Shewanella fodinae sp. nov., isolated from a coal mine and from a marine lagoon

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Strains JC15^T and JC19 were isolated from samples collected from different locations in India, including a coal mine and a marine lagoon. Both strains were Gram-stain-negative rods, motile by means of a single polar flagellum, catalase- and oxidase-positive, and hydrolysed casein, produced H₂S and showed β -haemolysis. Strain JC15^T grew optimally at pH 6 (range pH 5–8) while strain JC19 grew optimally at pH 7 (range pH 6–9) and both had a growth temperature optimum of 30–37 °C (range 22–40 °C). Both strains could grow chemo-organoheterotrophically and chemolithoautotrophically. Neither strain required NaCl for growth and both could tolerate up to 9% (w/v) NaCl, with optimum growth at 5% NaCl. Vitamin B₁₂ was required as a growth factor by both strains. The major fatty acids were iso-C_{15:0}, C_{17:1}ω8c and iso-C_{13:0} 3-OH. The DNA G + C contents of strains JC15^T and JC19 were 53.6 and 54.3 mol%, respectively. A phylogenetic tree based on 16S rRNA gene sequence analysis showed that strains JC15^T and JC19 were most closely related to *Shewanella haliotis* DW01^T (approximately 94% sequence similarity) and to other members of the genus *Shewanella*. Genomic relatedness (DNA–DNA hybridization) between strains JC15^T and JC19 is 88%. On the basis of phenotypic and molecular genetic evidence, strain JC15^T represents a novel species of the genus *Shewanella*, for which the name *Shewanella fodinae* sp. nov. is proposed. The type strain is JC15^T (=CCUG 57102^T =NBRC 105216^T =KCTC 22506^T).

The family *Shewanellaceae* of the class *Gammaproteobacteria* presently comprises only one genus, *Shewanella* (Ivanova *et al.*, 2004), with 48 species having validly published names as of 2008 (<http://www.bacterio.cict.fr/s/shewanella.html>). *Shewanella marina* (Park *et al.*, 2009), *Shewanella chilikensis* (Sucharita *et al.*, 2009) and *Shewanella xiamenensis* (Huang *et al.*, 2009) are recent additions. The majority of *Shewanella* species were isolated from aquatic/marine environments and most can grow by anaerobic respiration (Bowman, 2005). Although coal mine samples yield large numbers of methanogenic bacteria (Flores, 2008; Strąpoć *et al.*, 2008), *Shewanella putrefaciens* (EMBL accession number for 16S

rRNA gene sequence from strain B2C1-8 is EU481671) was isolated from a coal mine sample (Li *et al.*, 2008). In this communication, we propose a novel species of the genus *Shewanella*, which is represented by strain JC15^T isolated from a coal mine in India. An additional strain, JC19, was isolated from the Chilika marine lagoon of India.

Strain JC15^T was isolated from a coal mine sludge sample collected from a depth of 204.35 m below the surface on 25 May 2007 from the 8 Incline mine of Godavarikhani, Karimnagar District, Andhra Pradesh, India (18° 41' N 79° 32' E). Strain JC19 was isolated from a sediment sample collected on 7 March 2008 from the central sector of Chilika lagoon near Alupatna village (19° 39' N 85° 28' E). Enrichment for strains JC15^T and JC19 was carried out in Postgate's B medium broth (Postgate, 1984) with the following composition (g l⁻¹) in distilled water: sodium lactate (3.5), MgSO₄ · 7H₂O (2.0), NH₄Cl (1.0), CaCl₂ · 2H₂O (0.05), yeast extract (0.05), KH₂PO₄ (0.5), FeSO₄ · 7H₂O

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JC15^T and JC19 are FM203122 and FM887036, respectively.

A micrograph showing a cell of strain JC15^T, a graph depicting NaCl tolerance of strains JC15^T and JC19, and results of the GN2 microplate test for strain JC15^T are available with the online version of this paper.

