Halothiobacillus kellyi* sp. nov., a mesophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium isolated from a shallow-water hydrothermal vent in the Aegean Sea, and emended description of the genus *Halothiobacillus

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A new mesophilic, chemolithoautotrophic, sulfur-oxidizing bacterium, strain Milos-BII1^T, was isolated from a sediment sample taken from a shallow-water hydrothermal vent in the Aegean Sea with thiosulfate as electron donor and CO₂ as carbon source. Based on the almost complete sequence of the 16S rRNA gene, strain Milos-BII1^T forms a phylogenetic cluster with *Thiobacillus hydrothermalis*, *Thiobacillus neapolitanus*, *Thiobacillus halophilus* and *Thiobacillus* sp. W5, all of which are obligately chemolithoautotrophic bacteria. Because of their phylogenetic relatedness and their physiological similarities it is proposed to transfer these organisms to a newly established genus within the γ -subclass of the *Proteobacteria*, *Halothiobacillus* gen. nov. (Kelly and Wood 2000). Strain Milos-BII1^T represents a new species of this genus, named *Halothiobacillus kellyi*. Cells were Gram-negative rods and highly motile. 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. Growth was observed between pH values of 3.5 and 8.5, with an optimum at pH 6.5. The temperature limits for growth were 3.5 and 49 °C, with an optimum between 37 and 42 °C. Growth occurred between 0 and 2 M NaCl, with an optimum NaCl concentration between 400 and 500 mM. The mean maximum specific growth rate on thiosulfate was 0.45 h⁻¹.

Keywords: *Thiobacillus*, *Halothiobacillus*, sulfur-oxidizing bacteria, hydrothermal vent

INTRODUCTION

The genus *Thiobacillus* contains a wide range of Gram-negative, rod-shaped, colourless sulfur bacteria, which have in common the ability to use reduced sulfur compounds as electron donor for autotrophic growth (Kelly & Harrison, 1989; Kuenen *et al.*, 1992). In contrast to the original description of an obligately

autotrophic, sulfur-oxidizing organism given by Beijerinck (Beijerinck, 1904; Beijerinck & Minkman, 1910) several species growing heterotrophically or mixotrophically have also been named *Thiobacillus* because they could use reduced sulfur compounds as electron donors and produced sulfate as end product. The different physiological properties and the range of DNA base composition (45–70 mol % G + C) already indicated the need for a reclassification of members of this genus. This was even more obvious after the sequence data for the 16S rRNA genes showed that the species of *Thiobacillus* fall into the α -, β - and γ -subclasses of the *Proteobacteria* (Lane *et al.*, 1992). Since then, several species of this genus have been

Abbreviations: DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); MPN, most probable number.

The GenBank accession numbers for the nearly complete 16S rRNA sequences of strain Milos-BII1^T and *Halothiobacillus neapolitanus* (formerly *Thiobacillus neapolitanus*) are AF170419 and AF173169, respectively.

reclassified (Katayama *et al.*, 1995; Moreira & Amils, 1997; Hiraishi *et al.*, 1998). Because of their phylogenetic relatedness and their physiological similarities, Kelly & Wood (2000) proposed the transfer of *Thiobacillus hydrothermalis*, *Thiobacillus neapolitanus* and *Thiobacillus halophilus* to a new genus within the γ -subclass of the *Proteobacteria*, *Halothiobacillus* gen. nov. In this paper, we describe a new species of a marine, obligately chemolithoautotrophic, sulfur-oxidizing bacterium which, based on phylogenetic and physiological data, belongs to the genus *Halothiobacillus*. Together with *Halothiobacillus hydrothermalis*, its closest relative, this is the second example of a chemolithoautotrophic, sulfur-oxidizing bacterium isolated from a marine hydrothermal vent system which does not belong to the genus *Ventimicrospira*. In addition, the complete 16S rDNA sequence of *Halothiobacillus neapolitanus* (formerly *Thiobacillus neapolitanus*), the type strain of the proposed genus *Halothiobacillus*, was determined because the earlier sequence was incomplete.

METHODS

A mineral medium with 20 mM thiosulfate (added by sterile filtration to the autoclaved medium) as sole electron donor was used for enrichment, isolation and routine culture work. The composition of the mineral medium (TB medium) (in g l⁻¹) was: NaCl (29), (NH₄)₂SO₄ (1), MgSO₄·7H₂O (1.5), CaCl₂·2H₂O (0.42), K₂HPO₄ (0.5), KCl (0.7), vitamin B₁₂ (0.00005), with trace element solution containing EDTA (Widdel & Bak, 1992) (1 ml l⁻¹). Bromothymol blue was added as pH indicator at a concentration of 4 mg l⁻¹. K₂HPO₄ was autoclaved separately and added to the medium after autoclaving.

