Shewanella chilikensis sp. nov., a moderately alkaliphilic gammaproteobacterium isolated from a lagoon

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A Gram-negative, motile, rod-shaped, facultatively anaerobic bacterium was isolated from sediment of Chilika Lagoon, a brackish water lagoon in India. The strain, designated JC5^T, was able to grow in the presence of 0–8.0 % NaCl and at pH 7.0–10.0. The isolate was positive for oxidase and catalase and exhibited α -haemolysis. The major fatty acids were iso-C_{15:0} (18.3 %), C_{16:0} (11.3 %), C_{17:1 ω 8c} (13.1 %) and a summed feature, C_{16:1 ω 7c} and/or C_{16:1 ω 6c} (15.1 %). The genomic DNA G + C content was 54.6 mol%. A phylogenetic tree based on the 16S rRNA gene sequences showed that strain JC5^T forms a lineage within the genus *Shewanella* and is closely related to *Shewanella haliotis* DW01^T (98.0 %), *Shewanella algae* ATCC 51192^T (97.6 %) and *Shewanella marina* C4^T (95.8 %). Further, genomic DNA–DNA hybridization of strain JC5^T with *S. haliotis* DW01^T and *S. algae* ATCC 51192^T showed relatedness of only 42 and 23 %, respectively. On the basis of phenotypic and molecular genetic evidence, strain JC5^T represents a novel species of the genus *Shewanella*, for which the name *Shewanella chilikensis* sp. nov. is proposed. The type strain is JC5^T (=CCUG 57101^T =NBRC 105217^T =KCTC 22540^T).

The genus *Shewanella* is a member of the class *Gammaproteobacteria* (Anzai *et al.*, 2000) and comprises a group of Gram-negative, motile, rod-shaped, oxidase-positive, non-fermentative and facultatively anaerobic aquatic and marine bacteria (Park *et al.*, 2009; Gauthier *et al.*, 1995; MacDonell & Colwell, 1985; Venkateswaran *et al.*, 1999). At the time of writing, the genus *Shewanella* comprised 48 recognized species (<http://www.bacterio.cict.fr/s/shewanella.html>). The majority of *Shewanella* species have been isolated from aquatic and marine environments and most can grow by anaerobic respiration (Bowman, 2005). While studying the anoxygenic phototrophic bacterial diversity of Chilika Lagoon, India, strain JC5^T appeared as a pale-brown colony on anaerobic agar slants used for purifying cultures of purple anoxygenic

phototrophic bacteria. Strain JC5^T did not contain any bacterial chlorophyll or carotenoids and analysis of its 16S rRNA gene sequence indicated that the strain belongs to the genus *Shewanella*. In this communication, we propose a novel moderately alkaliphilic species of the genus *Shewanella*.

Strain JC5^T was isolated from a sediment sample taken from the southern part of Chilika Lagoon, near Barkul, Orissa, India (19° 47' N 85° 49' E), on 12 May 2007. The sample that yielded strain JC5^T had a pH of 8.5 and salinity of 2 % (w/v). The sediment sample was originally used for the enrichment of purple bacteria in Pfennig's mineral medium (Biebl & Pfennig, 1981) with (l⁻¹) 3.0 g sodium pyruvate as carbon source, 0.3 g yeast extract and 0.3 g Casamino acids as growth factors and 1.2 g ammonium chloride as nitrogen source. Phototrophic incubation (2400 lx) at 30 °C for 4–5 days resulted in the formation of pale-coloured enrichments, which were streaked directly onto agar slants (25 × 150 mm test tubes sealed with butyl rubber stoppers and the gas phase replaced with argon). Pale-brown colonies appeared after 2 days and the colonies

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JC5^T is FM210033.

Supplementary tables showing GN2 MicroPlate results for strain JC5^T and cellular fatty acid compositions for strain JC5^T and its closest phylogenetic relatives are available with the online version of this paper.

were purified by repeated streaking on agar slants. The purified culture was grown in nutrient broth and designated strain JC5^T. The pure culture of strain JC5^T was maintained under refrigeration at 4 °C and preserved as lyophilized cells.

