Bacillus selenatarsenatis sp. nov., a selenate- and arsenate-reducing bacterium isolated from the effluent drain of a glass-manufacturing plant

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A facultatively anaerobic, selenate- and arsenate-reducing bacterium, designated strain SF-1^T, was isolated from a selenium-contaminated sediment obtained from an effluent drain of a glass-manufacturing plant in Japan. The bacterium stained Gram-positive and was a motile, spore-forming rod capable of respiring with selenate, arsenate and nitrate as terminal electron acceptors. The major cellular fatty acids of the strain were iso-C_{15:0}, iso-C_{17:1}ω10c and C_{16:1}ω7c alcohol. The G + C content of the genomic DNA was 42.8 mol%. Though the nearest phylogenetic neighbour was *Bacillus jeotgali* JCM 10885^T, with a 16S rRNA gene sequence similarity of 99.6%, DNA–DNA hybridization studies showed only 14% relatedness between these strains, a level that is clearly below the value recommended to delimit different species. This, together with the phenotypic differences (utilization of electron acceptors, NaCl tolerance), suggests that strain SF-1^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus selenatarsenatis* sp. nov. is proposed. The type strain is SF-1^T (= JCM 14380^T = DSM 18680^T).

Selenium and arsenic, trace elements distributed widely in the Earth's crust, are required in trace amounts for growth and metabolism but are toxic at micromolar concentrations. In oxic environments, they occur primarily as the soluble oxyanions selenate [Se(VI)] and arsenate [As(V)]. In anoxic environments, these oxyanions can be utilized by prokaryotes as terminal electron acceptors for anaerobic respiration. The selenate- and/or arsenate-respiring prokaryotes isolated to date are diverse both physiologically and phylogenetically and are not confined to one particular phylum (Oremland & Stolz, 2000, 2003; Stolz *et al.*, 2002). In general, oxyanions, including selenate and arsenate, are actively cycled and play an important role in carbon mineralization processes in certain environments.

A Gram-positive, selenate-reducing bacterium was isolated from a selenium-contaminated sediment collected from an effluent drain that had been receiving selenium-containing discharge from a glass-manufacturing plant. The bacterium, designated strain SF-1^T (Fujita *et al.*, 1997), is capable of respiring with selenate as a terminal electron acceptor and lactate as an electron donor. Selenate is reduced to elemental selenium via the intermediate selenite (Fujita *et al.*, 1997; Kashiwa *et al.*, 2000). A laboratory-scale continuous reactor using strain SF-1^T had been constructed to remove selenate and selenite from wastewater (Fujita *et al.*, 2002). Our studies revealed that strain SF-1^T also grew anaerobically when arsenate was present as an electron acceptor (Yamamura *et al.*, 2003), and this ability for dissimilatory reduction of arsenate to arsenite could be utilized to extract arsenic from contaminated soil for the purpose of bioremediation (Yamamura *et al.*, 2005). Based on physiological, phylogenetic and molecular evidence presented here, the strain represents a novel species of the genus *Bacillus*.

Strain SF-1^T was grown on a basal salt medium supplemented with 0.1% yeast extract (pH 8.0) as described by

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SF-1^T is AB262082.

A figure showing a maximum-parsimony tree is available as supplementary material in IJSEM Online.

Yamamura *et al.* (2003). For aerobic growth, 10 g glucose l^{-1} was used as the electron donor, whereas sodium lactate (20 mM) was used instead of glucose for anaerobic growth with one of selenate, arsenate or nitrate (1 mM) as the electron acceptor. *Bacillus jeotgali* JCM 10885^T, obtained from the RIKEN BRC-JCM, was cultivated on an appropriate medium (Yoon *et al.*, 2001) or under the same condition as strain SF-1^T, except that the pH was adjusted to 7.5, for phenotypic comparison. Biochemical tests were carried out using API 20E and API 50CHB kits according to the instructions of the manufacturer (bioMérieux). To determine sensitivity to antibiotics, a culture of strain SF-1^T was inoculated onto LB agar plates (pH 8.0) containing kanamycin, tetracycline, chloramphenicol, ampicillin or erythromycin at 1–30 $\mu g\ ml^{-1}$ and incubated at 37 °C for 4 days. Fatty acid methyl esters were extracted and analysed following the standard protocol of the Sherlock Microbial Identification System (MIDI).

