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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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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, it is proposed that strain JB-A1^T (= DSM 12350^T) and strain JB-A2^T (= DSM 12351^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 pH 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 and 35 °C. The mean maximum growth rate on thiosulfate was 0·35 h^{-1} for strain JB-A1^T and 0·45 h^{-1} for strain JB-A2^T.

Keywords: *Thiomicrospira*, sulfur-oxidizing bacteria, Wadden Sea

INTRODUCTION

The genus *Thiomicrospira* was first described by Kuenen & Veldkamp (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 subclass of the *Proteobacteria* (Muyzer *et al.*, 1995; Brinkhoff & Muyzer, 1997).

The aim of this study was to isolate new *Thiomicrospira* spp. from intertidal marine flats and get a better understanding of their ecological role in comparison to species isolated from deep-sea hydrothermal vent systems.

Abbreviation: RuBisCO, ribulose-bisphosphate carboxylase.

The GenBank accession numbers for the 16S rRNA sequences of strains JB-A1^T and JB-A2^T are AF013978 and AF013974, respectively.

METHODS

Culture media. The medium used for enrichments, isolation and routine culture work was liquid medium based on the medium for *T. 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 single organic compounds and 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 8 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 colour 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 was 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 the 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 (DSMZ) (Braunschweig, Germany) and *Thiomicrospira 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 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 re-adjusted 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 was 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 colour 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 aluminium 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 30 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 10 d. The optimal growth temperature was determined within 24 h after inoculation.

The Na^+ requirement of the isolates was tested in TP medium supplied with 20 mM thiosulfate containing various 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) or 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%/20% (v/v) hydrogen/air 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 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. J. 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 TLC (Macherey-Nagel no. 805 203), using hexane:*tert*-butyl methyl ether as solvent (9:1). UV-absorbing bands corresponding to ubiquinones were removed from the plate and further analysed by HPLC. The latter was carried out on an LDC Analytical (Thermoseparation products) HPLC apparatus fitted with a reverse-phase column (Macherey-Nagel, 2 mm × 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 was purified on hydroxyapatite (Cashion *et al.*, 1977). The DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The resulting deoxyribonucleosides were analysed 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 Ley *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 Coomassie brilliant blue dye binding technique (Bradford, 1976) using a Bio-Rad protein assay kit.

Phylogenetic analysis. The 16S rRNA sequences of strains JB-A1^T (*Thiomicrospira kuenenii*; GenBank accession no. AF013978) and JB-A2^T (*Thiomicrospira frisia*; GenBank accession no. AF013974) and the phylogenetic position were 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.

RESULTS

Isolation of strains 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.

Morphology

Cells of strain JB-A1^T appeared as single motile vibrios of 0·3–0·4 × 1·0–2·5 μm in size (Fig. 1a), whereas cells of JB-A2^T were slightly bent rods of 0·3–0·5 × 1·0–2·7 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.

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 substrates 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 mean 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 0·3–0·4 h^{-1}), whereas strain JB-A2^T showed a slightly higher rate of 0·45 h^{-1} (range 0·4–0·5 h^{-1}). The rates were nearly the same for 100% and 20% air-saturated medium.

When the pH was re-adjusted 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 a 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 pH 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*

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*

The following accession numbers were used: *Chromatium vinosum* (M26629), *Thiothrix nivea* (M79435), *Piscirickettsia salmonis* (U36915), *Bathymodiolus thermophilus* (mussel) 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) and *Thiomicrospira* sp. JB-A2^T (AF013974).

	1	2	3	4	5	6	7	8	9	10
1 *Chromatium vinosum*										
2 *Thiothrix nivea*	88·5									
3 *Piscirickettsia salmonis*	85·2	87·1								
4 *Bathymodiolus thermophilus* sym.	85·5	86·9	84·6							
5 *Thiomicrospira pelophila*	84·9	84·3	83·9	86·0						
6 *Thiomicrospira thyasirae*	85·2	84·7	84·0	85·9	99·9					
7 *Thiomicrospira crunogena*	84·5	84·2	85·1	85·6	92·3	92·2				
8 *Thiomicrospira* sp. L-12	84·6	84·3	84·8	85·5	91·7	91·7	99·1			
9 *Thiomicrospira* sp. MA2-6	84·7	84·8	84·5	86·1	92·5	92·5	97·2	97·1		
10 *Thiomicrospira* sp. JB-A1^T	83·9	84·4	84·9	85·4	92·1	92·1	95·6	95·7	95·0	
11 *Thiomicrospira* sp. JB-A2^T	83·9	84·1	84·6	85·4	91·1	91·1	94·5	94·3	93·4	94·1