Isolation of bacteria. Most probable number (MPN) estimates and enrichment cultures were obtained by inoculating medium with 1 cm³ sediment from a shallow-water hydrothermal vent system located in the Bay of Palaeochori, Milos, Greece. A detailed description of the MPN counting procedure, the sampling site and the prevailing environmental conditions was given by Sievert *et al.* (1999). The cultures were incubated at 22 °C in the dark to avoid growth of phototrophic bacteria. After growth had occurred, as indicated by a change in the colour of the pH indicator and by sulfur deposition, 1 ml culture was transferred to 10 ml fresh medium. For isolation of pure cultures, 0.1 ml aliquots 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.

Growth experiments. Batch cultures were grown in 500 ml flasks containing 100 ml medium on a rotary shaker at 30 °C in the dark. Routine cultivation of the isolates and utilization of different substrates were investigated in 15 ml tubes containing 10 ml mineral medium. Large-scale cultivation was done in 3 and 20 l glass carboys supplied with 40 mM thiosulfate, in which the pH was monitored by a sterilized pH electrode (Ingold) and readjusted by titration with Na₂CO₃ (1 M) through a personal computer program controlling a peristaltic pump. The program was developed by V. Meyer at the Max-Planck-Institute for Marine Microbiology, Bremen, Germany.

The maximum specific growth rate in thiosulfate medium was determined at 22 and 37 °C by direct counts [DAPI (4,6-

diamidino-2-phenylindole) staining and epifluorescence microscopy; Porter & Feig, 1980], increase in optical density (at 420 nm) or protein concentration. Protein was determined by the Coomassie brilliant blue dye binding technique (Bradford, 1976) using a Bio-Rad protein assay kit. Growth kinetics on thiosulfate in continuous cultures were determined in home-made chemostats. The pH was controlled and adjusted as indicated above. Dissolved oxygen was supplied in excess at 50–100% air saturation. The oxygen concentration was monitored by an autoclavable oxygen electrode (Ingold) and adjusted via magnetic valves controlled by the same computer program as indicated above.

Estimates of the optimal pH value for growth of the new isolate and the lowest and highest pH values tolerated were determined by using medium adjusted to different initial pH values (3.5–10.0). A colour change of the pH indicator upon acidification indicated growth. The optimal pH was determined by measuring the oxygen consumption rate at different pH values. The chemostat was equilibrated under substrate limitation at a dilution rate, *D*, of 0.1 h⁻¹ (22 °C, pH 7.0). The addition of substrate was stopped, the dissolved oxygen concentration adjusted to 100% air saturation and the desired pH value of the medium adjusted. The cells were then supplied with fresh medium at *D* = 0.4 h⁻¹ and the oxygen consumption rate was determined. This procedure was carried out for each pH value from 5.0 to 8.0 at 0.5 unit intervals.

The optimal growth temperature of the new isolate was determined in a thermally insulated aluminium block which was heated electrically to +60 °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 4 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 NaCl requirement and tolerance of the isolates were tested in medium supplied with 20 mM sodium thiosulfate containing varying NaCl concentrations (in M: 0, 0.2, 0.4, 0.55, 0.8, 1.0, 2.0, 2.5 and 3.0) and incubated at 30 °C.

Utilization of inorganic electron donors. The ability to oxidize and grow on different reduced sulfur compounds was tested by using TB medium supplemented with one of the following compounds: thiosulfate (20 mM), tetrathionate (10 mM), sulfite (1, 3, 5, 10 and 20 mM), thio-cyanate (1, 3, 5 and 10 mM) and elemental sulfur (0.1%, w/v). The sulfite stock solution was prepared in 50 mM EDTA to prevent autooxidation. Substrate utilization and product formation was monitored according to Rethmeier *et al.* (1997). Sulfite oxidation was tested with a solution of Ellman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid); DTNB] (1 g l⁻¹ in 50 mM potassium phosphate buffer pH 7.0). DTNB is reduced by sulfite and thiol groups to a yellow-coloured product (maximum absorbance at 412 nm). 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 (Nishihara *et al.*, 1989).

Anaerobic growth. The use of nitrate as an electron acceptor in the absence of oxygen was tested under autotrophic and

heterotrophic conditions by using bicarbonate (30 mM)-buffered medium supplied with 20 mM KNO₃ and prepared anaerobically. Hungate tubes contained 10 ml liquid medium under a 90%/10% (v/v), N₂/CO₂ gas phase and the various electron donors indicated above.

Utilization of organic electron donors. Heterotrophic growth was checked in TB medium without pH indicator supplied with one of the following compounds: fructose (5 mM), glucose (5 mM), formate (20 and 40 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 TB 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 cultures as described previously (Brinkhoff *et al.*, 1999a).

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 hydrolysed with P1 nuclease and the nucleotides 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) at the DSMZ (Braunschweig, Germany) (Mesbah *et al.*, 1989).

PCR amplification and sequencing of the 16S rRNA gene. To amplify the almost complete 16S rRNA encoding gene (1500 bp) of strains MT-96, M40/2 CIV-2.3 and M40/2 CIV-3.2, primers GM3F and GM4R were used in a 35-cycle PCR with an annealing temperature of 40 °C. PCR products were purified by using the QIAquick Spin PCR purification kit (Qiagen) as described by the manufacturer. The Taq Dyedexy Terminator Cycle Sequencing kit (Applied Biosystems) was used to directly sequence the PCR products according to the protocol provided by the manufacturer. The sequencing primers were as described by Buchholz-Cleven *et al.* (1997). The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer.

