cultures.

Isolation of bacteria in pure cultures. Two isolates, which were both obtained from 10^{-5} dilutions of the enrichments, gave a positive signal with the *Thiomicrospira* specific PCR. Strain Milos T-1 was received from a sample taken in June 1996 from the upper sediment layer, underneath the white precipitate, at a distance of 123 cm of the vent center. Strain Milos T-2 was isolated directly from the white precipitate in June 1997. While isolate Milos T-1 is vibrio-shaped as most other *Thiomicrospira* spp., strain Milos T-2 is rod-shaped like two recently described new *Thiomicrospira* species, i.e., *T. frisia* (Brinkhoff et al., 1998a) and *T. chilensis* (Brinkhoff et al., 1998c). The two strains Milos T-1 and Milos T-2 were further characterized by sequencing of their 16S rRNA genes.

Detection of *Thiomicrospira* by DGGE and hybridization analysis. Figures 2A and 2C show DGGE profiles of 16S rDNA fragments obtained from extracted DNA from sediment and water samples after enzymatic amplification with primers specific for Bacteria. These samples were the same as those used for the MPN counts. The only exception is the water sample (surface layer) taken in June 1996 from the third zone (165 cm distance), for which no PCR product could be obtained. Figure 2A shows DGGE patterns of samples taken in June 1996, Figure 2C of samples from September 1996. PCR products of the new *Thiomicrospira* isolates Milos T-1 and Milos T-2 were used on both sides of the gels as markers (Fig. 2A, lanes 1 and 2, and lanes 18 and 19; Fig. 2C, lanes 1 and 2, and lanes 19 and 20).

The MPN counts indicated that *Thiomicrospira* species belong to the dominant SOB in this habitat. Hybridization analysis of DGGE profiles with a *Thiomicrospira* specific probe (Fig. 2B and 2D) was performed to determine the relative abundance of *Thiomicrospira* spp., and to try to confirm the presence of the *Thiomicrospira* isolates Milos T-1 and Milos T-2.

Figure 2B shows the hybridization analysis of the DGGE pattern of samples taken in June 1996. Positive signals were obtained with the bands from the two isolates and with bands from samples of different zones and layers. For the first zone, at a distance of 10 cm from the vent center, a signal was obtained for the water above the sediment (Fig. 2B, lane 3). For the second zone (123 cm distance) signals were obtained for the water above the sediment and the first sediment layer (Fig. 2B, lanes 7 and 8). No signals were obtained for the third zone (Fig. 2B, 165 cm distance). For the fourth zone (235 cm distance) a signal was obtained for the water above the sediment (Fig. 2B, lane 14) and a weak signal for the first sediment layer (Fig. 2B, lane 15).

Figure 2D shows the hybridization analysis of the DGGE pattern of samples taken in September 1996. Positive signals were here also obtained for the bands belonging to the two *Thiomicrospira* isolates and for bands of different zones and layers. For the first zone, at a distance of 30 cm from the vent center, signals were obtained for the first, second, and third sediment layer (Fig. 2D, lanes 4, 5, and 6), but not for the water above the sediment (Fig. 2D, lane 2). For the two cores taken in the second zone (117 cm distance) slightly different results were obtained. For the first core (Fig.

2D, lanes 7-10) signals for the surface (Fig. 2D, lane 7), and all sediment layers (Fig. 2D, lanes 8-10) were obtained (although for the second layer (lane 9) only a weak signal). For the second core (Fig. 2D, lanes 11-14) only weak signals were obtained for the surface and the second sediment layer (Fig. 2D, lanes 11 and 13, respectively). For the third zone (200 cm distance) a signal was only obtained for the first sediment layer (Fig. 2D, lane 16).

All hybridization signals obtained with the *Thiomicrospira* specific probe run in both gels at the same position, as the bands belonging to strain Milos T-2 (Fig. 2B, lanes 2 and 19, and Fig. 2D, lanes 2 and 20), what indicates identical or nearly identical sequences.

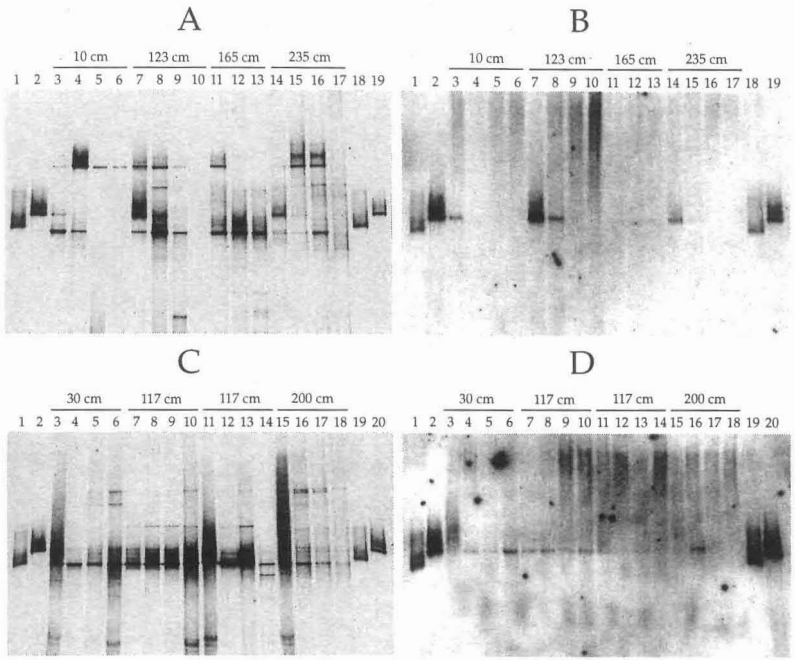

Fig. 2: Hybridization analysis of DGGE profiles of 16S rDNA fragments obtained with primers specific for Bacteria, and template DNA from environmental samples along a transect at a shallow-water hydrothermal vent of Milos and *Thiomicrospira* isolates obtained from the same location. A: DGGE patterns of samples taken in June 1996.Lanes 1 and 18, isolate *Thiomicrospira* sp. str. Milos T-1; lanes 2 and 19, isolate *Thiomicrospira* sp. str. Milos T-2; lanes 3 - 6, samples taken at 10 cm distance from the vent centre (3, surface; 4, 0 - 10 mm depth; 5, 10 - 20 mm depth; 6, 20 - 30 mm depth); lanes 7 - 10, equivalent samples taken at 123 cm distance; lanes 11 - 13, samples taken at 165 cm distance (11, 0 - 10 mm depth; 12, 10 - 20 mm depth; 13, 20 - 30 mm depth); lanes 14 - 17, equivalent samples to lanes 3 - 6, but taken at 235 cm distance. B: hybridization analysis of pattern A with a *Thiomicrospira* specific, Dig-labeled oligonucleotide, whose target sequence is located within the rDNA amplified.
C: DGGE patterns of samples taken in September 1996. Lanes 1 and 19, isolate *Thiomicrospira* sp. str. Milos T-1; lanes 2 and 20, *Thiomicrospira* sp. str. Milos T-2; lanes 3 - 6, samples taken at 30 cm distance from the vent centre (3, surface; 4, 0 - 5 mm depth; 5, 8 - 13 mm depth; 6, 16 - 26 mm depth); lanes 7 - 10, equivalent samples taken at 117 cm distance (Core I); lanes 11 - 14, equivalent samples taken at 117 cm distance (Core II); lanes 15 - 18, equivalent samples taken at 200 cm distance. D: hybridization analysis of pattern C with the *Thiomicrospira* specific probe.

Phylogenetic analysis of *Thiomicrospira* sequences. To receive phylogenetic information the nearly complete 16S rRNA encoding genes of the two new obtained bacterial isolates, i.e., *Thiomicrospira* sp. strain Milos T-1 and strain Milos T-2, were sequenced. Phylogenetic analysis confirmed the affiliation of the isolates to the genus *Thiomicrospira* (Fig. 3). However, the two new isolates are not closely related to each other and are located in different subclusters of the tree. Strain Milos T-1 is related to *Thiomicrospira kuenenii* , and *Hydrogenovibrio marinus* (Fig. 3, cluster 1). The latter differs from *Thiomicrospira* spp. only in the ability to use hydrogen as electron donor (Brinkhoff et al., 1998d). Strain Milos T-2 is grouped with sequences from the MPN cultures (see below) and the excised DGGE band ML-1, all obtained during this study (Fig. 3, cluster 2). Band ML-1, which run in the DGGE patterns at the same position as the band from strain Milos T-2, and hybridized with the *Thiomicrospira* specific probe was excised from a DGGE gel and subsequently sequenced. Comparison with the sequence of strain Milos T-2 resulted in one basepair difference out of a total of ca. 470 bp (99.8% sequence similarity).

Although the *Thiomicrospira* species from the highest dilutions of the MPN counts performed in September 1996 were not isolated, partial sequences of their 16S rDNA could be determined by using the *Thiomicrospira*-specific primer pair for amplification and sequencing. Both DNA strands of the about 700 bp long fragments were sequenced. Only for one culture a mixed sequence was obtained (Tms-MPN/Milos-DIV5). This sequence showed ambiguities at positions where differences in

Thiomicrospira 16S rDNA occur, what indicates that more then one *Thiomicrospira* sequence type was present in this culture.

