Rhodanobacter thiooxydans sp. nov., isolated from a biofilm on sulfur particles used in an autotrophic denitrification process

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A novel thiosulfate-oxidizing bacterium, designated strain LCS2^T, was isolated from a biofilm on sulfur particles used in an autotrophic denitrification process. The strain was found to comprise Gram-negative, non-motile, non-spore-forming rods that produced yellow-pigmented colonies on R2A agar. The strain contained Q-8 as the major ubiquinone and 17 : 1 iso ω 9c, 15 : 0 iso and 17 : 0 iso as the major fatty acids. The G + C content of the genomic DNA was 64.6 mol%. The 16S rRNA gene sequence of strain LCS2^T was found to be most similar to that of *Rhodanobacter fulvus* IAM 15025^T (97.4 % similarity). The results of DNA–DNA hybridization and phenotypic analysis showed that strain LCS2^T can be distinguished from all known *Rhodanobacter* species and therefore represents a novel species of the genus, for which the name *Rhodanobacter thiooxydans* sp. nov. is proposed. The type strain is LCS2^T (=DSM 18863^T =KCTC 12771^T).

At the time of writing, the genus *Rhodanobacter* accommodates three species: *Rhodanobacter lindaniclasticus* (Nalin *et al.*, 1999), isolated from a soil sampled from a wood-treatment site and which degrades lindane (γ -hexachlorocyclohexane); *Rhodanobacter fulvus* (Im *et al.*, 2004), isolated from soil mixed with rotten rice straw and which produces β -galactosidase; and *Rhodanobacter spathiphylli* (De Clercq *et al.*, 2006), isolated from the rhizospheres of *Spathiphyllum* plants grown in a compost-amended potting mix. Although the species were first isolated from widely separated geographical locations, all members of the genus *Rhodanobacter* have emerged from soil environments.

Various denitrification processes to remove nitrate from wastewater have been developed and studied. The autotrophic denitrification process can use sulfur particles as an energy source. This process is cheaper and easier to handle than the heterotrophic denitrification approach, involving organic chemicals (Batchelor & Lawrence, 1978; Claus & Kutzner, 1985; Koenig & Liu, 1996; Zhang & Lampe, 1999), but the exact microbial roles are not yet known.

During a study of the role of the bacterial communities that reside in the biofilms on sulfur particles used in an autotrophic denitrification process, a laboratory-scale upflow anaerobic sludge blanket reactor fed with such sulfur particles was used to enumerate and identify the bacteria involved. A biofilm from sulfur particles was

thoroughly suspended in 50 mM phosphate buffer (pH 7.0). The suspension was spread on one-tenth-strength R2A agar (Difco). The agar plates were incubated for 1 month at room temperature under both aerobic and anaerobic conditions, and each single colony was incubated once again using half-strength R2A agar. Consequently, seven strains were isolated under aerobic conditions. They were subjected to identification by means of conventional biochemical tests, analysis of the cellular fatty acids and partial 16S rRNA gene sequencing. Only one strain, LCS2^T, was shown to represent a novel species.

The effect of thiosulfate on growth of strain LCS2^T was tested in succinate/mineral medium containing (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. The medium was supplemented with various amounts of thiosulfate, resulting in final concentrations ranging from 0 to 20 mM. Growth in liquid medium at 30 °C with vigorous shaking was investigated. Autotrophic growth was tested by omitting succinate from the succinate/mineral medium and replacing the yeast extract with 0.5 ml vitamin solution l^{-1} (Frankel *et al.*, 1997). The ability to grow anaerobically was determined by supplementing the R2A or succinate/mineral medium with KNO_3 (2 g l^{-1}) or $Fe_4(P_2O_7)_3$ (3.72 g l^{-1}) as an alternative electron acceptor and then incubating the inoculated medium in anaerobic jars (Spring *et al.*, 2001). The reduction of nitrate and the oxidation of thiosulfate were monitored by using an ion chromatograph (model 790

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LCS2^T is AB286179.

personal IC; Metrohm) equipped with a conductivity detector and an anion-exchange column (Metrosep Anion Supp 4; Metrohm).

