Sulfuritalea hydrogenivorans gen. nov., sp. nov., a facultative autotroph isolated from a freshwater lake

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A novel facultatively autotrophic bacterium, designated strain sk43H^T, was isolated from water of a freshwater lake in Japan. Cells of the isolate were curved rods, motile and Gram-reaction-negative. Strain sk43H^T was facultatively anaerobic and autotrophic growth was observed only under anaerobic conditions. The isolate oxidized thiosulfate, elemental sulfur and hydrogen as sole energy sources for autotrophic growth and could utilize nitrate as an electron acceptor. Growth was observed at 8–32 °C (optimum 25 °C) and 6.4–7.6 (optimum pH 6.7–6.9). Optimum growth of the isolate occurred at NaCl concentrations of less than 50 mM. The G + C content of genomic DNA was around 67 mol%. The fatty acid profile of strain sk43H^T when grown on acetate under aerobic conditions was characterized by the presence of C_{16:0} and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) as the major components. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain was a member of the class *Betaproteobacteria* showing highest sequence similarity with *Georgfuchsia toluolica* G5G6^T (94.7 %) and *Denitratisoma oestradiolicum* AcBE2-1^T (94.3 %). Phylogenetic analyses were also performed using genes involved in sulfur oxidation. On the basis of its phylogenetic and phenotypic properties, strain sk43H^T (=DSM 22779^T =NBRC 105852^T) represents a novel species of a new genus, for which the name *Sulfuritalea hydrogenivorans* gen. nov., sp. nov. is proposed.

Members of the class *Betaproteobacteria* have highly diverse physiological properties and there are numerous combinations of electron donors and acceptors that can support their growth. Recently, it was demonstrated that toluene can serve as electron donor for Fe(III) reduction by a novel betaproteobacterial isolate, strain G5G6^T (Weelink *et al.*, 2009). This strain is closely related to the dominant phylotype in denitrifying enrichment cultures growing on *p*-xylene (Rotaru *et al.*, 2010). In the present study, a novel bacterium closely related to these organisms was isolated and characterized. The novel isolate, designated strain sk43H^T, was facultatively chemolithotrophic and was capable of growing on sulfur compounds and hydrogen under denitrifying conditions.

Strain sk43H^T was obtained from a water sample taken from Lake Mizugaki, a stratified artificial freshwater lake located in central Japan (Kojima *et al.*, 2009). The sample was obtained at a depth of 30 m, corresponding to the upper boundary of the hypolimnion. The basal medium

used for enrichment and isolation was bicarbonate-buffered low-salt defined medium (Kojima & Fukui, 2010), which comprised (l⁻¹): 0.25 g NaCl, 0.2 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 0.1 g NH₄Cl, 0.1 g KH₂PO₄, 0.1 g KCl, 1 ml trace element solution, 1 ml selenite–tungstate solution, 1 ml vitamin mixture solution, 1 ml vitamin B₁₂ solution, 1 ml thiamine solution, 30 ml NaHCO₃ solution and 1.5 ml Na₂S₂O₃ solution. All stock solutions were prepared as described previously (Widdel & Bak, 1992). To establish the first enrichment, 0.2 ml lake water was inoculated into a bottle containing 20 ml medium. Just before inoculation, anaerobic stock solution of NaNO₃ was added to the bottle to obtain a final concentration of 25 mM. The headspace of the bottle was filled with N₂/CO₂ (80:20, v/v) and incubation was performed at room temperature in the dark for 6 months. After this time, a sample of the culture was diluted 100–200-fold by transferring into medium of the same composition to obtain subsequent enrichment cultures. The isolate was obtained from the third enrichment by using the agar shake dilution method (Widdel & Bak, 1992). For the isolation procedure, thiosulfate concentration in the medium was raised to 20 mM. A well-separated colony was transferred to a slightly modified medium that contained 10 mM thiosulfate, 20 mM nitrate and no NaCl. Purity of

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *aprA* and *soxB* gene sequences of strain sk43H^T are AB552842, AB552843 and AB552844, respectively.

A supplementary table is available with the online version of this paper.

the isolate was tested by phase-contrast light microscopy and sequencing of 16S rRNA gene fragments amplified with several universal PCR primer pairs.

Cell morphology was observed using phase-contrast microscopy. Gram-staining was tested with a commercially available kit (Fluka). Catalase activity was assessed by pouring a 3 % H₂O₂ solution onto a pellet of cells obtained by centrifugation. Oxidase activity was also tested with a pellet of cells by using a differentiation disk (Fluka).