The phylogenetic analysis of the molecular isolates obtained from the MPN cultures showed that these sequences are also monophyletic with the known *Thiomicrospira* sequences (Fig. 3). The new sequences are present in different branches of the phylogenetic tree of *Thiomicrospira*. Cluster 2 contains the sequences of the bacterial isolate strain Milos T-2, the excised DGGE band ML-1, one sequence of the third zone (200 cm distance = D sequences), and with one exception the sequences from MPN cultures with the increased salinity from the second zone (117 cm distance, 55‰ salinity = C sequences). The remaining sequence from the latter zone was obtained from the deepest sediment layer (Tms-MPN/Milos-CIV1) and is the deepest branching *Thiomicrospira* sequence obtained so far (see Fig. 3). Cluster 3 contains the three sequences obtained from the MPN cultures of the second zone (117 cm distance) with 30‰ salinity (B sequences), and additionaly two sequences from the third zone.

Generally the obtained sequences were highly related. The only exceptions were the sequences of strain Milos T-1 and the deep branching sequence Tms-MPN/Milos-CIV1. Within each of the clusters 2 and 3 very high sequence similarities of more than 99% were found. Between these two clusters differences ranged from 1 to 2%. Only comparison of the sequence differences of the excised band ML-1 and sequences of cluster 3 revealed slightly lower values due to several sequence ambiguities of the former sequence.

Comparison of the sequences obtained from the two MPN counts of the second zone (B and C sequences) showed that all sequences from the MPN counts with 30‰ salinity (B sequences) were identical and are therefore grouped in cluster 3. With the only exception of the deep branching sequence Tms-MPN/Milos-CIV1 all sequences of the MPN counts with the increased salinity of 55‰ (C sequences) were also highly similar to each other (more than 99.3% similarity) and are grouped in cluster 2. The sequences obtained from the third zone (D sequences) are also identical or highly similar to the sequences of cluster 2 and 3, but they are scattered in these two clusters. Between the sequences of cluster 2 and 3 and earlier obtained sequences differences of at least 2.5% were determined.

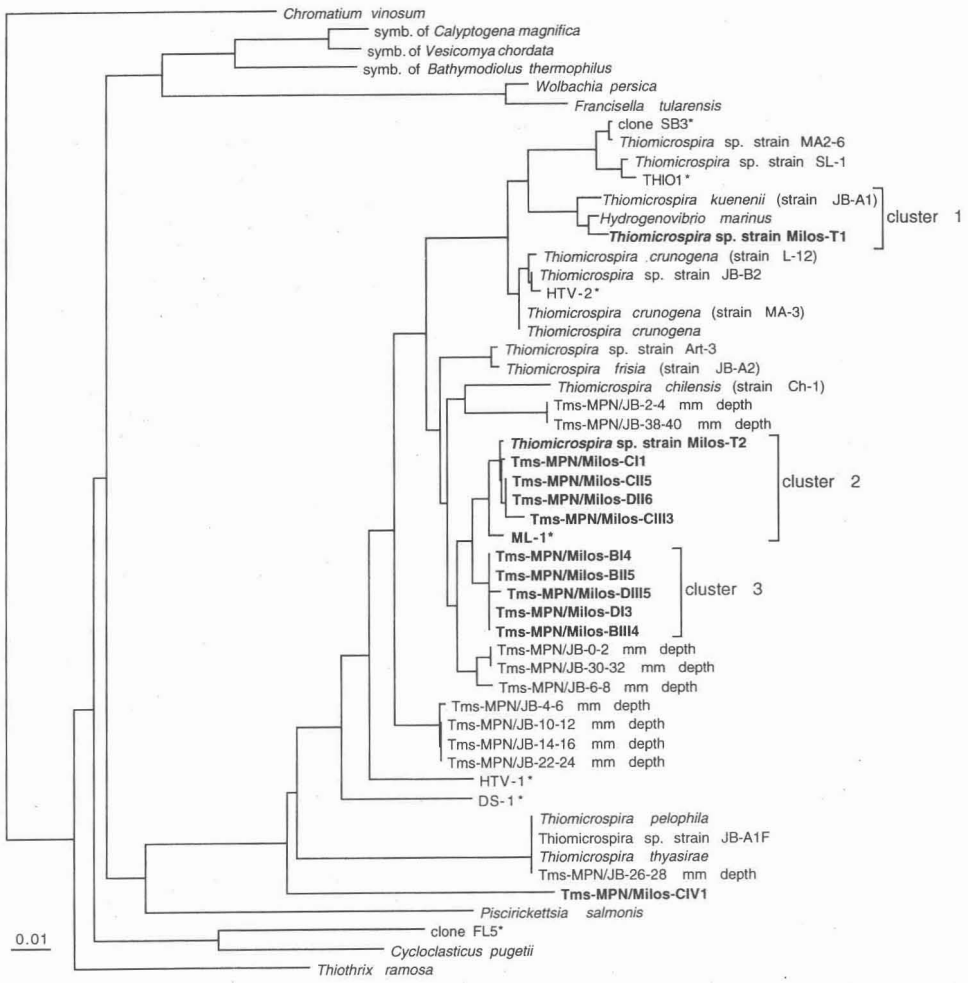

Fig. 3: Neighborjoining tree obtained after 16S rRNA sequence comparison showing the phylogenetic affiliation of two new *Thiomicrospira* isolates (strain Milos-T1 and Milos-T2) and MPN isolates. The sequences determined in this study are bold typed. Molecular isolates are marked with an asterisk. The scale bar represents 0.01 estimated nucleotide change per sequence position.

DISCUSSION

The high abundance of *Thiomicrospira* cells in some zones of the shallow-water hydrothermal vent system of Milos can probably be attributed to high concentrations of reduced sulfur compounds, like at the deep-sea vents (Jannasch and Mottl, 1985). Outflowing hot water from the vents at the Milos system contains among other reduced elements hydrogen sulfide (H_2S) in concentrations up to 1.5 mM (Dando et al., 1995b) what promotes the growth of chemoautotrophic sulfur-oxidizing bacteria. Additionally high amounts of CO_2 were measured for deep-sea hydrothermal vents (Welhan and Craig, 1983; Mottl and Holland, 1978) and the Milos system (Dando et al., 1995b; van den Ende, 1997) what might also favour the growth of chemolithoautotrophic bacteria compared to heterotrophic bacteria.

In the accompanying study (Sievert et al., 1998) temperature and pH values were determined at several distances from the vent center and for several sediment depths. From 20°C in the surrounding water and sediment the temperature raised towards the center, and the pH values were decreasing simultaneously. Temperatures of 25 to 55°C, and pH values between 5.7 and 5.0 were measured for the layers of the first zone (30 cm distance) investigated in this study. Oxygen penetration into the sediment increased with greater distance from the vent center (Sievert et al., 1998). These gradients might explain the low numbers of SOB in

general and the absence of *Thiomicrospira* cells at 30 cm distance as determined by the MPN approach.

At 117 cm distance numbers of SOB and *Thiomicrospira* cells were decreasing with depth. While the conditions of this zone are moderate at the sediment surface, temperatures of more than 40°C and pH values of 5.5 and lower at the deeper layers seem to inhibit growth of SOB and *Thiomicrospira*. The numbers of SOB and *Thiomicrospira* cells as determined in the parallel MPN count with an increased salinity of 55‰, were slightly lower for the sediment layers, than those obtained at 30‰, even though the higher value reflected the in situ concentration of the outflowing brine. This indicates that SOB adapted to a higher salinity are not dominant. The significant lower numbers of SOB and *Thiomicrospira* cells determined for the surface layer of the MPN counts with the increased salinity might be due to the absence of the white precipitate of the respective sample.

At 200 cm distance moderate conditions seem to allow the presence of higher numbers of SOB and *Thiomicrospira* cells also in deeper layers. The predominance of *Thiomicrospira* cells in the first sediment layer of the third zone shows that here the environmental parameters favor the growth specifically of members of this genus. *Thiomicrospira* cells were found here also in deeper layers, where no oxygen is present (Sievert et al., 1998), in nearly the same order of magnitude. This phenomenon was described earlier for this genus in sediment of an intertidal mud flat (Brinkhoff et al., 1998b). However, in the latter habitat *Thiomicrospira*

populations were found to be metabolically acitve only in the oxic part of the sediment.

Comparison of the informations obtained from the MPN results with those from the DGGE and hybridization analysis shows deviations for the presence of *Thiomicrospira*. No *Thiomicrospira* cells were detected with the MPN counts in the first zone, i.e., in 30 cm distance of the vent center. On the other hand, hybridization analysis of both DGGE patterns gave positive signals for this zone. For samples taken in June 1996 a signal was obtained for the sediment surface (Fig. 2B, lane 3). This could be due to different environmental conditions at this time, allowing growth of *Thiomicrospira*, compared to the conditions determined in September 1996 when the MPN counts were performed. However, the physicochemical parameters described by Sievert et al. (1998) should actually allow growth of *Thiomicrospira* spp. at the surface of this region.

The presence of *Thiomicrospira* in the sediment layers of the first zone of samples taken in September 1996, as determined by DGGE and hybridization analysis (Fig. 2D, lanes 4 to 6), is in contradiction to the MPN results and the environmental parameters, which were measured at the same time. It is unlikely that *Thiomicrospira* cells grew in these layers since the in situ temperature was above the upper growth limit of the known species (Kuenen et al., 1992; Brinkhoff et al., 1998d). Additionally oxygen was not present in these layers (Sievert et al., 1998).