Comparative analysis of 16S rRNA sequences. The 16S rRNA sequences used in this study were: *Thiomonas thermosulfata* ATCC 51520^T (U17839), *Thiomonas cuprina* DSM 5495^T (U67162), *Acidiphilium acidophilum* DSM 700^T (D86511), *Thiobacillus thioparus* DSM 505^T (M79426), *Thiobacillus thiooxidans* DSM 504^T (M79396, M79397, M79398), *Thiobacillus ferrooxidans* DSM 583^T (M79404, M79405, M79406), *Thiobacillus caldus* DSM 8584^T (Z29975), *Thiobacillus tepidarius* DSM 3134^T (M79399, M79419, M79420), *Thiomonas perometabolis* ATCC 23370^T (M79399, M79400), *Thiobacillus novellus* DSM 506^T (D32247), *Thiobacillus* sp. W5 (X97534), *Halothiobacillus halophilus* DSM 6132^T (U58020), *H. hydrothermalis* DSM 7121^T (M90662), *H. neapolitanus* DSM 581^T (M79418, M79419, 79420). Sequences that were not included in the 16S rRNA sequence database ARB of the Technical University Munich (O. Strunk and others, <http://www.mikro.biologie.tu>.

muenchen.de) were added from databases. The tool ARB-align was used for sequence alignment. The alignment was checked by eye and corrected manually. Tree topologies were evaluated by performing maximum-parsimony, neighbour-joining and maximum-likelihood analysis. Only at least 90% complete sequences were used for the calculation of different trees. Partial sequences were inserted into the reconstructed tree by applying the parsimony criteria without allowing for changes in the overall tree topology.

RESULTS

Isolation of strain Milos-BII1^T

Pure cultures were obtained by subculturing single colonies of the lowest dilution of the MPN series and from enrichments. After sequencing of the 16S rRNA genes of these isolates, it became obvious that they were all identical, but showed less than 93% similarity to all described *Thiobacillus* spp. (see Table 1). Several identical strains were obtained from different zones and sediment depths, indicating widespread occurrence at the vent site. However, *Thiobacillus* spp. were only isolated from the low dilutions of the MPN series, which could also be seen as enrichment cultures. The higher dilutions of the series lead to the isolation of other sulfur-oxidizing bacteria, for example *Thiomicrospira* spp. (Brinkhoff *et al.*, 1999b). One isolate, Milos B-II1^T, originating from sediment underlying a white precipitate (sediment layer 0–5 mm) that formed at a specific region around the vent site (Sievert *et al.*, 1999) was used for further characterization. The *in situ* temperature in this zone increased from 22 °C at the sediment surface to almost 50 °C at 5 cm sediment depth; the *in situ* pH was about 6.0–7.0 at the sediment surface and remained constant at about 5.5 in the sediment (Sievert *et al.*, 1999).

Morphology

Cells of strain Milos-BII1^T appeared singly or in pairs as motile rods 0.4–0.6 µm in width and 1.2–2.5 µm in length. The isolate was Gram-negative and spore-formation was absent.

Growth conditions

The isolate was strictly aerobic and grew autotrophically on thiosulfate, tetrathionate, sulfur and sulfide, but not on thiocyanate. Growth on thiosulfate lowered the pH from neutrality to 2.8–3.0. Thiosulfate was completely oxidized to sulfate, with a recovery of 90–99%. Formation of elemental sulfur was observed on solid media and in liquid media. Sulfite was oxidized to sulfate if the concentration in the medium did not exceed 3 mM. EDTA alone showed no toxic effect. In controls without bacteria, sulfite remained present as indicated by the reduction of DTNB. No growth occurred in medium supplemented with any of the organic substrates tested. Oxidation of thiosulfate was not inhibited by any of the organic substrates. Nitrate was not used as a terminal electron acceptor. Hydrogen

Table 1. Similarity values for the 16S rRNA sequences of *Halothiobacillus* sp. Milos-BII1^T and other micro-organisms originally classified as *Thiobacillus*

