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ORIGINAL PAPER

Desulfurispirillum alkaliphilum gen. nov. sp. nov., a novel obligately anaerobic sulfur- and dissimilatory nitrate-reducing bacterium from a full-scale sulfide-removing bioreactor

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Abstract Strain SR 1^T was isolated under anaerobic conditions using elemental sulfur as electron acceptor and acetate as carbon and energy source from the Thiopaq bioreactor in Eerbeek (The Netherlands), which is removing H_2S from biogas by oxidation to elemental sulfur under oxygen-limiting and moderately haloalkaline conditions. The bacterium is obligately anaerobic, using elemental sulfur, nitrate and fumarate as electron acceptors. Elemental sulfur is reduced to sulfide through intermediate polysulfide, while nitrate is dissimilatory reduced to ammonium. Furthermore, in the presence of nitrate, strain SR 1^T was able to oxidize limited amounts of sulfide to elemental sulfur during anaerobic growth with acetate. The new isolate is mesophilic and belongs to moderate haloalkaliphiles, with a pH range for growth (on acetate and nitrate) from 7.5 to 10.25 (optimum 9.0), and a salt range from 0.1 to 2.5 M Na^+ (optimum 0.4 M). According to phylogenetic analysis, SR 1^T is a member of a deep bacterial lineage, distantly related to *Chrysiogenes arsenatis* (Macy et al. 1996). On the basis of the phenotypic and genetic data, the novel isolate is placed into a new genus and species, *Desulfurispirillum alkaliphilum* (type strain SR^T = DSM 18275 = UNIQEM U250).

Keywords *Desulfurispirillum alkaliphilum* · Sulfur-reducing · Dissimilatory nitrate-reducing · Haloalkaliphilic

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Nucleotide sequence accession number: the GenBank/EMBL accession number of the 16S rRNA gene sequence of strain SR 1^T is DQ666683.

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Introduction

The full-scale Thiopaq bioreactor in Eerbeek (The Netherlands) is aimed for the removal of H_2S from biogas, which is produced during anaerobic treatment of wastewater from paper-mill factories. The process is based on recently developed biotechnology of preferential sulfide oxidation to insoluble sulfur by lithoautotrophic sulfide-oxidizing bacteria under oxygen limitation (Buisman et al. 1990; Janssen et al. 1998). During a first physico-chemical step of the process, H_2S from the biogas is absorbed at a pH of around 8.7 by an alkaline solution, containing $NaHCO_3$. The absorbed alkaline sulphide solution is fed into the bioreactor, operating at 35–45°C and –300 to –350 mV under oxygen-limiting conditions to facilitate incomplete oxidation of sulphide to elemental sulphur. This situation creates perfect conditions for a complete sulfur cycle, whereby both aerobic and anaerobic reactions involving sulfur compounds are possible. Lithoauto-

trophic sulfide-oxidizing bacteria are the dominant populations in such reactors. They are producing elemental sulfur, as a major product, and thiosulfate and sulfate, as by-products, from sulfide, and organic compounds from CO_2. The products then can be utilized by anaerobic sulfur-reducing, thiosulfate-reducing and sulfate-reducing bacteria, which are considered as unwanted components in such reactors (Buisman 1989). Furthermore, the development of a sulfur-reducing population in the reactor is facilitated by the special properties of biologically-produced sulfur, which, in contrast to chemically-produced sulfur, is hydrophilic and has properties of colloidal particles (Janssen et al. 1996). Indeed, anaerobic enrichments at moderately haloalkaline, mesophilic conditions indicated the presence of an active population of the latter type, and resulted in the isolation of a haloalkaliphilic sulfur-reducing (respiring) bacterium strain SR 1, which is described below. Despite the intensive characterization of different functional groups of haloalkaliphilic bacteria (Jones et al. 1998; Zavarzin et al. 1999; Sorokin and Kuenen 2005), sulfur-respiring and dissimilatory nitrate-reducing representatives have yet not been found among the haloalkaliphilic communities in soda lakes. The bacterium described below represents a first example of such a type.