(0.5), ascorbic acid (0.01) and thioglycolate (0.01). Cultures were incubated in fully filled screw cap test tubes (10 × 100 mm) at 28 ± 2 °C. Black enrichments were obtained after 5 days of incubation. Purification was achieved by repeated streaking of the culture on agar slants (25 × 150 mm test tubes) of Postgate B medium sealed with suba seals and flushed with argon gas to maintain anaerobic conditions. After five days of incubation, small, round, transparent colonies were observed; initially a black zone formed around them, turning the whole slant black within two days of growth. Pure cultures of both strains were maintained under refrigeration at 4 °C and preserved by lyophilization in vials using skim milk powder (20 % w/v) as a cryoprotectant.

Morphological properties of strains JC15^T and JC19 grown in nutrient broth were observed after 48 h of incubation at 30 °C. Morphological properties such as cell shape, cell size and motility were observed by phase-contrast light microscopy (Olympus BH-2). Flagellar position and number were determined by using a transmission electron microscope (H-7500; Hitachi). All physiological tests for both strains were performed in modified Postgate's B medium in which FeSO₄ was replaced with MgSO₄ to avoid interference of the black precipitate. Utilization of organic compounds as carbon sources/electron donors for organoheterotrophic growth was tested with specific organic compounds (0.35 % w/v or v/v) in the presence of yeast extract (0.05 % w/v). Utilization of sulfur sources was tested with various sulfur compounds as sole sulfur sources (MgSO₄, FeSO₄, Na₂SO₃, Na₂S₂O₃, thioglycollate, cystine and Na₂S) at 0.2 % (w/v). Nitrogen source utilization was tested by replacing ammonium chloride with different nitrogen sources [NaNO₃, NaNO₂, CO(NH₂)₂ (urea), NH₄Cl (at 0.1 % w/v) and N₂ gas (gas phase replaced with nitrogen in tube sealed with suba seal)]. Lithoautotrophic growth was determined with NaHCO₃ (3 mM) as sole carbon source, Na₂S₂O₃ as electron donor and NH₄Cl as nitrogen source. Vitamin requirement was tested by replacing yeast extract in the medium with a solution containing a mixture of different vitamins (B₁₂, biotin, niacin, PABA, pantotheine, pyridoxal phosphate, riboflavin, thiamine) except the one under test as a growth factor. Various biochemical tests were carried out in prescribed media to meet the requirements of the standard methods described by Cappuccino & Sherman (1998). Acid phosphatase was determined as described by Pikovskaya (1948) with tricalcium phosphate (0.05 % w/v) as the sole phosphorus source. Phylogenetically related strains (*Shewanella haliotis* DW01^T, *Shewanella algae* ATCC 51192^T, *S. chilikensis* JC5^T and *S. marina* C4^T) were tested under the same conditions. Growth was measured turbidometrically (Systronics colorimeter model 112) at 540 nm in cultures which were centrifuged (15 000 r.p.m. for 15 min) and resuspended in distilled water. Other biochemical tests were also performed using the GN2 microplate (Biolog) prepared according to the manufacturer's instructions, except that bacterial strains were suspended in

distilled water supplemented with 2 % (w/v) sea salts. Cellular fatty acids of the strains grown on nutrient agar for 2 days at 30 °C were prepared, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; MIDI) (Sasser, 1990), which was outsourced through Royal Research Laboratories, Secunderabad, India. Phylogenetically related strains (*S. haliotis* DW01^T, *S. algae* ATCC 51192^T, *S. chilikensis* JC5^T and *S. marina* C4^T) were tested under the same conditions.

Genomic DNA was extracted and purified according to the method of Marmur (1961) and the mol% G + C content of the DNA was determined by using HPLC (Mesbah *et al.*, 1989). The taxonomic relationship between strains JC15^T, JC19 and *S. chilikensis* JC5^T was examined by using DNA–DNA hybridization. Genomic relatedness was determined by using a membrane filter technique (Seldin & Dubnau, 1985) using a DIG High Prime DNA labelling and detection starter kit II (Roche).

