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Isolation of extremophiles with the detection and retrieval of *Shewanella* strains in deep-sea sediments from the west Pacific

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Abstract Tests to detect the presence of piezophilic *Shewanella* strains in the deep-sea sediments of the west, mid- and east Pacific at different depths were done by amplification of previously identified pressure-regulated operons (ORF1,2 and ORF3). The operon fragments were detected in all the deep-sea sediment samples, indicating the broad presence of piezophilic deep-sea *Shewanella* species or related species in the deep-sea sediments across the Pacific. Extremophiles were isolated from the deep-sea sediment of the west Pacific under atmospheric pressure. Two psychrophilic/psychrotrophic strains, WP2 and WP3, were assigned to the *Shewanella* genus as determined by their 16S rDNA sequences. WP2 and WP3 were both capable of amplifying pressure-regulated operons; the sequences of the pressure-regulated operons of WP2 and WP3 share high identity between each other, but have more differences from those of *S. benthica* and *S. violacea*. The major fatty acids of WP2 and WP3 are 3OH-i-13:0, 14:0, i-15:0, 16:0, 16:1, 18:1, and 20:5. Combined phenotypic analysis, 16S rDNA sequences, and DNA–DNA hybridization results suggest that WP2 and WP3 are two new deep-sea *Shewanella* species.

Keywords Deep-sea · Eicosapentaenoic acid · Extremophile · Pressure-regulated operon · *Shewanella* · West Pacific

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All piezophilic, psychrophilic bacteria isolated from deep-sea environments fall into five genera and include *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella*, and *Psychromonas* within the *γ-Proteobacteria* group (DeLong et al. 1997; Nogi et al. 2002). The *Shewanella* genus includes two piezophilic species – *S. benthica* and *S. violacea* – which have been isolated from deep-sea animals and sediments (Deming et al. 1984; Kato et al. 1998; Nogi et al. 1998). From both *S. benthica* and *S. violacea*, two pressure-regulated operons located close together were cloned and partially characterized (Kato et al. 1996, 1997a). These two operons (ORFs) were found only in the piezophilic branch of the *Shewanella* genus, and thus were suggested for use as molecular markers for identifying piezophilic *Shewanella* strains(Li et al. 1998). By using these markers, Kato et al. have reported the direct detection of deep-sea adapted *Shewanella* bacteria from DNA extracted from Mariana Trench sediments (Kato et al. 1997b).

Although *Shewanella* strains have frequently been reported to be isolated from deep-sea environments at depths of 2,485–10,898 m, all of them have been found to be either *S. benthica* or *S. violacea* (Kato et al. 1998; Nogi et al. 1998) and our knowledge of the deep-sea *Shewanella* strains including their distribution is limited to these two species. In this paper, the distribution of the deep-sea *Shewanella* strains in the sediment from the west Pacific warm pool area with a depth of 1,914 m, as well as in deep-sea sediments from the mid- and east Pacific, were assayed by checking for the presence of the pressure-regulated operons. The diversity of extremophiles in the deep-sea sediment of the west Pacific was investigated, and two morphologically different *Shewanella* strains were isolated under atmospheric pressure. Further studies suggest that the two strains are new deep-sea *Shewanella* species.

Sediment samples were collected during the cruise of DaYang No. 1 in 2001 at the west Pacific site WP (142°30′08″E, 8°00′11″N, 1,914 m), east Pacific A station (153°52′19″W, 7°33′46″N, 5,027 m) and mid-Pacific station MP (177°42′20″W, 10°35′06″N, 5,774 m). The

sediments were collected with a multi-core sampler and transferred aseptically in the clean bench to sterile falcon tubes. The sediment samples were shipped to the laboratory at −20°C and stored at −20°C in the laboratory until use.

PCR amplification of the piezophilic *Shewanella*-specific pressure-regulated operons was done as described by Li et al. (1998), using DNAs isolated from the three sediments from the west, mid-, and east Pacific as templates. The pressure-regulated operons could all be amplified from the three deep-sea sediment samples, but not from the shallow seashore sediment and the Antarctic soil sediment sample which were assayed for comparison (photo not shown). This result indicated the wide presence of the piezophilic *Shewanella* species in geographically different deep-sea sediments of varying depths across the Pacific.

