Sulfuricella denitrificans gen. nov., sp. nov., a sulfur-oxidizing autotroph isolated from a freshwater lake

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A novel facultatively anaerobic, sulfur-oxidizing bacterium, strain skB26^T, was isolated from anoxic water of a freshwater lake in Japan. The cells were rod-shaped, motile and Gram-negative. Strain skB26^T oxidized elemental sulfur and thiosulfate to sulfate as sole energy sources. Strain skB26^T was microaerobic and could also utilize nitrate as an electron acceptor, reducing it to nitrogen. Growth was observed at temperatures below 28 °C; optimum growth was observed at 22 °C. The pH range for growth was 6.0–9.0, and the optimum pH was 7.5–8.0. Optimum growth of the isolate was observed in medium without NaCl, and no growth was observed in medium containing more than 220 mM NaCl. The G + C content of genomic DNA was around 59 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the strain was a member of the class *Betaproteobacteria*, and the closest cultivated relative was '*Thiobacillus plumbophilus*' DSM 6690, with 93 % sequence similarity. Phylogenetic analyses were also performed using sequences of genes involved in sulfur oxidation, inorganic carbon fixation and nitrate respiration. On the basis of its phylogenetic and phenotypic properties, strain skB26^T (=NBRC 105220^T =DSM 22764^T) is proposed as the type strain of a novel species of a new genus, *Sulfuricella denitrificans* gen. nov., sp. nov.

Nitrogen and sulfur are essential for all organisms as major components of cell materials. There are also a variety of abundant inorganic compounds of these elements in the biosphere, with a wide range of redox states. These chemical species have specific properties, and biogeochemical cycling of the elements is largely dependent on dissimilatory and assimilatory activities of prokaryotes. Compounds of nitrogen and sulfur act as electron acceptors or donors for diverse types of respiration, and cycles of these elements are coupled directly by sulfur-oxidizing bacteria, which utilize oxidized forms of nitrogen as electron acceptors. Phylogenetically diverse bacteria and archaea are capable of chemolithotrophic growth on reduced forms of sulfur, but the majority of isolated mesophilic species belong to the phylum *Proteobacteria*.

One of the major sources of reduced sulfur compounds in natural environments is sulfate reduction occurring under

oxygen- and nitrate-depleted conditions. This terminal process of mineralization takes place mainly in marine and lake sediments. These environments are often associated with low temperature, and the presence of cold-adapted sulfur oxidizers is expected in such habitats.

In the present study, a novel psychrotolerant, sulfur-oxidizing, nitrate-reducing bacterium, strain skB26^T, was isolated from cold anoxic water obtained from a freshwater lake. Based on the phylogenetic analysis, it was suggested that this strain represents a novel taxon in the class *Betaproteobacteria*.

Strain skB26^T was obtained from the hypolimnion of a meromictic freshwater lake, Lake Mizugaki, an artificial lake located in central Japan (Kojima *et al.*, 2009). The water sample was obtained from a depth of 40 m, approximately 3 m above the sediment surface. The water was characterized by depletion of oxygen, a smell of sulfide and low temperature (5 °C). The basal medium used for enrichment and isolation was a carbonate-buffered low-salt defined medium, modified from the medium for sulfate-reducing bacteria (Widdel & Bak, 1992). The modification included the elimination of sulfate and replacement of sulfide with thiosulfate as an alternate reductant and sulfur source. The composition of the medium was (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

Abbreviation: RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain skB26^T is AB506456; accession numbers for the partial *aprA*, *soxB*, *cbbL*, *cbbM* and *nirS* sequences of strain skB26^T are AB506457–AB506461.

Phylogenetic trees based on partial deduced amino acid sequences derived from *cbbL*, *cbbM* and *nirS* gene sequences are available as supplementary material with the online version of this paper.

solution, 1 ml selenite-tungstate solution, 1 ml vitamin mixture, 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 by Widdel & Bak (1992), and the preparation procedure for the medium was virtually the same as for the original medium. Before dispensing into glass bottles, the medium was adjusted to pH 7.0–7.2. Just before inoculation, anaerobic stock solutions of NaNO₃ and Na₂S₂O₃ were added to the bottle to obtain final concentrations of 20 and 10 mM, respectively. To establish the first enrichment, 0.1 ml lake water was inoculated into 50 ml medium. The headspace of the bottle was filled with N₂/CO₂ (80:20, v/v) and incubation was performed in the dark at 22 °C. A well-grown culture (1% volume of fresh medium) was transferred to medium of the same composition to obtain subsequent enrichment cultures. Throughout the cultivation, N₂/CO₂-flushed syringes were used to transfer cultures. A pure culture of the strain was obtained from the fifth enrichment by using agar shake dilution (Widdel & Bak, 1992). Purity of the isolate was tested by phase-contrast light microscopy, transferring to various media containing organic compounds and sequencing of 16S rRNA gene fragments amplified with several universal PCR primer pairs.