Methods

Cultivation

Enrichment and routine cultivation of haloalkaliphilic sulfur-reducing bacteria was performed at 30°C on a mineral medium containing sodium-bicarbonate, 0.6–0.8, 0.1 M NaCl, and 0.5 g l^{-1} of K_2HPO_4. The pH was adjusted to 9.0 by addition of Na_2CO_3. After sterilization, the medium was supplemented with 20 mM acetate as carbon and energy source, 50 mg l^{-1} of yeast extract, 4 mM NH_4Cl, 1 mM $MgSO_4$, and 1 ml l^{-1} of trace metal solution (Pfenning and Lippert 1966). Elemental sulfur was obtained from the Eerbeek Thiopaq reactor sludge as a thick suspension. It was separated from the cells by low-speed centrifugation, washed several times with distilled water, sterilized as 20% (v/v) suspension in distilled water, washed again several times with sterile distilled water, and used at a final concentration of 30 mM. Other electron acceptors used were KNO_3 (20 mM), KNO_2, Na_2SO_3, sodium selenate and selenite, sodium arsenate, DMSO (5 mM each), sodium fumarate (20 mM; alone or with 2 mM acetate as carbon source), and freshly prepared ferrihydrite (20 mM). Growth at microoxic conditions was tested with an oxygen concentration in the gas phase from 1 to 5%. Anaerobic cultivation was performed in 60–100 ml serum bottles with 40–80 ml medium whereby air was replaced with argon. In some cases argon was replaced by H_2 with 0.5 bar overpressure. Solid alkaline media with a final salt concentrations of 0.5 M Na^+ was prepared by 1:1 mixing of 4% (w/v) agarose and 1 M Na^+ mineral medium at 50°C. The plates were incubated in closed jars under argon atmosphere with an oxygen-scavenging catalyzer (Oxoid). The pH dependence was examined at Na^+ content of 0.6 M, using the following filter-sterilized mineral medium: for pH 6–8, 0.1 M HEPES and NaCl; for pH 8–11, a mixture of sodium bicarbonate/sodium carbonate containing 0.1 M NaCl. Growth resulted in a shift of initial pH values, especially in highly alkaline region; so only final pH values were used as suitable range for growth. To study the influence of salt concentration on growth, mineral media, containing 0.1 and 3.0 M of total Na^+ were mixed in different proportions.

Analytical procedures

Chemical analysis of sulfur (sulfide, polysulfide, sulfur) and nitrogen (nitrate, nitrite, ammonium) compounds, and cell protein were performed as described previously (Sorokin et al. 2001, 2004). Phase contrast microphotographs were obtained with a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). For electron microscopy, the cells were fixed with glutaraldehyde (final 3% v/v) and positively contrasted with 1% (w/v) uranyl acetate. Respiratory lipoquinones and polar lipids were extracted from 100 mg of freeze-dried cells using the method described by Tindall (1990a, b). Respiratory lipoquinones were separated into their structural classes (menaquinones, ubiquinones, etc.) by TLC, bands eluted and further separated and identified by HPLC, using an RP_{18} column (Tindall 1996). Polar lipids were separated by two-dimensional chromatography and identified on the basis of their R_f values in combination with their reaction with specific staining reagents (Tindall 1990a, b). Fatty acid methyl esters were released from 20 mg freeze-dried cells using methodologies, which release only ester linked fatty acids or ester and amide linked fatty acids (Labrenz et al. 2005; Strömpl et al. 1999).