Cell material for 16S rRNA gene sequencing was taken from the pure and isolated cultures. DNA was extracted and purified by using the Qiagen genomic DNA extraction kit. PCR amplification and 16S rRNA gene sequencing were performed as described by Imhoff *et al.* (1998). Recombinant Taq polymerase was used for PCR with the primers 5'-GTTTGATCCTGGCTCAG-3' and 5'-TACCT-TGTTACGACTTCA-3' (*E. coli* positions 11–27 and 1489–1506, respectively). PCR amplification of the *dsrAB* genes was performed as described by Leloup *et al.* (2004) with primers (*dsr*-1F) 5'-AC[C/G]CACTGGAAGCACG-3' and (*dsr*-4R) 5'-GTGTAGCAGTTACCGCA-3'. Sequences were obtained by cycle sequencing with the SequiTherm sequencing kit (Biozym) and the chain termination reaction (Sanger *et al.*, 1977) using an automated laser fluorescence sequencer (Pharmacia). Nearest relatives and associated 16S rRNA gene sequence similarities were determined by NCBI BLAST search (Altschul *et al.*, 1990) and EzTaxon (Chun *et al.*, 2007). For phylogenetic analysis, 16S rRNA gene sequences of representative type strains of the order *Alteromonadales* and of strains JC15^T and JC19 were aligned using the CLUSTAL X program (Multiple alignment option; Thompson *et al.*, 1997). Alignment was checked manually using BioEdit software (Hall, 1999) and the file saved in .fas format. Phylogenetic trees were reconstructed by the neighbour joining (Fig. 1) and maximum-parsimony methods (data not shown) using MEGA 4.0 software (Tamura *et al.*, 2007).

Morphological and physiological properties of strains JC15^T and JC19 are shown in Table 1 and Supplementary Table S1 (available in IJSEM Online). On nutrient agar, aerobically grown colonies of strains JC15^T and JC19 are pale pink, circular and convex with entire margins and dark centres. Individual cells of both strains are rod-shaped, 2.0–3.0 µm long and 0.5–1.0 µm wide. Electron microscopy showed that both strains have a single polar flagellum (Supplementary Fig. S1). Both strains could grow chemo-organotrophically [with lactate (0.35 % v/v) as

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strains JC15^T and JC19 within the order Alteromonadales. The tree was reconstructed using the neighbour-joining method. Numbers at nodes are bootstrap values (values below 50 not shown). Bar, 1 substitution per 100 nucleotides.

carbon source/electron donor and NH₄Cl as nitrogen source] and chemolithoautotrophically [with NaHCO₃ (3 mM) as carbon source, Na₂S₂O₃ as electron donor and NH₄Cl as nitrogen source; growth yield determined by optical density of culture at 540 nm was 0.4 (strains JC15^T and JC19) compared with 0.02 (control)]. Ammonium chloride alone was used as the nitrogen source, while nitrate, nitrite, molecular nitrogen and glutamate were not utilized by either strain. Both strains showed good growth in sulfate when supplied in the form of FeSO₄·7H₂O, Na₂SO₄, MgSO₄, MnSO₄, (NH₄)₂SO₄ or sulfite. Sulfate respiration was not studied in either strain; however, they formed black colonies on sulfate-iron medium. Amplification of the desulforeductase (*dsrAB*) genes in both strains was unsuccessful, in contrast to a control strain belonging to the genus *Desulfovibrio*. Both strains showed poor growth on thiosulfate, cystine and thioglycollate and

no growth in sulfide when used as sulfur sources. Sodium chloride was not required for growth, but both strains showed optimum growth at 4–5 % and could tolerate up to 9 % (w/v) NaCl (Supplementary Fig. S2). Strain JC15^T had a pH optimum at 6.0 (range 5–8), while strain JC19 had a pH optimum at 7.0 (range 6–9). The temperature range for growth was 20–40 °C with an optimum at 30–37 °C. Vitamin B₁₂ was required as a growth factor for both strains. Whole-cell fatty acid analysis revealed that iso-C_{15:0}, C_{17:1}ω8c and iso-C_{13:0} 3-OH predominated in both strains (Table 2). The DNA base composition of strain JC15^T was 53.6 % G + C, and of strain JC19 was 54.3 % G + C.