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. <i>Tm. perometabolis</i> ATCC 23370 ^T	—														
2. <i>Tm. thermosulfata</i> ATCC 51520 ^T	95.0	—													
3. <i>Tm. cuprina</i> DSM 5495 ^T	93.7	93.8	—												
4. <i>Tb. thioparus</i> DSM 505 ^T	87.7	87.7	89.0	—											
5. <i>Thiobacillus</i> sp. W5	85.7	83.1	82.9	83.4	—										
6. <i>Ht. hydrothermalis</i> DSM 7121 ^T	85.3	83.4	83.6	84.3	90.2	—									
7. <i>Tb. thiooxidans</i> DSM 504 ^T	83.0	82.3	82.6	83.2	84.1	83.8	—								
8. <i>Tb. ferrooxidans</i> DSM 583 ^T	83.8	84.0	84.1	83.9	84.5	84.5	98.2	—							
9. <i>Tb. caldus</i> DSM 8584 ^T	83.2	82.5	82.7	83.9	83.6	84.5	94.8	96.3	—						
10. <i>Tb. tepidarius</i> DSM 3134 ^T	83.7	82.8	83.1	83.5	84.3	83.6	90.2	90.7	89.6	—					
11. <i>Tb. novellus</i> DSM 506 ^T	83.0	81.1	81.0	80.3	81.1	81.1	80.4	82.6	80.1	82.1	—				
12. <i>Ap. acidophilum</i> DSM 700 ^T	83.2	82.7	83.0	82.0	83.7	84.2	83.5	84.4	84.0	82.6	85.3	—			
13. <i>Ht. halophilus</i> DSM 6132 ^T	85.2	83.1	83.3	84.1	90.1	98.7	83.8	84.3	84.4	83.5	81.0	83.7	—		
14. <i>Ht. sp.</i> Milos-BII1 ^T	87.5	84.9	85.1	83.5	91.7	93.4	84.2	85.2	85.1	84.4	81.7	84.2	93.0	—	
15. <i>Ht. neapolitanus</i> DSM 581 ^T	85.8	83.3	83.5	83.4	98.5	90.8	84.3	85.0	83.8	84.5	81.2	83.7	90.6	92.2	—

was not used as an electron donor for autotrophic growth. Addition of vitamin B₁₂ was not essential for growth. Maximum specific growth rates on thiosulfate at 37 °C and at optimal pH were obtained from the mean of three different procedural determinations, i.e. direct counts, optical density and protein production. Growth in batch cultures with 20 mM thiosulfate showed a maximum specific growth rate ($\mu_{\max}$) of 0.4 h⁻¹ at pH 6.5 and 37 °C, whereas the rate at pH 7.0 and 22 °C was 0.25–0.3 h⁻¹. The maximum specific growth rate in chemostat cultures with 20 mM thiosulfate was estimated from washout kinetics after raising the dilution rate of the culture in steady state from 0.2 to 0.4. At pH 7.0 and 22 °C, the $\mu_{\max}$ was between 0.25 and 0.3. Under optimal conditions (pH 6.5 and 37 °C) the values for $\mu_{\max}$ estimated from washout kinetics were in the range 0.5–0.6. The rates were nearly the same for 100 % and 20 % air-saturated medium.

The pH range for growth of strain Milos-BII1^T on thiosulfate was between 3.5 and 8.5. At a pH of 9.0, no growth was observed.

The temperature range for growth was between 3.5 and 49 °C, with an optimum between 37 and 42 °C. Growth was determined by measurement of acidification of the medium over a period of 10 d.

The isolate showed no specific requirement for NaCl (0–2 M), although best growth occurred at NaCl concentrations between 400 and 500 mM. Nevertheless, it should be noted that the medium without NaCl contained at least 40 mM Na⁺ from the addition of sodium thiosulfate.

DNA base ratio and ubiquinone content

The G+C content of strain Milos-BII1^T was 62 ± 0.2 mol %. The isolate contained Q-8 as the only major ubiquinone.

Phylogenetic analysis

Similarity matrix values and phylogenetic affiliation are shown in Table 1 and Fig. 1, respectively. Comparison of the nearly complete 16S rRNA genes showed that strain Milos-BII1^T belongs to the γ -subclass of the *Proteobacteria* and exhibits less than 93.4 % similarity to all other described *Thiobacillus* spp. The closest relative is *H. hydrothermalis* with a value of 93.4 %. The new, nearly complete sequence of the 16S rDNA gene from *H. neapolitanus* corresponded well with previous results obtained with the incomplete sequence (Kelly & Wood, 2000).

DISCUSSION

Strain Milos-BII1^T is a chemolithoautotrophic, sulfur-oxidizing bacterium. Based on the almost complete sequence of the 16S rRNA gene, it forms a phylogenetic cluster with *H. hydrothermalis*, *H. neapolitanus*, *H. halophilus* and *Thiobacillus* sp. W5 (Fig. 1), which are also obligately chemolithoautotrophic. The similarity values of the 16S rRNA sequences for the new isolate as listed in Table 1 are below 97 %. According to the definition of Stackebrandt & Goebel (1994), this itself indicates that strain Milos-BII1^T does not belong to a presently described species. In addition, although strain Milos-BII1^T shares many physiological proper-