Genetic and phylogenetic analysis

The isolation of the DNA and determination of the G + C content of the DNA was performed according to Marmur (1961). Genomic DNA was extracted from

the cells using the UltraClean Soil DNA Extraction Kit (MolBio Laboratories, USA), following the manufacture's instructions. The nearly complete 16S rRNA gene was obtained using general bacterial primers GM3f (5′-AGAGTTTGATCCTGGCTCAG-3′) and GM4r (5′-TACGGTTACCTTGTTACGACTT-3′). Sequencing was performed by the company BaseClear (Leiden, The Netherlands). The sequence was first compared to sequences stored in the GenBank database using BLAST search tool. Subsequently, the sequence was imported into the ARB software program (Ludwig et al. 2004), automatically aligned and manually checked. A tree was then generated using maximum likelihood.

Results and discussion

Enrichment and isolation of pure culture

Primary anaerobic enrichment cultures with acetate/sulfur were positive for a pH range from 8 to 10, and salt concentrations from 0.4 to 1.5 M total Na^+, closely matching the reactor conditions. During growth, two stages of sulfur transformation were observed: in the beginning, when sulfur was in excess, greenish-yellow polysulfide (S_n^{2-}) was produced, which accumulated until all sulfur was dissolved. After that, the color started to disappear and all sulfur was finally converted to sulfide. MPN dilution series indicated the presence of up to 10^6 viable cells in 1 ml of reactor sample. From the highest positive dilution, a pure culture was isolated using sulfur-containing plates. Sulfur-reducing colonies formed clearing of the insoluble sulfur around them with yellow polysulfide as an intermediate stage (Fig. 1a). The strain was designated SR 1^T. The new isolate is a thin spirillum (Fig. 1b), highly motile with bipolar flagella (Fig. 1c).

Growth characteristics and metabolism

Strain SR 1^T grew anaerobically at pH 9 and salt concentration of 0.6 M Na^+ using biologically-produced sulfur as electron acceptor, which was first fully dissolved as polysulfide due to a chemical reaction with produced sulfide under alkaline conditions. At a later stage, polysulfide disappeared and sulfide was the final product (Fig. 2a). Chemical analysis demonstrated the presence of S_3^{2-} (greenish) and S_4^{2-} (yellow) as the dominant polysulfide species. The strain also could initiate growth with polysulfide (S_6^{2-}) instead of sulfur, but at a concentration of sulfan (terminal sulfide atoms) not higher than 10 mM. The μ_{max} for growth under sulfur-reducing conditions was 0.12 h^{-1}. From other tested electron acceptors, nitrate and fumarate were utilized. In case of nitrate, ammonia was identified as the final product, so the novel isolate belongs to dissimilatory nitrate reducers. Growth with acetate and nitrate as electron acceptor, instead of sulfur, was faster (μ_{max} = 0.19 h^{-1}), but the culture stopped growing