For genomic DNA isolation, strain SF-1^T and *B. jeotgali* JCM 10885^T were grown aerobically in 1 litre batches to mid-exponential phase and cells were harvested by centrifugation at 8000 *g* (10 min, 4 °C). Genomic DNA was prepared using the DNeasy Tissue kit or DNeasy Plant kit (Qiagen) according to the manufacturer's instructions.

The G + C content of the DNA was determined by HPLC as described previously (Katayama-Fujimura *et al.*, 1984). DNA–DNA hybridization of strain SF-1^T and *B. jeotgali* JCM 10885^T was carried out by the method of Ezaki *et al.* (1989).

Gene fragments specific to the 16S rRNA-encoding regions were amplified by PCR using primers 20F (5'-GAGTTTGATCCTGGCTCAG-3'; positions 9–27) and 1500R (5'-GTTACCTTGTTACGACTT-3'; positions 1509–1492) (Kawasaki *et al.*, 1993). Positions in the 16S rRNA gene fragment are based on the *Escherichia coli* numbering system (GenBank accession no. V00348) of Brosius *et al.* (1981).

Amplified 16S rRNA genes were sequenced directly using the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit and an ABI PRISM model 310 Genetic Analyzer (Applied Biosystems). The following primers were used for sequencing: 20F, 1500R, 520F (5'-CAGCAGCCGCGGTA-ATAC-3'; positions 519–536), 520R (5'-GTATTACCGCG-GCTGCTG-3'; positions 536–519), 920F (5'-AAACTCA-AATGAATTGACGG-3'; positions 907–926) and 920R (5'-CCGTCAATTCATTGAGTTT-3'; positions 926–907).

The 16S rRNA gene sequence of strain SF-1^T determined was compared with reference sequences using BLAST similarity searches (Altschul *et al.*, 1997) and the closely related sequences were obtained from GenBank. Multiple alignments were generated and the calculation of distance matrices for the aligned sequences (Kimura, 1980) was carried out using CLUSTAL X (Thompson *et al.*, 1997) and MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) methods. The phylogenetic tree topology was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985). Sequence similarity values were calculated using GENETYX version 8 (Genetyx Corporation).

Strain SF-1^T is a Gram-positive, oxidase-negative, catalase-positive, motile, spore-forming, facultatively anaerobic and rod-shaped bacterium (Fujita *et al.*, 1997). Phylogenetic analysis based on the 16S rRNA gene sequence (1413 bp) of strain SF-1^T indicated that it fell within the low-G + C-content, Gram-positive, aerobic, spore-forming bacilli (Fig. 1 and Supplementary Fig. S1 available in IJSEM Online). The closest phylogenetic relative of strain SF-1^T was *B. jeotgali* JCM 10885^T, with a sequence similarity of 99.6%; lower similarity was observed with some phylogenetically related *Bacillus* type strains, including *Bacillus vireti* LMG 21834^T (96.7%), *Bacillus novalis* LMG 21837^T (96.6%), *Bacillus drentensis* LMG 21831^T (96.5%),

Fig. 1. Neighbour-joining tree derived from 16S rRNA gene sequences showing the relationships between strain SF-1^T and related *Bacillus* species. Bootstrap percentages (based on 1000 replications) greater than 50% are given at branching points. Bar, 0.01 substitutions per nucleotide position. *Paenibacillus polymyxa* DSM 36^T was used as the outgroup to root the tree. Accession numbers are given in parentheses. A maximum-parsimony tree is available as Supplementary Fig. S1 in IJSEM Online.