The morphology of the isolate was observed under phase-contrast microscopy. Gram staining was conducted with a commercially available kit (Fluka). Activity of catalase 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 disc (Fluka).

The basal medium containing 20 mM NaNO₃ and 10 mM Na₂S₂O₃ was used throughout the characterization of the strain, and cultures were incubated at 22 °C unless otherwise specified. For testing of electron donor utilization, the concentration of Na₂S₂O₃ in the medium was reduced to 0.4 mM and 20 mM NaNO₃ was used as an electron acceptor. Utilization of electron acceptors was tested in medium with nitrate omitted. The effect of NaCl concentration was tested with modified media containing varying concentrations of NaCl. To test the effect of pH on growth, modified media (pH 5.5–9.0) were also prepared with HCl or Na₂CO₃. Growth under different conditions was assessed by monitoring changes in concentrations of nitrate, sulfate and thiosulfate with ion chromatography. Sensitivity to antibiotics was tested for 100 µg kanamycin and ampicillin ml⁻¹.

The G+C content of the genomic DNA was determined with the fluorescence monitoring method (Gonzalez & Saiz-Jimenez, 2002) by using real-time PCR apparatus (MiniOpticon; Bio-Rad).

The nearly full-length 16S rRNA gene was amplified with the primers 27F and 1492R (Lane, 1991) and the PCR product was then directly sequenced. Partial fragments of functional genes were also amplified with appropriate

primer pairs and then sequenced directly. The analysed genes tested are involved in sulfur oxidation (*soxB*, encoding sulfate thioesterase/sulfate thiohydrolase, and *aprA*, encoding adenosine-5'-phosphosulfate reductase), inorganic carbon fixation [*cbbL* and *cbbM*, encoding forms I and II, respectively, of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)] and nitrate respiration (*nirS*, encoding cytochrome *cd*₁ nitrite reductase). The *soxB* and *aprA* gene fragments were amplified with the primer pairs *soxB*693F/*soxB*1446B (Meyer *et al.*, 2007) and *Apr*-1-FW/*Apr*-5-RV (Meyer & Kuever, 2007a), respectively. Amplification of the genes encoding the two forms of RuBisCO was performed as described previously (Elsaied & Naganuma, 2001). For amplification of *nirS*, the primer pair *cd3aF*/*R3cd* (Throbäck *et al.*, 2004) was used. The 16S rRNA gene sequence of *skB26*^T was aligned with related sequences retrieved from the DDBJ/EMBL/GenBank databases using the program CLUSTAL_X (Thompson *et al.*, 1997). Genetic distances were calculated using the program MEGA3 (Kumar *et al.*, 2004). In the phylogenetic analyses of the functional genes, nucleotide sequences were translated to amino acid sequences and the deduced sequences were aligned with related sequences from the databases. Based on the resulting alignment, genetic distances were calculated using the model of Poisson correction. Phylogenetic trees were constructed using the neighbour-joining, minimum-evolution and maximum-parsimony methods and the robustness of the each tree was examined with bootstrap tests of 1000 replicates.

Cells of isolate *skB26*^T were Gram-negative, straight rods (0.8–2.0 µm long and 0.4–0.6 µm wide) and exhibited motility. Spore formation was not observed. The catalase test was negative, while the oxidase test was positive. Growth of the isolate was inhibited by kanamycin and ampicillin. The G+C content of the genomic DNA of the isolate was 59.0 mol%.

In the presence of nitrate, heterotrophic growth of strain *skB26*^T on the following substrates was tested and none of them supported growth: methanol, formate, pyruvate, citrate, lactate, acetate (all 5 mM), lactose, propionate, glucose, succinate, ethanol, fumarate, xylose, malate, benzoate, butyrate, isobutyrate (all 2.5 mM) and yeast extract (0.02%; Difco). The isolate could grow chemolithotrophically on thiosulfate (10 mM) and S⁰ (0.5 g l⁻¹), generating sulfate as the end product. Growth on inorganic sulfur compounds other than thiosulfate was tested for sulfide (1.0, 2.0, 2.9 and 7.4 mM), sulfite (5 mM) and tetrathionate (10 mM), but none of these supported growth of strain *skB26*^T. Autotrophic growth on other inorganic electron donors was tested [H₂ (H₂/N₂/CO₂, 50:40:10 by vol.; 200 kPa total pressure) and FeSO₄ (20 mM)], but no growth was observed.

