***Limnobacter thiooxidans* gen. nov., sp. nov., a novel thiosulfate-oxidizing bacterium isolated from freshwater lake sediment**

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Two novel thiosulfate-oxidizing strains were isolated from sediment of the littoral zone of a freshwater lake (Lake Chiemsee, Bavaria, Germany). The new isolates, designated CS-K1 and CS-K2^T, were Gram-negative, slightly curved rods with pointed ends that were motile by means of single polar flagella. Both strains were obligately aerobic and grew on a variety of organic substrates, but not autotrophically. The utilization of thiosulfate led to an increase in the growth yield, indicating that these strains were able to grow chemolithoheterotrophically by oxidation of thiosulfate to sulfate. The optimum thiosulfate concentrations for growth were determined to be 10 mM for strain CS-K1 and 20 mM for strain CS-K2^T. Phylogenetically, both strains were affiliated to the β -Proteobacteria. Their characterization by a polyphasic approach resulted in the placement of both strains into a single species that is related only distantly to any known type species. Thus, the creation of a novel taxon is proposed, with the name *Limnobacter thiooxidans* gen. nov., sp. nov., to include the novel strains. In addition, the phylogenetic position of the chemolithoheterotrophic strain 'Thiobacillus' Q was determined.

Keywords: Freshwater sediment, chemolithoheterotrophy, thiosulfate, *Thiobacillus* sp.

INTRODUCTION

The biological oxidation of reduced sulfur compounds in sulfidic environments by chemolithoautotrophic bacteria is a well-studied process and many representative micro-organisms have been described. In contrast, most of the biological oxidation of sulfur compounds in habitats characterized by low sulfide concentrations may be due to heterotrophic bacteria (Gottschal & Kuenen, 1980). To date, only a few obligately heterotrophic bacteria have been studied in detail and adequately described that are able to generate metabolically useful energy from the oxidation of reduced sulfur compounds. Bacteria capable of this type of metabolism have been designated

chemolithoheterotrophs. They can be classified into two types according to the mode of thiosulfate oxidation, which can lead to either tetrathionate or sulfate. *Catenococcus thiocycli* has been shown to oxidize thiosulfate to tetrathionate aerobically (Sorokin *et al.*, 1996), whereas some strains allied to *Pseudomonas stutzeri* are also able to oxidize thiosulfate to tetrathionate anaerobically using nitrate, nitrite or N₂O as electron acceptors (Sorokin *et al.*, 1999). Both species are located within the γ -Proteobacteria.

Bosea thiooxidans, on the other hand, a member of the α -Proteobacteria, represents the second metabolic type, oxidizing thiosulfate to sulfate (Das *et al.*, 1996). This type of thiosulfate oxidation was studied thoroughly with 'Thiobacillus' strain Q, but no species description was given (Gommers & Kuenen, 1988). Recently, a novel chemolithoheterotrophic member of the β -Proteobacteria was isolated from a hot spring in Portugal. The thermophilic isolate was classified as *Tepidimonas ignava* and oxidized thiosulfate to sulfate (Moreira *et al.*, 2000).

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Abbreviations: PHB, polyhydroxybutyrate; pNA, para-nitroanilide; pNP, para-nitrophenyl.

The EMBL/GenBank accession numbers for the 16S rRNA gene sequences of *Limnobacter thiooxidans* CS-K2^T and strain 'Thiobacillus' Q are AJ289885 and AJ289884, respectively.

In this study, we describe the isolation and characterization of two novel strains of heterotrophic sediment bacteria that may be ecologically significant and could play an important role in the oxidative part of the sulfur cycle in the littoral zone of freshwater lakes.

