Shewanella psychrophila sp. nov. and *Shewanella piezotolerans* sp. nov., isolated from west Pacific deep-sea sediment

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Two *Shewanella*-like bacterial strains, WP2^T and WP3^T, which were isolated from west Pacific deep-sea sediment, were studied to determine their taxonomic position. Cells of the two bacteria were facultatively anaerobic, Gram-negative rods and motile by means of a single polar flagellum. Strain WP2^T was psychrophilic, growing optimally at about 10–15 °C, whereas strain WP3^T was psychrotolerant, growing optimally at 15–20 °C. The two strains grew in the pressure range 0.1–50 MPa, with optimal growth at 20 MPa. Strain WP3^T was able to use nitrate, fumarate, trimethylamine *N*-oxide (TMAO), DMSO and insoluble Fe(III) as terminal electron acceptors during anaerobic growth, whereas strain WP2^T was able to use only nitrate, TMAO and DMSO. The 16S rRNA gene sequences of strains WP2^T and WP3^T were 97% identical, and showed highest similarity (97%) to those of *Shewanella fidelis* KMM 3589 and *Shewanella benthica* ATCC 43992^T, respectively. The *gyrB* gene sequences of strains WP2^T and WP3^T were also determined, and showed highest similarity to those of *Shewanella violacea* JCM 10179^T (90%) and *Shewanella sairae* SM2-1^T (87%), respectively. Contrary to the 16S rRNA gene sequence results, the phylogeny based on *gyrB* gene sequence analysis placed strain WP2^T, *S. violacea* and *S. benthica* in one group, while strain WP3^T grouped with *S. fidelis* and *S. sairae*. DNA–DNA hybridization experiments supported the placement of strain WP2^T with *S. violacea* and *S. benthica*. Phylogenetic evidence, together with DNA–DNA relatedness and phenotypic characteristics, indicated that the two new strains represented two novel deep-sea *Shewanella* species. The names *Shewanella psychrophila* sp. nov. (type strain WP2^T = JCM 13876^T = CGMCC 1.6159^T) and *Shewanella piezotolerans* (type strain WP3^T = JCM 13877^T = CGMCC 1.6160^T) are proposed.

Shewanella species are widely distributed in nature, especially associated with aquatic and marine environments (MacDonell & Colwell, 1985; Skerratt *et al.*, 2002; Venkateswaran *et al.*, 1998, 1999; Satomi *et al.*, 2003). At the time of writing the genus comprises more than 20 recognized species, among which *Shewanella benthica*, *Shewanella violacea* and *Shewanella profunda* have been isolated from deep-sea environments (Deming *et al.*, 1984; MacDonell & Colwell, 1985; Nogi *et al.*, 1998; Toffin *et al.*,

2004). Kato & Nogi (2001) divided the genus *Shewanella* into two major branches, groups 1 and 2. Most *Shewanella* group 1 species are psychrophilic/psychrotolerant and piezophilic/piezotolerant and produce eicosapentanoic acid (EPA); by contrast, species in group 2 are mesophilic, pressure-sensitive and produce no or only trace amounts of EPA. *Shewanella* group 1 species include *S. benthica*, *S. violacea*, *S. hanedai*, *S. gelidimarina* and *S. pealeana*; *Shewanella* group 2 species include *S. putrefaciens*, *S. oneidensis*, *S. algae*, *S. baltica*, *S. amazonensis* and *S. frigidimarina*.

Shewanella strains are the most abundant *Proteobacteria* in the deep sea (Kato & Nogi, 2001; Kato, 1999). We have previously reported the isolation of two psychrotolerant/psychrophilic *Shewanella* strains, designated WP2^T and

Abbreviations: EPA, eicosapentanoic acid; TMAO, trimethylamine *N*-oxide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of *Shewanella psychrophila* WP2^T are AJ551089 and AM229307, and those of *Shewanella piezotolerans* WP3^T are AJ551090 and AM229308, respectively.

WP3^T, from a deep-sea sediment (Wang *et al.*, 2004). In the present study, these two strains were further characterized based on combined phenotypic, phylogenetic and chemotaxonomic analyses. They were determined to represent two novel deep-sea *Shewanella* species.