Bacillus niacini NBRC 15566^T (96.1 %), *Bacillus soli* LMG 21838^T (95.9 %), *Bacillus bataviensis* LMG 21833^T (95.9 %) and *Bacillus fumarioli* LMG 17489^T (95.5 %).

The optimal pH for aerobic growth of strain SF-1^T is 8.0 and the optimal temperature is 40 °C. Strain SF-1^T was able to grow on LB agar plates containing kanamycin (3 µg ml⁻¹), tetracycline (3 µg ml⁻¹) and chloramphenicol (5 µg ml⁻¹), whereas it was highly sensitive to ampicillin and erythromycin. Several other biochemical and physiological characteristics of strain SF-1^T were compared with those of its nearest phylogenetic relative on the basis of 16S rRNA gene sequence analysis, *B. jeotgali* JCM 10885^T (Table 1). Strain SF-1^T can grow not only aerobically but also anaerobically using selenate, arsenate and nitrate as terminal electron

Table 1. Comparison of phenotypic characteristics between strain SF-1^T and *B. jeotgali* JCM 10885^T

Unless otherwise specified, data for strain SF-1^T were obtained from Fujita *et al.* (1997) and Yamamura *et al.* (2003) and data for *B. jeotgali* JCM 10885^T were obtained from Yoon *et al.* (2001) and Imada *et al.* (2005). +, Positive; -, negative; w, weakly positive. Both strain SF-1^T and *B. jeotgali* JCM 10885^T are positive for growth in the presence of 2 and 5 % NaCl, use of oxygen as an electron acceptor, catalase, β-galactosidase, nitrate reduction, decomposition of starch and acid production from glucose, fructose, aesculin, cellobiose, maltose, sucrose, trehalose, starch, glycogen, gentiobiose and 5-ketogluconate. Both strains are negative for oxidase, formation of indole and acid production from glycerol, erythritol, D-arabinose, L-arabinose, ribose, L-xylose, adonitol, methyl β-D-xyloside, galactose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylglucosamine, lactose, melibiose, inulin, melezitose, raffinose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate and 2-ketogluconate.

Characteristic	Strain SF-1 ^T	<i>B. jeotgali</i> JCM 10885 ^T
Gram stain	Positive	Variable
Temperature range for growth (°C)	25–40	10–45
pH range for growth	7.5–9.0	7.0–8.0
H ₂ S production	+	–*
Acid production from:		
D-Xylose	+	–
Arbutin	+	–
Salicin	+	–
Amygdalin	+	–
NaCl tolerance:		
7 %	–	+
13 %	–	+
Electron acceptors:		
Selenate	+	–*
Arsenate	+	–*
Nitrate	+	w*

*Results obtained in this study.

acceptors; the selenate is reduced to elemental selenium via selenite, arsenate to arsenite and nitrate to ammonia via nitrite (Fujita *et al.*, 1997; Kashiwa *et al.*, 2000; Yamamura *et al.*, 2003, 2004). *B. jeotgali* JCM 10885^T, however, was able to grow with neither selenate nor arsenate as terminal electron acceptors, although nitrate supported weak growth. *B. jeotgali* JCM 10885^T can grow in the presence of 13 % (w/v) NaCl, whereas strain SF-1^T did not grow in the presence of more than 7 % (w/v) NaCl. Additionally, strain SF-1^T was positive for acid production from D-xylose, arbutin, salicin and amygdalin, whereas *B. jeotgali* JCM 10885^T could not produce acid from these carbon compounds. Production of H₂S was also observed only in strain SF-1^T. These phenotypic differences seemed to distinguish SF-1^T from its phylogenetic relative. The cellular fatty acid profile of strain SF-1^T, in comparison with that of *B. jeotgali* JCM 10885^T, is shown in Table 2. The major cellular fatty acid of strain SF-1^T was found to be iso-C_{15:0} (47.3 mol%), as is the case for *B. jeotgali* JCM 10885^T.