METHODS

Enrichment and isolation procedure. In August 1997, cores of sediment were taken by scuba divers from Lake Chiemsee at a depth of 14 m. Samples taken from the top layer of the sediment core were serially diluted in filter-sterilized lake water, obtained from the site of sampling. Aliquots of the serial dilution were inoculated at room temperature in enrichment medium, resulting in dilutions ranging from 10^{-4} to 10^{-9} . The enrichment medium was essentially the thiosulfate/mineral medium used by Frankel *et al.* (1997) to culture magnetotactic bacteria with the exception that no agar was added to solidify the medium. The medium contained 10 mM thiosulfate as the principal energy source and 2.4 mM NaHCO_3 as the carbon source. A very small amount of quinate (20 μM) and traces of organic material in the sterilized lake water represented additional sources of organic carbon in the medium. After 14 d, aliquots of the various dilutions were checked microscopically for growth of bacteria. Isolates were obtained from positive enrichment cultures by streaking aliquots onto agar plates of the enrichment medium. After several days, small pinpoint colonies developed, which were picked and inoculated into R2A broth (Reasoner & Geldreich, 1985) supplemented with $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (5 g l^{-1}) and phenol red (0.01 g l^{-1}), added as a pH indicator in order to test the ability of the isolates to produce acid from thiosulfate. The growth yield obtained in R2A medium was much higher than that in the original mineral medium used for the enrichment, so the isolates were purified further by repeated streaking on R2A agar.

Bacterial strains and growth media. Two strains were isolated from the enrichment cultures and designated CS-K1 and CS-K2^T. They were maintained on R2A agar and transferred to fresh medium at intervals of 3–4 weeks. The effect of thiosulfate on growth of the newly isolated strains was tested in succinate/mineral medium that contained (l^{-1}): 0.4 g NH_4Cl , 0.8 g K_2HPO_4 , 0.3 g KH_2PO_4 , 2.7 g sodium succinate hexahydrate, 0.1 g yeast extract and 5 ml mineral solution (Frankel *et al.*, 1997). The pH was adjusted to 7.5 with NaOH. This medium was supplemented with varying amounts of thiosulfate, resulting in final concentrations ranging from 2 to 50 mM. Growth experiments in liquid media were performed at 30 °C with vigorous shaking. For some experiments, the carbon source in the succinate/mineral medium was replaced by sodium acetate trihydrate. Autotrophic growth of the novel strains was tested by omitting succinate from the succinate/mineral medium and replacing yeast extract with 0.5 ml vitamin solution l^{-1} (Frankel *et al.*, 1997). The ability to grow anaerobically was determined by supplementing R2A or succinate/mineral medium with KNO_3 (2 g l^{-1}) or $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ (3.72 g l^{-1}) as alternative electron acceptors and incubation of the inoculated medium in anaerobic jars using the Anaerogen system (Unipath).

'*Thiobacillus*' strain Q (LMD 81.11) was obtained from the Netherlands Culture Collection of Bacteria (NCCB, Utrecht, The Netherlands) and grown on R2A medium.

Phylogenetic analyses. Whole cells of bacteria were used directly for the *in vitro* amplification of almost full-length

16S rRNA gene fragments (Wallner *et al.*, 1997). The resulting PCR products were purified by using the Prep-A-Gene DNA purification kit (Bio-Rad) and sequenced using a LICOR automated sequencer (MWG Biotech). Cycle-sequencing protocols based on the chain-termination technique were applied using a Thermo Sequenase fluorescent-labelled primer cycle-sequencing kit (Amersham). The newly determined sequences were added to an alignment of about 14000 published and unpublished homologous primary structures from bacteria using the alignment tool of the ARB program package (Ludwig & Strunk, 1997). Phylogenetic analyses were performed by applying maximum-parsimony (ARB, PHYLIP), distance matrix (ARB, PHYLIP) and maximum-likelihood methods (fastDNAm1; Maidak *et al.*, 1996) on different datasets.

DNA base composition and DNA–DNA hybridization. Chromosomal DNA was isolated and purified according to the method of Meyer & Schleifer (1975). The G + C content of the DNA was determined from the midpoint value of the thermal denaturation profile obtained with a Gilford 2600 spectrophotometer (Mandel & Marmur, 1968). The G + C content was calculated by using the equation of De Ley (1970) and *Escherichia coli* ATCC 11775^T DNA as a reference (G + C content, 51 mol %).

DNA–DNA hybridization experiments were carried out by using a quantitative dot-blot membrane-filter technique, according to the protocol of Owen & Pitcher (1985) as modified by Schulze *et al.* (1999). The results obtained in the quantitative dot-blot DNA–DNA hybridization experiments were compared with an estimate of DNA–DNA relatedness obtained by applying a method based on the quantitative measurement of DNA renaturation rates with a Gilford 2600 photometer as described by De Ley *et al.* (1970).