Cells of strain JC5^T grown on nutrient agar were observed by phase-contrast microscopy (Olympus BH-2) for shape, size and motility and by transmission electron microscopy (H-7500; Hitachi) for flagella. Utilization of organic compounds as carbon sources/electron donors was tested in Pfennig's medium (Biebl & Pfennig, 1981) containing the specific organic compound (0.35 %, w/v) in the presence of yeast extract (0.01 %, w/v). Fermentative growth was tested using triple-sugar iron medium and also with the methyl red-Voges-Proskauer method. Nitrogen source utilization was tested by replacing ammonium chloride with different nitrogen sources [sodium nitrate, sodium nitrite, urea and ammonium chloride (at 0.1 %, w/v) and N₂ (gas phase replaced with nitrogen in tubes sealed with butyl rubber stoppers)]. Requirements for vitamins were tested by replacing yeast extract with different vitamins [vitamin B₁₂, biotin, niacin, *p*-aminobenzoic acid, pantothenate, pyridoxal phosphate, riboflavin, thiamine or a cocktail of all these vitamins

(0.02 %, w/v)] as growth factors. Biochemical tests (Table 1) were carried out in prescribed media to meet the requirements of the standard methods as mentioned by Cappuccino & Sherman (1998). Phylogenetically related strains (*Shewanella haliotis* DW01^T, *S. algae* ATCC 51192^T and *S. marina* C4^T) were tested under the same conditions. Growth was measured turbidometrically at 540 nm from cultures after centrifugation at 15 000 r.p.m. for 15 min and resuspension in distilled water. Other biochemical tests were performed using GN2 MicroPlates (Biolog) (Supplementary Table S1, available in IJSEM Online) according to the manufacturer's instructions except that strains were suspended in distilled water supplemented with 2 % (w/v) sea salts. Antibiotic resistance was determined using the disc diffusion method with commercial antibiotic-impregnated discs (BBL Becton Dickinson). The results were interpreted according to the guidelines set down by Clinical Laboratory Standards Institute (CLSI, 2003). Haemolytic activity was tested on trypticase soy agar supplemented with 5 % (v/v) defibrinated sheep blood (Benson, 2002).

Fatty acid methyl esters were prepared from cells grown in the above medium and identified according to the instructions of the Microbial Identification System

Table 1. Phenotypic characteristics that differentiate strain JC5^T from its closest phylogenetic neighbours

Strains: 1, *Shewanella chilikensis* sp. nov. JC5^T; 2, *S. algae* ATCC 51192^T (data from this study unless indicated); 3, *S. haliotis* DW01^T (Kim *et al.*, 2007); 4, *S. marina* C4^T (Park *et al.*, 2009). All strains are motile by single polar flagellum, tolerant of 6 % NaCl, positive for catalase, oxidase and H₂S production and negative for starch hydrolysis, glucose fermentation, citrate and fructose utilization and indole production. +, Positive; w, weakly positive; −, negative; R, resistant; S, sensitive.

Characteristic	1	2	3	4
Isolation source	Marine lagoon sediment	Salt marsh ^{a*}	Abalone	Seawater
Colony colour	Pale brown	Pink	Pink–orange	Pale brown
Growth at:				
4 °C	−	−	−	+
42 °C	+	+	+	−
pH range (optimum)	7–10 (8)	5–11 (7–8)	5–11 (7)	5–10 (7)
Carbon source utilization				
Malate	+	−	+	−
Mannose	−	+	−	−
Glucose	−	+	−	w
Casein hydrolysis	+	+	−	+
Gelatin liquefaction	−	+	+	+
Nitrate reduction	−	+	+	+
Arginine dihydrolase	−	+	+	+
Lysine decarboxylase	−	−	−	+
Ornithine decarboxylase	+	+	+	−
Urease	+	−	−	−
Haemolysis	+ (α)	+	−	+
Antibiotic sensitivity				
Penicillin	S	R	R	R
Vancomycin	S	R	R	R
DNA G + C content (mol%)	54.6	52–54 ^b	53.7	40.8

*Data taken from: *a*, Simidu *et al.* (1990); *b*, Nozue *et al.* (1992) (range of values for 36 strains, including the type strain).