From the west Pacific site WP, 13 morphologically distinct strains were isolated under atmospheric pressure. Among the strains, three were alkaliphilic halophiles and four were psychrotrophs/psychrophiles. The 16S rDNA fragments around 1.5 kb of the extremophilic strains were amplified, sequenced, and analyzed (see Table 1). All the seven strains belonged to the *Proteobacteria* including *Shewanella*, *Halomonas*, *Psychrobacter*, and *Hyphomicrobium*. Two psychrotrophic/psychrophilic strains designated WP2 and WP3 were assigned to the genus *Shewanella*. The extremophilic strains were also isolated from the mid- and east Pacific sites MP and A; no *Shewanella* strains were isolated in these two sediments of 5,027 m and 5,774 m by the isolation methods we used under atmospheric pressure (unpublished data). The 16S rDNA sequence of WP2 (AJ551089) and WP3 (AJ551090) had 97% similarity; WP2 had the highest similarity with *S. fidelis* (97%); WP3 had the highest similarity with *S. benthica* (97%). The phylogenetic relationships of WP2 and WP3 with other *Shewanella* species are depicted in Fig. 1.

The presence of the deep-sea piezophilic *Shewanella*-specific pressure-regulated operons was further investigated in WP2 and WP3. Both strains were found to be as capable of amplifying the specific bands for the pressure-regulated operons as *S. benthica* and *S. violacea* (photo not shown). The amplified fragments from WP2 and WP3 were cloned and sequenced. The DNA sequences of the amplified fragments of WP2, WP3, *S. benthica* strains and *S. violacea* were aligned and are shown in Fig. 2. The ORF1,2 operons of WP2 and WP3 are identical; the ORF3 operons of WP2 and WP3 have 94% identity. ORF1,2 and ORF3 of WP2 and WP3 show 77%–90% and 89%–94% identity with those of *S. benthica* and *S. violacea* strains, respectively.

The growth temperature ranges of WP2 and WP3 were determined. WP2 could grow at temperatures between

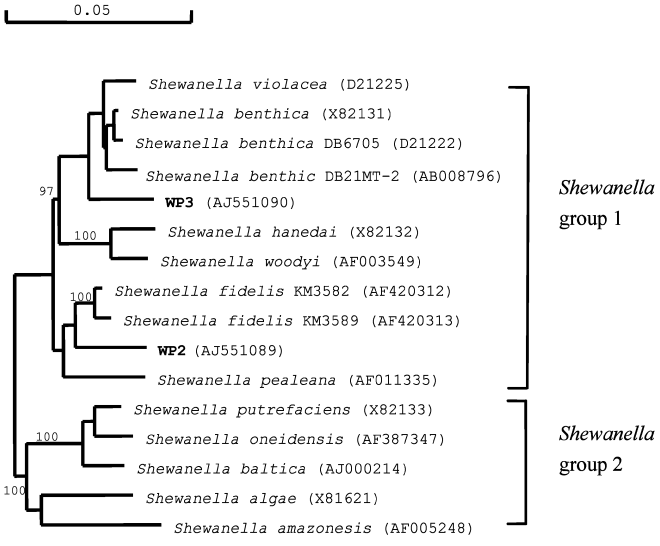

Fig. 1 Phylogenetic tree showing the relationships of WP2 and WP3 with other related *Shewanella* reference strains. The dendrogram was constructed from a matrix of pairwise genetic distances by the neighbor-joining method (Saitou and Nei 1987) using the DNAMAN program. The bootstrap values above 90% from 1,000 replicates are shown. The *scale bar* represents 0.05 substitutions per amino-acid site

Table 1 Isolated extremophiles from the west Pacific station WP[a]

Isolate no.	Closest related species (16S rDNA accession number, similarity %)	Enzyme production
Alkaliphilic halophiles		
WP1	*Halomonas* sp. (AF316143, 99%)	Esterase
WP4	*Halomonas meridiana* (AJ306891, 99%)	Esterase
WP5	*Halomonas aquamarina* (AJ306888, 99%)	Esterase
Psychrotrophs/psychrophiles		
WP2	*Shewanella fidelis* (AF420313, 97%)	β-Galactosidase
WP3	*Shewanella benthica* (D21221, 97%)	Esterase, β-galactosidase, gelatinase, amylase
WP8	*Psychrobacter pacificensis* (AB016059, 99%)	Esterase, gelatinase
WP13	*Hyphomicrobium* sp. (AB055793, 98%)	Esterase, gelatinase, amylase, lecithinase

[a] The sediment sample was melted at 2°–4°C and diluted in a ratio of approximately 1:5 in artificial sea water, 100 μl of the suspension was spread on Marine 2216 agar plates and incubated at 10°C. Modified Marine agar plates of different pH (3, 7.6, 9.7) and NaCl concentrations (0%, 5%, 15%) were used for the isolation of acidophiles, alkaliphiles, and halophiles as described previously (Takami et al. 1999). Strains were selected on the basis of differing morphology of colonies. Enzyme production of the strains, including esterase, protease, β-galactosidase, chitinase, gelatinase, amylase, and lecithinase, were checked using published procedures (see Bowman 2001)