The presence or absence of bands belonging to *Thiomicrospira* is not the only difference between the two DGGE patterns. Comparison of Figure 2A with Figure 2C shows several differences in the banding patterns,

indicating differences in the bacterial communities. Most likely resuspension of the upper sediment layers due to a storm, previous to the sampling was responsible for this difference (Sievert et al., 1998).

A positive hybridization result from a DGGE pattern indicating the presence of a particular bacterial group can not be compared quantitatively with a MPN result. In previous studies it was shown that the sensitivity of DGGE analysis of PCR products obtained with bacterial primers was about 1%, meaning that a particular bacterial population representing 1% of the total community could still be dectected in the DGGE profile (Muyzer et al., 1993; Murray et al., 1996). The apparent contradiction between the MPN results, which indicate high numbers of *Thiomicrospira* for the outer zone, and a single positive hybridization signal for this zone from the samples taken each in June and in September 1996, might be explained with the general higher amount of other bacterial cells in this zone (Sievert et al., 1998) in relation to the *Thiomicrospira* population. On the other hand indicates the hybridization signal of the strong band in the Figure 2A lane 7 a high abundance of *Thiomicrospira* cells in relation to the remaining bacterial population of this sample.

The hybridization analysis of DGGE patterns with the environmental samples from June and September 1996 showed that all bands, which gave positve signals, run at the same height as those from the new isolated *Thiomicrospira* strain Milos T-2. Based on these data it can be derived that this is apparently the dominant *Thiomicrospira* sequence type of this ecosystem. This was confirmed by the isolation of this organism from a high dilution enrichment of a sample taken in June 1997

and additionally the sequence similarity of a band excised from a DGGE gel, having 99.8% sequence homology to strain Milos T-2. However, sequence homology of 16S rDNA does not always mean that organisms are also physiologically equal. Recently Wirsen et al. (1998) demonstrated that three *Thiomicrospira* strains with almost identical 16S rDNA sequences show clear physiological differences. Surprinsingly no band corresponding to the band of strain Milos T-1 was found in the DGGE patterns, and no similar sequence was obtained from the MPN cultures, even though strain Milos T-1 was also isolated from a high dilution enrichment as strain Milos T-2. This finding can not be explained by the present data.

Most described *Thiomicrospira* strains are vibrio- or spiral shaped (Brinkhoff et al., 1998d). However, strain Milos T-2 is rod-shaped and falls phylogenetically in one subcluster of the *Thiomicrospira* branch, which contains recently described rod-shaped isolates, i.e., *T. frisia*, *T. chilensis* (Brinkhoff et al., 1998a, c) and *Thiomicrospira* sp. str. Art-3 (Brinkhoff and Muyzer, 1997). This indicates a common ancestor for strains sharing this morphotype. Within this cluster even most of the sequences obtained from the MPN cultures of this study can be found and also several MPN sequences, which were obtained from an intertidal mud flat habitat (Brinkhoff et al., 1998b) (see Figure 3). This indicates the presence of many non isolated rod-shaped *Thiomicrospira* strains in the environment.

Almost all sequences presented in this study show differences much lower than 2%. Therefore physiological adaptations to the different zones of the studied vent system might be possible without greater differences in the 16S rDNA. An indication for this is, that all sequences obtained from

the MPN cultures of the second zone (117 cm distance), grown at a salinity of 30‰ are identical. Most sequences obtained from the MPN counts of the same zone, grown at salinity of 55‰ are also nearly identical among each other. This might reflect an adaptation of a *Thiomicrospira* population to a higher salinity.

One exception is sequence Tms-MPN/Milos-CIV1, which was obtained from the MPN cultures with a salinity of 55‰, with sediment from the deepest layer of the second zone. The phylogenetic analysis revealed that the sequence is the deepest branching found for this genus so far (see Fig. 3). This finding and the extreme environment where the sequence was obtained from might mean that the corresponding organism has a different physiology compared to the known *Thiomicrospira* species.

The more moderate conditions in the outer zone, correlate with a higher phylogenetic diversity of the obtained *Thiomicrospira* sequences from this zone. Similar results for the general bacterial diversity, as reflected by DGGE analysis, were described in the accompanying publication of Sievert et al. (1998).

The phylogenetic analysis of the present study and the results from the earlier investigation of an intertidal mud flat (Brinkhoff et al., 1998b) show a high diversity within closely related sequences for both ecosystems. Sequences obtained from the intertidal mud flat (Tms-MPN/JB-x-y m m depth) formed clusters. Additionally they are in general not closely related with other *Thiomicrospira* sequences, and show more than 3% difference to other sequences (Brinkhoff et al., 1998b). The same is true for the

sequences from the Milos hydrothermal vent, which have at least 2.5% sequence difference.

Due to the presence of light chemolithoautotrophic SOB were found to be not the only primary producers of the shallow-water vent system off Milos (Dando et al., 1995a; Thiermann et al., 1997). However, results from this study clearly indicate that even though differences between the deep-sea and the shallow-water hydrothermal vent systems exist, *Thiomicrospira* is in both cases an important member of the sulfur-oxidizing community and has to be taken into account in further ecological and microbiological investigations of these ecosystems.

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REFERENCES

American Public Health Association. 1969. Standard methods for the examination of water and wastewater, including bottom sediments and sludge, p. 604-609. American Public Health Association, Washington, D. C.

Benson, D.A., Boguski, M.S., Lipman, D.J., and Ostell J. 1997 Genbank. Nucleic Acids Res. **25:1-6.**

Brinkhoff, T. and G. Muyzer. 1997. Increased Species Diversity and Extended Habitat Range of Sulfur-Oxidizing *Thiomicrospira* spp. Appl. Environ. Microbiol. **63**:3789-3796.

Brinkhoff, T., G. Muyzer, C. O. Wirsen, and J. Kuever. 1998a. Characterization of *Thiomicrospira kuenenii* sp. nov. and *Thiomicrospira frisia* sp. nov., two mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacteria isolated from an intertidal mud flat. Int. J. Syst. Bacteriol. In press.

Brinkhoff, T., C. M. Santegoeds, K. Sahm, J. Kuever, and G. Muyzer. 1998b. A polyphasic approach to study the diversity and vertical distribution of sulfur-oxidizing *Thiomicrospira* species in coastal sediments of the German Wadden Sea. Appl. Environ. Microbiol. **64**:4650-4657.

Brinkhoff, T., G. Muyzer, C. O. Wirsen, and J. Kuever. 1998c. Characterization of *Thiomicrospira chilensis* sp. nov., a mesophilic obligately chemolithoautotrophic sulfur-oxidzing bacterium isolated from a *Thioploca* mat. Int. J. Syst. Bacteriol. In press.

Brinkhoff, T., J. Kuever, G. Muyzer, and H. W. Jannasch. 1998d. The genus *Thiomicrospira*. *In* Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. Submitted.

Buchholz-Cleven, B. E. E., B. Rattunde, and K. L. Straub. 1997. Screening for genetic diversity of isolates of anaerobic Fe(II)-oxidizing bacteria using DGGE and whole cell hybridization. Syst. Appl. Microbiol. **20**:301-309.

Dando., P. R., J. A. Hughes, and F. Thiermann. 1995a. Preliminary observations on biological communities at shallow hydrothermal vents in the Aegean Sea. p. 303-317. *In* L.M. Parson, C.L. Walker, and D.R. Dixon (eds.), Hydrothermal Vents and Processes. Geological Society Special Publication 87, London.

Dando, P. R., J. A. Hughes, Y. Leahy, S. J. Niven, L. J. Taylor, and C. Smith. 1995b. Rates of gas venting from submarine hydrothermal areas around the island of Milos in the Hellenic Volcanic Arc. Cont. Shelf Res. **15**:913-929.

Don, R. H., P. T. Cox, B. Wainwright, K. Baker, and J. S. Mattick. 1991. ``Touchdown´´PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. **19**:4008.

Gilbert, D. G. 1992. SeqApp - a biosequence analysis application. Indiana University, Bloomington.

Hashimoto, J., T. Miura, K. Fujikura, and J. Ossaka. 1993. Discovery of vestimentiferan tube-worms in the euphotic zone. Zoolog. Science. **10**:1063-1067.

Jannasch, H. W., and C. O. Wirsen. 1979. Chemosynthetic primary production at East Pacific sea floor spreading centers. BioScience **29**:592-598.

Jannasch, H. W., and M. J. Mottl. 1985. Geomicrobiology of deep-sea hydrothermal vents. Science **229**:717-725.

Kuenen, J. G. and H. Veldkamp. 1972. *Thiomicrospira pelophila,* gen. n., sp. n., a new obligately chemolithotrophic colorless sulfur bacterium. Antonie van Leeuwenhoek **38**: 241-256.

Kuenen, J. G., L. A. Robertson, and O. H. Tuovinen. 1992. The genera *Thiobacillus, Thiomicrospira,* and *Thiosphaera,* p. 2638-2657. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (eds.), The prokaryotes, 2nd ed. Springer, Berlin, Germany.

Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek R., McCaughey, M.J. and Woese, C.R. 1997. The RDP (Ribosomal Database Project). Nucleic Acids Res. **25:** 109-110.