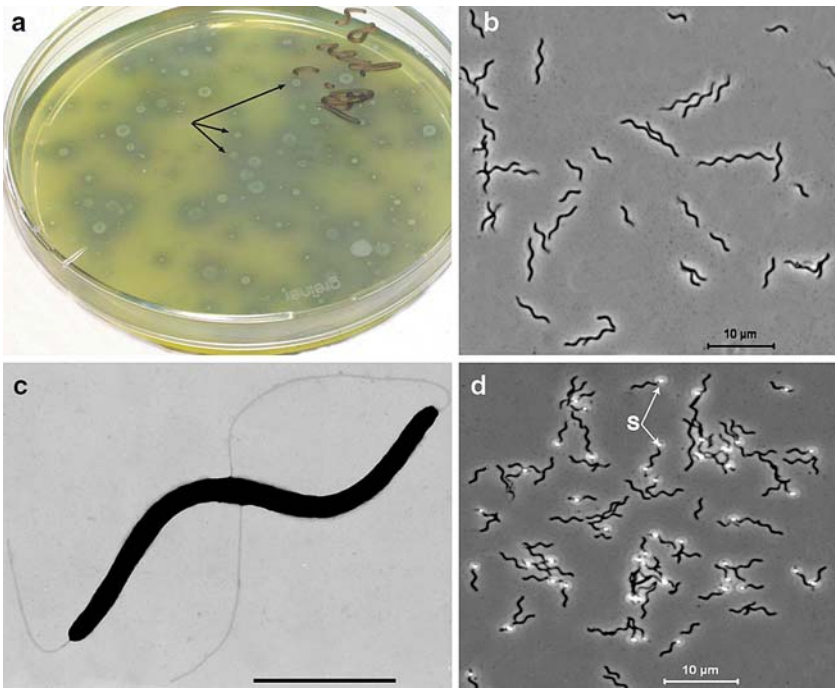

Fig. 1 Morphology of SR 1. **a** Colonies obtained from enrichment culture on sulfur-containing alkaline agar; sulfur was first converted to polysulfide (greenish-yellowish background) and finally to sulfide indicated by clearing zone around the active colonies. **b** Phase-contrast microphotograph of cells grown with acetate and nitrate. **c** Electron microphotograph of positively stained cell, bar = 1 µm. **d** Phase-contrast microphotograph of cells accumulating intracellular sulfur (*S*) during oxidation of sulfide

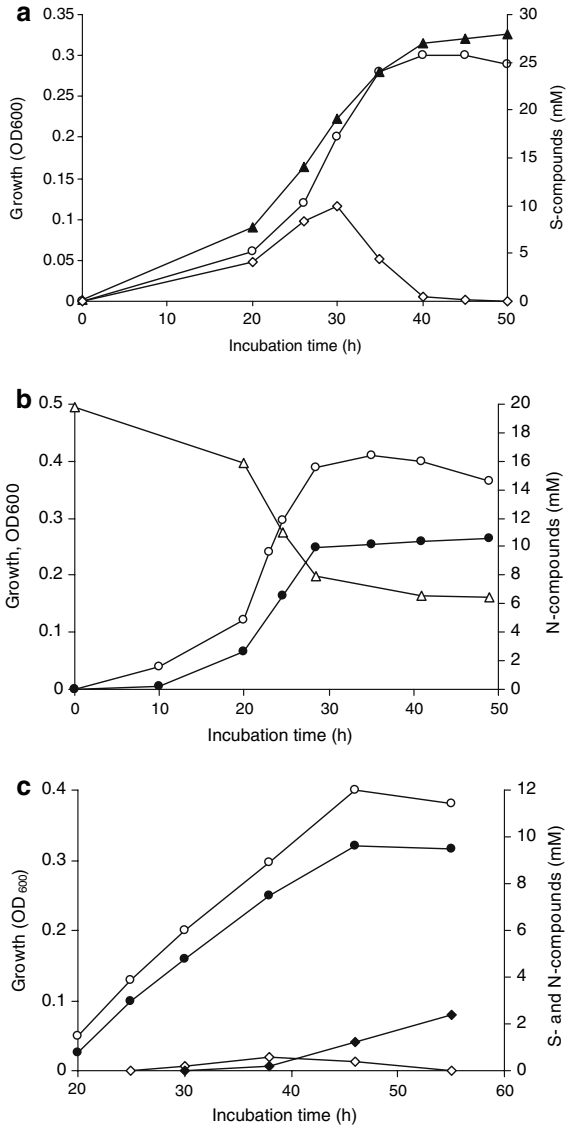

Fig. 2 Anaerobic growth and product accumulation of strain SR 1 at pH 9 and 0.6 M Na^+. **a** Growth with acetate + sulfur; **b** growth with acetate + nitrate; **c** oxidation of sulfide in culture growing with acetate + nitrate. Symbols: *open circles* biomass; *closed circles* NH_3; *open triangles* nitrate; *closed triangles* total sulfane from HS^-/S_n^{2-}; *open diamonds* elemental sulfur dissolved in polysulfide; *closed diamonds* insoluble elemental sulfur