Although the 16S rRNA gene sequence of strain SF-1^T was more than 99 % similar to that of *B. jeotgali* JCM 10885^T, the DNA–DNA reassociation value between strain SF-1^T and *B. jeotgali* JCM 10885^T was 14 % (mean from three independent determinations), which is lower than the threshold value (70 %) recommended for delimiting different species (Wayne *et al.*, 1987). Thus, strain SF-1^T can be classified as representing a novel species of the genus *Bacillus*. The DNA G+C content of strain SF-1^T (42.8 mol%) was comparable to that of *B. jeotgali* JCM 10885^T (41 mol%) (Yoon *et al.*, 2001).

Table 2. Cellular fatty acid compositions (%) of strain SF-1^T and *B. jeotgali* JCM 10885^T

Data for strain SF-1^T were obtained in this study, whereas data for *B. jeotgali* JCM 10885^T were taken from Yoon *et al.* (2001).

Fatty acid	Strain SF-1 ^T	<i>B. jeotgali</i> JCM 10885 ^T
iso-C _{14:0}	4.5	1.9
C _{14:0}	0.6	1.3
Summed feature 1*	1.0	–
iso-C _{15:0}	47.3	49.3
anteiso-C _{15:0}	4.2	8.8
C _{16:1} ω7c alcohol	8.6	4.5
iso-C _{16:1} H	2.7	–
iso-C _{16:0}	2.9	2.3
C _{16:1} ω11c	2.6	5.0
C _{16:0}	0.8	3.2
iso-C _{17:1} ω10c	10.1	7.5
Summed feature 4*	8.7	6.6
iso-C _{17:0}	2.6	4.1
anteiso-C _{17:0}	1.4	3.7

*Summed features represent groups of two or three fatty acids that could not be separated by GC with the MIDI system. Summed feature 1 contained iso-C_{15:1} H and/or C_{13:0} 3-OH; summed feature 4 contained anteiso-C_{17:1} B and/or iso-C_{17:1} I.

The results from biochemical characterizations and DNA–DNA hybridization strongly indicated that strain SF-1^T can be distinguished from its closest phylogenetic relative, although they were not differentiated on the basis of cellular fatty acid profiles or 16S rRNA gene sequence analysis. Based on the evidence presented above, we describe a novel species within the genus *Bacillus*, *Bacillus selenatarsenatis* sp. nov.

Description of *Bacillus selenatarsenatis* sp. nov.

Bacillus selenatarsenatis (se'le.nat.ar.se.na'tis. N.L. gen. n. *selenatis* of selenate; N.L. gen. n. *arsenatis* of arsenate; N.L. gen. n. *selenatarsenatis* of selenate and arsenate).

The following description is based on data from this study and from Fujita *et al.* (1997) and Yamamura *et al.* (2003). Cells stain Gram-positive and are spore-forming, motile rods (1 × 3–6 µm). Colonies are round and white. Growth occurs at 25–40 °C and at pH 7.5–9.0. Growth occurs in the presence of 2–5 % NaCl, but not in the presence of 7 % NaCl. Positive results are obtained for catalase, β-galactosidase, H₂S production and nitrate reduction and negative results are obtained for oxidase, the Voges–Proskauer test, indole production and phenylalanine deamination. Gelatin and starch are hydrolysed. Acid is produced from D-xylose, glucose, fructose, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, sucrose, trehalose, starch, glycogen, gentiobiose and 5-ketogluconate, but not from glycerol, erythritol, D-arabinose, L-arabinose, ribose, L-xylose, adonitol, methyl β-D-xyloside, galactose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylglucosamine, lactose, melibiose, inulin, melezitose, raffinose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate or 2-ketogluconate. Cells are resistant to 3 µg kanamycin ml⁻¹, 3 µg tetracycline ml⁻¹ and 5 µg chloramphenicol ml⁻¹, but highly sensitive to ampicillin and erythromycin. The bacterium is a facultative anaerobe that respire oxygen, selenate, arsenate and nitrate as terminal electron acceptors. Selenate is reduced to elemental selenium via the intermediate selenite, arsenate to arsenite and nitrate to ammonia via the intermediate nitrite. The dominant fatty acids are iso-C_{15:0} (47.3 mol%), iso-C_{17:1ω10c} (10.1 mol%) and C_{16:1ω7c} alcohol (8.6 mol%). The DNA G+C content of the type strain is 42.8 mol%.