Fig. 1. Maximum-likelihood tree based on 1308 positions of nearly full-length 16S rRNA sequences from 23 bacteria, using the α -subclass of the *Proteobacteria* as an outgroup. Trees constructed with other reconstruction algorithms (neighbour-joining and parsimony) resulted in the same overall topology. In bootstrap analysis using neighbour-joining and parsimony criteria, the branching of the proposed *Halothiobacillus* cluster was supported with values of 100. Partial sequences of *Acidiphilium acidophilum*, *Thiomonas thermosulfata*, *Thiomonas perometabolis*, *Thiomonas cuprina*, *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Thiobacillus tepidarius* were inserted into the tree by applying parsimony criteria without allowing for changes in the overall topology. Bacteria of the α -, β - and γ -subclasses of the *Proteobacteria* are indicated by ' α ', ' β ' and ' γ ', respectively. The sequences of *Halothiobacillus* sp. Milos-BII1^T and *Halothiobacillus neapolitanus* (Parker strain X^T) were determined in this study.

ties with *H. hydrothermalis*, there are distinct differences (Table 2). For example, the pH optimum and the lower pH limit for growth are shifted to lower values. This might indicate an adaptation to a more acidic environment (Sievert *et al.*, 1999). Therefore, we propose that the isolate can be considered as a new species within the aforementioned cluster.

Ecological significance

From its growth characteristics, strain Milos-BII1^T seemed to be well adapted to the environment from which it was isolated. However, it was apparently not among the dominant sulfur-oxidizing bacteria in this habitat. It was not isolated from the highest dilutions of MPN series that showed positive growth. Instead, other sulfur-oxidizing bacteria, for example *Thiomicrospira* strains Milos T-1 and T-2 (Brinkhoff *et al.*,

1999b), were isolated. In addition, *Thiomicrospira* populations could be detected in a denaturing gradient gel electrophoresis (DGGE) analysis of the bacterial community (Brinkhoff *et al.*, 1999b), whereas no DGGE bands were found that exhibited the same electrophoretic behaviour as the 16S rDNA fragment of strain Milos-BII1^T (S. M. Sievert, unpublished data). Results obtained from a competition experiment carried out in a chemostat between strain Milos-BII1^T and *Thiomicrospira* strain Milos T-2 indicated that *Thiomicrospira* strain Milos T-2 was superior to strain Milos-BII1^T at high dilution rates, i.e. $D > 1.7 \text{ h}^{-1}$. In contrast, at low dilution rates, i.e. low substrate concentrations, strain Milos-BII1^T displaced *Thiomicrospira* strain Milos T-2 (unpublished data). In relation to the actual environment, this might indicate that the sulfur-oxidizing bacterial community was not limited by substrate availability *in situ*. This seems

Table 2. Morphological and physiological characteristics among *Halothiobacillus* spp.

All species were obligately chemolithotrophic motile rods; oxidized sulfide, sulfur, thiosulfate and tetrathionate, but not thioisocyanate; did not use nitrate as a terminal electron acceptor; and contained ubiquinone Q-8. Data were obtained from Hutchinson *et al.* (1965, 1969); Smith & Kelly (1979); Kelly & Harrison (1989); Durand *et al.* (1993); Wood & Kelly (1991); McDonald *et al.* (1997); Visser *et al.* (1997); Kelly *et al.* (1998); and own results. ND, Not determined.

Character	<i>Halothiobacillus</i> sp. Milos-BII1 ^T	<i>H. hydrothermalis</i> DSM 7121 ^T	<i>H. halophilus</i> DSM 6132 ^T	<i>H. neapolitanus</i> DSM 581 ^T	<i>Thiobacillus</i> sp. W5
Width (µm)	0.4–0.6	0.4–0.5	0.3–0.5	0.3–0.5	0.5
Length (µm)	1.2–2.5	1.2–1.5	1.0–1.2	1.0–1.5	1.0–1.5
G + C content (mol %)	62.0	67.4	64.2	56.0	56.0
Maximum specific growth rate on thiosulfate (h ⁻¹)	0.45	0.6	ND	0.28	ND
Optimum pH	6.5	7.5–8.0	7.0–7.3	6.5–6.9	7.0–7.5
pH range for growth	3.5–8.5	6.0–9.0	ND	4.5–8.5	3.5–8.5
Lowest pH produced in thiosulfate medium	2.8	4.8	5.5–6.0	2.8	ND
Optimum temperature (°C)	37–42	35–40	30–32	28–32	25–30
Maximum temperature (°C)	48–49	48–49	35–36	39	42
NaCl requirement	–	–	+	–	ND
Optimal NaCl concentration (mM)	400–500	430	800–1000	ND	ND
NaCl tolerance (mM)	2500	2000	4000	> 860	ND

reasonable considering a constant resupply of reduced sulfur compounds by the expelled hydrothermal fluid. Another possible explanation might be a limitation of iron or other metals caused by the formation of metal sulfides as a result of high sulfide levels at the vent site. In chemostat studies under iron-limiting conditions, *Thiomicrospira pelophila* outcompeted *Thiobacillus thioparus* (Kuenen *et al.*, 1977). Both observations could also provide an explanation for the prevalence of *Thiomicrospira* at hydrothermal vent systems in general (Ruby *et al.*, 1981; Ruby & Jannasch, 1982; Jannasch *et al.*, 1985; Muyzer *et al.*, 1995; Brinkhoff *et al.*, 1999b). However, more studies are required to confirm these hypotheses, for example by varying other relevant environmental parameters in the chemostat and by enumeration of the respective cells with culture-independent techniques such as fluorescent *in situ* hybridization (FISH). Since both organisms fall into coherent phylogenetic clusters, the design of specific probes should be feasible. It is interesting to note that at the hydrothermal vent system in the Fiji Basin, both *H. hydrothermalis* and *Thiomicrospira* spp. were present (Durand *et al.*, 1994).