Mottl, M. J., and H. D. Holland. 1978. Chemical exchange during hydrothermal alteration of basalt by seawater. Geochim. Cosmochim. Acta. **42**:1103-1116.

Murray, A. E., J. T. Hollibaugh, and C. Orrego. 1996. Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl. Environ. Microbiol. **62**:2676-2680.

Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**: 695-700.

Muyzer, G., A. Teske, C. O. Wirsen, H. W. Jannasch. 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea

hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. **164**: 165-172.

Muyzer, G., T. Brinkhoff, U. Nübel, C. Santegoeds, H. Schäfer, and C. Wawer. 1998. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. 3.4.4:1-27. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (eds.). Molecular microbial ecology manual, 3rd ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Olafsson, J., K. Thors, and J. Cann. 1991. A sudden cruise off Iceland. RIDGE Events. **2**:35-38.

Sievert, S., T. Brinkhoff, G. Muyzer, W. Ziebis, and J. Kuever. 1998. Spatial heterogeneity of bacterial populations at a shallow-water hydrothermal vent in the Aegean Sea (Milos, Greece) as studied by the application of molecular and cultivation-based techniques. Appl. Environ. Microbiol. Submitted.

Sorokin, D. Y. 1991. Oxidation of reduced sulphur compounds in volcanic regions in the Bay of Plenty (New Zealand) and Matupy Harbour (New Britian, Papua-New Guinea). Proceedings of the USSR Academy of Science, Series Biological. **3**:376-387 (In Russian).

Tarasov, V. G., M. V. Propp, L. N. Propp, A. V. Zhirmunsky, B. B. Namsaraev, V. M. Gorlenko, D. A. Starynin. 1990. Shallow-water gasohydrothermal vents of Ushishir Volcano and the ecosystem of Kraternaya Bight (the Kurile Islands). P. S. Z. N. I. Mar. Ecol. **11**:1-23.

Thiermann, F., I. Akoumianaki, J. A. Hughes, and O. Giere. 1997. Benthic fauna of a shallow-water gasohydrothermal vent area in the Aegean Sea (Milos, Greece). Mar. Biol. **128**:149-159.

Trager, G. C., and M. J. DeNiro. 1990. Chemoautotrophic sulfur bacteria as a food source for mollusks at intertidal hydrothermal vents: evidence from stable isotopes. Veliger. **33**:359-362.

van den Ende, F. P. 1997. Microbial ecology of oxygen-sulfide interfaces in marine benthic ecosystems. PhD thesis, Rijksuniversiteit Groningen, Groningen, Netherlands.

Vidal, V. M., F. M. Vidal, and J. D. Isaacs. 1978. Coastal submarine hydrothermal activity off northern Baja California. J. Geophys. Res. **83**:1757-1774.

Welhan, J. A., and H. Craig. 1983. Methane, hydrogen and helium in hydrothermal fluids at 21 degrees N on the East Pacific Rise. *In* Hydrothermal processes at seafloor spreading centers. Rona, P. A., Bostroem, K., Laubier, L., and Smith, K. L. Jr. (eds.), NATO conference series, IV, Marine sciences, Plenum Press, New York, **12**:391-409.

Wirsen, C. O., T. Brinkhoff, J. Kuever, G. Muyzer, S. Molyneaux, and H. W. Jannasch. 1998. A new *Thiomicrospira* strain from the Mid-Atlantic ridge compared to known hydrothermal vent isolates. Appl. Environ. Microbiol. **64**:4057-4059.

Wood, A. P., and D. P. Kelly. 1989. Isolation and physiological characterization of *Thiobacillus thyasiris* sp. nov., a novel marine facultative autotroph and the putative symbiont of *Thyasira flexuosa*. Arch. Microbiol. **152**:160-166.

Wood, A. P., and D. P. Kelly. 1993. Reclassification of *Thiobacillus thyasiris* as *Thiomicrospira thyasirae* comb. nov. an organism exhibiting

pleomorphism in response to environmental conditions. Arch. Microbiol. **159**:45-47.

Zhou, J., M. A. Bruns, and J. M. Tiedje. 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62**:316-322.

Chapter 8

General discussion

Discussion of the used strategy and the obtained results

To assess the ecological relevance of a particular group of bacteria the habitat range of these organisms must be determined. For such a horizontal survey it is useful to have fast and reliable detection method. Culturing of microorganisms is laborious and time consuming, and it is not always possible to isolate the relevant organisms of an ecosystem (Amann et al., 1995). The rRNA approach (Olsen et al., 1986) made it possible to give evidence for the presence of uncultured microorganisms in the environment (e.g., Ward et al., 1992; Akkermans et al., 1994; Amann et al., 1995; Muyzer and Ramsing, 1996). Therefore was the first attempt of this dissertation the development of two *Thiomicrospira* specific oligonucleotides. The design of these oligonucleotides based on the five known 16S rDNA sequences of the *Thiomicrospira* species present in the monophyletic branch of the gamma subclass of the Proteobacteria (Distel and Wood, 1992; Lane et al., 1992; Muyzer et al., 1995). The oligonucleotides were used in a specific PCR and several hybridization techniques. These methods were the condition for the following detection of *Thiomicrospira* spp. and their DNA, respectively, in enrichment cultures or in DNA extracted from environmental samples, and thus for the demonstration of a worldwide distribution and an extended habitat range of this genus. However, it has to be taken into account that it is always possible that rRNA or rDNA probes do not match all target organisms present in the environment and therefore do not detect the whole distribution and diversity. In addition is the development of

specific 16S rRNA oligonucleotides generally restricted to monophyletic taxa of bacteria. Groups like the present genus *Thiobacillus*, which is scattered within the Proteobacteria make it difficult or impossible to design probes.

As mentioned above, several molecular biological methods mainly based on 16S rRNA analysis were developed (e.g., Giovannoni et al., 1988; Muyzer et al., 1993), which enabled scientists to study microbial ecology without the necessity of culturing microorganisms (e.g., Barns et al., 1994; Borneman et al., 1996). However, for a better understanding of the function of microorganisms in the environment it is necessary to isolate and characterize them. Finally molecular microbial ecologists infer properties of organisms on the basis of homologous properties in cultivated relatives (Pace, 1996). Therefore it was one aim of this study to obtain new isolates belonging to the genus *Thiomicrospira*.

From the enrichment cultures used for the horizontal survey, several new strains were obtained. Using sequence differences as an indication for new species (Stackebrand and Goebel, 1994) three of these strains were chosen for further characterization. Table 1 gives an overview about the characteristics of all described *Thiomicrospira* spp., including the new species, *T. kuenenii*, *T. frisia*, and *T. chilensis*. The detection of new physiological and morphological properties of the new species demonstrated that there is still a need for the isolation of new organisms.

Table 1: Morphological and physiological characteristics of the species comprising the genus *Thiomicrospira, Hydrogenovibrio marinus* and *"Thiomicrospira denitrificans"*[a]

	T. crunogena	***T. kuenenii***	***H. marinus***	***T. frisia***	***T. chilensis***	***T. pelophila***	***T. thyasirae***	***"T. denitrificans"***
- Subgroup of the Proteobacteria	Gamma	Gamma	Gamma	Gamma	Gamma	Gamma	Gamma	Epsilon
- Shape	Vibrio	Vibrio	Vibrio	Rod	Rod	Vibrio	Vibrio	Spiral
- Width (µm)	0.4	0.3-0.4	0.2-0.5	0.3-0.5	0.3-0.5	0.2-0.3	0.3	0.3
- Length (µm)	1.5	1.5-2.5	1-2	1-2.7	0.8-2	1-2	0.8-2.3	variable
- Motility	+	+	+	+	+	+	-	-
- G+C content (mol%)	44.2 (44)[b]	42.4	44.1	39.6	49.9	45.7 (45)[b]	45.6 (52)[b]	31.7
- Major ubiquinone	Q-8	Q-8	Q-8	Q-8	Q-8	Q-8	Q-8[c]	nr
- Maximum growth rate with thiosulfate (h^{-1})	0.8	0.35	0.6	0.45	0.4	0.3	0.06[d]	0.06
- Optimal pH	7.5-8.0	6.0	6.5	6.5	7.0	7.0[e]	7.5	7.0
- pH range	5.0-8.5	4.0-7.5	nr	4.2-8.5	5.3-8.5	5.6-9.0[e]	7.0-8.4	nr
- Optimal temperature (°C)	28-32	29-33.5	37	32-35	32-37	28-30	35-40	22
- Temperature range (°C)	4-38.5	3.5-42	nr	3.5-39	3.5-42	3.5-42	10-45	nr
- Na^+ requirement	+	+	+	+	+	+	+	-
- Optimal Na^+-concentration (mM)	nr	470	500	470	470	470	430	nr

	T. crunogena	*T. kuenenii*	*H. marinus*	*T. frisia*	*T. chilensis*	*T. pelophila*	*T. thyasirae*	*"T. denitrificans"*
- Na^+-concentration range (mM)	at least 45	100-640	nr	100-1240	100-1240	40-1240[e]	250-3000	nr
- Vitamin B_{12}-dependent	-	-	-	-	-	+	-	nr
- Calvin Benson cycle present	+	+	+	+	+	+	+	+
- Formation of sulfur from thiosulfate at pH 7.0 in liquid medium	+	-	+	-	+	+	+	-
- Growth on H_2 as the sole electron donor	-[f]	-	+	-	-	-[f]	-[f]	nr
- Fully aerobic growth	+	+	+	+	+	+	+	-
- Denitrification	-	-	-	-	-	-	-	+
- Heterotrophic growth	-	-	-	-	-	-	-[g]	nr

[a] Symbols: +, 90% or more strains are positive, -, 90% or more strains are negative, nr: not reported; data from Kuenen and Veldkamp, 1972; 1973; Timmer-ten Hoor, 1975; Ruby and Jannasch, 1982; Jannasch et al. 1985; Kuenen and Robertson, 1989; Wood and Kelly 1989, 1993; Nishihara et al., 1991, 1998; Brinkhoff et al. 1998a, 1998b; and Kuever unpublished data.