Phenotypic characterization. The cellular morphology of the newly isolated bacteria was studied by phase-contrast microscopy (Axioplan; Zeiss). For the observation of flagella, the staining method of Heimbrook *et al.* (1989) was applied. The presence of polyhydroxybutyrate (PHB) granules was demonstrated by their orange fluorescence after staining with Nile Blue A, as described by Rees *et al.* (1992).

The pH range for growth was determined by adjusting the final pH of R2A medium to values between 5.0 and 11.0 with NaOH or HCl. For the determination of the temperature range for growth, cells were incubated on R2A agar at temperatures between 4 and 40 °C.

The ability to utilize and degrade various substrates (Kämpfer *et al.*, 1997) was tested as described previously (Kämpfer *et al.*, 1991). Tests were read after 72 h of incubation at 25 °C. For comparison, nutritional profiles were obtained by using the Biotype 100 system with medium 1 (bioMérieux). Growth in the Biotype 100 strips was recorded after 6 d of incubation at 28 °C.

For analyses of fatty acid compositions, the isolates were grown on different media for 72 h prior to the analysis. Fatty acid methyl esters were extracted and prepared by the standard protocol of the Microbial Identification System (MIDI; Microbial ID). Extracts were analysed as described previously (Kämpfer & Kroppenstedt, 1996; Kämpfer *et al.*, 1997). The location of the position of the double bond in unsaturated fatty acid methyl esters was determined by using dimethyl disulfide adducts and was carried out by B. J. Tindall (DSMZ). Dimethyl disulfide adducts were formed from the fatty acid methyl esters released from 20 mg freeze-

dried cells by using the method of Nichols *et al.* (1986). GC-MS analysis of the fatty acids was carried out using a Finnigan MAT GCQ GC-MS. The GC was equipped with a DB-5 column (30 m × 0.22 mm, film thickness 0.25 µm) and helium, at a linear velocity of 40 cm s⁻¹, was used as the carrier gas. Samples of 1 µl were injected in the splitless mode and separated using a temperature program starting at 80 °C (held for 1 min) followed by a rise of 10 °C min⁻¹ to 300 °C. The MS was run in the EI mode, with a source temperature of 175 °C and a transfer line temperature of 275 °C.

Analytical techniques. Growth curves of bacterial cultures were determined by measuring the optical density at 430 nm with a UV/Vis spectrophotometer (Lambda 20; Perkin Elmer). Dry weights of cellular biomass of cultures grown to stationary phase were measured by centrifugation of 80–100 ml cell suspension at 5000 g and drying the pellet at 80 °C under vacuum to constant weight.

Thiosulfate and sulfate concentrations in supernatants of centrifuged culture medium were measured by ion chromatography (Dionex 2000i/SP) using an AS4A separation column combined with a conductivity detector (Salas-Auvert *et al.*, 1995). The eluent contained 1.7 mM NaHCO₃ and 1.8 mM Na₂CO₃.

RESULTS AND DISCUSSION

Enrichment and isolation of pure cultures

It was our objective in this study to retrieve ecologically significant bacteria from the upper layers of a freshwater lake sediment. Natural freshwater habitats are often characterized by a low content of organic carbon, so many indigenous micro-organisms are oligotrophic and have adapted to the use of alternative sources of energy. Consequently, the medium that we used for the enrichment of fastidious sediment bacteria contained only traces of organic carbon and thiosulfate as the principal energy source in order to avoid the selection of fast-growing but allochthonous bacteria adapted to high concentrations of organic carbon. The enrichment medium was originally designed for the isolation and growth of the microaerophilic, chemolithoautotrophic, magnetotactic coccus MC-1, which gains energy by the oxidation of reduced sulfur compounds (Frankel *et al.*, 1997). Magnetotactic cocci phylogenetically related to strain MC-1 have been identified previously in sediment from Lake Chiemsee (Spring *et al.*, 1994), leading to the assumption that this medium might be suitable for the enrichment of ecologically relevant micro-organisms from this habitat. In order to isolate the numerically abundant bacteria, the enrichment medium was inoculated with serially diluted sediment samples. Growth was detected up to a dilution of 10⁻⁷. Two isolates, CS-K1 and CS-K2^T, were purified from this dilution step of Lake Chiemsee littoral zone sediment. The cell density in the 10⁻⁷ dilution was very low and only very small colonies were obtained on enrichment medium agar. In the course of this study, it turned out that both strains were obligately heterotrophic. Growth of these obviously oligotrophic bacteria in a medium designed originally for autotrophic bacteria may be explained