Similarities between *H. neapolitanus*, *H. hydrothermalis*, *H. halophilus* and strain Milos-BII1^T

As has already been reported in previous publications on the taxonomic position of various *Thiobacillus* spp., there is a high level of physiological and phylogenetic similarity between *H. neapolitanus*, *H. hydrothermalis* and *H. halophilus* (McDonald *et al.*, 1997; Kelly *et al.*, 1998). As shown in Fig. 1, all these organisms form a monophyletic group within the γ -subclass of the

Proteobacteria as supported by a bootstrap value of 100 and can clearly be distinguished from other strains of the genus belonging to the same subgroup or to the γ - and β -subclasses of the *Proteobacteria* (McDonald *et al.*, 1997). The identity of the different species was confirmed by DNA–DNA hybridization for organisms showing a high level of similarity at the 16S rRNA gene level (Kelly *et al.*, 1998). The organism described in this paper and another obligate chemolithoautotrophic sulfur-oxidizing bacterium tentatively named *Thiobacillus* sp. W5 (Visser *et al.*, 1997) are phylogenetically and physiologically closely related to these species (see Tables 1 and 2), although some data and a formal description of the latter is lacking. Combining the available physiological data on all species belonging to this cluster, it becomes obvious that they share several properties. They are all motile, obligately chemolithoautotrophic, sulfur-oxidizing bacteria containing ubiquinone Q-8. A very unusual feature compared to other *Thiobacillus* spp. is the NaCl tolerance of all species and a NaCl requirement of some species. *H. neapolitanus* has not been shown to have a strict NaCl requirement, but it tolerates high NaCl concentrations of more than 860 mM. The available data in this respect for *Thiobacillus* sp. W5 are lacking. Because of the phylogenetic and physiological relatedness of the aforementioned organisms, it was suggested that they are transferred to the new genus *Halothiobacillus* to clarify the confused taxonomic situation within the genus *Thiobacillus* (Kelly & Wood, 2000). The new, nearly complete 16S rDNA sequences obtained in the present study confirm the results already obtained by McDonald *et al.* (1997) and Kelly *et al.* (1998). The proposal by Kelly & Wood

(2000) for the new genus *Halothiobacillus* to accommodate the halotolerant, obligately chemolithoautotrophic organisms *T. neapolitanus*, *T. halophilus* and *T. hydrothermalis* is justified with respect to our data. Their proposal to designate *H. neapolitanus* (NCIMB 8539^T, DSM 581^T) as the type species of the genus *Halothiobacillus* is appropriate. It was the first species to be described and was the subject of many early studies on the oxidation of reduced sulfur compounds by autotrophic organisms. In addition, historical reasons should be considered because Nathansohn (1902) isolated a very similar organism from the Bay of Naples, Italy. *H. neapolitanus* is also well suited to reflect the halotolerance of most species of this genus.

Emended description of the genus *Halothiobacillus* (Kelly and Wood 2000)

Halothiobacillus (Hal.o.thi.o.ba.cil'lus. Gr. n. *hals* sea, salt; Gr. n. *thios* sulfur; L. n. *bacillus* a small rod; L. masc. n. *Halothiobacillus* salt-loving sulfur rodlet).

Cells are rod-shaped, 0.3–0.6 µm in diameter and 1.0–2.5 µm in length. They are Gram-negative, occur singly or in pairs and are motile. Spore formation is absent. All members of the genus are strictly aerobic and grow chemolithoautotrophically with thiosulfate, tetrathionate, sulfur and sulfide, but not with thiocyanate, as electron donor, and with carbon dioxide as carbon source. Heterotrophic growth is never observed. Addition of acetate can increase the amount of biomass produced if a reduced sulfur compound is provided as electron donor. Sulfate is the end product of sulfur compound oxidation, but sulfur, sulfite or polythionates may be accumulated, sometimes transiently, by most species. During growth on reduced sulfur compounds, the pH decreases from neutrality to a pH as low as 2.5–3.0 depending on the species. Optimal growth occurs between pH 6.5 and 8.0 at temperatures of 30–42 °C. The optimal NaCl concentration for growth is 400–500 mM for most strains, but many tolerate much higher concentrations. A requirement for NaCl is found for some species. Carbon dioxide is fixed by means of ribulose-bisphosphate carboxylase. Nitrate can be reduced to nitrite, but is not reduced further. On thiosulfate agar, cells produce small, white–yellowish, smooth, entire colonies (1–3 mm in diameter) 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 of members of this genus varies between 56 and 67 mol%. As determined by 16S rRNA gene sequence analysis, the genus *Halothiobacillus* belongs to the γ -subclass of the *Proteobacteria*. Members of the genus can be isolated from freshwater, soil and frequently from marine environments. Traditional enrichment culture techniques seem to favour their isolation because of their higher acid tolerance compared to other marine, sulfur-oxidizing bacteria such as *Thiomicrospira* spp. The type species is *Halo-*

thiobacillus neapolitanus (formerly *Thiobacillus neapolitanus*) strain NCIMB 8539^T (= DSM 581^T = Parker strain X^T).

