**Shewanella pneumatophori** sp. nov., an eicosapentaenoic acid-producing marine bacterium isolated from the intestines of Pacific mackerel (*Pneumatophorus japonicus*)

Kikue Hirota,¹ Yoshinobu Nodasaka,² Yoshitake Orikasa,³ Hidetoshi Okuyama³ and Isao Yumoto¹,⁴

1Research Institute of Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology (AIST), Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan
2Laboratory of Electron Microscopy, Graduate School of Dentistry, Hokkaido University, Kita-ku, Sapporo 060-8586, Japan
3Laboratory of Environmental Molecular Biology, Graduate School of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan
4Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan

An eicosapentaenoic acid-producing bacterium, previously described as *Shewanella* sp. strain SCRC-2738, was classified by phenotypic characterization, chemotaxonomic analysis, 16S rRNA gene sequence analysis and DNA–DNA hybridization. The isolate was Gram-negative, rod-shaped and motile by using polar flagella. The strain grew at 4–32 °C; the optimum growth temperature was 27 °C. NaCl was required for growth. The major isoprenoid quinones were ubiquinone-7 and ubiquinone-8 and its DNA G+C content was 42 mol%. The whole-cell fatty acids mainly (above 5%) consisted of iso-C₁₃ : ₀, iso-C₁₅ : ₀, C₁₆ : ₀, C₁₆ : ₁₀⁷c, C₁₈ : ₁₀⁷c and C₂₀ : ₅₃ (eicosapentaenoic acid). Phylogenetic analysis based on the 16S rRNA gene sequence showed that strain SCRC-2738T was related closely (sequence similarities above 99%) to *Shewanella marinintestina* (99.3%), *Shewanella sairae* (99.3%) and *Shewanella schlegeliana* (99.2%). DNA–DNA hybridization and phenotypic characteristics confirmed that strain SCRC-2738T merited classification as a novel species of the genus *Shewanella*, for which the name *Shewanella pneumatophori* sp. nov. is proposed. The type strain is SCRC-2738T (= JCM 13187T = NCIMB 14060T).

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are kinds of polyunsaturated fatty acid (PUFA) present in fish, e.g. tuna, mackerel and sardines. Such PUFAs exhibit physiological and pharmacological effects (Terano *et al*., 1984; Harris, 1989; Abbey *et al*., 1990; Radwan, 1991) and are commercially very important as additives in food, cosmetics and products of the health industries (Uauy-Dagach & Valenzuela, 1992). In most cases, the main source of commercially available EPA and DHA is fish. However, several problems exist in terms of their production from fish, such as difficulty in obtaining highly pure products and an unpleasant smell. Several strains belonging to the genus *Shewanella* have been reported as being high-level PUFA-producing micro-organisms. Besides these, strains belonging to the genera *Colwellia*, *Photobacterium*, *Flexibacter*, *Vibrio* and *Moritella* are also PUFA-producing micro-organisms (Bowman *et al*., 1998; Russell & Nichols, 1999; Allen & Bartlett, 2002). Some of these strains produce such PUFAs in relation to their piezophilic or psychrophilic natures (Yano *et al*., 1997; Russell & Nichols, 1999).

In 1988, Yazawa *et al*. (1988a, b) first discovered two bacterial strains, from fish intestines, able to produce EPA; the strains were tentatively identified as *Alteromonas* spp. One of the two strains, named *Alteromonas* sp. SCRC-2738 (Yazawa *et al*., 1988a), was isolated from Pacific mackerel, *Pneumatophorus japonicus*. This strain produces 36-3% EPA with respect to the total fatty acid content under appropriate culture conditions. The strain was renamed *Shewanella* sp. SCRC-2738 concomitant with the dissociation of the genus...
Shewanella from the genus Alteromonas (MacDonell & Colwell, 1985). The 38 kb EPA-synthesis gene cluster was first isolated from strain SCRC-2738 (Yazawa, 1996). This gene cluster transformed Escherichia coli cells, enabling them to synthesize significant amounts of EPA. The gene cluster consisted of five open reading frames as essential components of EPA production. The function of these gene clusters has also been examined (Orikasa et al., 2004). Although, as described above, strain SCRC-2738 is very important in studies on bacterial EPA production, it has not been identified to species level. In the present study, taxonomic studies of strain SCRC-2738 were performed.

Shewanella sp. SCRC-2738 was obtained from the Sagami Chemical Research Center (Sagamihara, Kanagawa Prefecture, Japan). The micro-organism was cultivated until the late-exponential phase of growth by reciprocal shaking (140 r.p.m.) at 27 °C in marine broth 2261 (Difco), for chemotaxonomic studies, or for 1–2 days at 27 °C in marine agar 2216 (Difco), for preparation of inoculum and maintenance of the culture. In addition to strain SCRC-2738, Shewanella fidelis ATCC BAA-318T, Shewanella sairae JCM 11563T, Shewanella marinintestina JCM 11558T and Shewanella schlegeliana JCM 11561T were used as reference strains for DNA–DNA hybridization. These microorganisms were cultivated in marine broth 2216 by reciprocal shaking (140 r.p.m.) at 20 °C.