The type strain, SF-1^T (=JCM 14380^T=DSM 18680^T), was isolated from an effluent drain in a glass-manufacturing plant in Japan.

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References

- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Brosius, J., Dull, T. J., Sleeter, D. D. & Noller, H. F. (1981). Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J Mol Biol* **148**, 107–127.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fujita, M., Ike, M., Nishimoto, S., Takahashi, K. & Kashiwa, M. (1997). Isolation and characterization of a novel selenate-reducing bacterium, *Bacillus* sp. SF-1. *J Ferment Bioeng* **83**, 517–522.
- Fujita, M., Ike, M., Kashiwa, M., Hashimoto, R. & Soda, S. (2002). Laboratory-scale continuous reactor for soluble selenium removal using selenate-reducing bacterium, *Bacillus* sp. SF-1. *Biotechnol Bioeng* **80**, 755–761.
- Imada, C., Harada, Y., Kobayashi, T., Hamada-Sato, N. & Watanabe, E. (2005). Degradation of ferric chelate of ethylenediaminetetraacetic acid by bacterium isolated from deep-sea stalked barnacle. *Mar Biotechnol* **7**, 21–25.
- Kashiwa, M., Nishimoto, S., Takahashi, K., Ike, M. & Fujita, M. (2000). Factors affecting soluble selenium removal by a selenate-reducing bacterium *Bacillus* sp. SF-1. *J Biosci Bioeng* **89**, 528–533.
- Katayama-Fujimura, Y., Komatsu, Y., Kuraishi, H. & Kaneko, T. (1984). Estimation of DNA base composition by high performance liquid chromatography of its nuclease P1 hydrolysate. *Agric Biol Chem* **48**, 3169–3172.
- Kawasaki, H., Hoshino, Y., Hirata, A. & Yamasato, K. (1993). Is intracytoplasmic membrane structure a generic criterion? It does not coincide with phylogenetic interrelationships among phototrophic purple nonsulfur bacteria. *Arch Microbiol* **160**, 358–362.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kluge, A. G. & Farris, F. S. (1969). Quantitative phyletics and the evolution of anurans. *Syst Zool* **18**, 1–32.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Oremland, R. S. & Stolz, J. F. (2000). Dissimilatory reduction of selenate and arsenate in nature. In *Environmental Microbe-Metal Interactions*, pp. 199–224. Edited by D. R. Lovley. Washington, DC: American Society for Microbiology.
- Oremland, R. S. & Stolz, J. F. (2003). The ecology of arsenic. *Science* **300**, 939–944.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Stolz, J. F., Basu, P. & Oremland, R. S. (2002). Microbial transformation of elements: the case of arsenic and selenium. *Int Microbiol* **5**, 201–207.
- 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.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Yamamura, S., Ike, M. & Fujita, M. (2003). Dissimilatory arsenate reduction by a facultative anaerobe, *Bacillus* sp. strain SF-1. *J Biosci Bioeng* **96**, 454–460.

Yamamura, S., Terashi, S., Ike, M., Yamashita, M. & Fujita, M. (2004). Characterization of arsenate-, selenate- and nitrate-reducing activities in *Bacillus* sp. SF-1. *Jpn J Water Treat Biol* **40**, 161–168.

Yamamura, S., Yamamoto, N., Ike, M. & Fujita, M. (2005). Arsenic extraction from solid phase using a dissimilatory arsenate-reducing bacterium. *J Biosci Bioeng* **100**, 219–222.

Yoon, J.-H., Kang, S.-S., Lee, K.-C., Kho, Y. H., Choi, S. H., Kang, K. H. & Park, Y.-H. (2001). *Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood. *Int J Syst Evol Microbiol* **51**, 1087–1092.