Fig. 1. Phase-contrast micrograph of strain CS-K2^T. Bar, 10 µm.

by traces of organic carbon in the inoculum and agar impurities. The two strains differed in the amount of acid produced during growth with thiosulfate in R2A medium. Growth of strain CS-K2^T induced a change of the colour of the pH indicator to yellow, whereas the lowering of the pH by strain CS-K1 was apparently not sufficient to have this effect.

Phenotypic characterization

Morphological and cultural characteristics. Cells of strains CS-K1 and CS-K2^T occurred singly or in short chains. They were Gram-negative, slightly curved rods with pointed ends, having dimensions of 0.6–1.5 × 1.5–3.7 µm. Cells were motile by means of single polar flagella. PHB was identified as an intracellular reserve material under conditions of phosphate limitation. The intra- or extracellular deposition of elemental sulfur was not detected during growth in medium supplemented with thiosulfate. A phase-contrast micrograph of cells of strain CS-K2^T is shown in Fig. 1.

In broth cultures, especially at the beginning of the exponential growth phase, cells aggregated and formed flocs. Colonies grown on R2A agar were around 0.8 mm (CS-K1) or 1.0 mm (CS-K2^T) in diameter, circular, smooth, translucent and flat with entire margins.

Both strains grew at temperatures ranging from 4 to 38 °C; temperatures below 4 °C were not tested. The pH range for growth was pH 5.5–10.5 for both strains. The forming of aggregates during growth in liquid culture prevented a reliable determination of the temperature and pH optima for these strains by measuring optical densities, so we assumed a temperature of 30 °C and a pH of 7.5 as optimal.

Whole-cell fatty acid composition. The cellular fatty acid compositions of cells of CS-K1 and CS-K2^T were very similar, but depended largely on the medium used for the growth of cells. The major fatty acid components of both strains cultivated on R2A medium were

Table 1. Cellular fatty acid composition of strains CS-K1 and CS-K2^T after growth on R2A medium for 72 h prior to analysis

Values are percentages of total fatty acids. The position of the double bond in unsaturated fatty acids is located by counting from the methyl (ω) end of the carbon chain; *cis* and *trans* isomers are indicated by the suffixes *c* and *t*. Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 4 contained one or more of the following fatty acids: 16:1 ω 7*c* and 15:0 iso 2-OH. Summed feature 7 contained one or more of the following isomers: 18:1 ω 7*c*, 18:1 ω 9*t* and/or 18:1 ω 12*t*. Summed feature 9 contained one or more of the following isomers: 19:1 ω 6*c*, 19:0 cyclo and/or an unknown compound with the equivalent chain length of 18:846.

Fatty acid	CS-K1	CS-K2 ^T
10:0	0.3	0.2
10:0 3-OH	8.3	7.8
14:0	4.9	4.5
16:0 iso	1.0	—
Summed feature 4	27.0	27.9
16:0	11.7	13.1
18:1 ω 9 <i>c</i>	0.5	0.4
Summed feature 7	40.5	40.1
18:0	1.5	1.4
Summed feature 9	4.4	4.5

Fig. 2. Changes in the fatty acid profile of strain CS-K2^T after growth on succinate/mineral medium (shaded bars) instead of R2A medium (open bars). Minor fatty acids with proportions below 1% of the total cellular fatty acid composition are not shown. Nomenclature of fatty acids is the same as in Table 1.