(Sasser, 1990). The DNA G + C content was determined by HPLC using the methods of Mesbah *et al.* (1989). The taxonomic relationships between strain JC5^T and *S. algae* ATCC 51192^T and *S. haliotis* DW01^T were examined further using DNA–DNA hybridization. Genomic relatedness was determined using a membrane filter technique (Seldin & Dubnau, 1985) using a DIG High Prime DNA Labelling and Detection starter kit II (Roche).

Genomic DNA was obtained from 1–2 ml well-grown culture from nutrient broth (dehydrated medium; HiMedia) using a genomic DNA extraction kit (Qiagen). PCR amplification was performed as described by Imhoff *et al.* (1998) with recombinant *Taq* polymerase and the primers F'-27 (5'-GTTTGATCCTGGCTCAG-3') and R'-1489 (5'-TACCTTGTTACGACTTCA-3') (positions 11–27 and 1489–1506, respectively, according to the *Escherichia coli* 16S rRNA gene sequence numbering system of the International Union of Biochemistry). Sequencing of the amplification products was outsourced to MWG Biotech Pvt Ltd (Bangalore, India). The resultant sequence of strain JC5^T was aligned manually against sequences obtained from the GenBank database. Phylogenetic trees were inferred from bases that were available for all sequences (positions 42–1450; *E. coli* numbering system) using neighbour-joining (Saitou & Nei, 1987), Fitch–Margoliash (Fitch & Margoliash, 1967) and maximum-likelihood (Guindon & Gascuel, 2003) methods. Evolutionary distance matrices were generated according to Jukes & Cantor (1969). The resultant neighbour-joining tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out using the jPHYDIT program (available at <http://plaza.snu.ac.kr/~jchun/jphydit/>) and PAUP 4.0 (Swofford, 1998) as described by Chun *et al.* (2000).

On nutrient agar, aerobically grown colonies of strain JC5^T are pale brown, circular and convex with entire margins. Individual cells are rods, 1.5–2.0 µm long and 0.5–1.0 µm wide. Electron microscopy showed that the strain has a single polar flagellum. Substrates that were utilized as carbon sources/electron donors under organoheterotrophic conditions are given in Table 1. Ammonium chloride, nitrate and glutamate were used as nitrogen sources for growth, while nitrite and molecular nitrogen were not utilized. Addition of NaCl to the medium was not required for growth of strain JC5^T, but the strain could tolerate NaCl up to a concentration of 8.0%. Strain JC5^T could grow at pH 7.0–10.0 (optimum pH 8.0) (Fig. 1) and at 25–42 °C (optimum 28–30 °C). There is no vitamin requirement for strain JC5^T; however, addition of yeast extract (0.01%, w/v) enhanced growth. Strain JC5^T was catalase-positive and α-haemolytic. Starch was not hydrolysed; casein was hydrolysed. Gelatin was not liquefied and indole was not produced from L-tryptophan. Although lipase and acid phosphatase tests were negative, strain JC5^T showed urease activity. Acid formation from glucose and lactose

Fig. 1. Effect of pH on the growth of strain JC5^T. Values are means ± SD from 2 determinations.

was negative. These results show clear phenotypic differences from recognized *Shewanella* species (Table 1).

The whole-cell fatty acid profile of strain JC5^T (Supplementary Table S2) included C_{12:0} (3.5%), C_{16:0} (11.3%), iso-C_{13:0} (4.3%), iso-C_{15:0} (18.3%), C_{17:1ω8c} (13.1%) and summed feature 4 (C_{16:1ω7c} and/or C_{16:1ω6c}; 15.1%). Significant differences in the contents of predominant fatty acids between strain JC5^T and type strains of related species were found: e.g. summed feature 4 was found only in strain JC5^T and summed feature 3 was found only in the type strains of other *Shewanella* species (although both summed features include C_{16:1ω7c}). The DNA G + C content of strain JC5^T was 54.6 mol% (HPLC), which is similar to values reported for *S. algae* and *S. haliotis*.