Description of *Halothiobacillus kellyi*

Halothiobacillus kellyi (kel'ly.i. M.L. gen. n. *kellyi* of Kelly; named after Donovan P. Kelly, a British microbiologist who has made important contributions to research on sulfur-oxidizing bacteria and their physiology).

Cells are Gram-negative, motile and rod-shaped (0.4–0.6 × 1.2–2.5 µm). They are strictly aerobic and grow autotrophically on thiosulfate, tetrathionate, sulfur and sulfide, but not on thiocyanate. Sulfite is oxidized to sulfate if the concentration does not exceed 3 mM. The organism does not grow heterotrophically. When thiosulfate is used as the primary energy source a transient formation of sulfur occurs. During growth on reduced sulfur compounds, the pH decreases from neutrality to around 2.8. Thiosulfate is completely oxidized to sulfate. Autotrophic growth on thiosulfate occurs between pH 3.5 and 8.5 and at temperatures of 3.5–49 °C; optimum growth occurs at pH 6.5 and at 37–42 °C. The optimal NaCl concentration for growth is 0.4–0.5 M; growth is possible between NaCl concentrations of 0 and 2 M. Nitrate is not used as terminal electron acceptor. The mean maximum specific growth rate on thiosulfate is 0.45 h⁻¹. On thiosulfate agar, cells produce white–yellowish, smooth, entire colonies (diameter on 1.2% agar is 1–4 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 62 mol%. As determined by 16S rRNA gene sequence analysis, the organism belongs to the γ -subclass of the *Proteobacteria* and is closely related to other members of this genus. The type strain is Milos-BII1^T, which was isolated from a marine shallow-water hydrothermal vent system and is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, under accession number DSM 13162. The GenBank accession number for the nearly complete 16S rRNA gene sequence of *H. kellyi* is AF170419.