In our experiments, *R. fulvus* IAM 15025^T and *R. spathiphylli* LMG 23181^T were obtained from culture collection centres, but the type strain of the type species of the genus, *R. lindaniclasticus* LMG 18385^T (the only known strain of this species), could not be included, as the strain no longer exists (Mergaert *et al.*, 2002). All strains were cultivated identically and tested under the same conditions for comparative studies. For the analysis of fatty acids, the strains were cultivated in tryptic soy broth (Difco) at 30 °C for 4 days because of the slow growth of strain LCS2^T.

The Gram reaction was performed as described by Gerhardt *et al.* (1994). Cell morphology and motility were observed under a phase-contrast microscope (Optiphot; Nikon) at 1000× magnification with cells grown for 1–7 days on R2A agar. Oxidase activity was tested using 1% (w/v) tetramethyl-*p*-phenylenediamine, and catalase activity was tested using 3% H₂O₂. Growth was investigated at temperatures ranging from 5 to 45 °C (using increments of 5 °C), at pH 4–10 (using increments of 1 pH unit) and in different salt concentrations (1, 2, 3 and 5% NaCl). Hydrolysis of casein and starch was tested on casein agar and starch (Difco). An H₂S-production test was performed on triple-sugar-iron agar (BBL). Carbon-source-utilization tests and additional physiological tests were performed using API 20NE, API 32GN, API 50 CH and API ZYM galleries according to the instructions of the manufacturer (bioMérieux).

Fatty acid methyl esters were prepared and analysed as described previously (Klatte *et al.*, 1994) using the standard Microbial Identification System (MIDI) for automated GC analysis (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted and purified as described previously (Tindall, 1990) and dried preparations were dissolved in 200 µl 2-propanol; 1–10 µl samples were separated by HPLC without further purification.

Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Rainey *et al.* (1996). The 16S rRNA gene sequence was aligned with published sequences retrieved from EMBL by using CLUSTAL_X (Thompson *et al.*, 1997) and was edited using BioEdit (Hall, 1999). The phylogenetic tree was constructed on the basis of the neighbour-joining method (Saitou & Nei, 1987); distances were estimated using the Kimura two-parameter model (Kimura, 1983) with MEGA version 3.1 (Kumar *et al.*, 2004). The resultant neighbour-joining tree topology was evaluated by using bootstrap analysis (Felsenstein, 1985) based on 1000 resampled datasets. The DNA G+C content was determined by HPLC after hydrolysis, as described by Tamaoka & Komagata (1984), and non-methylated λ DNA (Sigma) was used as a standard. DNA–DNA hybridization to determine genomic relatedness was performed fluorometrically by following

the method of Ezaki *et al.* (1989) using DNA probes labelled with photobiotin (A1935; Sigma) and 96-well microdilution plates (Greiner Bio-One) at 50.8 °C.

The effect of thiosulfate on the growth of strain LCS2^T in succinate/mineral medium was quantified by determining the cellular dry weight (Table 1). It is clear that the growth yield was dependent on the amount of added thiosulfate. The optimal thiosulfate concentration was 10 mM and the increase in biomass yield was obtained with the optimal concentration of thiosulfate. This result was similar to that reported for *Limnobacter thiooxidans* CS-K2^T (Spring *et al.*, 2001). Increases in the sulfate concentration, coupled with decreasing thiosulfate concentration, were observed. The concentration of thiosulfate in the medium decreased from 10 to 2 mM and the sulfate concentration reached 12 mM at stationary phase.

Strain LCS2^T formed visible colonies (0.2–0.5 mm in diameter) on R2A agar at 30 °C within 48 h. No growth was observed at 5 °C or at temperatures above 40 °C within 14 days. The colonies were yellowish, translucent and shiny with entire edges. The cells were Gram-negative, non-motile, non-spore-forming rods (0.6–0.8 × 1.5–3.0 µm).

Analysis of the 16S rRNA gene sequence of strain LCS2^T, consisting of 1482 nt, indicated its membership of the genus *Rhodanobacter* (Fig. 1), the intragenomic relatedness of which ranges from 96.0 to 98.5% among the type strains of species. Strain LCS2^T showed 97.4, 96.9 and 96.8% sequence similarity with respect to the type strains of *R. fulvus*, *R. lindaniclasticus* and *R. spathiphylli*, respectively.

When DNA–DNA hybridizations were performed between strain LCS2^T and *R. fulvus* IAM 15025^T, a DNA relatedness of 31% was revealed, confirming that LCS2^T represents a distinct genomic species (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987).