summed feature 7 (18:1 ω 7*c*, 18:1 ω 9*t* and/or 18:1 ω 12*t*; 40.1–40.5%), summed feature 4 (16:1 ω 7*c* and/or 15:0 iso 2-OH; 27.0–27.9%), 16:0 (11.7–13.1%) and 10:0 3-OH (7.8–8.3%). Other fatty acid components were present in amounts below 5% (Table 1). Analysis of the dimethyl disulfide adducts of the unsaturated fatty acids of strain CS-K2^T indicated that the two major unsaturated fatty acids have fragmentation patterns characteristic of the 16:1 ω 7*c* and

18:1 ω 7*c* fatty acid methyl esters. Thus, it was possible to confirm the structure of the unsaturated hexadecenoic acid present and to show that the unresolved mixture of unsaturated octadecenoic fatty acids detected by the MIDI system contained only one component, which was shown to be 18:1 ω 7*c*. The presence of the hydroxylated fatty acid 10:0 3-OH distinguishes this genus from members of the two closely related genera *Burkholderia* and *Ralstonia*, which can contain 14:0 3-OH or 14:0 2-OH, but not 10:0 3-OH (Yabuuchi *et al.*, 1992, 1995).

Interestingly, the fatty acid profiles of both strains as shown in Table 1 changed significantly after growth on mineral medium supplemented with succinate (Fig. 2). The amount of the fatty acid 16:0 increased on succinate/mineral medium, from 13.1 to 23.8%, whereas the amount of 18:1 ω 7*c* decreased from 40.1 to 28.6%. This shift in the fatty acid profile may be explained by adaptation to growth on a single carbon source. This assumption is supported by the observation that the addition of thiosulfate to succinate/mineral medium had only a small effect on the fatty acid profiles of cells grown on either medium (data not shown). Guezennec & Fiala-Medioni (1996) proposed the monounsaturated fatty acids 18:1 ω 7 and 16:1 ω 7 as biomarkers for thio-oxidizing bacteria represented by genera like *Thiothrix*, *Thiomicrospira* or *Thiobacillus*, in which one or both of these components represent the predominant fatty acid of the membrane lipids. It seems that this is only partly true for the chemolithoheterotrophic thiosulfate-oxidizing bacteria. *Bosea thiooxidans* contains 61.6% 18:1 ω 7*c* as cellular fatty acid (Das *et al.*, 1996), strain CS-K2^T contains between 28.6 and 40.1% of this fatty acid and the recently isolated species *Tepidimonas ignava* contains only 6.1% 18:1 ω 7*c* and 12.4% 16:1 ω 7*c* (Moreira *et al.*, 2000).

Physiology and metabolic properties. Strains CS-K1 and CS-K2^T were both oxidase- and catalase-positive. They could not grow anaerobically in R2A medium or succinate/mineral medium containing thiosulfate with or without the addition of nitrate or iron(III) pyrophosphate. Nitrate was not reduced by strain CS-K2^T and was reduced only weakly by strain CS-K1. Neither organism was able to grow autotrophically, but both clearly needed only small amounts of organic carbon for growth. In thiosulfate/mineral medium supplemented with a vitamin mixture, no growth was observed and no significant amount of thiosulfate was oxidized, whereas the organic carbon contained in mineral medium supplemented with 0.1 g yeast extract l⁻¹ allowed some oligotrophic growth. Both organisms could grow well in succinate/mineral medium without added vitamins or yeast extract, so the failure to grow autotrophically in medium supplemented with the vitamin mixture should not be due to missing growth factors present in the yeast extract.

Two different systems were used to test the utilization of organic carbon sources by strains CS-K1 and CS-K2^T. With the microplate assay described by Kämpfer

Table 2. Physiological characteristics of strain CS-K2^T

Substrate utilization data were obtained with the miniaturized assay system of Kämpfer *et al.* (1997) and with the Biotype 100 system. Symbols: +, good growth; –, no growth; w, weak growth; +/–, growth in the microplate system, but not in the Biotype strip; –/+, growth in the Biotype strip, but not in the microplate system. The following carbon sources could not be utilized for growth: L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannose, L-rhamnose, D-ribose, sucrose, D-trehalose, D-xylose, adonitol, maltitol, D-mannitol, D-sorbitol, putrescine, *cis*-aconitate, *trans*-aconitate, 4-aminobutyrate, citrate, glutarate, itaconate, propionate, L-alanine, L-histidine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate.