Preliminary sequence comparison with 16S rRNA gene sequences held in GenBank indicated that our isolate was related closely to the genus *Shewanella*. After manual alignment of the newly determined sequence (1442 nt) with those of representatives of the genus *Shewanella*, strain JC5^T showed highest 16S rRNA gene sequence similarity to sequences from *S. haliotis* DW01^T (98.0%) and *S. algae* ATCC 51192^T (97.6%). To elucidate the phylogenetic relationship between the isolate and other species of the genus *Shewanella*, phylogenetic trees were constructed using three different tree-making algorithms. The neighbour-joining tree (Fig. 2) showed that strain JC5^T formed a distinct branch with the clade comprising *S. haliotis* DW01^T and *S. algae* ATCC 51192^T, and this was also observed in the Fitch–Margoliash tree (not shown).

Genomic DNA–DNA hybridization of strain JC5^T with *S. haliotis* DW01^T and *S. algae* ATCC 51192^T showed relatedness values of only 42 and 23%, respectively. Thus, levels of genetic relatedness according to

Fig. 2. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain JC5^T and members of the genus *Shewanella*. Percentages at nodes are levels of bootstrap support based on 1000 resampled datasets. The sequence of *Vibrio cholerae* ATCC 14035^T was used as an outgroup. Bar, 0.01 nucleotide substitutions per position.

DNA–DNA hybridization experiments were less than 70 %, which supports the conclusion that strain JC5^T represents a novel and distinct species.

On the basis of the phylogenetic, genomic and phenotypic data, it is clear that strain JC5^T represents a novel species within the genus *Shewanella* (Bowman, 2005), for which the name *Shewanella chilikensis* sp. nov. is proposed.

Description of *Shewanella chilikensis* sp. nov.

Shewanella chilikensis (chi.li.ken'sis. N.L. fem. adj. *chilikensis* pertaining to Chilika, referring to the isolation of the type strain from Chilika lagoon, Orissa, India).

On nutrient agar, aerobically grown colonies are pale brown, circular and convex with entire margins. Cells are rod-shaped, 1.5–2.0 µm long and 0.5–1.0 µm wide and motile by single polar flagellum. Gram-negative, non-fermentative, non-spore-forming and facultatively

anaerobic. Moderately alkaliphilic (range pH 7.0–10.0, optimum pH 8.0). Growth occurs with 0–8 % (w/v) NaCl and at 25–42 °C (optimum 28–30 °C). No vitamin requirement but yeast extract enhances growth. Oxidase- and catalase-positive. Negative for nitrate reduction and glucose fermentation. Produces urease and ornithine decarboxylase, but not lipase, arginine dihydrolase or lysine decarboxylase. Produces H₂S but not indole. Hydrolyses casein but not gelatin or starch. Acid production from carbohydrates (glucose, lactose) and acid phosphatase activity are negative. The carbon sources that are utilized include fumarate, malate, pyruvate, succinate, glycogen, Tweens 40 and 80, *N*-acetylgalactosamine, *N*-acetylglucosamine, pyruvic acid methyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, α - and β -hydroxybutyric acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, propionic acid, succinic acid, bromosuccinic acid, L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-aspartic acid, glycyl

L-glutamic acid, L-leucine, L-proline, L-serine, L-threonine, inosine, uridine and thymidine. Citrate is not utilized. Exhibits α -haemolysis. The predominant fatty acids (>5%) are iso-C_{15:0}, C_{16:0}, C_{17:1} ω 8c and summed feature 4 (C_{16:1} ω 7c and/or C_{16:1} ω 6c). The DNA G + C content of the type strain is 54.6 mol% (HPLC).

The type strain, JC5^T (=CCUG 57101^T =NBRC 105217^T =KCTC 22540^T), was isolated from sediment of Chilika Lagoon, Orissa, India.

Acknowledgements

We thank Dr J. Euzby for his expert suggestion on the correct species epithet and Latin etymology. Funding received for this work from MoES is acknowledged. Facilities used under the DST, FIST-II are acknowledged from University of Hyderabad.

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