Electron acceptor utilization was tested with 10 mM thiosulfate as an electron donor. During growth by nitrate reduction, gas production was observed and accumulation of nitrite was not detected. Aerobic and microaerobic

Fig. 1. Phylogenetic position of skB26^T within the class *Betaproteobacteria*, based on 16S rRNA gene sequence analysis. The sequence of *Thiovirga sulfuroxydans* SO07^T (*Gamma-proteobacteria*) was included as an outgroup. The tree was constructed with the minimum-evolution method using 1298 positions. Numbers at nodes represent percentage values from 1000 bootstrap resamplings (values >50 % are shown). Bar, 0.02 substitutions per nucleotide position.

growth were tested with varying concentrations of O₂ (20, 10 and 2 %) in the headspace. Growth was observed under all tested conditions, but growth under 20 % O₂ was considerably slower than that under the other conditions. Growth of strain skB26^T was also supported by N₂O (4 % in the headspace). Utilization of nitrite (5 and 10 mM) was also tested, but it could not support growth of the isolate. Electron acceptor utilization was also tested with the alternative electron donor S⁰, and the same results were obtained.

Growth of strain skB26^T was observed at temperatures lower than 28 °C, and optimum growth was observed at 22 °C. The lower limit of temperature for growth was not determined, but psychrotolerance of the isolate was demonstrated by growth at 0 °C. The range of initial pH for growth was 6.0–9.0, and the optimum pH was 7.5–8.0. Optimum growth of the isolate was observed in medium without NaCl, and no growth was observed in medium containing more than 220 mM NaCl.

Phylogenetic analysis of the 16S rRNA gene sequence revealed strain skB26^T to belong to the class *Betaproteobacteria*. The closest cultivated relative of the novel strain was 'Thiobacillus plumbophilus' DSM 6690, an aerobic

bacterium capable of chemolithotrophic growth on H₂S, H₂ and PbS (Drobner *et al.*, 1992). The sequence similarity between skB26^T and 'T. plumbophilus' DSM 6690 was 93 %. Several environmental clones closely related to the novel strain have been reported from cold sediment of a meromictic lake (Nelson *et al.*, 2007); these sequences formed a phylogenetic cluster distinct from the cluster comprising species of the genus *Thiobacillus* with validly published names (Fig. 1).

Highest similarity to 'T. plumbophilus' DSM 6690 was also observed in phylogenetic analyses of genes involved in sulfur oxidation, *soxB* and *aprA* (Figs 2 and 3). In the analysis of *soxB*, the phylogenetic relationship among strain skB26^T, 'T. plumbophilus' DSM 6690 and *Thiobacillus* species with validly published names was similar to that revealed by the 16S rRNA gene sequence analysis (Fig. 2). For *aprA*, 'T. plumbophilus' DSM 6690 possesses two loci of differing sequences, which hindered direct sequencing of PCR products (Meyer & Kuever, 2007b). In contrast, direct sequencing of the PCR product obtained from strain skB26^T resulted in successful acquisition of a single sequence, without ambiguity. The obtained sequence belonged to the cluster referred to as 'Apr lineage II' (Meyer & Kuever, 2007b) (Fig. 3).

Fig. 2. Phylogenetic position of skB26^T based on *soxB* gene sequence analysis. This minimum-evolution tree was constructed from amino acid sequences deduced from *soxB* gene sequences (239 amino acid positions were used). Numbers at nodes are percentage values from 1000 bootstrap resamplings (values >50 % are shown). Bar, 0.1 substitutions per amino acid position.

Fig. 3. Phylogenetic position of skB26^T based on *aprA* gene sequence analysis. This minimum-evolution tree was constructed from amino acid sequences deduced from *aprA* gene sequences (119 amino acid positions were used). Numbers at nodes are percentage values from 1000 bootstrap resamplings (values >50% are shown). Bar, 0.05 substitutions per amino acid position.

Genes for two forms of RubisCO were detected and successfully sequenced from genomic DNA of strain skB26^T. In the phylogenetic analysis of the large subunit of form I RubisCO, encoded by the *cbbL* gene, the novel isolate clustered with beta- and gammaproteobacterial chemolithotrophs (Supplementary Fig. S1, available in IJSEM Online). A similar result was obtained from analysis of form II RubisCO, encoded by *cbbM* (Supplementary Fig. S2). Sequences of these genes were not available for '*T. plumbophilus*' in the public databases.

Denitrification ability of the isolate was supported by detection of the *nirS* gene. It has been shown that the phylogeny of this gene is only partially consistent with that based on the 16S rRNA gene, and the novel strain belonged to the cluster comprising betaproteobacteria (Supplementary Fig. S3).

