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

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A new member of the genus *Thiomicrospira*, which utilizes thiosulfate as the electron donor and CO_2 as the carbon source, was isolated from a sediment sample dominated by the filamentous sulfur bacterium *Thioploca*. Although the physiological properties investigated are nearly identical to other described species of the genus, it is proposed that strain Ch-1^T is a member of a new species, *Thiomicrospira chilensis* sp. nov., on the basis of differences in genotypic characteristics (16S rRNA sequence, DNA homology, G+C content). Strain Ch-1^T was highly motile 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 an electron acceptor. Chemolithoautotrophic growth was observed with thiosulfate, tetrathionate, sulfur and sulfide. The isolate was not able to grow heterotrophically. Growth of strain Ch-1^T was observed between pH 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 optimal growth temperature was between 32 and 37 °C. The mean maximum growth rate on thiosulfate was 0·4 h^{-1}. This is the second *Thiomicrospira* species described that has a rod-shaped morphology; therefore discrimination between vibrio-shaped *Thiomicrospira* and rod-shaped *Thiobacilli* is no longer valid.

Keywords: *Thiomicrospira chilensis* sp. nov., sulfur-oxidizing bacteria, *Thioploca* mat

INTRODUCTION

Most *Thiomicrospira* species 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.*, 1999). All members of this genus are obligately chemolithoautotrophic sulfur-oxidizing bacteria. The ecological importance of *Thiomicrospira* species in marine habitats rich in reduced-sulfur compounds has already been demonstrated (Muyzer *et al.*, 1995; Brinkhoff & Muyzer, 1997) but very little is known about their role in sulfide-influenced habitats that 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* species was isolated from a sediment sample from the Solar Lake, Egypt, which can be regarded as an model system for such an ecosystem (Brinkhoff & Muyzer, 1997). In the work described in this paper, we used samples from a different mat-like community, the *Thioploca* mats on the Chilean coastal shelf, to obtain *Thiomicrospira* isolates.

Abbreviation: RuBisCO, ribulose-bisphosphate carboxylase/oxygenase.

The GenBank accession number for the 16S rDNA sequence of strain Ch-1^T reported in this paper is AF013975.

METHODS

Culture media and isolation of bacteria. The medium used (TP) and the procedure for isolation were as described previously (Brinkhoff *et al.*, 1999).

Growth experiments and utilization of electron donors. All growth experiments and tests for electron donors were carried out as described previously (Brinkhoff *et al.*, 1999); the results are summarized in Table 3.

Chemical, biochemical and molecular-biological analysis. The formation of intermediates and final products during growth on thiosulfate was monitored by cyanolysis (Kelly *et al.*, 1969) and HPLC (Rethmeier *et al.*, 1997). The ubiquinones were analysed by B. J. Tindall (DSMZ Identification Service, Braunschweig, Germany) and the DNA base composition analysis and the DNA-DNA hybridizations were carried out by J. Burghardt (DSMZ), as described previously (Brinkhoff *et al.*, 1999).

The ribulose-bisphosphate carboxylase/oxygenase (RuBisCO) activity in cell-free extracts was measured at 30 °C as described before (Brinkhoff *et al.*, 1999). 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* species and closely related organisms.

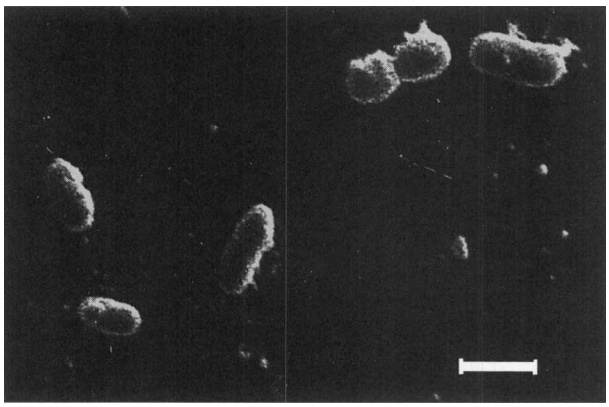

Fig. 1. Electron micrograph of strain Ch-1^T, showing the typical rod-shaped cell form. Bar, 1 μm.