Characteristic	Score
Anaerobic growth	–
Autotrophic growth	–
Growth at 4 °C	+
Growth at 40 °C	–
Reduction of nitrate	–
Presence of:	
Oxidase	+
Catalase	+
PHB	+
Hydrolysis of:	
Aesculin	–
pNP β -glucopyranoside	–
<i>bis</i> -pNP phosphate	+
L-Alanine pNA	+
L-Proline pNA	–
Utilization of:	
Acetate	+
Fumarate	+
DL-3-Hydroxybutyrate	+ / –
2-Ketoglutarate	+ / –
DL-Lactate	+ / –
L-Malate	– / +
Pyruvate	w
Succinate	+
L-Aspartate	+
L-Glutamate	+

et al. (1997), growth was detected on the following substrates: acetate, fumarate, DL-3-hydroxybutyrate, DL-lactate, 2-oxoglutarate and L-aspartate. None of the carbohydrates or polyols tested was used for growth. The chromogenic substrates *bis*-*para*-nitrophenyl (*bis*-pNP) phosphate and L-alanine *para*-nitroanilide (L-alanine pNA) were hydrolysed. The only difference between the two strains was that CS-K1 could not use pyruvate, whereas CS-K2^T was able to utilize it very weakly. Several minor differences in the substrate utilization pattern appeared when the Biotype 100 system, originally developed for the differentiation of members of the *Enterobacteriaceae*, was used. In contrast to the microplate system, both strains could use L-malate, but could not grow with DL-

Fig. 3. Effect of thiosulfate on the growth of strain CS-K2^T in succinate/mineral medium: ■, growth in medium without thiosulfate; ●, growth in medium supplemented with 10 mM thiosulfate; △, decrease in pH during growth with thiosulfate, expressed as the difference between pH values determined during growth with thiosulfate and pH values measured during growth without thiosulfate.

lactate, DL-3-hydroxybutyrate or 2-oxoglutarate. In addition, succinate and L-glutamate, which were not included in the microplate assay, could be identified as substrates. The results of the physiological characterization of strain CS-K2^T are summarized in Table 2.

Effect of thiosulfate on growth. In batch cultures, significant increases in the growth yields of strains CS-K1 and CS-K2^T were observed after the addition of thiosulfate to mineral medium containing a suitable carbon source such as acetate or succinate. The two modes of thiosulfate oxidation can be distinguished easily in batch cultures due to the pH change in the medium. The formation of tetrathionate from thiosulfate is accompanied by an alkalization of the culture medium, whereas oxidation to sulfate leads to a decrease in the pH. A typical growth experiment with strain CS-K2^T is shown in Fig. 3, illustrating the formation of acid during growth on succinate/mineral medium containing 10 mM thiosulfate. At the end of this experiment, the thiosulfate concentration in the medium had decreased to 4 mM and the concentration of sulfate formed reached 12 mM. The observed stoichiometry is in line with the following equation for thiosulfate oxidation: $S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$, for which $\Delta G'_0 = -818.42 \text{ kJ mol}^{-1}$ (Sorokin *et al.*, 1996).

The effect of various amounts of thiosulfate on the growth of the two strains in succinate/mineral medium was quantified by determination of the cellular dry weight in cultures grown to stationary phase (Table 3), because the spectrophotometric method gave unreliable results due to the spontaneous formation of aggregates in batch cultures. It is clear from Table 3 that the increase in growth yield was dependent on the amount of added thiosulfate. The optimal thiosulfate concentration for strain CS-K1 was 10 mM and for

Table 3. Growth yields of strains CS-K1 and CS-K2^T in succinate/mineral medium containing various amounts of thiosulfate

For each thiosulfate concentration, the mean value for dry weight of two independent measurements and the standard deviation are given. The maximum values are highlighted in bold.