Strains WP2^T and WP3^T were isolated from west Pacific deep-sea sediment (142° 30' 08" E 8° 00' 11" N) at a depth of 1914 m. The reference strain *Shewanella fidelis* LMG 20551^T was obtained from the Belgian Co-ordinated Collections of Microorganisms (BCCM/LMG), *S. benthica* ATCC 43992^T from the American Type Culture Collection (ATCC) and *S. violacea* JCM 10179^T from the Japan Collection of Microorganisms (JCM). These bacteria were grown in marine 2216E medium (0.5 % tryptone, 0.1 % yeast extract, 3.4 % NaCl and 0.01 % FePO₄, pH 7.6–7.8). Strain WP2^T was grown at 15 °C, strain WP3^T at 20 °C, *S. benthica* and *S. violacea* at 10 °C, and *S. fidelis* at 25 °C unless otherwise indicated. High-pressure cultivation was performed using a hand-operated pump and a quick-fit connector to the high-pressure vessels (Yayanos & Dietz, 1982; Li *et al.*, 2006). Marine 2216E medium was inoculated with 1 % inoculum culture in exponential growth phase; 60 ml of inoculated medium was dispersed in sterile injection syringes. Duplicate syringes were placed into the high-pressure vessels and pressurized. Growth of the cultures was checked by decompressing the vessels and was monitored by measuring the optical absorption at OD₆₀₀. Growth tests at different pressures were repeated three times.

Morphological, biochemical and physiological tests followed the general procedures described by Dong & Cai (2001). The ability of the strains to utilize a broad range of carbon sources was determined by using Biolog GN2 microplates. Cell morphology of the bacteria was observed by using light and transmission electron microscopy. After around 6–7 days incubation on marine 2216E agar plates at 10 °C, the two new strains formed pale-yellow colonies of 0.5 mm in diameter with rounded edges. After 5 days incubation in marine 2216E broth, the cell aggregates showed a pinkish colour. Cells of strains WP2^T and WP3^T were Gram-negative rods, 0.5–0.8 µm wide and 2–5 µm long, and motile by means of a single polar flagellum (Fig. 1). When incubated in liquid culture, strain WP2^T grew over a temperature range of 0–20 °C, and optimally at 10–15 °C; strain WP3^T was able to grow at 0–28 °C, with optimal growth at 15–20 °C. The strains were also cultivated in 2216E media modified with the addition of NaCl (0–12 %). The two new strains required NaCl for growth. They were able to grow at salinities of 1–7.2 %, with optimum growth at 3–4 % NaCl. The strains were tested for their ability to grow at pressures of 0.1, 10, 20, 30, 40 and 50 MPa. Strains WP2^T and WP3^T showed growth within the pressure range 0.1–50 MPa and displayed optimal growth at 20 MPa (Fig. 2).

Strain WP2^T was able to use citrate, maltose, *N*-acetyl-D-glucosamine, sucrose and D-trehalose as sole carbon sources. Nitrate was reduced to nitrite. Hydrogen sulfide was not

Fig. 1. Transmission electron micrographs of cells of strain WP2^T (a) and WP3^T (b). Cells were grown on 2216E agar plates at 15 °C for 16 h. One colony was picked and dispersed in 100 µl PBS buffer, and 5 µl liquid was placed on a copper grid for negative staining using 1 % uranyl acetate. Bars, 0.5 µm.

produced. Strain WP2^T was positive for cytochrome oxidase and catalase, and negative for the production of lipase, gelatinase, chitinase and amylase (Table 1). Strain WP3^T used acetate, D-glucose, maltose and *N*-acetyl-D-glucosamine as sole carbon sources. Nitrate was reduced to nitrite. Hydrogen sulfide was produced. Strain WP3^T was positive for cytochrome oxidase, lipase, gelatinase, amylase and catalase, but negative for the production of chitinase (Table 1).

Dissimilatory iron reduction was tested using lactate as the carbon and energy source (Bozal *et al.*, 2002). Anaerobic growth was also evaluated using trimethylamine *N*-oxide (TMAO), DMSO, sodium fumarate, sodium nitrite and sodium nitrate in the same medium in which Fe(III) citrate was omitted. Strain WP3^T was able to utilize nitrate, fumarate, TMAO, DMSO and insoluble Fe(III) as terminal electron acceptors for anaerobic growth, whereas strain WP2^T used only nitrate, TMAO and DMSO.