[b] the values in brackets are from the original description, determined by thermal denaturation.

[c] in the original description Q-10 was found (Wood and Kelly, 1989); we could only detect Q-8 and traces of Q-7.

[d] in the original description the maximum growth rate on acetate was 0.53 (h^{-1}) (Wood and Kelly, 1989). Growth rates on thiosulfate seemed to be similar to *T. pelophila* in the range of 0.2 to 0.3 (Kuever, unpublished results).

[e] data from Brinkhoff et al., 1998a.

[f] Kuever, unpublished.

[g] different to the original description (Wood and Kelly, 1989) heterotrophic growth was not obtained, even after incubation for more than six weeks (Kuever, unpublished)

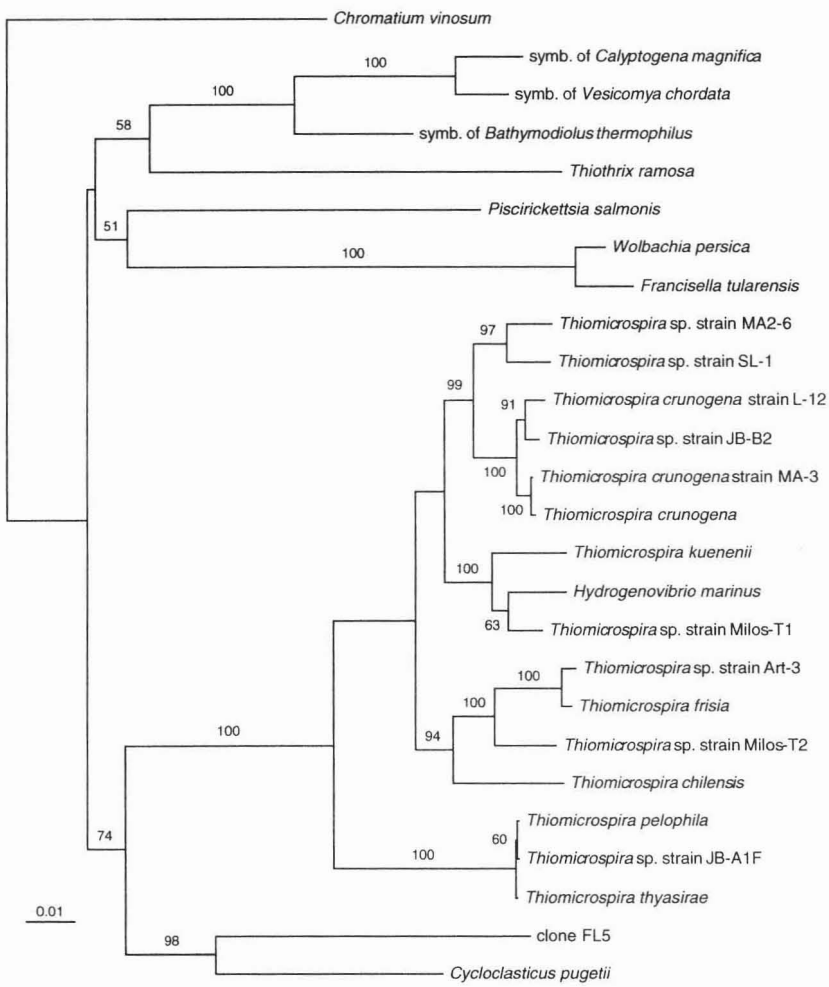

Figure 1: Neighbor joining tree based on nearly complete 16S rRNA sequences, showing the phylogenetic relationships between different *Thiomicrospira* strains and closely related bacteria. The *Thiomicrospira* sequences were aligned to pre-aligned sequences taken from the Ribosomal Database Project (RDP; Maidak et al., 1997). *Chromatium vinosum* was taken as the outgroup. The numbers on the branches refer to bootstrap values; only values of 50% and higher are shown. The scale bar represents 0.01 estimated nucleotide change per sequence position.

During the 23 years, since the detection of the first *Thiomicrospira* species, *T. pelophila* (Kuenen and Veldkamp, 1972), and the beginnig of this study, organisms belonging to this genus were only isolated from two different ecosystems, i.e., intertidal mud flats and deep-sea hydrothermal vents (Kuenen and Veldkamp, 1972; Ruby et al., 1981; Ruby and Jannasch, 1982; Jannasch et al., 1985; Wood and Kelly, 1989, 1993; Muyzer et al., 1995). Only three of the obtained isolates were fully described as new species during the same time period (Kuenen and Veldkamp, 1972; Jannasch et al., 1985; Wood and Kelly, 1989, 1993). The strategy used in this dissertation resulted in the finding that *Thiomicrospira* spp. are worldwide distributed and present in many different ecosystems. Furthermore several new isolates belonging to this genus were obtained, indicating the advantage of using a combined approach of molecular biological and microbiological methods.

For an overview Figure 1 shows the phylogenetic positions of all *Thiomicrospira* strains and species isolated so far, most which were obtained during the studies of this dissertation. *Thiomicrospira denitrificans* (Timmer-ten Hoor, 1975), which awaits reclassification as a new genus (Muyzer et al., 1995), is not included in this tree, because it belongs to the epsilon subdivision of the Proteobacteria (Muyzer et al., 1995). However, *Hydrogenovibrio marinus* (Nishihara et al., 1991, 1998) falls within this cluster and is therefore also included in Table 1. The only significant difference of this organism to *Thiomicrospira* spp. is the ability of the former to use hydrogen as electron donor (Tab. 1). Since hydrogen

utilization is a ubiquitous trait among many phylogenetically diverse bacteria, hydrogen utilization alone may be insufficient to separate this organism as an own genus (Brinkhoff et al., 1998c).

Before this study also little was known about the ecological relevance of *Thiomicrospira* spp. in particular habitats. Kuenen and Veldkamp (1972) speculated about the ecological role of *Thiomicrospira pelophila* in the Wadden Sea sediment, and Muyzer et al. (1995) indicated a high abundance of *Thiomicrospira* spp. in deep-sea hydrothermal vents by the use of molecular biological methods. However, no investigations were performed to determine the number and the spatial distribution of these organisms.

We know that ecosystems are complex systems, in which microorganisms occur in hetereogenous communities (Akkermans et al., 1994). These communities are dependent on the environmental parameters. To understand the role of particular bacterial populations it is necessary to determine the physicochemical parameters, too. Amann and Kühl (1998) summarized three factors, which are important for the study of microbial ecology: a) diversity - what organisms are present in an ecosystem, b) structure - how many cells of a certain species are in a defined spatial element at a given time, and c) function - what is the in situ activity of microbial cells in an environment defined by physicochemical parameters. It is impossible to retrieve these informations with use of a single method and there is a need for the combination of several techniques and concepts.

In the investigations of an intertidal mud flat and a shallow-water hydrothermal vent during this study, tools from different disciplines were used to determine the number, distribution, diversity, and in one case the metabolical state of the present *Thiomicrospira* cells. Additionally physicochemical parameters, which possibly influence these factors were measured, e.g., by the use of microsensor measurements.

Samples of both habitats were investigated by the use of MPN counts. The number of *Thiomicrospira* cells was determined by screening the MPN cultures with a specific PCR, which again demonstrated the usefulness of the combination of microbiological and molecular biological methods. The obtained results showed a high abundance in some regions of the shallow-water hydrothermal vent, and a possible high relevance of *Thiomicrospira* spp. within the sulfur-oxidizing community. However, the role of this genus appears to be minor in the intertidal mud flat, compared with other sulfur-oxidizing bacteria. In both cases it was found that the determined environmental parameters had a strong impact on the *Thiomicrospira* populations. Without knowing these parameters it would have been impossible to understand the spatial distribution of this genus.

16S rRNA sequences obtained from the highest MPN dilutions for *Thiomicrospira* revealed the presence of many unknown species, while only one sequence, which was similar to the sequence of a known isolate was found. All *Thiomicrospira* species, which have been isolated so far were obtained from batch cultures. It has long been recognized that enrichment cultures limit the number and the relative growth rates of

organisms, which can be obtained from the environment and a serious limitation is the fact that liquid batch culture enrichments typically select for fast-growing organisms (Harder and Dijkhuizen, 1982). Additionally enrichment in liquid batch culture yields only one or a few strains possessing a specific phenotype (Dunbar et al., 1997). The results of this dissertation confirm these findings already on the genus level and speak for isolation techniques based on dilution series, to obtain the dominant organisms, like performed for the shallow-water hydrothermal vent.