before all the nitrate was reduced, probably due to toxicity of accumulating ammonia at high pH (Fig. 2b). Nitrite was not detected as an intermediate, however, it was the main product of nitrate reduction in experiments with washed cells. Most probably the nitrite-reductase was partially inactivated during cell harvesting under aerobic conditions. In case when both sulfur and nitrate were present as electron acceptors, sulfur reduction was initiated first and only after complete conversion of sulfur to sulfide nitrate reduction started slowly, in contrast to what might be expected from the rule of red-ox potentials. Most probably, the accumulation of polysulfide blocked nitrate reduction. A noticeable difference in color was observed for cells grown at sulfur-reducing and nitrate-reducing conditions: the sulfur-reducing biomass was black, while the nitrate-reducing biomass was bright red, due to a high concentration of a cytochrome *c554* (data not shown), probably associated with the presence of dissimilatory nitrite reductase *ccNIR* (Simon 2002).

The bacterium could grow anaerobically in presence of fumarate as the only substrate (μ_{max} = 0.05 h^{-1}). Although we did not analyze the products, the only possible explanation for the growth at such conditions is fumarate fermentation. Addition of acetate as carbon source did not enhanced the final growth yield in fumarate-grown cultures. Addition of nitrate also did not affect the growth efficiency on fumarate, although up to 5 mM nitrate was finally reduced to ammonia.

Strain SR 1^T can utilize H_2 as an alternative electron donor when acetate was supplied in limited amount (2 mM instead of 20 mM) as the C source both with sulfur or nitrate as electron acceptors. In the presence of H_2, the biomass yield increased 4–5 times with sulfur and 2–3 times with nitrate, respectively. With fumarate as the only substrate, addition of H_2 did not stimulate the growth.

In experiments with washed cells, grown with acetate, nitrate and sulfide, it was H_2, but not acetate, which was utilized as the preferable electron donor, and it was especially active in case of elemental sulfur as the electron acceptor. In case of nitrate, the product was nitrite.

Successful initiation of nitrate-reducing cultures was facilitated by adding sulfide as a reductant at 0.5–1 mM concentrations. At the end of growth, sulfide was converted to elemental sulfur. So it seemed that SR 1^T could reverse sulfur reduction reaction. This ability was proven by growing the isolate with 20 mM acetate, 20 mM nitrate and 3 mM sulfide (Fig. 2c). Sulfide oxidation started in the late logarithmic phase with polysulfide as an intermediate and elemental sulfur as the final product. First, all elemental sulfur can be seen as tiny droplets inside the cells (Fig. 1d), which, on later stage, were excreted into the medium. With fumarate as the electron acceptor, the oxidation of sulfide, either in growing cultures or by washed cells, was not observed. The ability to oxidize sulfide to elemental sulfur was clearly manifested in the experiments with washed cells in presence of both nitrate and nitrite as electron acceptors. The estimated rate of sulfide oxidation in the presence of nitrate at pH 9 was 26 nmol (mg protein $min)^{-1}$.

The results suggest an interesting combination of sulfur and nitrogen red-ox metabolism in SR 1^T as summarized in Fig. 3. The dissimilatory nitrate- and sulfur-reducing Epsilonpoteobacteria *Wolinella succinogenes* (Bokranz et al. 1983; Macy et al. 1986; Klimmeck et al. 1991) and *Sulfurispirillum deleyianum* (Schumaher et al. 1992; Eisenmann et al 1995) are also capable of reversed reaction of sulfide oxidation to sulfur, for which polysulfide reductase is held responsible (Krafft et al. 1995), but the oxidation of sulfide was observed both in the presence of nitrate and fumarate as the electron acceptor. This indicates that the conditions whereby sulfur and nitrogen species can be interconverted within the same ecological niche might be common in certain natural habitats as well as in the industrial bioreactors.

Influence of pH and salts on the growth of strain SR 1^T was examined using nitrate as electron acceptor. The results demonstrated that the reactor isolate is a moderate, but obligate haloalkaliphile (Fig. 4). Growth at a salt content between 1.5 and 2.5 M Na^+ was only possible after gradual adaptation, but not directly using the cells from the low-salt cultures.