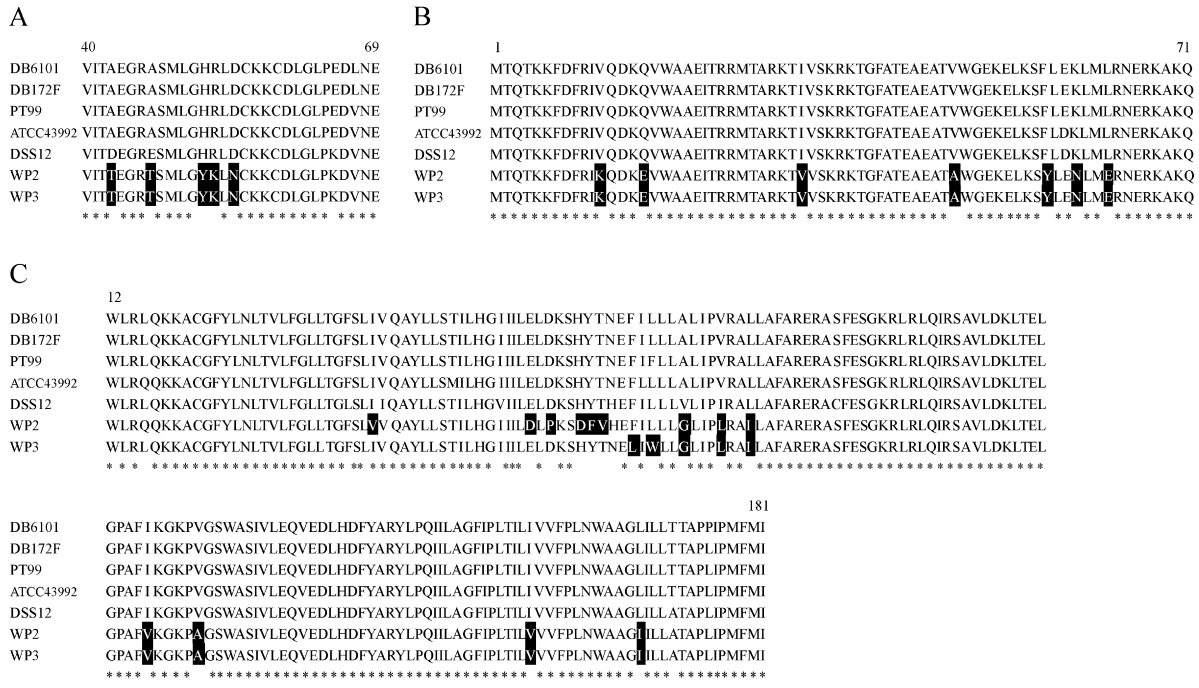

Fig. 2 Alignment of the deduced amino acids of ORF1 (**A**), ORF2 (**B**) and ORF3 (**C**). The identical residues are marked by an *asterisk*; the amino acids in WP2 and WP3 which are different from their counterparts in *S. benthica* or *S. violacea* strains are indicated by *white characters on a black background.* The nucleotide sequences of the operons from WP2 and WP3 were sent to the EMBL, GenBank, and the DDBJ databank, the accession numbers are AJ566371, AJ566373 for *orf1–orf2*, *orf3* of WP2; AJ566372, AJ566374 for *orf1–2*, *orf3* of WP3. The DNA sequence accession numbers of the pressure-regulated operons (*orf1,2–orf3*) for the *S. benthica* strains DB6101, DB172F, PT99, ATCC43992 and *S. violacea* DSS12 are D88780–D88786, D88782–D88788, D88783–D88789, and D88784–D88790, respectively

Table 2 Major fatty-acid composition of strains.*Shewanella benthica* was grown at 10°C, WP2 at 10°C, and WP3 at 15°C

Fatty acid	WP2	WP3	*S. benthica* ATCC43992^T	*S. fidelis*[a] KMM3582^T
OH-i-13:0	8	8	4	–[b]
14:0	8	3	6	2.3
i-15:0	3	10	3.5	22.3
16:0	13	10	10	12.9
16:1	36	24	33	20
18:1	8	9	8	5.6
20:5	7	13	16	–[b]

[a] Data from Ivanova et al. (2003)
[b] –, not detected

0° and 20°C; its optimal growth temperature was around 10–15°C. WP3 could grow at a temperature range of 0° to 28°C; best growth temperature was 15–20°C. Both of the strains are neutrophiles. After around 6–7 days' incubation on Marine 2216 agar plates at 10°C, both strains formed 0.5-mm diameter, round-edged, faint yellowish colonies. After 5 days' incubation in Marine 2216 broth, the cell aggregates had turned a pinkish color. Using light microscopy observation, the two strains were shown to be rod-shaped (photo not shown).