Even though the presence of *Thiomicrospira* was demonstrated for many habitats there are still many types of ecosystems which were not investigated during this study, but would be worth to check for the presence or distribution of this genus. Jannasch et al. (1991) obtained several chemolithoautotrophic sulfur-oxidizing bacteria from the Black Sea and affiliated them to the genus *Thiomicrospira*, due to their G + C contents of 37-40 mol% (Kuenen and Robertson, 1989; Kuenen et al., 1992; Brinkhoff et al., 1998c). Thus, e.g., the Black Sea would probably be an interesting habitat for further studies on this genus.

Finally it should be tried in further studies of the genus *Thiomicrospira* to isolate species, those existence was shown in this dissertation, but which were not obtained in pure culture, like the freshwater *Thiomicrospira* species.

References

Akkermans, A. D. L., M. S. Mirza, H. J. M. Harmsen, H. J. Blok, P. R. Herron, A. Sessitsch, and W. M. Akkermans. 1994. Molecular ecology of microbes: A review of promises, pitfalls and true progress. FEMS Microbiol. Rev. **15**:185-194.

Amann, R. I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews **59**:143-169.

Amann R., and M. Kühl. 1998. In situ methods for assessment of microorganisms and their activities. Current Opinion in Microbiol. **1**:352-358.

Barns S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc. Natl. Acad. Sci. USA **91**:1609-1613.

Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62**:1935-1943.

Brinkhoff, T., G. Muyzer, C. O. Wirsen, and J. Kuever. 1998a. Characterization of *Thiomicrospira kuenenii* sp. nov. and *Thiomicrospira frisia* sp. nov., two mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacteria isolated from an intertidal mud flat. Int. J. Syst. Bacteriol. In press.

Brinkhoff, T., Muyzer, G., Wirsen, C. O., and Kuever, J. 1998b. Characterization of *Thiomicrospira chilensis* sp. nov., a mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacterium isolated from a *Thioploca* mat. Int. J. Syst. Bacteriol. In press.

Brinkhoff, T., J. Kuever, G. Muyzer, and H. W. Jannasch. 1998c. The genus *Thiomicrospira*. *In* Bergey`s manual of systematic bacteriology, 2nd ed. vol. 2. Submitted.

Distel, D. L., and A. P. Wood. 1992. Characterization of the gill symbiont of *Thyasira flexuosa* (Thyasiridae: Bivalvia) by use of polymerase chain reaction and 16S rRNA sequence analysis. J. Bacteriol. **174**:6317-6320.

Dunbar, J., S. White, and L. Forney. 1997. Genetic diversity through the looking glass: effect of enrichment bias. Appl. Environ. Microbiol. **63**:1326-1331.

Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace. 1988. Phylogenetic group-specific probes for identification of single microbial cells. J. Bacteriol. **170**:720-726.

Harder, W., and L. Dijkhuizen. 1982. Strategies of mixed substrate utilization in microorganisms. Philos. Trans. R. Soc. London Ser. B **297**:459-480.

Jannasch, H. W., C. O. Wirsen, D. C. Nelson, and L. A. Robertson. 1985. *Thiomicrospira crunogena* sp. nov., a colorless, sulfur-oxidizing bacterium from a deep-sea hydrothermal vent. Int. J. System. Bacteriol. **35**:422-424.

Jannasch, H. W., C. O. Wirsen, and S. J. Molyneaux. 1991. Chemoautotrophic sulfur-oxidizing bacteria from the Black Sea. Deep-Sea Research **38**:1105-1120.

Kuenen, J. G., and H. Veldkamp. 1972. *Thiomicrospira pelophila* nov. gen., nov. sp., a new obligately chemolithotrophic colorless sulfur bacterium. Antonie Van Leeuwenhoek **38**:241-256.

Kuenen, J. G. and H. Veldkamp. 1973. Effects of organic compounds on growth of chemostat cultures of *Thiomicrospira pelophila, Thiobacillus thioparus* and *Thiobacillus neapolitanus*. Arch. Microbiol. **94**: 173-190.

Kuenen, J. G., and L. A. Robertson. 1989. The genus *Thiomicrospira*. *In* Bergey´s manual of systematic bacteriology, Staley, J. T., Bryant, M. P., Pfennig, N., and Holt, J. G. (eds.), Vol. **3**, p. 1858-1861, Williams and Wilkins, Baltimore.

Kuenen, J. G., L. A. Robertson, and O. H. Tuovinen. 1992. The genera *Thiobacillus, Thiomicrospira,* and *Thiosphaera*, p. 2638-2657. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (eds.), The prokaryotes, 2nd ed. Springer, Berlin, Germany.

Lane, D. J., A. P. Harrison, D. A. Stahl, B. Pace, S. J. Giovannoni, G. J. Olsen, and N. R. Pace. 1992. Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. J. Bacteriol. **174**:269-278.

Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese. 1997. The RDP (Ribosomal Database Project). Nucleic Acids Res. **25**:109-110.

Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695-700.

Muyzer, G., A. Teske, C.O. Wirsen, H.W. Jannasch. 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. **164**: 165-172.

Muyzer, G., and N. Ramsing. 1996. Molecular methods to study the organization of microbial communities. Water Sci. Technol. **32**:1-9

Nishihara, H., Y. Igarashi, and T. Kodama. 1991. *Hydrogenovibrio marinus* gen. nov. sp. nov., a marine obligately chemolithoautotrophic hydrogen-oxidizing bacterium. Int. J. Syst. Bacteriol. **41**:130-133.

Nishihara,, H., T. Yaguchi, S. Y. Chung, K.-I. Suzuki, M. Yanagi, K. Yamasoto, T. Kodama, and Y. Igarashi. 1998. Phylogenetic position of an obligately chemoautotrophic, marine hydrogen-oxidizing bacterium, *Hydrogenovibrio marinus*, on the basis of 16S rRNA sequences and two forms 1 RuBisCO gene sequences. Arch. Microbiol. **169**:364-368.

Olsen, G. J., D. J. Lane, S. J. Giovannoni, and N. R. Pace. 1986. Microbial ecology and evolution: a ribosomal RNA approach. Ann. Rev. Microbiol. **40**:337-365.

Pace, N. R. 1996. New perspective on the natural microbial world: molecular microbial ecology. ASM news **62**:463-470.

Ruby, E. G., C. O. Wirsen, and H. W. Jannasch. 1981. Chemolithotrophic sulfur-oxidizing bacteria from the Galapagos Rift hydrothermal vents. Appl. Environ. Microbiol. **42**:317-324.

Ruby, E. G., and H. W. Jannasch. 1982. Physiological characteristics of *Thiomicrospira* sp. strain L-12 isolated from deep-sea hydrothermal vents. J. Bacteriol. **149**:161-165.

Stackebrand, E., and B. M. Goebel. 1994. Taxonomic note: A place for DNA:DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44**:846-849.

Timmer-ten Hoor, A. 1975. A new type of thiosulphate oxidizing, nitrate-reducing microorganism: *Thiomicrospira denitrificans* sp. nov. Netherlands J. Sea Res. **9**:343-351.

Ward, D. M., M. M. Bateson, R. Weller, and A. L. Ruff-Roberts. 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. *In* Advances in Microbial Eology, Marshall, K. C. (eds.), Vol. 12, p. 219-286, Plenum Press, New York.

Wood, A. P., and D. P. Kelly. 1989. Isolation and physiological characterization of *Thiobacillus thyasiris* sp. nov., a novel marine facultative autotroph and the putative symbiont of *Thyasira flexuosa*. Arch. Microbiol. **152**:160-166.

Wood, A. P., and D. P. Kelly. 1993. Reclassification of *Thiobacillus thyasiris* as *Thiomicrospira thyasirae* comb. nov. an organism exhibiting pleomorphism in response to environmental conditions. Arch. Microbiol. **159**:45-47.

Summary

In this dissertation the ecological role of sulfur-oxidizing bacteria of the genus *Thiomicrospira* was investigated, with a combination of molecular biological and microbiological methods.

At the beginning two oligonucleotides, specific for the 16S rRNA encoding genes of organisms belonging to the genus *Thiomicrospira* were developed, to allow fast and reliable detection. These probes were used in a specific PCR and in different hybridization techniques. Enrichment cultures for sulfur-oxidizing bacteria or extracted DNA from very different environmental samples were screened for the presence of *Thiomicrospira*, by using the specific approach. Thus a worldwide distribution and an extended habitat range of this genus was shown. The presence of *Thiomicrospira* spp. was demonstrated for different marine habitats, like intertidal mud flats, deep-sea and shallow-water hydrothermal vent systems, a continental shelf, and hypersaline microbial mats. Additionally the genus was found in a saline spring, and also one freshwater habitat. The main requirement for the presence of *Thiomicrospira* spp. seems to be the presence of reduced sulfur compounds.

The worldwide distribution of one species, *Thiomicrospira crunogena*, was shown by a high similarity of the 16S rRNA sequences and also high levels of DNA-DNA similarities of several isolates, which were obtained from different habitats.

From many habitats, which were investigated during this project, *Thiomicrospira* spp. were isolated. Phylogenetic analysis of their 16S

rRNA sequences indicated an increased species diversity within this genus and confirmed that the genus *Thiomicrospira* is a monophyletic group in the gamma subunit of the Proteobacteria. Closely related species and strains were often obtained from very different habitats.