For characterization of the strain, an NaCl-free version of the basal medium containing 20 mM NaNO₃ was used, unless otherwise specified, and cultures were incubated at 25 °C without shaking. Each experiment was performed in duplicate. Sensitivity to antibiotics was tested with kanamycin and ampicillin at 100 µg ml⁻¹.

Utilization of electron acceptors was tested in medium without nitrate. Electron donor utilization was first tested under anoxic conditions by using basal medium with lowered thiosulfate concentration (0.4 mM). Utilization of electron donors was also tested under aerobic conditions using a medium containing no NaNO₃ or NaHCO₃, which was buffered with 20 mM MOPS/KOH. The effects of salt concentration (0, 20, 50, 100, 150, 180, 200, 220, 240, 260, 280 mM NaCl) and temperature (2, 5, 8, 13, 20, 22, 25, 28, 30, 32, 34, 37, 42 °C) on growth of the strain were tested under aerobic conditions by using MOPS-buffered medium supplemented with 16 mM sodium acetate. Growth was tested at pH 6.0, 6.4, 6.5, 6.7, 6.8, 6.9, 7.1, 7.3, 7.6, 7.9, 8.2 and 9.0 in media that contained CAPSO (11 g l⁻¹), MOPS (10 g l⁻¹) and the required amount of KOH to adjust the pH level.

Changes in concentrations of nitrite, nitrate, sulfate and thiosulfate were monitored with an ion chromatograph (DX-120; Dionex) equipped with a column for anion analyses (IonPac AS4ASC; Dionex).

The genomic G + C content of the DNA was determined by the fluorescence monitoring method (Gonzalez & Saiz-Jimenez, 2002). The fatty acid profile of the strain was analysed from biomass obtained after aerobic growth on MOPS-buffered medium with sodium acetate. Fatty acid analysis was performed at Techno Suruga (Shizuoka, Japan) by using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's instructions.

A fragment of nearly full-length 16S rRNA gene was amplified with the primer pair 27F and 1492R (Lane, 1991) and then sequenced directly. Based on the obtained sequence, phylogenetic affiliation of the novel strain was deduced by using RDP Classifier (Wang *et al.*, 2007) with a confidence threshold of 95 %. The 16S rRNA gene sequence of sk43H^T was aligned with related sequences retrieved from DDBJ/EMBL/GenBank using the program CLUSTAL_X (Thompson *et al.*, 1997). Genetic distances were calculated using the program MEGA3 (Kumar *et al.*, 2004). Partial fragments of genes required for sulfur oxidation, *soxB* encoding sulfate thioesterase/sulfate thiohydrolase

and *aprA* encoding adenosine-5'-phosphosulfate reductase, were also amplified by PCR and then sequenced directly. The fragments of the *soxB* and *aprA* genes were amplified with the primer pairs *soxB*432F/*soxB*1446B (Meyer *et al.*, 2007) and *Apr*-1-FW/*Apr*-5-RV (Meyer & Kuever, 2007), respectively. In the phylogenetic analyses of the functional genes, nucleotide sequences were translated to amino acid sequences. The deduced sequences were aligned with related sequences from the database. Based on the resulting alignment, genetic distances were calculated using the Poisson correction model.

Although strain sk43H^T was enriched and isolated by using thiosulfate as sole electron donor, growth on this energy source was rather slow. Hydrogen was tested as an alternative growth substrate, which enhanced lithotrophic growth of the isolate. After that, the isolate was cultured with hydrogen and its properties were determined.

Cells of the isolate were Gram-negative, curved rods (1.0–3.5 × 0.3–0.5 µm). Spore formation was not observed. The isolate was catalase-negative and oxidase-positive. Growth was inhibited by kanamycin and ampicillin. The genomic DNA G + C content of the isolate was 67 mol%.

The isolate could grow anaerobically and heterotrophically in medium containing nitrate on the following substrates (mM): pyruvate (5), lactate (5), acetate (5), propionate (2.5), succinate (2.5), fumarate (2.5), malate (2.5), benzoate (2.5), butyrate (2.5) and isobutyrate (2.5). All these substrates also supported aerobic growth of the strain. The following substrates (mM) could not support aerobic or anaerobic growth of the novel strain: methanol (5), ethanol (2.5), formate (5), citrate (5), lactose (2.5), glucose (2.5), xylose (2.5) and phenol (2). Under denitrifying conditions, the isolate could grow chemolithotrophically on H₂ (H₂/N₂/CO₂; 50 : 40 : 10, v/v/v; 200 kPa total pressure), thiosulfate (20 mM) and S⁰ (0.5 g l⁻¹). The end product of sulfur oxidation was sulfate. Growth on other inorganic compounds, including sulfide (2 mM), sulfite (5 mM) and FeSO₄ (20 mM), was tested but these substrates did not support growth of strain sk43H^T. Autotrophic growth under aerobic conditions was tested with bicarbonate-buffered medium that contained no nitrate with various concentrations (20, 10 and 2 %) of O₂ in the headspace. In all cases, growth on hydrogen or thiosulfate was not observed.