The main fatty acid composition of WP2 and WP3 is given in Table 2. Both have a similar fatty acid profile typical of *Shewanella*. 3OH-i-13:0, 14:0, i-15:0, 16:0, 16:1, and 18:1 are the main components of their fatty acids. At the same time, substantial amounts of eicosapentaenoic acid (EPA, 20:5) were found in both WP2 and WP3. Furthermore, the relation of EPA content in the cell with the growth temperature of WP2 and WP3 was investigated and is shown in Fig. 3. This shows that the percentage of EPA in the WP2 cells was stable between 10° and 20°C (its optimum growth temperature range); it could produce 7% EPA at its highest growth temperature (20°C), which is nearly the same amount as at its optimal growth temperature around 10–15°C; the EPA composition increased dramatically at 4°C and further increased to reach its highest value at 0°C. Although WP3 could only produce traces (1.6%) of EPA at its highest growth temperature (28°C), it increased its EPA production dramatically at its optimal growth temperature (15–20°C), and the EPA concentration increased gradually from 20° to 4°C, reaching its highest value at 0°C (Fig. 3).

The 16S rDNA sequence of WP2 had the highest similarity (97%) with *S. fidelis*. *S. fidelis* strains are newly characterized mesophilic *Shewanella* species isolated from shallow water sediments of the South China Sea and the seawater of the Japan Sea (Ivanova et al. 2003). From published descriptions, *S. fidelis* has a growth temperature range from 4° to 30°C, with an optimal growth temperature of 20–25°C. Its predominant fatty acids are i-13:0, i-15:0, 16:0, and 16:1, and it could not produce EPA. WP2 showed substantially different characteristics from *S. fidelis*: it is psychrophilic, grows at a temperature range of 0°–20°C, with

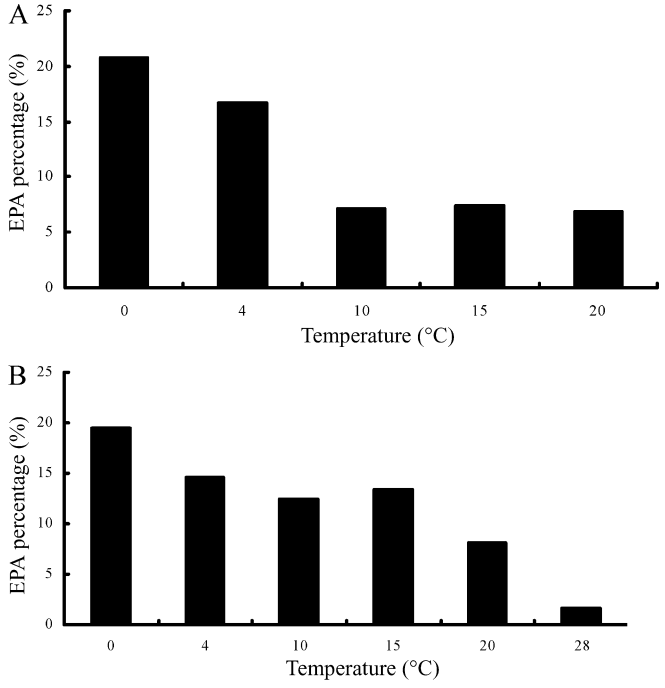

Fig. 3 EPA percentage composition in WP2 (**A**) and WP3 (**B**) at different temperatures. Cells were grown at different temperatures in Marine 2216 broth to early stationary phase, washed twice with 3% NaCl at 4°C, centrifuged at 8,000 *g*, then freeze-dried. 10 mg of dried cells was put in a Teflon-lined, screw-capped tube with 2 ml anhydrous methanolic HCl. The tubes were tightly closed and sonicated for 5 min, then heated at 100°C for 40 min. After cooling, 1 ml of *n*-hexane was added for extracting the fatty acid methyl esters. The extracting procedures were repeated three times. The analysis of fatty acid (*FA*) methyl ethers was performed by gas–liquid chromatography (model Agilent 6890)

optimal growth occurring at 10–15°C; 3OH-i-13:0, 14:0, 16:0, 16:1, and 18:1 are the predominant fatty acid components and it could produce large quantities of EPA at each growth temperature. Our results strongly suggest that WP2 does not belong to *S. fidelis*, but is a new deep-sea *Shewanella* species. The 16S rDNA sequence of WP3 showed the highest similarity (97%) with *S. benthica* and *S. violacea*. Total DNA of WP3 showed 24%, 19%, and 40% homology with that of WP2, *S. benthica* ATCC43992^T and *S. violacea* JCM10179^T, respectively, indicating that they are different species. The 16S rDNA sequence analysis and DNA–DNA hybridization results confirm that WP3 is a new deep-sea *Shewanella* species. Both WP2 and WP3 could be assigned to *Shewanella* group 1 as classified by Kato and Nogi (2001; see Fig. 2).

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