RESULTS

Isolation of strain Ch-1^T

Enrichment cultures were inoculated with mud from the continental shelf off Chile at the Bay of Concepcion at 36°32′ S (Fossing *et al.*, 1995). Using sulfide gradients, we observed a large 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 more recent publication revised the taxonomic position of this taxon and described it as '*Aquaspirillum bipunctata*' (Dubinina *et al.*, 1993). After several attempts to enrich and isolate these organisms, a good enrichment culture was only obtained by using thiosulfate as the electron donor. Differing from the original enrichment on sulfide, this culture was dominated by short vibrios and rods without sulfur globules. Using solid modified medium TP, as described recently (Brinkhoff *et al.*, 1999), a vibrio-shaped organism (Ch-2) was isolated. After filtration of the enrichment culture through a 0·45 μm filter, a rod-shaped, sulfur-oxidizing bacterium (Ch-1^T) was obtained in pure culture. Both isolates were identified as *Thiomicrospira* species by using the specific PCR approach (Brinkhoff & Muyzer, 1997). The two isolates could be distinguished on the basis of nearly complete sequences of the 16S rRNA-encoding genes. The sequence obtained from the vibrio-shaped *Thiomicrospira* isolate was nearly identical (99·5%) to that of *Thiomicrospira crunogena*, whereas the sequence obtained from the rod-shaped isolate showed less than 96% identity to all *Thiomicrospira* species described (see Table 1). Therefore, only this latter isolate, known as *Thiomicrospira* sp. Ch-1^T, was characterized further.

Morphology

Cells of strain Ch-1^T appeared as single, motile rods of 0·3–0·5 × 0·8–2 μm (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 *Thiomicrospira frisia* (Brinkhoff *et al.*, 1999). Spore formation was absent and the Gram reaction was negative.

Table 1. 16S rDNA identities between strain Ch-1^T and related taxa of the γ-subclass of the *Proteobacteria*

Sequences with the following accession numbers were compared: *Chromatium* (*Chr.*) *vinosum*, M26629; *Calyptogena* (*Cal.*) *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 chilensis* strain Ch-1^T, AF013975.

Taxon	*Chr. vinosum*	*Cal. magnifica* gill symbiont	*T. pelophila*	*T. thyasirae*	*T. crunogena*	*Thiomicrospira* sp. L-12	*Thiomicrospira* sp. MA2-6	*T. kuenenii*	*T. frisia*
Chromatium vinosum									
Calyptogena magnifica gill symbiont	84·0								
Thiomicrospira pelophila	85·0	84·7							
Thiomicrospira thyasirae	85·6	85·1	99·9						
Thiomicrospira crunogena	84·9	84·2	92·5	92·5					
Thiomicrospira sp. L-12	86·0	84·6	92·4	92·4	99·2				
Thiomicrospira sp. MA2-6	84·9	84·7	92·6	92·7	97·3	97·4			
Thiomicrospira kuenenii	84·1	83·1	94·1	92·3	95·7	96·1	95·1		
Thiomicrospira frisia	84·1	84·0	91·1	91·3	94·7	94·8	93·5	92·2	
Thiomicrospira chilensis Ch-1^T	83·8	84·8	92·1	92·3	94·9	94·9	93·9	93·1	95·3

Table 2. Levels of DNA–DNA similarity for *Thiomicrospira* species

Taxon	Relatedness (%)			
	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	
T. chilensis Ch-1^T	31	27	16	18

Growth conditions

The isolate was strictly aerobic and grew autotrophically on thiosulfate, tetrathionate, sulfur and sulfide but not on sulfite or thiocyanate. Growth on thiosulfate reduced the pH from 7·2 to 4·5. Thiosulfate was completely oxidized to sulfate, with a recovery of 92–99%. 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 substrates 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; nitrite was not tested. Hydrogen was not used as an electron donor for autotrophic growth. Addition of vitamin B_{12} enhanced growth but was not essential for growth. For strain Ch-1^T, growth was observed between pH 5·3 and 8·5 with an optimum at pH 7·0. At 30 °C and optimal pH, the maximum growth rate on thiosulfate was 0·4 h^{-1}.

When the pH was readjusted to the optimal pH during growth on thiosulfate, the new isolate caused 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. Tetrathionate was not detected.

RuBisCO activity

RuBisCO activity was found in cell-free extracts of the new isolate. The specific activity for strain Ch-1^T was 8·2 nmol carbon fixed mg protein^{-1} min^{-1}.

DNA base ratio and ubiquinone content

The G+C content of DNA of 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 Tables 1 and 2. These results show clearly that strain Ch-1^T can be considered as a new *Thiomicrospira* species.

DISCUSSION

Thioploca species are well adapted to use nitrate as a terminal electron acceptor. All *Thiomicrospira* species, with the exception of *Thiomicrospira denitrificans*, are dependent on oxygen as the terminal electron acceptor. Although still included within the genus, *T. denitrificans* should be reclassified because it belongs to the ε-subclass of the *Proteobacteria* (Muyzer *et al.*, 1995). Using DNA extracted from these *Thioploca* mats, the specific PCR approach was negative for *Thiomicrospira* species belonging to the γ-subclass of the *Proteobacteria* (Brinkhoff & Muyzer, 1997), suggesting that the numbers of individuals from this genus are negligible in this habitat compared with hydrothermal vent systems. Nevertheless, we were able to isolate two *Thiomicrospira* species 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 *et al.* (1998) that this species can be found worldwide and seems to be favoured by using enrichment techniques.