By using bicarbonate-buffered medium, utilization of electron acceptors was tested using 16 mM acetate as electron donor. The strain could utilize oxygen, at all concentrations, and nitrate (20 mM) as electron acceptors. During growth on nitrate, gas production was observed and nitrite did not accumulate. The following electron acceptors (mM) could not support (or only poorly support) growth of the strain: nitrite (5), sulfite (5), sulfate (10), crystalline iron(III) oxide (10), iron(III) nitrilotriacetic acid (10).

Strain sk43H^T was able to grow between 8 and 32 °C, with optimum growth at 25 °C. The pH range for growth was

Fig. 1. Phylogenetic position of strain sk43H^T within the family Rhodocyclaceae based on 16S rRNA gene sequence analysis. The tree was constructed using the minimum-evolution method based on 1405 nucleotide sites. An identical tree was obtained with the neighbour-joining method. *Escherichia coli* ATCC 11775^T (*Gammmaproteobacteria*) is included as outgroup. Bootstrap values >50% (based on 1000 resamplings) are given at nodes. Bar, 0.02 substitutions per nucleotide position.

6.4–7.6, with optimum growth at pH 6.7–6.9. Optimum growth of the isolate was observed in media containing 0–50 mM NaCl; only very faint growth was observed in medium containing 240 mM NaCl.

16S rRNA gene sequence analysis revealed that strain sk43H^T belonged to the family Rhodocyclaceae of the class Betaproteobacteria (Fig. 1) and did not belong to any known lineage of sulfur-oxidizing bacteria. The closest relative of the isolate was *Georgfuchsia toluolica* G5G6^T (94.7% sequence similarity), a strict anaerobe that degrades aromatic compounds by reducing Fe(III), Mn(IV) or nitrate as electron acceptors (Weelink *et al.*, 2009). It has been reported that *G. toluolica* cannot utilize carboxylic acids or hydrogen as electron donors. Another closely related strain was *Denitratisoma oestradiolicum* AcBE2-1^T (94.3% sequence similarity), a denitrifying bacterium capable of degrading 17 β -oestradiol (Fahrbach *et al.*, 2006). It has been shown that *D. oestradiolicum* cannot grow on benzoate or thiosulfate, both of which support growth of strain sk43H^T as sole electron donors. Isolates closely related to strain sk43H^T have been identified from nitrifying–denitrifying activated sludge (Juretschko *et al.*, 2002), coal tar waste-contaminated groundwater (Yagi *et al.*, 2010) and enrichment cultures degrading *p*-xylene (Rotaru *et al.*, 2010) (Fig. 1). Following these results, three primer pairs were tested to detect genes involved in degradation of monoaromatic hydrocarbons. The primer pairs used were *bssA_f*/*bssA_r* to detect the *bssA* gene (Botton *et al.*, 2007), *BamA*-SP9/*BamA*-ASP1 to

detect the *bamA* gene (Kuntze *et al.*, 2008) and *bzAQ4R*/*bzAQ4R* to detect the *bcrA* gene (Song & Ward, 2005). With these primer pairs, however, no PCR product was obtained from the genomic DNA of strain sk43H^T (*Thauera aromatica* K172^T was used as a positive control and fragments of the expected sizes were obtained in all cases).

The novelty of strain sk43H^T was also apparent in the phylogenetic analyses of genes involved in sulfur oxidation, *soxB* and *aprA* (Fig. 2, Fig. 3). As a trial to identify pathways for inorganic carbon fixation, PCR amplifications with primer pairs for the detection of genes involved in the Calvin–Benson–Bassham cycle were also tested. The primer pair *cbbLG1F*/*cbbLG1R* (Slesi *et al.*, 2005) and two other primer pairs (Elsaied & Naganuma, 2001) were used to detect genes *cbbL* and *cbbM*, encoding forms I and II of ribulose-1,5-bisphosphate carboxylase/oxygenase. None of these primer pairs yielded fragments of the expected size from the genomic DNA of strain sk43H^T (*Sulfuricella denitrificans* skB26^T was used as a positive control).