The phylogenetic relationship of strains JC15^T and JC19 with each other and with other members of the genus *Shewanella* was examined by 16S rRNA gene sequencing. The near complete (1458 bases for JC15^T and 1439 bases

Table 1. Comparison of the morphological and physiological properties of strains JC15^T and JC19 with those of the nearest phylogenetic neighbours

Strains: 1, JC15^T; 2, JC19; 3, *Shewanella algae* IAM 14159^T; 4, *Shewanella haliotis* JCM 14758^T; 5, *Shewanella chilikensis* JC5^T; 6, *Shewanella marina* C4^T. All strains are Gram-stain-negative, rod-shaped, motile, catalase- and oxidase-positive, tolerant of 6 % NaCl, produce H₂S and are negative for starch hydrolysis, glucose fermentation, utilization of citrate and indole production. +, Positive/utilized; –, negative/no growth. Data from the present study except where indicated.

Characteristics	1	2	3	4	5	6
Habitat	Coal mine	Sediment of a marine lagoon	Red alga	Gut microflora of abalone (snail)	Marine lagoon sediment	Sea water
Growth at 4 °C	–	–	–	–	–	+
Growth at 42 °C	–	–	+	+	+	–
pH range (optimum)	5–8 (6)	6–9 (7)	5–11 (7–8)	5–11 (7)	7–9 (8)	5–10 (7)
DNA G + C content (mol%; HPLC)	53.6	54.3	52–54*	53.7*	54.6	40.8
Carbon substrates						
Sorbitol	+	+	–	–	–	–
Malate	–	–	+	+	+	–
Biochemical tests						
Casein hydrolysis	+	+	+	–	+	+
Urease	–	–	–	–	+	–
Haemolysis	+	+	+	–	+	+
Acid phosphatase	–	–	+	+	–	+
Nitrate reduction	–	–	+	+	–	+
Gelatin liquefaction	–	+	+	+	+	+
Arginine dihydrolase	–	–	–	–	–	+
Lysine decarboxylase	–	–	–	–	–	+
Ornithine decarboxylase	–	–	+	+	+	–

*Data from Holt *et al.* (2005) and Kim *et al.* (2007).

for JC19) 16S rRNA gene sequences demonstrated the similarity between strains JC15^T and JC19 and members of the genus *Shewanella*. Strains JC15^T and JC19 showed highest 16S rRNA gene sequence similarity with the type strain of *S. haliotis* (94.2 %) followed by other species of the genus *Shewanella*, including the recently described *S. xiamenensis* (Huang *et al.*, 2009). Between strain JC15^T and JC19 there was a similarity of 99.5 %. The genomic DNA–DNA hybridization of strain JC15^T with strain JC19 and *S. chilikensis* showed relatedness of 88 % and 42 %, respectively. To elucidate the phylogenetic relationship between the novel isolates and other species of the genus *Shewanella*, phylogenetic trees were reconstructed by using two different tree-making algorithms. The neighbour-joining tree (Fig. 1) showed that strains JC15^T and JC19 formed a distinct branch with the clade comprising *S. algae* ATCC 51192^T, *S. haliotis* DW01^T and *S. chilikensis* JC5^T, as was found in the maximum-parsimony tree (data not shown). On the basis of 16S rRNA gene sequence similarity data and the results of the phylogenetic analysis, it is clear that strain JC15^T represents a novel species within the genus *Shewanella*, for which the name *Shewanella fodinae* sp. nov. is proposed, while strain JC19 represents an additional strain based on genomic relatedness and phenotypic similarities with strain JC15^T.

Description of *Shewanella fodinae* sp. nov.

Shewanella fodinae (fo.di'nae. L. gen. n. *fodinae* of a mine).

On nutrient agar, aerobically grown colonies are pale pink, circular and convex with entire margins and dark centres. Cells are rod-shaped, 2.0–3.0 µm long and 0.5–1.0 µm wide and motile by a single polar flagellum. Gram-negative, non-fermentative and facultatively anaerobic. The type strain prefers acidic pH (optimum pH 6.0; range 5.0–8.0), while an additional strain grows optimally at neutral pH. Chemo-organoheterotrophy with various organic compounds is the preferred mode of growth. No obligate requirement for NaCl, but can tolerate up to 9 %, with optimum growth at 4–6 % NaCl. Good carbon sources are acetate, butyrate, lactate, pyruvate and valerate. Growth also occurs in benzoate, butanol, ethanol, formate, fumarate, sorbitol and succinate. Fructose, glycerol, malate, maltose, mannitol and propionate cannot support growth. Sulfate and sulfite are good sulfur sources for growth. Can also use thiosulfate, thioglycollate and cystine to some extent but cannot utilize sulfide. Ammonium chloride is used as sole nitrogen source; cannot use nitrate, glutamine, glutamate, urea, nitrogen gas or nitrite. Vitamin B₁₂ is required as a growth factor. Positive for catalase and oxidase. H₂S is produced. Casein is hydrolysed. Gelatin is