Three new isolates were chosen for further characterization, based on significant differences of their 16S rRNA sequences compared with those of the known species. Two of the new isolates, which were obtained from an intertidal mud flat of the German Wadden Sea, were introduced as *Thiomicrospira frisia* and *Thiomicrospira kuenenii*. The third organism was isolated from the continental shelf of the coast of Chile and was named *Thiomicrospira chilensis*. *Thiomicrospira frisia* and *Thiomicrospira kuenenii* showed a lower pH optimum than all other described species. *Thiomicrospira chilensis* showed significant differences to the other species only on the DNA level (16S rRNA sequence similarity, DNA homology, and G + C content). However, metabolical differences, which were not found during the standard characterization can not be excluded, and are possible because of the high genetical differences.

Thiomicrospira frisia, *Thiomicrospira chilensis*, and the new strains *Thiomicrospira* sp. str. Art-3, and *Thiomicrospira* sp. str. Milos T-2 have a rod-shaped morphology, like thiobacilli. Therefore discrimination between the genus *Thiomicrospira* and the genus *Thiobacillus* based on the morphology is no longer possible.

To determine the role of *Thiomicrospira* in two different habitats, sediment of an intertidal mud flat of the Jadebusen Bay, which is part of the German Wadden Sea, and a shallow-water hydrothermal vent in the

Aegean Sea (Milos, Greece) were investigated. The former was chosen because the highest number of different species was obtained from this habitat. During an earlier study a high abundance of *Thiomicrospira* spp. was demonstrated for deep-sea vents. To determine the abundance of this genus in other hydrothermal vents the shallow-water vent system of Milos was investigated. Furthermore a systematic comparison of a hydrothermal vent system with an intertidal mud flat should be performed.

By MPN counts for sulfur-oxidizing bacteria, in combination with the specific PCR approach, the numbers of cells belonging to the genus *Thiomicrospira* were determined for different sediment layers of the intertidal mud flat. The numbers of *Thiomicrospira* cells were low, compared with the numbers of other sulfur-oxidzing bacteria. From this finding it was derived that the ecological role in this habitat is probably minor, even though a high diversity of this genus was found within the MPN cultures.

Thiomicrospira spp. are described as obligate aerobic organisms. Therefore it was surprising that the cell numbers of the *Thiomicrospira* populations, which were present in different sediment layers down to a depth of 4 cm, were all in the same range, while oxygen was only present in the first two mm of the sediment, as determined by microsensor measurements. For this reason rRNA was isolated from several sediment layers, and hybridized with a *Thiomicrospira* specific probe. The results revealed much higher amounts of *Thiomicrospira* rRNA in the oxic part of the sediment than in the anoxic. Combination of the results obtained

from this polyphasic approach showed that the present *Thiomicrospira* spp. are a small, but active population in the oxic layers of the sediment, while the *Thiomicrospira* populations in the anoxic part are probably physiologically not active.

Using a similar approach as for the intertidal mud flat it could be demonstrated that the ecological role of the genus *Thiomicrospira* in the shallow-water vent in the Aegean Sea appears to be high. In some sediment layers of different zones around the investigated vent *Thiomicrospira* spp. belong to the dominant sulfur-oxidizers. By comparing results from MPN counts with environmental parameters it was found that the numbers of *Thiomicrospira* cells were probably mainly influenced by the existing temperature gradient. Further new *Thiomicrospira* strains were isolated from high dilution enrichments, one of which appears to be the dominant *Thiomicrospira* species within this habitat.

Zusammenfassung

In dieser Dissertation wurde die ökologische Relevanz von schwefel-oxidierenden Bakterien der Gattung *Thiomicrospira* mit einer Kombination von molekularbiologischen und mikrobiologischen Methoden untersucht.

Zu Beginn wurden zwei Oligonukleotide, spezifisch für die 16S rRNA kodierenden Gene von Organismen der Gattung *Thiomicrospira*, entwickelt, um eine schnelle und verläßliche Nachweismethode zu erhalten. Diese Sonden wurden in einer spezifischen PCR und in verschiedenen Hybridisierungstechniken eingesetzt. Anreicherungskulturen für schwefel-oxidierende Bakterien oder aus verschiedenen Umweltproben extrahierte DNA wurden mittels dieser spezifischen Nachweismethoden auf die Anwesenheit von *Thiomicrospira* untersucht. Somit konnte eine weltweite Verbreitung dieser Gattung sowie ihre Anwesenheit in einer Vielzahl von Habitaten nachgewiesen werden. Das Vorkommen von *Thiomicrospira* spp. konnte für verschiedene marine Habitate, wie dem Wattenmeer, Tiefsee- und Flachwasser-Hydrothermalsystemen, einem Kontinentalschelf und hypersalinen mikrobiellen Matten nachgewiesen werden. Außerdem wurde diese Gattung in einer Salzquelle und in einem Süßwasserhabitat gefunden. Die Hauptanforderung für die Anwesenheit von *Thiomicrospira* spp. scheint das Vorhandensein von reduzierten Schwefelverbindungen zu sein.

Die weltweite Verbreitung der Art *Thiomicrospira crunogena* konnte durch die Übereinstimmung von 16S rRNA Sequenzen und

DNA-Homologien einiger untersuchter Isolate, die aus verschiedenen Habitaten stammten, gezeigt werden.

Von vielen Habitaten, die während dieses Projektes untersucht wurden, konnten *Thiomicrospira* spp. isoliert werden. Die phylogenetische Analyse ihrer 16S rRNA Sequenzen ergab eine erhöhte Diversität dieser Gattung und bestätigte, daß es sich bei *Thiomicrospira* um eine monophyletische Gruppe innerhalb der Gamma-Untereinheit der Proteobakterien handelt. Nahe verwandte Arten und Stämme wurden häufig aus sehr verschiedenen Habitaten isoliert.

Drei der neuen Isolate wurden für eine weitere Charakterisierung ausgewählt. Diese unterschieden sich signifikant in ihren 16S rRNA Sequenzen von denen bekannter Arten. Zwei der neuen Isolate, *Thiomicrospira frisia* und *Thiomicrospira kuenenii*, stammten aus dem Deutschen Wattenmeer. Der dritte Organismus wurde aus einer Probe, die von der Küste Chiles stammte, isoliert und *Thiomicrospira chilensis* genannt. *Thiomicrospira frisia* und *Thiomicrospira kuenenii* zeigten ein niedrigeres pH-Optimum als alle anderen bisher beschriebenen Arten. *Thiomicrospira chilensis* zeigte lediglich auf der DNA-Ebene signifikante Unterschiede zu anderen Arten (Ähnlichkeit der 16S rRNA Sequenz, DNA-Homologie und G+C Gehalt). Unterschiede im Stoffwechsel, die während der Standardcharakterisierung nicht gefunden werden konnten, können jedoch aufgrund der erheblichen genetischen Unterschiede nicht ausgeschlossen werden

Thiomicrospira frisia, *Thiomicrospira chilensis* und die beiden neuen Stämme *Thiomicrospira* sp. str. Art-3 und *Thiomicrospira* sp. str.

Milos T-2 haben eine stäbchenförmige Zellform, ebenso wie Thiobacillen. Daher ist eine Unterscheidung der Gattungen *Thiomicrospira* und *Thiobacillus* basierend auf der Morphologie nicht länger möglich.

Um die Bedeutung von *Thiomicrospira* in zwei unterschiedlichen Habitaten zu bestimmen, wurde Sediment des Jadebusens, welcher Teil des Deutschen Wattenmeeres ist, sowie ein Flachwasser-Hydrothermalsystem der Ägäis (Milos, Griechenland) untersucht. Das Wattenmeer wurde ausgewählt, da von hier die größte Anzahl unterschiedlicher Arten isoliert wurde. In einer früheren Studie wurde ein hohes Vorkommen von *Thiomicrospira* spp. in Tiefsee-Hydrothermalsystemen gezeigt. Um das Vorkommen dieser Gattung auch in anderen Hydrothermalsystemen zu untersuchen, wurde das Flachwasser-Hydrothermalsystem von Milos zu diesem Zweck herangezogen. Weiterhin sollte ein systematischer Vergleich von einem Hydrothermalsystem mit dem Habitat Wattenmeer durchgeführt werden.

Mittels MPN-Zählungen für schwefel-oxidierende Bakterien, in Kombination mit der spezifischen PCR, konnte die Anzahl der Zellen der Gattung *Thiomicrospira* in verschiedenen Sedimentschichten des Wattenmeeres bestimmt werden. Die Anzahl der *Thiomicrospira*-Zellen war im Vergleich zu den Zellzahlen anderer schwefel-oxidierender Bakterien gering. Hieraus konnte abgeleitet werden, daß die ökologische Relevanz in diesem Habitat aller Wahrscheinlichkeit nach gering ist, obwohl eine hohe Diversität dieser Gattung in den MPN-Kulturen gefunden wurde.