Strain LCS2^T differed significantly from the type strains of *Rhodanobacter* species in terms of the acid-production, carbon-source-utilization and substrate-hydrolysis profiles, and also with regard to nitrate reduction. The phenotypic

Table 1. Effect of the initial concentration of thiosulfate on growth of strain LCS2^T

For each thiosulfate concentration, the mean ± SD for dry cell weight is given. The maximum value is highlighted in bold. Maximum growth of strain LCS2^T was reached after cultivation with 10 mM thiosulfate at 30 °C for 90 h.

Na ₂ S ₂ O ₃ concentration (mM)	Dry cell weight (mg l ⁻¹)
0	64.6 ± 5
5	75.9 ± 7
10	103.9 ± 10
15	97.3 ± 10
20	101.2 ± 11

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, constructed using the neighbour-joining method (Saitou & Nei, 1987), showing the position of strain LCS2^T among members of the family Xanthomonadaceae. *Escherichia coli* ATCC 11775^T was used as an outgroup. Numbers at branching points refer to bootstrap percentages above 50 % (based on 1000 resamplings). Bar, 2 substitutions per 100 nucleotide positions.

characteristics are summarized in Table 2 and the species description.

The major ubiquinone of strain LCS2^T was Q-8. The fatty acids 17:1 iso ω 9c (22.0 %), 15:0 (21.5 %) and 17:0 iso (13.2 %) were predominant; the detailed fatty acid composition is shown in Table 3. Strain LCS2^T has a fatty acid profile similar to those of the type strains of *Rhodanobacter* species, but it has a larger proportion of 17:1 iso ω 9c and a smaller proportion of 11:0 iso. Interestingly, the fatty acid profiles were rather different from those described by De Clercq *et al.* (2006) because of differences in the conditions employed. Strain LCS2^T and the two *Rhodanobacter* type strains were cultured under identical conditions, as mentioned above, for 4 days in order to secure sufficient quantities of fatty acids because of the slow growth of the novel strain.

On the basis of the phenotypic and genotypic data, strain LCS2^T merits recognition as a novel species within the genus *Rhodanobacter*, for which the name *Rhodanobacter thiooxydans* sp. nov. is proposed.

Description of *Rhodanobacter thiooxydans* sp. nov.

Rhodanobacter thiooxydans (thi.o.ox'y.dans. Gr. n. *thion* sulfur; Gr. adj. *oxus* sharp, acid; N.L. v. *oxydo* to make acid, to oxidize; N.L. part. adj. *thiooxydans* oxidizing sulfur).

Cells are aerobic, non-motile, non-spore-forming rods (0.6–0.8 × 1.5–3.0 μ m). Gram-negative and oxidase- and catalase-positive. Good growth is observed on R2A agar at 25–35 °C and pH 5–8. Growth occurs in the presence of 1 and 2 % NaCl. Colonies are yellowish, convex and circular

Table 2. Characteristics that differentiate strain LCS2^T from the type strains of *Rhodanobacter* species

Strains: 1, LCS2^T; 2, *R. fulvus* IAM 15025^T; 3, *R. spathiphylli* LMG 23181^T; 4, *R. lindaniclasticus* LMG 18385^T. Data for *R. lindaniclasticus* were taken from Nalin *et al.* (1999). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4
G + C content (mol%)	64.6	65.3	67.6	63.0
Motility	–	+	–	–
Nitrate reduction	+	–	–	–
Protease (gelatin)	+	–	–	ND
Acid production from:				
D-Xylose	–	+	–	ND
Galactose	–	+	–	ND
Glucose	–	+	–	ND
Fructose	–	+	–	ND
Mannose	–	+	–	ND
Amygdalin	–	+	–	ND
Salicin	–	+	–	ND
Maltose	–	+	+	ND
Lactose	–	+	–	ND
Melibiose	–	+	–	ND
Starch	–	+	–	ND
Utilization of:				
D-Mannose	–	+	–	–
D-Melibiose	–	+	–	–
D-Ribose	+	–	–	–
Sucrose	–	+	–	–
Glycogen	+	–	–	–
Salicin	–	+	–	–
Valerate	+	–	+	ND
Hydrolysis of:				
2-Naphthyl butyrate	+	–	–	ND
2-Naphthyl caprylate	–	+	+	ND
6-Bromo-2-naphthyl α -D-galactopyranoside	–	+	–	ND
1-Naphthyl N-acetyl- β -D-glucosaminide	–	+	+	ND