S ₂ O ₃ ²⁻ concentration (mM)	Cellular dry weight (mg l ⁻¹)	
	CS-K1	CS-K2 ^T
0	344 ± 10	369 ± 10
2	373 ± 28	364 ± 37
5	361 ± 19	393 ± 12
10	444 ± 12	419 ± 7
20	413 ± 21	461 ± 8
50	371 ± 24	430 ± 4

strain CS-K2^T it was 20 mM. The increase in biomass yields obtained with optimal concentrations of thiosulfate were similar to results reported for '*Thiobacillus*' strain Q (Gommers & Kuenen, 1988) and the obligate chemolithoautotroph *Thiobacillus denitrificans* (about 13 mg dry weight mmol⁻¹ thiosulfate; Timmer-ten Hoor, 1976). It could be shown with cultures grown in acetate/mineral medium that the consumption of thiosulfate is coupled to the assimilation of an organic substrate. Strain CS-K2^T oxidized approximately 0.7 mmol thiosulfate mmol⁻¹ acetate consumed.

Concentrations of thiosulfate above the optimum inhibited growth (Table 3) and thiosulfate oxidation (data not shown). The medium that was used initially to check the ability of the newly isolated strains to oxidize thiosulfate was R2A containing 5 g Na₂S₂O₃ · 5H₂O l⁻¹ (20.1 mM). Under these conditions, strain CS-K1 produced much less acid than did CS-K2^T, enabling easy discrimination of the two otherwise physiologically almost identical organisms.

Genomic characterization

The G + C contents of the total DNA of strains CS-K1 and CS-K2^T were both 55 mol %. The levels of DNA similarity between the two strains revealed by DNA–DNA hybridization experiments were around 100 % with the membrane filter method and 87–95 % when the spectrophotometric method was applied. The high level of DNA–DNA similarity between the two strains indicates that they belong to a single species.

Phylogeny and ecology

The sequences of the 16S rRNA genes of strains CS-K1 and CS-K2^T were determined and compared with sequences of other related bacteria. The two strains had identical 16S rRNA sequences and could be

Fig. 4. Phylogenetic tree showing the positions of the newly isolated strain CS-K2^T and the partly characterized strain '*Thiobacillus*' Q (LMD 81.11) among representatives of the β -Proteobacteria. The GenBank/EMBL accession numbers for each reference strain are shown in parentheses. *, No strain designation provided. The tree is based on a collection of over 100 16S rRNA gene sequences representing members of the β -Proteobacteria. With the exception of the sequences of *Lautropia mirabilis* (1073 nt) and *Thermothrix thiopara* (551 nt), almost complete sequences were used to reconstruct the tree with the neighbour-joining method of Saitou & Nei (1987). Phylogenetic distances were calculated as described by Jukes & Cantor (1969). The sequence of *E. coli* K-12 (L10328) was used as an outgroup (not shown). For the sake of clarity, only known type species within the *Burkholderia* group and some species closely related to chemolithoheterotrophic (in bold) or autotrophic colourless sulfur bacteria within the β -Proteobacteria are shown in this figure. Bar, 10 % estimated sequence divergence.

affiliated phylogenetically to the *Burkholderia* group of the β -Proteobacteria. The most closely related described species was *Burkholderia cepacia*, sharing a sequence similarity of 92.7 % with the novel strains. In addition, the 16S rRNA gene sequence of '*Thiobacillus*' strain Q was determined, in order to clarify the phylogenetic affiliation of this partly described chemolithoheterotrophic micro-organism. The similarity of the sequence of '*Thiobacillus*' Q to the sequence of CS-K1 and CS-K2^T was only 89.1 %. The phylogenetic positions of '*Thiobacillus*' Q and strain CS-K2^T within the β -Proteobacteria are shown in Fig. 4. The physiological properties and the large phylo-

genetic distance of the newly isolated strains from any known type species within this group indicate that they represent a novel genus within the β -*Proteobacteria*.