Fig. 2. Growth of strains WP2^T (○) and WP3^T (■) at different pressures (at 15 °C). For strain WP2^T, 100% growth rate: $1/t_d = 0.405$; for strain WP3^T, 100% growth rate: $1/t_d = 0.5$, where t_d is doubling time. Bars indicate SD of three experiments.

Strains WP2^T and WP3^T were grown in 250 ml marine 2216E broth. Cultures were incubated with shaking at 150 r.p.m. for 24 h. The cell membrane was extracted via the TCA method and purified with trypsin according to Schleifer & Kandler (1972). Isoprenoid quinones were extracted and purified according to Collins *et al.* (1977). The

Table 1. Phenotypic characteristics of strains WP2^T and WP3^T and related *Shewanella* species

Taxa: 1, strain WP2^T; 2, strain WP3^T; 3, *S. benthica* ATCC 43992^T; 4, *S. violacea* JCM 10179^T; 5, *S. fidelis* LMG 20551^T. +, Positive reaction; –, negative reaction; V, variable reaction; ND, not determined. All are positive for catalase and oxidase.

Characteristic	1	2	3	4	5
Optimal growth temperature (°C)	10–15	15–20	4–15	8	20–25
DNA G+C content (mol%)	51	49	48	47	45
Production of H ₂ S	–	+	+	–	+
Gelatinase	–	+	+	+	+
Chitinase	–	–	ND	ND	–
Amylase	–	+	–	–	–
Lipase	–	+	ND	ND	V
Use as sole carbon sources:					
Acetate	–	+	ND	ND	ND
Cellobiose	–	–	+	+	ND
Citrate	+	–	ND	ND	–
D-Galactose	–	–	–	+	–
D-Glucose	–	+	+	+	+
Maltose	+	+	–	–	ND
N-acetyl-D-glucosamine	+	+	ND	ND	ND
Sucrose	+	–	–	–	–
D-Trehalose	+	–	–	–	ND

purified menaquinones were analysed by HPLC-MS (LCQ MAT; Finnigan) (Nishijima *et al.*, 1997) with an APCI (atmospheric pressure chemical ionization) ion source using a solution of 2-propanol and acetonitrile (1.25:1, v/v). Ubiquinones and menaquinones were found in the two new strains. The major ubiquinones in strains WP2^T and WP3^T were ubiquinone 7 (Q7) (59 and 60 %, respectively) and ubiquinone 8 (Q8) (41 and 40 %, respectively). Traces of menaquinone 7 (MK7) and monomethylmenaquinone 7 (MMK7) were also detected.

Cells for cellular fatty acid analysis were likewise harvested after cultivation for 24 h. Fatty acids were extracted and analysed following the instructions of the Microbial Identification System operating manual (MIDI Inc.). The fatty acid profiles of strains WP2^T and WP3^T were typical of members of the genus *Shewanella* and included iso-13:0, 14:0, iso-15:0, 16:0, 16:1, 18:1 and 20:5 (Table 2).

Genomic DNA was extracted as described by Chen *et al.* (2005). The G+C content of the DNA was determined using HPLC analysis of hydrolysed DNA according to Tamaoka & Komagata (1984). The obtained nucleoside mixture was then separated by reversed-phase HPLC using a C₁₈ column (Kromasil ODS, 5 µm, 250 × 4.6 mm inner diameter) at room temperature. The solvent was 0.05 mol l⁻¹ NH₄H₂PO₄, pH 4.0, with 7 % acetonitrile (Tamaoka & Komagata, 1984). The G+C content was calculated based on determination of the G/(G+T) content according to Mesbah *et al.* (1989). The DNA G+C content of strains WP2^T and WP3^T was 51 and 49 mol%, respectively.

Table 2. Fatty acid composition (%) of strains WP2^T and WP3^T

Strain WP2^T was grown at 10 °C and strain WP3^T at 15 °C.