Genetic analysis

The G + C content in the genomic DNA of strain SR 1^T was 44.8 ± 0.5 mol% (T_m). Phylogenetic analysis based on nearly complete sequence of the 16S rRNA gene placed the novel isolate into a deep bacterial lineage with the arsenate- and nitrate-respiring anaerobic bacterium *Chrysiogenes arsenatis* as the closest described relative (Fig. 5). Low sequence similarity (91%) indicated that SR 1^T represents a new genus within the family *Chrysiogenetes*. Among the unrecognized members of this group, SR 1^T had a much closer relative, strain TE37, which has recently been isolated as an iron reducer from the deep-subsurface alkaline fluids within the Chinese Continental Drilling project (Zhang et al. 2005). Sequence similarity of 98% indicated relation on the species level (Fig. 5). It seems not a mere coincidence, since the dominant sulfur-oxidizing bacterium from the same bioreactor (*Thioalkalivibrio* sp.) turned out to have a very close relative among one of the clones (CCSD_DF730_B8) identified within the Chinese Continental Drilling project (our unpublished results).

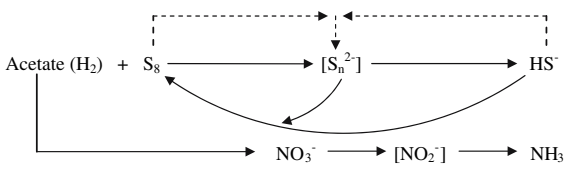

Fig. 3 Scheme of red-ox interaction of sulfur and nitrogen catabolism in SR 1. *Dashed lines* indicate spontaneous reaction

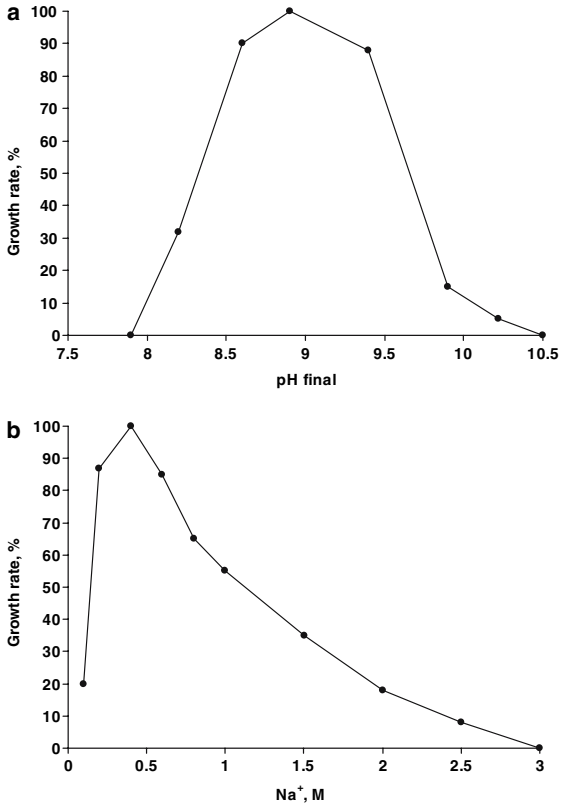

Fig. 4 Influence of pH at 0.6 M Na^+ (**a**) and sodium carbonate/bicarbonate at pH 9 (**b**) on anaerobic growth of strain SR 1 with acetate and nitrate

Chemotaxonomy

Examination of the respiratory lipoquinone composition in SR 1 indicated that menaquinones were the sole respiratory lipoquinones present, and were dominated by the MQ-8. The major polar lipids were phospholipids, no evidence being found for glycolipids or amino acid based lipids. The major components were phosphatidylglycerol, and phosphatidylethanolamine, with smaller amounts of an unidentified phopsholipid, diphosphatidylglycerol, together with traces of two additional unidentified phospholipids and an aminophospholipid. The fatty acids were dominated by the 18:1w7c species. Interestingly, SR 1 also contained multiple isomers of unsaturated 16:1 fatty acids (Table 1). In addition a single unidentified derivative of a 3-OH amide-linked fatty acid was detected, which might be an indicative of the presence of a cell wall-bound lypopolysaccharide.