Comparison of the nearly complete 16S rRNA-gene sequence shows that the sequence of strain Ch-1^T is at least 4% different from other *Thiomicrospira* species that have been described. According to the definition of Stackebrandt & Goebel (1994), this indicates that strain Ch-1^T does not belong to a species already described. 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 species described previously. The DNA base ratio of 49·9% for strain Ch-1^T is much higher than those for the other species of the genus. Based on the great genotypic differences, it can be speculated that strain Ch-1^T may also have some metabolic properties that separate it from other *Thiomicrospira* species, but which were not detected using the general descriptive methods. Like *T. frisia*, strain Ch-1^T has a rod-like shape, which is not found for the other *Thiomicrospira* species. Differentiation of *Thiomicrospira* species from *Thiobacillus* species on the basis of morphology is no longer valid. Together with *T. frisia* and *Thiomicrospira* sp. strain Art-3, strain Ch-1^T falls into a sub-cluster of rod-shaped organisms (J. Kuever, unpublished results) within the *Thiomicro-*

Table 3. Morphological and physiological characteristics among *Thiomicrospira* species

Data were obtained from the present study and from Kuenen & Veldkamp (1972, 1973), Kuenen & Robertson (1989), Jannasch *et al.* (1985) and Brinkhoff *et al.* (1999).

Character	*T. pelophila*	*T. crunogena*	*T. kuenenii* JB-A1^T	*T. frisia* JB-A2^T	*T. chilensis* Ch-1^T
Shape	Vibrio	Vibrio	Vibrio	Rod	Rod
Width (μm)	0·2–0·3	0·4–0·5	0·3–0·4	0·3–0·5	0·3–0·5
Length (μm)	1–2	1·5–3·0	1–2·5	1–2·7	0·8–2
Motility	+	+	+	+*	+
G+C content (mol%)†	45·7 (44)	44·2 (42)	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
Growth pH:					
Optimal	7·0‡	7·5–8·0	6·0	6·5	7·0
Range	5·6–9·0	5·0–8·5	4·0–7·5	4·2–8·5	5·3–8·5
Growth temperature (°C):					
Optimal	28–30	28–32	29–33·5	32–35	32–37
Range§	3·5–42	4–38·5	3·5–42	3·5–39	3·5–42
Na^+ concentration (mM):					
Optimal	470	ND	470	470	470
Range	40–1240‖	> 45	100–640	100–1240	100–1240
Vitamin B_{12} dependence	+	−	−	−	−
RuBisCO activity	+	+	+	+	+
Formation of sulfur from thiosulfate at pH 7·0 in liquid medium	+	+	−	−	+

* Motility can decrease rapidly during growth.

† Determined by HPLC; values in parentheses were determined previously by thermal denaturation.

‡ Data from present study, determined by CO_2 incorporation.

§ Growth of all taxa except *T. crunogena* 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.

spira cluster, which was notable in the phylogenetic tree published by Brinkhoff & Muyzer (1997). Therefore, we propose that the isolate Ch-1^T should be considered as a new species of this genus and be given the name *Thiomicrospira chilensis* sp. nov.

Thiomicrospira chilensis (chi.len'sis. M.L. adj. *chilensis* from Chile, South America).

Cells are Gram-negative, motile and rod-shaped (0·3–0·5 × 0·8–2 μm). Strictly aerobic and grows autotrophically on thiosulfate, tetrathionate, sulfur and sulfide but not on sulfite or thiocyanate. 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 3·5–42 °C; optimal growth occurs at pH 7·0 and at 32–37 °C. The optimal Na^+ concentration for growth is 470 mM; growth is possible between Na^+ concentrations of 100 and 1240 mM. Carbon dioxide is fixed by means of ribulose-bisphosphate carboxylase/oxygenase. Nitrate is not used as a 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 49·9 mol%. As determined by a 16S rRNA gene sequence analysis, *Thiomicrospira chilensis* belongs to the γ-subclass of the *Proteobacteria* and is closely related to previously described members of the genus *Thiomicrospira*. The type strain of the species, Ch-1^T, has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen as strain DSM 12352^T. The GenBank accession number for the nearly complete 16S rRNA gene sequence of *T. chilensis* is AF013975.

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