Fatty acid	WP2 ^T	WP3 ^T
12:0	5.9	2.6
iso-13:0	7.3	8.0
13:0	–	0.6
3-OH 12:0	–	0.6
14:0	7.9	2.6
iso-13:0 3-OH	1.8	1.4
iso-15:0	4.0	10.3
anteiso-15:0	–	0.4
15:0	–	3.0
16:1 ω 7 _c	38.2	23.5
16:0	12.7	10.0
17:1 ω 8 _c	1.6	5.6
17:0	–	2.2
18:1 ω 7 _c	–	2.5
18:1 ω 7 _t	9.4	9.3
18:0	2.0	1.7
20:5 ω 3	7.1	13.4

PCR was used to amplify a portion of the *gyrB* gene according to the procedure of Yamamoto & Harayama (1995). PCR products of 1200 bp of the *gyrB* gene fragment (positions 274–1525 according to the *Escherichia coli* numbering scheme) were cloned into the pGEM-T vector by using a 2 × rapid ligation kit according to the instructions of the manufacturer (Promega). Ligation mixtures were used to transform competent cells of *E. coli* XL1 Blue according to the manufacturer's recommendations (Promega). Positive clones were picked for sequencing (Sangon). The resulting sequences were searched in the NCBI database. Related sequences were aligned using the program DNAMAN (Lynnon Biosoft, v. 5.1). A phylogenetic tree was constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm (Fitch, 1971) and the neighbour-joining method (Saitou & Nei, 1987) using the DNAMAN program, and bootstrap analyses (1000 trials) were used to provide estimates of confidence for phylogenetic tree topologies.

The *gyrB* gene sequences of strains WP2^T and WP3^T shared 84 % similarity. Strain WP2^T showed highest *gyrB* gene sequence similarity with the type strains of *S. violacea* (90 %) and *S. benthica* (89 %), and strain WP3^T showed highest similarity with the type strain of *Shewanella saireae* (87 %). The phylogenetic relationship of strains WP2^T and WP3^T with other *Shewanella* species based on *gyrB* gene sequences is shown in Fig. 3(b). Strain WP2^T clustered with *S. violacea* and *S. benthica* whereas strain WP3^T clustered with *S. fidelis* and *S. saireae*. This is not consistent with the results based on 16S rRNA gene sequence analyses (Fig. 3a; Wang *et al.*, 2004).

Levels of DNA–DNA relatedness were determined by DNA–DNA slot-blot hybridization as described by Chen *et al.* (2005). Triplicate target DNA (50–200 ng) was denatured in 0.4 M NaOH and transferred onto a nylon membrane (Amersham, Pharmacia) and hybridized with sheared probes labelled with digoxin (DIG High Prime DNA Labeling and Detection Starter KitII; Roche). Hybridization was performed at 55 °C, and high-stringency washes and signal detection were performed according to the manufacturer's instructions. Intensities of the signals were analysed using the GelBase/GelBlot-Pro software system. Signals produced by hybridization of the probe with homologous target DNA were considered to represent 100 % hybridization. Levels of DNA–DNA hybridization between strains WP2^T and WP3^T and recognized *Shewanella* species are given in Table 3. Levels ranged from 60 % (WP2^T/*S. benthica*) to 19 % (WP3^T/*S. benthica*) (Table 3), well below the 70 % threshold used to define a genomic species (Wayne *et al.*, 1987).

The use of *gyrB* sequences for phylogenetic placement of bacterial strains is well established (Yamamoto & Harayama, 1995; Venkateswaran *et al.*, 1999). The more rapid evolution of the *gyrB* gene compared with that of the 16S rRNA gene makes it a more appropriate choice for differentiating between closely related strains. The 16S rRNA gene sequence

Fig. 3. Phylogenetic trees showing the relationships of strains WP2^T and WP3^T with other related *Shewanella* reference species based on 16S rRNA (a) and *gyrB* (b) gene sequences. Numbers at nodes are percentage bootstrap values based on 1000 trials. Bars, 0.05 % sequence divergence.

of strain WP2^T had highest similarity (97 %) with *S. fidelis* KMM 3589, and strain WP3^T highest similarity (97 %) with *S. benthica* ATCC 43992^T. By contrast, the *gyrB* gene sequence of strain WP2^T exhibited highest similarity with those of the type strains of *S. violacea* and *S. benthica* (90 and 89 %, respectively), and the *gyrB* gene sequence of strain WP3^T exhibited highest similarity with that of the type strain of *S. saireae* (87 %). Discrepancies between *gyrB* and 16S rRNA gene sequences were previously evident among the *Shewanella* species examined by Venkateswaran *et al.* (1999). DNA–DNA hybridization results indicated that strain WP2^T is most closely related to *S. benthica*. DNA–DNA hybridization and *gyrB* sequence analysis both placed strain WP2^T, *S. violacea* and *S. benthica* within a single phylogenetic clade. The results of the polyphasic taxonomic investigations performed in this study suggest that strains WP2^T and WP3^T represent two novel *Shewanella* species. The names *Shewanella psychrophila* sp. nov. and *Shewanella*

Table 3. Levels of DNA–DNA relatedness between strains WP2^T and WP3^T and related *Shewanella* species

ND, Not determined. Values in parentheses are SD of three experiments.