Interestingly, the sequences of CS-K1 and CS-K2^T clustered together with a group of cloned sequences retrieved from large limnetic organic aggregates ('lake snow') from Lake Constance (Huber, 1997). The similarity value to the almost complete sequence LST3091, representing this cluster of 'lake snow' clones, was 98.6%. The abundance of sequences belonging to this cluster in clone libraries constructed from 'lake snow' varied with the depth of the sample. In a clone library constructed from material collected at a depth of 15 m, over 20% of the sequences were affiliated to this cluster, whereas, in a sample from 25 m, these sequences were found to represent less than 1% of the clones obtained (Huber, 1997). These results indicate that bacteria closely related to the newly isolated strains are indigenous to 'lake snow' aggregates and may be significantly abundant there. A characteristic trait of the novel strains is the formation of flocs in liquid cultures, which may be a further hint of the possible role of these bacteria in the formation of 'lake snow' aggregates.

Conclusions

Two strains of chemolithoheterotrophic, thiosulfate-oxidizing bacteria were isolated from a highly diluted sample of a littoral freshwater sediment. These bacteria formed a novel phylogenetic branch within the β -*Proteobacteria*, together with a cluster of 16S rRNA gene sequences retrieved from 'lake snow' aggregates from Lake Constance. The results of a polyphasic taxonomic study indicated that the two strains are members of a single species, which should be placed in a new genus. Based on the results presented in this paper, we describe a new taxon that includes strains CS-K1 and CS-K2^T, for which we propose the name *Limnobacter thiooxidans* gen. nov., sp. nov.

Description of *Limnobacter* gen. nov.

Limnobacter (Lim.no.bac'ter. Gr. n. *limnos* lake; M.L. n. *bacter* rod; M.L. masc. n. *Limnobacter* lake rod, referring to the isolation of the type species from lake sediment).

Cells are Gram-negative, slightly curved rods that are motile by single polar flagella. Polyhydroxybutyrate is stored as a reserve material. Endospores are not formed. Strictly aerobic; oxidase- and catalase-positive. Growth occurs between 4 and 38 °C. Carboxylic acids and amino acids are used as energy and carbon sources, but no carbohydrates or polyols are used. Not able to grow autotrophically. Thiosulfate is oxidized to sulfate in the presence of an organic carbon source. Major fatty acids are 18:1 ω 7c, 16:1 ω 7c, 16:0 and 10:0 3-OH. Phylogenetically affiliated to the *Burkholderia* group within the β -*Proteobacteria*. The type species is *Limnobacter thiooxidans*.

Description of *Limnobacter thiooxidans* sp. nov.

Limnobacter thiooxidans (thi.o.ox'i.dans. Gr. n. *thion* sulfur; M.L. v. *oxido* to make acid, oxidize; M.L. part. adj. *thiooxidans* oxidizing sulfur).

Cells are slightly curved rods with pointed ends, 1.5–3.7 μ m long with a diameter of 0.6–1.5 μ m. They occur singly or in short chains and are motile by single polar flagella. Elemental sulfur is not deposited intra- or extracellularly during growth on media containing thiosulfate. Colonies on R2A medium reach a diameter of 0.8–1.0 mm and are circular, smooth, translucent and flat with entire margins. Aggregates are formed during growth in liquid culture. The pH range for growth is pH 5.5–10.5. Yeast extract and vitamins are not required for growth. Nitrate is not reduced to nitrite by strain CS-K2^T. Several carboxylic acids and amino acids are used as sources of carbon and energy. The following compounds were identified as substrates for growth by the Biotype 100 system: fumarate, succinate, L-malate, L-aspartate and L-glutamate. In the presence of an organic substrate, thiosulfate can be used as an energy source, enabling chemolithoheterotrophic growth.

The G+C content of the DNA is 55 mol% (T_m). Isolated from the littoral sediment of a freshwater lake. The type strain is strain CS-K2^T (= DSM 13612^T = LMG 19593^T).

ACKNOWLEDGEMENTS

We would like to thank Dr M. Nimtz (GBF, Braunschweig) for access to the GC-MS facilities, A. Tiepold (GBF, Braunschweig) for running the GC-MS and Dr B. J. Tindall for preparation of the DMDS adducts and interpretation of the mass spectra. The thiosulfate and sulfate determinations were performed at the Lehrstuhl für Bodenkunde (Technische Universität München, Freising) with the friendly help of M. Hübner. Professor N. Petersen is acknowledged for organizing the sampling of littoral sediment at Lake Chiemsee. This project was supported financially by DFG grant Schl 120/10-5.

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