Thiomicrospira spp. sind als obligat aerobe Organismen beschrieben. Daher war es überraschend, daß die Zellzahlen der *Thiomicrospira* Populationen der unterschiedlichen Sedimentschichten bis zu einer Tiefe von 4 cm in der gleichen Größenordnung lagen, während Sauerstoff lediglich in den ersten beiden mm mittels Mikrosensormessungen nachgewiesen werden konnte. Aus diesem Grunde wurde rRNA aus verschiedenen Sedimentschichten isoliert und mit einer *Thiomicrospira*-spezifischen Sonde hybridisiert. Das Ergebnis zeigte deutlich höhere Mengen von *Thiomicrospira* rRNA in dem aeroben Teil des Sedimentes als in dem anaeroben. Die Kombination der Ergebnisse, die durch das vielschichtige Vorgehen erzielt wurden ergab, daß die anwesenden *Thiomicrospira* spp. eine kleine aber aktive Population im aeroben Teil des Sedimentes darstellen, während die *Thiomicrospira*-Populationen im anaeroben Teil vermutlich physiologisch nicht aktiv sind.

Durch den Einsatz einer ähnlichen Vorgehensweise wie für das Wattenmeersediment konnte gezeigt werden, daß die ökologische Relevanz der Gattung *Thiomicrospira* in dem Flachwasserhydrothermal System der Ägäis hoch zu sein scheint. In einigen Sedimentschichten verschiedener Zonen um die untersuchte Hydrothermalquelle gehörten *Thiomicrospira* spp. zu den dominanten Schwefeloxidierern. Durch Vergleich von Ergbnissen aus MPN-Zählungen mit Umweltparametern wurde festgestellt, daß die Zahl der *Thiomicrospira*-Zellen wahrscheinlich vorwiegend durch den vorhandenen Temperaturgradienten beeinflußt wird. Zusätzlich wurden neue *Thiomicrospira* Stämme aus

Verdünnungsreihen isoliert, von denen einer die dominante *Thiomicrospira* Spezies dieses Habitats zu sein scheint.

Share of scientific contributions to the publications presented in this dissertation

1. Increased species diversity and extended habitat range of sulfur-oxidizing *Thiomicrospira* spp. T. Brinkhoff, and G. Muyzer.

Concept by G. Muyzer and T. Brinkhoff. Execution of all experiments by T. Brinkhoff. Writing of the manuscript by G. Muyzer and T. Brinkhoff.

2. A new *Thiomicrospira* strain from the Mid-Altlantic Ridge compared to known Hydrothermal Vent isolates. C. O. Wirsen, T. Brinkhoff, J. Kuever, G. Muyzer, S. Molyneaux, and H. W. Jannasch.

Microbiological work done by C. O. Wirsen and J. Kuever. Sequencing and phylogenetic analysis by T. Brinkhoff and G. Muyzer. Writing of the manuscript by C. O. Wirsen and the co-authors.

3. Characterization of *Thiomicrospira kuenenii* sp. nov. and *Thiomicrospira frisia* sp. nov., two mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacteria isolated from an intertidal mud flat. T. Brinkhoff, G. Muyzer, C. O. Wirsen, and J. Kuever.

Development of the concept by T. Brinkhoff, J. Kuever, and G. Muyzer. Execution of the experiments by T. Brinkhoff, J. Kuever, and C. O. Wirsen. Writing of the manuscript by J. Kuever, T. Brinkhoff, and G. Muyzer.

4. Characterization of *Thiomicrospira chilensis* sp. nov., a mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacterium isolated from a *Thioploca* mat. T. Brinkhoff, G. Muyzer, C. O. Wirsen, and J. Kuever.

Development of the concept by T. Brinkhoff, J. Kuever, and G. Muyzer. Execution of the experiments by T. Brinkhoff, J. Kuever, and C. O. Wirsen. Writing of the manuscript by J. Kuever, T. Brinkhoff, and G. Muyzer.

5. A polyphasic approach to study the diversity and vertical distribution of sulfur-oxidizing *Thiomicrospira* species in coastal sediments of the German Wadden Sea. T. Brinkhoff, C. M. Santegoeds, K. Sahm, J. Kuever, and G. Muyzer.

Concept developed by T. Brinkhoff and G. Muyzer. Execution of all molecular and microbiological experiments by T. Brinkhoff supported by K. Sahm during the rRNA hybridizations. Writing by T. Brinkhoff and G. Muyzer.

6. Distribution and diversity of sulfur-oxidizing *Thiomicrospira* spp. at a shallow water hydrothermal vent in the Aegean Sea (Milos, Greece). T. Brinkhoff, S. Sievert, J. Kuever, and G. Muyzer.

Concept developed by T. Brinkhoff, G. Muyzer, J. Kuever, and S. Sievert. Execution of the molecular biological experiments by T. Brinkhoff. Execution of the microbiological experiments by S. Sievert and J. Kuever. Writing by T. Brinkhoff and G. Muyzer.

Further Publications

1. The genus *Thiomicrospira*. *In* Bergey's manual of systematic bacteriology. Brinkhoff, T., J. Kuever, G. Muyzer, and H. W. Jannasch. Submitted.

2. Xanthan degradation by an anaerobic, salt-tolerant co-culture. Friedrich, A. B., A. Pini, R. Rabus, T. Brinkhoff, and G. Muyzer. Submitted to Appl. Environ. Microbiol.

3. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. Muyzer G., T. Brinkhoff, U. Nübel, C. Santegoeds, H. Schäfer, and C. Wawer. 3.4.4: 1-27. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (eds.). Molecular microbial ecology manual, 3rd ed. Kluwer Academic Publishers, Dordrecht, The Netherlands. 1998.

4. Giant sulphur bacteria discovered in Namibian shelf sediments. Schulz, H. N., T. Brinkhoff, T. G. Ferdelman, M. Hernandez Marine, A. Teske, and B. B. Jørgensen. Submitted to Science.

5. Spatial heterogeneity of bacterial populations at a shallow-water hydrothermal vent in the Aegean Sea (Milos, Greece) as studied by the application of molecular and cultivation based techniques. Sievert, S., T. Brinkhoff, G. Muyzer, W. Ziebis, and J. Kuever. In preparation.

6. Isolation and characterization of a novel facultatively alkaliphilic, *Nitrobacter* species, *N. alkalicus* sp. nov. Sorokin, D. Y., G. Muyzer, T. Brinkhoff, J. G. Kuenen, and M. S. M. Jetten. 1998. Arch. Microbiol. 170:345-352.

7. Thiosulfate-oxidizing bacteria from North Atlantic deep-sea sediments. Teske, A., T. Brinkhoff, G. Muyzer, D. P. Moser, and H. W. Jannasch. In preparation.

8. Bacteriophage diversity in the North Sea. Wichels, A., S. S. Biel, H. R. Gelderblom, T. Brinkhoff, G. Muyzer, and C. Schütt. Appl. Environ. Microbiol. In press.

Additionally this dissertation led to flanking studies: The diploma thesis of Johanne Lüchtenborg, `Umsetzung von reduzierten Schwefelverbindungen durch neu isolierte autotrophe *Thiomicrospira* Spezies´, 1997, Fachhochschule Ostfriesland, Emden, Germany, and the diploma thesis of Thorsten Heidorn, `Kompetitionsversuche zwischen *Thiobacillus* sp. BII1 und *Thiomicrospira* sp. $t_1$5 im Chemostaten´, in preparation, TU Braunschweig, Braunschweig, Germany.

List of abbreviations

A	adenine
ATCC	american type culture collection
bp	base pairs
BSA	bovine serum albumin
C	cytosine
°C	degree Celsius
CFU	colony forming unit
CTAB	hexa-decyl-trimethyl-ammonium bromide
dA	deoxyadenosine
dG	deoxyguanosine
DGGE	denaturing gradient gel electrophoresis
Dig.	digoxygenin
DNA	desoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkuturen
E	Einstein
ed.	editor
eds.	editors
EDTA	ethylene diamine tetraacetic acid
e.g.	exempli gratia (for instance)
EMBL	European molecular biology laboratory
et. al.	et alii
g	gram
g	gravitational acceleration
G	guanine
Fig.	figure
h	hour
HPLC	high performance liquid chromatography
i.e.	id est (that is)
l	liter

µ	micro-
m	milli-
M	molar
min	minute
mm	millimeter
MPa	mega Pascal
MPN	most-probable-number
n	nano-
n.d.	not determined
no.	number
nov.	novum
n.r.	not reported
p	pico-
PAUP	phylogenetic analysis using parsimony
PCI	phenol-chloroform-isoamylalcohol
PCR	polymerase chain reaction
PVPP	polyvinylpolypyrrolidone
rDNA	rRNA encoding gene
RDP	Ribosomal Database Project
rRNA	ribosomal ribonucleic acid
RuBisCO	ribulose bisphosphate carboxylase
SDS	sodium-dodecyl-sulfate
SOB	sulfur-oxidizing bacteria
sp.	species (singular)
spp.	species (pural)
SSC	salt-sodiumcitrate
str.	strain
symb.	symbiont
T	thymine
T.	*Thiomicrospira*
TAE	Tris-Acetate-EDTA
T_d	temperature of dissociation
TE	Tris-EDTA

temp.	temperature
TP	*Thiomicrospira pelophila*
Tris	Trishydroxymethylaminoethan
U	uracil
UV	ultra violet
V	voltage
vol	volume
(vol/vol)	volume per volume
(v/v)	volume per volume
wt	weight
(wt/vol)	weight per volume
(wt/wt)	weight per weight
(w/v)	weight per volume

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