Strain	Hybridization (%) with labelled DNA from:			
	1	2	3	4
1. WP2 ^T	100	24 (±2.02)	55 (±1.99)	38 (±2.27)
2. WP3 ^T	29 (±1.91)	100	38 (±3.74)	33 (±5.08)
3. <i>S. benthica</i> ATCC 43992 ^T	60 (±1.52)	19 (±5.36)	100	46.4 (±4.69)
4. <i>S. violacea</i> JCM 10179 ^T	41 (±3.00)	40 (±4.28)	48.8 (±3.58)	100
<i>S. fidelis</i> KMM 3589	32 (±3.64)	38 (±5.11)	ND	ND

piezotolerans sp. nov. are proposed for strains WP2^T and WP3^T, respectively.

Description of *Shewanella psychrophila* sp. nov.

Shewanella psychrophila (psy.chro'phi.la. Gr. adj. *psychros* cold; Gr. adj. *philos* loving; N.L. fem. adj. *psychrophila* cold loving).

Cells are Gram-negative rods that are 0.5–0.8 µm wide and 2–5 µm long when grown at 0.1 MPa. Motile by means of a single polar flagellum. Facultatively anaerobic, with nitrate, TMAO and DMSO as electron acceptors. Colonies on agar plates are circular with a round edge and pale-yellow in colour. Growth occurs within the temperature range 0–20 °C, with optimal growth at 10–15 °C. Growth occurs within the pressure range 0.1–50 MPa, with optimal growth at 20 MPa. Growth occurs at NaCl concentrations between 1 and 7.2 % (optimum at 3–4 %), and at pH 6–8 (optimum at pH 7). Positive for oxidase and catalase. Hydrogen sulfide is not produced. The following compounds are utilized for respiration (Biolog): Tweens 40 and 80, *N*-acetyl-D-glucosamine, *myo*-inositol, maltose, sucrose, D-trehalose, *cis*-aconitic acid, α -ketovaleric acid, bromosuccinic acid, succinamic acid, L-alaninamide, D-alanine, L-glutamic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, D-serine L-serine, L-threonine, DL-carnitine, thymidine and DL- α -glycerol phosphate. Produces EPA. Major quinones are Q7 and Q8; MK7 and MMK7 are also present. The G + C content of the DNA is 51 mol%.

The type strain, WP2^T (= JCM 13876^T = CGMCC 1.6159^T), was isolated from west Pacific deep-sea sediment.

Description of *Shewanella piezotolerans* sp. nov.

Shewanella piezotolerans (pie.zo'to.le.rans. Gr. v. *piezo* to press; L. part. adj. *tolerans* tolerating; N.L. fem. part. adj. *piezotolerans* pressure tolerating).

Cells are Gram-negative rods, 0.5–0.8 µm wide and 2–5 µm long, and motile by means of a single polar flagellum. Facultatively anaerobic, with nitrate, fumarate, TMAO, DMSO and insoluble Fe(III) as terminal electron acceptors. Grows at temperatures of 0–28 °C, with optimal growth

occurring at 15–20 °C. Growth occurs within the pressure range 0.1–50 MPa, with optimal growth at 20 MPa. Growth occurs at NaCl concentrations between 1 and 7.2 % (optimum at 3–4 %), and at pH 6–8 (optimum at pH 7). Lipase, oxidase, catalase, gelatinase and amylase are produced. Hydrogen sulfide is produced from thiosulfate. Cells are able to reduce nitrate to nitrite. The following carbon sources are used: α -cyclodextrin, dextrin, Tweens 40 and 80, *N*-acetyl-D-glucosamine, gentiobiose, α -D-glucose, maltose, acetic acid, DL-lactic acid, propionic acid, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-leucine, L-serine, L-threonine, inosine, uridine and thymidine. Produces EPA. Major quinones are Q7 and Q8; MK7 and MMK7 are also present. The G + C content of the DNA is 49 mol%.

The type strain, WP3^T (= JCM 13877^T = CGMCC 1.6160^T), was isolated from west Pacific deep-sea sediment.

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