The fatty acid profile of strain sk43H^T, grown on acetate under aerobic conditions, was characterized by high concentrations of C_{16:0} (33.7%) and summed feature 3 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH; 61.2%). Other fatty acids detected were C_{14:0}, C_{18:1} ω 7c, C_{16:1} ω 7c alcohol, C_{16:0} N alcohol and C_{16:1} ω 5c (Supplementary Table S1, available in IJSEM Online). The two major components, C_{16:0} and summed feature 3, were also reported to be major fatty acids of closely related strain *D. oestradiolicum*

Fig. 2. Phylogenetic position of strain sk43H^T based on *soxB* gene analysis. The minimum evolution tree was constructed from amino acid sequences deduced from *soxB* gene sequences (235 aa positions were used). '*Aquifex aeolicus*' VF5 was used as an outgroup. Bootstrap values >50% (based on 1000 resamplings) are given at nodes. Bar, 0.1 substitutions per amino acid position.

Fig. 3. Phylogenetic position of strain sk43H^T based on *aprA* gene analysis. The minimum evolution tree was constructed from amino acid sequences deduced from *aprA* gene sequences (119 aa positions were used). *Chlorobium limicola* DSM 257 is included as outgroup. Bootstrap values >50% (based on 1000 resamplings) are given at nodes. Bar, 0.05 substitutions per amino acid position.

AcBE2-1^T grown on acetate under denitrifying conditions. On the other hand, the fatty acid C_{18:1}ω7c, the third most abundant fatty acid in *D. oestradiolicum* AcBE2-1^T (11.8%), was present in strain sk43H^T only as a minor component (1.2%). Differences in these two strains were also apparent in the levels of other minor fatty acids (Supplementary Table S1).

Strain sk43H^T showed high levels of sequence divergence from related genera. In addition, its physiological characteristics, including chemolithotrophy, differentiated it from related taxa (Table 1). On the basis of these phylogenetic and phenotypic properties, strain sk43H^T represents a novel species of a new genus, for which the

name *Sulfuritalea hydrogenivorans* gen. nov., sp. nov. is proposed.

Description of *Sulfuritalea* gen. nov.

Sulfuritalea (Sul.fu.ri.ta'le.a. L. neut. n. *sulfur* sulfur; L. fem. n. *talea* a rod; N.L. fem. n. *Sulfuritalea* sulfur-oxidizing rod).

Grows chemolithoautotrophically under anoxic conditions by the oxidation of reduced sulfur compounds and hydrogen. Heterotrophic growth occurs on organic acids. The major fatty acids are C_{16:0} and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH). Phylogenetically affiliated with the class *Betaproteobacteria* based on 16S rRNA gene sequence analysis. The type species is *Sulfuritalea hydrogenivorans*.

Description of *Sulfuritalea hydrogenivorans* sp. nov.

Sulfuritalea hydrogenivorans (hy.dro.ge.ni.vo'rans. N.L. n. *hydrogenum* hydrogen; L. part. adj. *vorans* consuming; N.L. part. *hydrogenivorans* hydrogen-consuming).

Cells are Gram-reaction-negative, curved rods, 1.0–3.5 × 0.3–0.5. Facultatively anaerobic and can reduce nitrate to nitrogen. Autotrophic growth occurs with oxidation of thiosulfate, elemental sulfur and hydrogen. Catalase-negative and oxidase-positive. Heterotrophic growth occurs on acetate, benzoate, butyrate, fumarate, isobutyrate, lactate, malate, propionate, pyruvate and succinate. Grows at 8–32 °C and pH 6.4–7.6, with optimum growth at 25 °C and pH 6.7–6.9.

The type strain, sk43H^T (=DSM 22779^T =NBRC 105852^T), was isolated from water of a stratified freshwater lake. The DNA G + C content is 67 mol%.

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Table 1. Differential physiological properties of strain sk43H^T and closely related strains

Strains: 1, sk43H^T; 2, *Georgfuchsia toluolica* G5G6^T; 3, *Denitratisoma oestradiolicum* AcBE2-1^T; 4, *Sterolibacterium denitrificans* Chol-1S^T. +, Growth; –, no growth; ND, not determined. Data were compiled from Weelink *et al.* (2009), Fahrback *et al.* (2006) and Tarlera & Denner (2003). Nitrate was used as an electron acceptor by all strains studied.

Property	1	2	3	4
Electron donors				
Acetate	+	–	+	+
Lactate	+	–	+	–
Succinate	+	–	+	–
Fumarate	+	–	+	–
Pyruvate	+	–	+	–
Isobutyrate	+	–	+	–
Benzoate	+	–	–	–
Phenol	–	+	–	–
Hydrogen	+	–	ND	ND
Thiosulfate	+	ND	–	ND
Electron acceptors				
Oxygen	+	–	+	+
Iron(III)	–	+	ND	–

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