Overall, strain SR 1^T, isolated from sulfide-removing haloalkaline bioreactor, represents a first example of

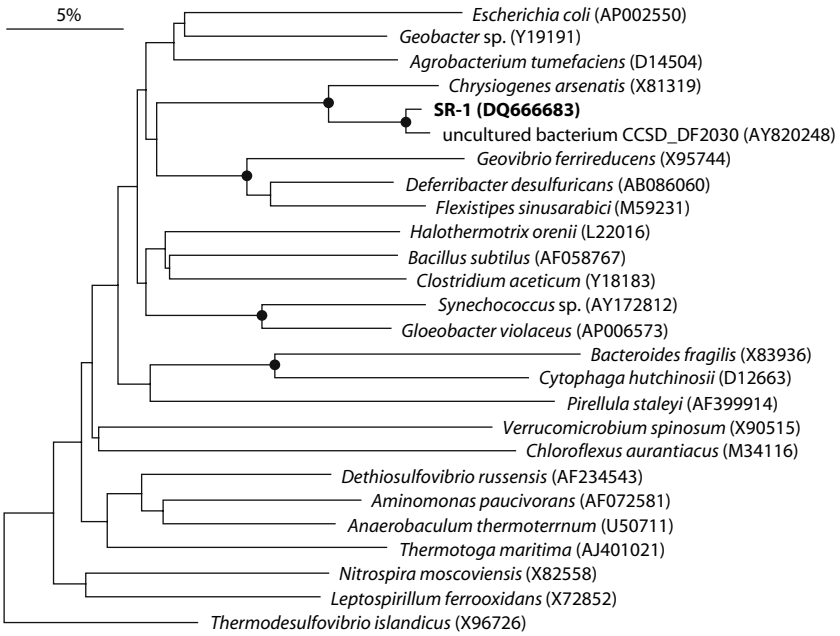

Fig. 5 Phylogenetic position of strain SR 1^T based on 16S rDNA gene sequence analysis. The tree was constructed using maximum likelihood method and filter. The *scale bar* represents 10 nucleotide changes per 100 nucleotides. The percentage of bootstraps was derived from 1,000 resampling using neighbour joining algorithm, only values greater than 95 are given. The sequence of *Thermotoga maritima* was used as an outgroup

Table 1 Fatty acid composition of polar lipids in SR 1

Fatty acid	Percentage from total
12:1	3.6
12:0	2.3
3OH 12:1[a]	2.3
3OH 14:1 [a,b]	2.3
16:1a	1.3
16:1w7c	5.3
16:1b	3.9
16:0	15.3
Cyclo 17:0	1.3
18:1w7c	57
Methyl 18:1	2.9
18:0	3.7

[a] Indicates uncertain identification

[b] Indicates amide-linked fatty acid

an obligatory anaerobic sulfur- and dissimilatory nitrate- reducing alkaliphile. Although it shares several key physiological properties with some mesophilic members of the Epsilonproteobacteria, its distant phylogenetic position and haloalkaliphily makes it unique. Therefore, the new isolate is proposed to be assigned into a new genus and species *Desulfurispirillum alkaliphilum* within the family *Chrysiogenetes*. The phenotypic comparison of SR 1^T with its closest culturable relative and Epsilonproteobacteria with similar metabolism is given in Table 2.

Description of *Desulfurispirillum* gen. nov. sp. nov.

Desulfurispirillum (De.sul.fu.ri.spi.ril' lum. L. pref. *de* from; L. n. *sulfur* sulfur; Gr. n. *spira* spiral; M. L. dim. neut. n. *Spirillum* a small spiral. M. L. neut. n. *Desulfurispirillum* a spirillum that reduces elemental sulfur).