Table 2. Cellular fatty acid composition (%) of strains JC15^T and JC19 and their closest phylogenetic neighbours

Strains: 1, JC15^T; 2, JC19; 3, *Shewanella algae* IAM 14159^T; 4, *Shewanella haliotis* JCM 14758^T; 5, *Shewanella chilikensis* JC5^T; 6, *Shewanella marina* C4^T. —, Fatty acid not present; tr, trace amount (<1%). Data from the present study except where indicated.

Fatty acid	1	2	3*	4*	5	6
Straight-chain saturated						
C _{12:0}	2.6	2.8	1.9	0.21	3.5	3.7
C _{12:0} aldehyde	—	—	—	—	2.6	—
C _{13:0}	1.6	1.4	tr	—	1.2	tr
C _{14:0}	0.4	0.2	1.3	1.9	1.2	1.4
C _{15:0}	—	—	6.5	2.7	—	2.5
C _{16:0}	5.0	4.2	16.8	13.4	11.3	16.3
C _{17:0}	—	—	4.1	2.5	3.4	2.3
C _{18:0}	0.4	0.2	0.4	6.5	tr	2.4
Branched saturated						
iso-C _{10:0}	0.8	1.0	—	—	—	—
iso-C _{13:0}	4.3	3.2	0.5	4.1	4.3	4.4
iso-C _{14:0}	0.4	0.4	1.4	1.1	tr	2.0
iso-C _{15:0}	20.1	18.6	27.4	17.7	18.3	7.7
iso-C _{17:0}	2.5	2.0	0.5	1.6	13.1	tr
Monounsaturated						
C _{16:1} ω7c	—	—	15.3	—	—	—
C _{16:1} ω9c	1.6	1.5	2.8	1.1	1.6	2.0
C _{17:1} ω8c	15.6	18.2	10.9	10.7	13.1	9.5
C _{18:1} ω7c	2.4	2.2	5.2	5.4	4.2	5.3
C _{18:1} ω9c	3.5	3.2	5.0	4.5	4.2	5.1
Hydroxylated						
C _{12:0} 3-OH	1.7	1.1	—	2.0	3.4	2.0
iso-C _{13:0} 3-OH	9.3	9.2	—	—	3.1	1.3
iso-C _{15:0} 3-OH	2.2	2.1	—	—	—	—
Summed features†						
1	3.7	3.4	—	1.5	2.3	tr
2	3.5	3.2	—	1.1	—	2.6
3	6.8	8.0	—	12.5	—	19.4
4	—	—	—	—	15.1	—

*Data from Holt *et al.* (2005) and Kim *et al.* (2007).

†Summed features represent groups of two fatty acids that could not be separated by GLC with the MIDI system. Summed feature 1, C_{13:0} 3-OH and/or iso-C_{15:1} I/H; 2, C_{14:0} 3-OH and/or iso-C_{16:1}; 3, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 4, C_{16:1}ω7c and/or C_{16:1}ω6c.

not liquefied and indole is not produced from tryptophan. Amylase, lipase and urease activity are negative; acids are not produced from carbohydrates. Negative for citrate utilization and nitrate reduction. β-Haemolysis on blood agar. Predominant fatty acids include iso-C_{15:0}, C_{17:1}ω8c and iso-C_{13:0} 3-OH. DNA base composition is 53.6–54.3 mol% G + C (by HPLC).

The type strain JC15^T (=CCUG 57102^T =NBRC 105216^T =KCTC 22506^T) was isolated from 8 Incline mine, Godavarikhani, Karimnagar District, Andhra Pradesh, India. An additional strain, JC19, was isolated from a sediment sample of Chilika marine lagoon, Orissa, India.

This strain differs from the type strain by growing optimally at pH 7 (range 6–9).

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