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REFERENCES

- Beijerinck, M. W. (1904). Ueber die Bakterien, welche sich im Dunkeln mit Kohlensäure als Kohlenstoffquelle ernähren können. *Zentbl Bakteriologie Abt II* **11**, 593–599.
- Beijerinck, M. W. & Minkman, D. C. (1910). Bildung und Verbrauch von Stickoxydul durch Bakterien. *Zentbl Bakteriologie Abt II* **25**, 30–63.
- Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Brinkhoff, T., Muyzer, G., Wirsén, C. O. & Kuever, J. (1999a). *Thiomicrospira kuenenii* sp. nov. and *Thiomicrospira frisia* sp. nov., two mesophilic obligately chemolithotrophic sulfur-oxidizing bacteria isolated from an intertidal mud flat. *Int J Syst Bacteriol* **49**, 385–392.
- Brinkhoff, T., Sievert, S. M., Kuever, J. & Muyzer, G. (1999b). Distribution and diversity of sulfur-oxidizing *Thiomicrospira* spp. at a shallow-water hydrothermal vent in the Aegean Sea (Milos, Greece). *Appl Environ Microbiol* **65**, 3843–3849.
- Buchholz-Cleven, B. E. E., Ratunde, B. & Straub, K. L. (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.
- Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.
- Durand, P., Reysenbach, A.-L., Prieur, D. & Pace, N. (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**, 39–44.
- Durand, P., Benyagoub, A. & Prieur, D. (1994). Numerical taxonomy of heterotrophic sulfur-oxidizing bacteria isolated from southwestern Pacific hydrothermal vents. *Can J Microbiol* **40**, 690–697.
- Hiraishi, A., Nagashima, K. V. P., Matsuura, K., Shimada, K., Takaichi, S., Wakao, N. & Katayama, Y. (1998). Phylogeny and photosynthetic features of *Thiobacillus acidophilus* and related acidophilic bacteria: its transfer to the genus *Acidiphilium* as *Acidiphilium acidophilum* comb. nov. *Int J Syst Bacteriol* **48**, 1389–1398.
- Hutchinson, M., Johnstone, K. I. & White, D. (1965). The taxonomy of certain Thiobacilli. *J Gen Microbiol* **41**, 357–366.
- Hutchinson, M., Johnstone, K. I. & White, D. (1969). Taxonomy of the genus *Thiobacillus*: the outcome of numerical taxonomy applied to the group as a whole. *J Gen Microbiol* **57**, 397–410.
- Jannasch, H. W., Wirsén, C. O., Nelson, D. C. & Robertson, L. A. (1985). *Thiomicrospira crumigena* sp. nov., a colorless sulfur-oxidizing bacterium from a deep-sea hydrothermal vent. *Int J Syst Bacteriol* **35**, 422–424.
- Katayama, Y., Hiraishi, A. & Kuraishi, H. (1995). *Paracoccus thiocyanatus* sp. nov., a new species of thiocyanate-utilizing facultative chemolithotroph, and transfer of *Thiobacillus versutus* to the genus *Paracoccus* as *Paracoccus versutus* comb. nov. with emendation of the genus. *Microbiology* **141**, 1469–1477.
- Kelly, D. P. & Harrison, A. P. (1989). Genus *Thiobacillus* Beijerinck. In *Bergey's Manual of Systematic Bacteriology*, 1st edn, vol. 3, pp. 1842–1858. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.
- Kelly, D. P. & Wood, A. P. (2000). Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *Int J Syst Evol Microbiol* **50**, 511–516.
- Kelly, D. P., Stackebrandt, E., Burghardt, J. & Wood, A. P. (1998). Confirmation that *Thiobacillus halophilus* and *Thiobacillus hydrothermalis* are distinct species within the γ -subclass of the Proteobacteria. *Arch Microbiol* **170**, 138–140.
- Kuenen, J. G., Boonstra, J., Schröder, H. G. J. & Veldkamp, H. (1977). Competition for inorganic substrates among chemo-organotrophic and chemolithotrophic bacteria. *Microb Ecol* **3**, 119–130.
- Kuenen, J. G., Robertson, L. A. & Tuovinen, O. H. (1992). The genera *Thiobacillus*, *Thiomicrospira*, and *Thiosphaera*. In *The Prokaryotes*, 2nd edn, pp. 2638–2657. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.
- Lane, D. L., Harrison, A. P., Stahl, D., Pace, B., Giovannoni, S. J., Olsen, G. J. & Pace, R. (1992). Evolutionary relationships among sulphur- and iron-oxidizing eubacteria. *J Bacteriol* **174**, 269–278.
- McDonald, I. R., Kelly, D. P., Murrell, J. C. & Wood, A. P. (1997). Taxonomic relationships of *Thiobacillus halophilus*, *Thiobacillus aquesulii*, and other species of *Thiobacillus*, as determined using 16S rRNA sequencing. *Arch Microbiol* **166**, 394–398.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Moreira, D. & Amils, R. (1997). Phylogeny of *Thiobacillus cuprinus* and other mixotrophic thiobacilli: proposal for *Thiomonas* gen. nov. *Int J Syst Bacteriol* **47**, 522–528.
- Muyzer, G., Teske, A., Wirsén, 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.
- Nathansohn, A. (1902). Über eine neue Gruppe von Schwefelbakterien und ihren Stoffwechsel. *Mitt Zool Stat Neapel* **15**, 655–680.
- Nelson, D. C. & Jannasch, H. W. (1983). Chemoautotrophic growth of a marine *Beggiatoa* in sulfide-gradient cultures. *Arch Microbiol* **136**, 262–269.
- Nishihara, H., Igarashi, Y. & Kodama, T. (1989). Isolation of an obligately chemolithoautotrophic, halophilic and aerobic hydrogen-oxidizing bacterium from marine environment. *Arch Microbiol* **152**, 39–42.
- Porter, K. G. & Feig, Y. S. (1980). The use of DAPI for identifying and counting aquatic microflora. *Limnol Oceanogr* **25**, 943–948.
- 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 Chromatogr 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., Wirsén, C. O. & Jannasch, H. W. (1981). Chemolithoautotrophic sulfur-oxidizing bacteria from the Galapagos Rift hydrothermal vents. *Appl Environ Microbiol* **42**, 317–342.
- Sievert, S. M., Brinkhoff, T., Muyzer, G., Ziebis, W. & Kuever, J. (1999). Spatial heterogeneity of bacterial populations along an environmental gradient at a shallow submarine hydrothermal

vent near Milos Island (Greece). *Appl Environ Microbiol* **65**, 3834–3842.

Smith, A. L. & Kelly, D. P. (1979). Competition in the chemostat between an obligately and a facultatively chemolithotrophic *Thiobacillus*. *J Gen Microbiol* **115**, 377–384.

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.

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

Visser, J. M., Stefess, G. C., Robertson, L. A. & Kuenen, J. G. (1997). *Thiobacillus* sp. W5, the dominant autotroph oxidizing sulfide to sulfur in a reactor for aerobic treatment of sulfidic wastes. *Antonie Leeuwenhoek* **72**, 127–134.

Widdel, F. & Bak, F. (1992). Gram-negative mesophilic sulfate-reducing bacteria. In *The Prokaryotes*, 2nd edn, vol. IV, pp. 3352–3378. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Wood, A. P. & Kelly, D. P. (1991). Isolation and characterisation of *Thiobacillus halophilus* sp. nov., a sulphur-oxidising autotrophic eubacterium from a Western Australian hypersaline lake. *Arch Microbiol* **156**, 277–280.