Gram-negative, motile spirillas. Obligately anaerobic with respiratory metabolism. Use elemental sulfur and nitrate as electron acceptors, and short-chain fatty acids and hydrogen as electron donors. Do not grow autotrophically. Alkaliphilic and slightly halophilic. Menaquinones are the dominant respiratory lipoquinones. Phosphatidylglycerol and phosphatidylethanolamine are the dominant polar lipids and belongs to the family *Chrysiogenetes*. The type species is *D. alkaliphilum*.

Description of *Desulfurispirillum alkaliphilum* sp. nov.

alkaliphilum (al.ka.li.phi'lum M.L. n. *alkali* soda ash; Gr. adj. phylum *loving*; M.L. adj. *alkaliphilum* loving alkaline conditions)

Cells are slender spirals, 0.15–0.20 × 2–5 μm, motile by single bipolar flagella, gram-negative and strictly anaerobic with respiratory metabolism. Use elemental sulfur, polysulfide, nitrate and fumarate as electron acceptors. The final products are sulfide and ammonium. Obligatory heterotrophic. Utilizes short-chain fatty acids, such as acetate, lactate, pyruvate, propionate, citrate and fumarate as electron donor, and carbon source; H_2 can be used as electron donor. Formate and methanol are not utilized. Can oxidize sulfide/polysulfide to elemental sulfur intracellularly in the presence of nitrate as electron acceptor. Moderately

Table 2 Phenotypic comparison of strain SR 1 and related bacteria

Characteristics	*Chrysiogenetes*		Epsylonproteobacteria	
	SR 1	*Chrysiogenes arsenatis*	*Wolinella succinogenes*	Genus *Sulfurispirillum*
Cell morphology	Spirillum, single polar flagellum	Vibrio, single polar flagellum	Spirillum, single polar flagellum	Spirillum, single polar flagellum
Autotrophic growth	–	–	–	+
Electron donors				
H_2	+	–	+	+
Formate	–	–	+	+
HS^-	+	n.d.	+	+
Acetate, fumarate, succinate, lactate, pyruvate	+	+	+	+
Electron acceptors				
O_2	–	–	+	+
Sulfur (polysulfide)	+	–	+	+
Nitrate (>> NO_2^- >>NH_3)	+	+	+	+
N_2O	–	–	+	–
Fumarate	+	–	+	+
Arsenate	–	+	–	+/–
DMSO	–	–	+	+/–
Selenate	–	–	–	+/–
Fe^{3+}	–	–	–	+/–
$S_2O_3^{2-}/SO_3^{2-}$	–	–	+	+/–
Oxidation of HS^- with				
Nitrate	+	n.d.	+	+
Fumarate	–	n.d.	+	+
pH range (optimum)	8.0–10.2 (9.0)	neutrophilic	neutrophilic	neutrophilic
Salt range (M Na^+)	0.1–2.5	Na-independent	Na-independent	Up to 0.5
Habitat	Bioreactor	Gold mine	Rumen fluid	Marine sediments

n.d. not determined, = present, +/– present in some species, – absent

alkaliphilic with a pH range for growth between 8.0 and 10.2 and an optimum at pH 9.0 and slightly halophilic with a salt range from 0.1 to 2.5 M Na^+ (optimum at 0.4 M). Mesophilic with a maximum temperature for growth at 45 and an optimum at 35°C. The main respiratory lipoquinone is MQ-8. The predominant fatty acids in the membrane lipids include 12:1, 12:0, 16:1a, 16:1b, 16:1w7c, cyclo 17:0, 18:1w7c, 18:0 and a methyl 18:1. The G + C content of the genomic DNA is 44.8 ± 0.5 mol% (T_m).

The type strain is SR 1^T (=DSM 18274 = UNIQEM U250). Isolated from a full-scale sulfide-removing bioreactor in the Netherlands. The GenBank 16S rDNA gene sequence accession number is DQ666683.

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