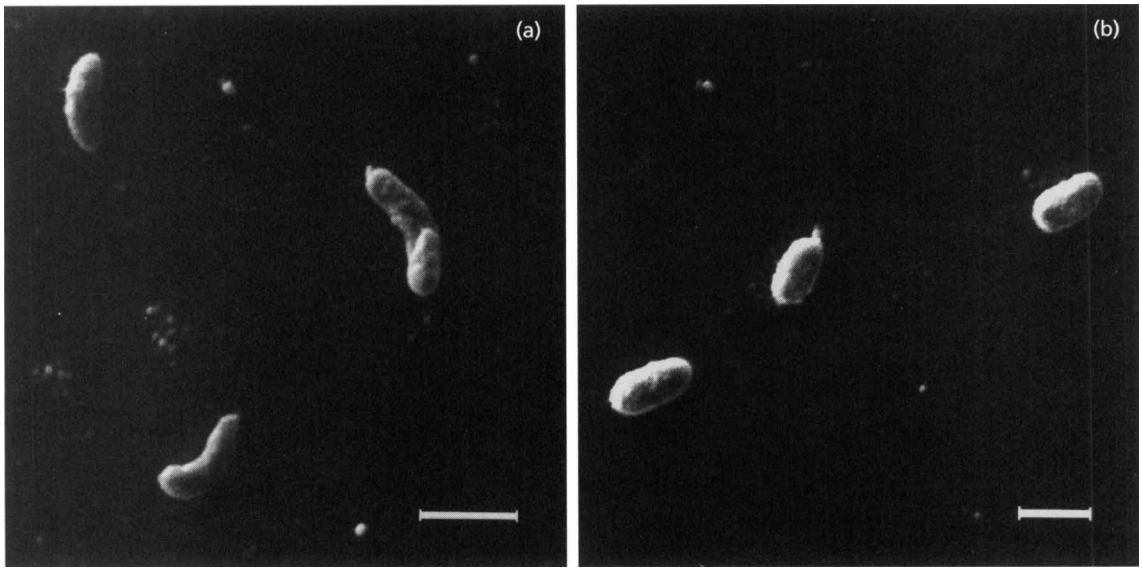

Fig. 1. Electron micrographs of both strains. (a) Typical morphology of strain JB-A1^T, very similar to all other described *Thiomicrospira* spp.; (b) typical rod-like morphology of strain JB-A2^T, which is different to the other *Thiomicrospira* spp. Bars, 1 μm.

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 3 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 and 35 °C. Growth was determined by acidification of the medium over a period of 10 d.

Both isolates showed a requirement for Na^+ 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,

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

Organism	Homology (%)		
	T. pelophila	*T. crunogena*	*T. kuenenii* (JB-A1^T)
T. pelophila	100·0		
T. crunogena	33·5	100·0	
T. kuenenii (JB-A1^T)	25·0	29·3	100·0
T. frisia (JB-A2^T)	27·0	27·0	25·0

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 described previously (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

RuBisCO was found in cell-free extracts of both isolates. The specific activity for strain JB-A1^T was 9·75 nmol C fixed (mg protein)$^{-1}$ min^{-1}, and strain JB-A2^T showed a specific activity of 12·65 nmol C fixed (mg protein)$^{-1}$ min^{-1}. Values for *T. pelophila* and *T. crunogena* were in a similar range and agreed well with previously published data (Kuenen & Veldkamp, 1973).

DNA base ratio and ubiquinone content

The G+C content was 42·4±0·2 mol% for strain JB-A1^T and 39·6±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 Tables 1 and 2, respectively. Comparison of the nearly complete 16S rRNA genes shows that strain JB-A1^T has at least a 4% difference and JB-A2^T has at least a 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 than 30% for JB-A1^T and JB-A2^T.

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 & 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 by 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 re-adjusted 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.

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

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

Data from Kuenen & Veldkamp (1972, 1973); Kuenen & Robertson (1989); Jannasch *et al.* (1985); and own data.

Character	*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	1·0–2·5	1·0–2·7
Motility	+	+	+	+*
G+C content (mol%)	45·7† (45)	44·4† (44)	42·4†	39·6†
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‡	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 temp. (°C)	28–30	28–32	29–33·5	32–35
Temp. range (°C)	3·5–42§	4–38·5	3·5–42§	3·6–39§
Optimal Na^+ concn (mM)	470	ND	470	470
Na^+ concn range (mM)	40–1240‖	At least 50	100–640	100–1240
Vitamin-B_{12}-dependent	+	−	−	−
RuBisCO	+	+	+	+
Formation of sulfur from thiosulfate at pH 7·0 in liquid medium	+	+	−	−

* Motility can rapidly decrease during growth.

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

‡ Data from present study, determined by CO_2 incorporation.

§ Growth would be likely to occur below 3·6 °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.

(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 favouring 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). All these compounds, except sulfite and sulfate, 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 6·5–7·0 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 and 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 slow 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 × 1·0–2·5 μm). *T. kuenenii* is strictly aerobic and grows autotrophically on thiosulfate, tetrathionate, sulfur and sulfide, but not on sulfite or 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 3·5–42 °C; optimum growth occurs at pH 6·0 and at 29–33 °C. The optimal Na^+ concentration for growth is 470 mM; growth is possible between Na^+ concentrations of 100 and 640 mM. CO_2

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, *T. kuenenii* belongs to the gamma subclass of the *Proteobacteria* and is closely related to previously described members of the genus *Thiomicrospira*. The type strain of the species is JB-A1^T (= DSM 12350^T). 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 north-west of Germany and the Netherlands).

Cells are Gram-negative, motile and bent-rod shaped (0·3–0·5 × 1·0–2·7 μm). During late-exponential growth cells have a strong tendency to clump and to form aggregates. *T. frisia* is strictly aerobic and grows autotrophically on thiosulfate, tetrathionate, sulfur and sulfide, but not on sulfite or 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 3·5–39 °C; optimum growth occurs at pH 6·5 and at 32–35 °C. The optimal Na^+ concentration for growth is 470 mM; growth is possible between a Na^+ concentration of 100 and 1240 mM. CO_2 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, *T. frisia* belongs to the gamma subclass of the *Proteobacteria* and is closely related to previously described members of the genus *Thiomicrospira*. The type strain of the species is JB-A2^T (= DSM 12351^T). The GenBank accession number for the nearly complete 16S rRNA gene sequence of *T. frisia* is AF013974.

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