*Data from API ZYM tests. The intensity of the colour was measured on a scale from 0 to 5 and was interpreted as negative when values were 0 or 1 and positive when values ranged from 2 to 5 (Mudarris *et al.*, 1994).

with entire edges. Uses carboxylic acids and amino acids as energy and carbon sources, but cannot grow autotrophically. Thiosulfate is oxidized to sulfate in the presence of an organic carbon source. Elemental sulfur is not deposited intracellularly or extracellularly during growth on media containing thiosulfate. β -Galactosidase activity is present. Nitrate is reduced, but nitrite is not. Indole and H₂S are not produced. Aesculin and gelatin are hydrolysed, but casein, starch and urea are not. Acid is produced from aesculin and D-fucose, but not from glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β -D-xylose, galactose, glucose, fructose, mannose,

Table 3. Cellular fatty acid profiles of strain LCS2^T and available type strains of the genus *Rhodanobacter*

Strains: 1, LCS2^T; 2, *R. fulvus* IAM 15025^T; 3, *R. spathiphylli* LMG 23181^T. Fatty acids are listed using standard abbreviations (no. of carbon atoms: no. double bonds). All strains were grown in tryptic soy broth at 30 °C for 4 days. –, Not detected.

Fatty acid	1	2	3
11:0 iso	4.1	13.7	10.7
Unknown (ECL 11.798)*	2.7	4.1	3.5
11:0 iso 3-OH	3.5	8.6	8.9
14:0 iso	1.5	1.7	3.2
13:0 iso 3-OH	2.9	10.6	6.8
15:0 iso	21.5	24.4	22.8
15:0 anteiso	8.3	4.9	5.3
16:0 iso	6.5	5.7	8.2
Summed feature 4†	10.4	4.6	4.5
16:0	3.7	5.7	3.2
17:1 iso ω 9c	22.0	4.1	16.7
17:0 iso	13.2	9.1	6.2
17:0 cyclo	–	2.7	–

*ECL, Equivalent chain-length (i.e. the identity of the fatty acid is unknown).

†Summed feature 4 contains 15:0 iso 2-OH and/or 16:1 ω 7c.

sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The following compounds are utilized as sole carbon sources: D-glucose, *N*-acetyl-D-glucosamine, maltose, D-ribose, glycogen and valerate. The following carbon sources are not utilized: L-arabinose, mannose, gluconate, caprate, adipate, D-mannitol, citrate, phenylacetate, L-rhamnose, inositol, sucrose, itaconate, suberate, malonate, acetate, DL-lactate, L-alanine, 5-ketogluconate, 3-hydroxybenzoate, L-serine, salicin, D-melibiose, L-fucose, D-sorbitol, propionate, L-histidine, 2-ketogluconate, 3-hydroxybutyrate, 4-hydroxybenzoate and L-proline. 2-Naphthyl phosphate (pH 8.5), 2-naphthyl butyrate, L-leucyl 2-naphthylamide, 2-naphthyl phosphate (pH 5.4), naphthol-AS-BI-phosphate and 6-bromo-2-naphthyl β -D-glucopyranoside are hydrolysed, but 2-naphthyl caprylate, 2-naphthyl myristate, L-valyl 2-naphthylamide, L-cystyl 2-naphthylamide, *N*-benzoyl-DL-arginine 2-naphthylamide, *N*-glutaryl-phenylalanine 2-naphthylamide, 6-bromo-2-naphthyl α -D-galactopyranoside, 2-naphthyl β -D-galactopyranoside, naphthol-AS-BI- β -D-glucuronide, 2-naphthyl α -D-glucopyranoside, 1-naphthyl *N*-acetyl- β -D-glucosaminide, 6-bromo-2-naphthyl α -D-mannopyranoside and 2-naphthyl α -L-fucopyranoside are not hydrolysed. The major quinone is ubiquinone Q-8. The

predominant fatty acids are 17:1 iso ω 9c (22.0%), 15:0 iso (21.5%) and 17:0 iso (13.2%). The G+C content of the DNA of the type strain is 64.6 mol%.

The type strain, LCS2^T (=DSM 18863^T =KCTC 12771^T), was isolated from a biofilm of sulfur particles used in an autotrophic denitrification process.

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