Phylogenetic analyses of multiple genes indicated the novelty of strain skB26^T. Among genera of sulfur-oxidizing bacteria, the genus *Thiobacillus* is the closest relative of the novel strain. However, strain skB26^T and its closest relative, '*T. plumbophilus*' DSM 6690, are phylogenetically distinct from *Thiobacillus* species with validly published names (Fig. 1). In addition, these two organisms are quite different in their utilization of electron donors and acceptors. As electron donors to sustain growth, the novel strain could utilize thiosulfate and elemental sulfur; '*T. plumbophilus*' DSM 6690 can grow on neither of these, and utilizes H₂ and H₂S, which could not sustain growth of skB26^T. Furthermore, the novel isolate is facultatively anaerobic, whereas '*T. plumbophilus*' DSM 6690 is strictly aerobic. On the basis of its phylogenetic and phenotypic properties, strain skB26^T is proposed to represent a novel species of a new genus, *Sulfuricella denitrificans* gen. nov., sp. nov.

Description of *Sulfuricella* gen. nov.

Sulfuricella [Sul.fu.ri.cel'la. L. neut. n. *sulfur* sulfur; L. fem. n. *cella* a small room and, in biology, a cell; N.L. fem. n. *Sulfuricella* sulfur(-oxidizing) cell].

Chemolithoautotrophic and grow by the oxidation of reduced sulfur compounds. Facultatively anaerobic. Based on 16S rRNA gene sequence analysis, affiliated phylogenetically to the class Betaproteobacteria. The type species is *Sulfuricella denitrificans*.

Description of *Sulfuricella denitrificans* sp. nov.

Sulfuricella denitrificans (de.ni.tri'fi.cans. N.L. v. *denitrifico* to denitrify; N.L. part. adj. *denitrificans* denitrifying).

Displays the following properties in addition to those described for the genus. Cells are Gram-negative rods, 0.8–2.0 µm long and 0.4–0.6 µm wide. Reduces nitrate to nitrogen. Autotrophic growth occurs with oxidation of thiosulfate and elemental sulfur to sulfate. Catalase-negative and oxidase-positive. Grows at temperatures lower than 28 °C, with optimum growth at 22 °C. The pH range for growth is 6.0–9.0; optimum growth occurs at pH 7.5–8.0. Does not require NaCl for growth, and does not grow at above 220 mM NaCl. The G+C content of genomic DNA of the type strain is 59 mol%.

The type strain, skB26^T (=NBRC 105220^T =DSM 22764^T), was isolated from anoxic lake water of a stratified freshwater lake.

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References

- Drobner, E., Huber, H., Rachel, R. & Stetter, K. O. (1992). *Thiobacillus plumbophilus* spec. nov., a novel galena and hydrogen oxidizer. *Arch Microbiol* 157, 213–217.
- Elsaied, H. & Naganuma, T. (2001). Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes

from deep-sea microorganisms. *Appl Environ Microbiol* **67**, 1751–1765.

Gonzalez, J. M. & Saiz-Jimenez, C. (2002). A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. *Environ Microbiol* **4**, 770–773.

Kojima, H., Iwata, T. & Fukui, M. (2009). DNA-based analysis of planktonic methanotrophs in a stratified lake. *Freshw Biol* **54**, 1501–1509.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Meyer, B. & Kuever, J. (2007a). Molecular analysis of the diversity of sulfate-reducing and sulfur-oxidizing prokaryotes in the environment, using *aprA* as functional marker gene. *Appl Environ Microbiol* **73**, 7664–7679.

Meyer, B. & Kuever, J. (2007b). Molecular analysis of the distribution and phylogeny of dissimilatory adenosine-5'-phosphosulfate

reductase-encoding genes (*aprBA*) among sulfur-oxidizing prokaryotes. *Microbiology* **153**, 3478–3498.

Meyer, B., Imhoff, J. F. & Kuever, J. (2007). Molecular analysis of the distribution and phylogeny of the *soxB* gene among sulfur-oxidizing bacteria – evolution of the Sox sulfur oxidation enzyme system. *Environ Microbiol* **9**, 2957–2977.

Nelson, D. M., Ohene-Adjei, S., Hu, F. S., Cann, I. K. & Mackie, R. I. (2007). Bacterial diversity and distribution in the holocene sediments of a northern temperate lake. *Microb Ecol* **54**, 252–263.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Throbäck, I. N., Enwall, K., Jarvis, A. & Hallin, S. (2004). Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol Ecol* **49**, 401–417.

Widdel, F. & Bak, F. (1992). Gram-negative mesotrophic sulfatereducing bacteria. In *The Prokaryotes*, 2nd edn, vol. 4, pp. 3352–3378. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.