For morphological and phenotypic characterization, marine broth 2216 was used as the basal medium for aerobic cultivation. Samples for transmission electron microscopy were prepared and observed as described previously (Yumoto et al., 2001), using an H-800 Hitachi microscope. The culture was incubated at 27 °C for 2 weeks and experiments were performed three times to confirm the reproducibility of the results. Carbohydrate metabolism was tested for according to the method of Leifson (1963). The results were checked daily for 2 weeks after inoculation. The hydrolysis of macromolecular substances, the test for various substrates as sole sources of carbon and energy and determination of the growth temperature were performed as described previously (Kawasaki et al., 2002; Yumoto et al., 2003). Other morphological, physiological and biochemical tests were performed as described by Barrow & Feltham (1993). The results obtained for the morphological, physiological and biochemical characteristics of strain SCRC-2738 are given in the species description.

Cells for chemotaxonomic analysis were prepared by using cultivation in marine broth 2216 with reciprocal shaking (140 r.p.m.) at 27 °C. Whole-cell fatty acids and isoprenoid quinones were analysed as described previously (Yumoto et al., 2001). GLC analysis (with GC-353 apparatus; GL Science) revealed that the cellular fatty acid composition of strain SCRC-2738 was as follows (means of two datasets obtained from two independent samples): C12 : 0(4·3 %), iso-C13 : 0(5·4 %), C14 : 0(3·5 %), iso-C15 : 0(8·3 %), anteiso-C15 : 0(1·3 %), C15 : 0(0·5 %), C16 : 0(19·9 %), C16 : 1o9c (1·3 %), C16 : 1o7c (27·7 %), C17 : 1(0·9 %), C18 : 0(9·9 %), C18 : 1o9c (3·1 %), C18 : 1o7c (10·0 %), C18 : 1o11c (0·8 %), C20 : 4(0·8 %), C20 : 5(9·5 %) and others (1·8 %).

The fatty acid composition of strain SCRC-2738 was similar to those of S. marinintestina, S. schlegeliana, S. sarae (Satomi et al., 2003) and Shewanella gelidimarina (Bowman et al., 1997). Yazawa et al. (1988a) reported that strain SCRC-2738 produces 27·8–36·6 % EPA with respect to the total fatty acid content. The difference between that value and the EPA content determined in the present study may due to differences in the medium and culture conditions used. The major isoprenoid quinones detected were ubiquinone-7 and ubiquinone-8.

Bacterial DNA was prepared according to the method of Marmur (1961). The DNA G + C content was determined by using the method of Tamaoka & Komagata (1984) and was found to be 42·8 mol%.

The 16S rRNA gene was amplified by using the PCR method with primers 9F (5’-GAGTTTGTGATCCTGCTCAG-3’) and 1541R (5’-AAGGAGGTGATCCAG-3’). The PCR product, approximately 1·5 kb in size, was sequenced by the dideoxynucleotide chain-termination method, using a BigDye terminator cycle sequencing kit (Applied Biosystems) and a DNA sequencer (ABI Prism 3100). Primers 9F, 339F, 785F, 1223R and 357R were used in the gene-sequencing reaction. Multiple alignments of the sequences were performed and the nucleotide-substitution rate (K_m value) was calculated. A phylogenetic tree was constructed by the neighbour-joining method (Kimura, 1980; Saitou & Nei, 1987) using the CLUSTAL W program (Thompson et al., 1994). Sequence similarity was calculated by using the GENETYX computer program (Software Development). The phylogenetic position, based on the determined sequence of strain SCRC-2738 and comparison with sequences in nucleotide databases, indicated that strain SCRC-2738 belonged to the genus Shewanella. Strain SCRC-2738 was found to have highest levels of similarity with S. fidelis ATCC BAA-318T (98·5 %), S. schlegeliana JCM 11561T (98·5 %), S. sarae JCM 11563T (99·3 %) and S. marinintestina JCM 11558T (99·3 %) (Fig. 1). These strains (with the exception of S. fidelis ATCC BAA-318T) produce large amounts of EPA.

The results of the 16S rRNA gene sequence analysis showed that strain SCRC-2738 is related closely to S. fidelis ATCC BAA-318T, S. schlegeliana JCM 11561T, S. sarae JCM 11563T and S. marinintestina JCM 11558T. These strains were used as reference strains for DNA–DNA hybridization. The level of DNA–DNA relatedness was determined fluorometrically by the method of Ezaki et al. (1989), using photobiotin-labelled DNA probes (Photoprobe biotin; Vector Laboratories) and black microplates (F16 Black Maxisorp; Nage Nunc International). The DNA–DNA hybridization results indicate that strain SCRC-2738 belongs to a species distinct from S. fidelis ATCC BAA-318T (37·1 % DNA–DNA relatedness), S. schlegeliana JCM 11558T (38·7 %), S. sarae JCM 11563T (13·9 %) and S. marinintestina JCM 11558T (8·1 %). These values were reproducible. Strain SCRC-2738 was thus confirmed to
belong to a species different from other phylogenetically related *Shewanella* strains.

Strain SCRC-2738 differed phenotypically and chemotaxonomically from phylogenetically closely related species and from other *Shewanella* strains, as shown in Table 1.

The growth characteristics and EPA production of strain SCRC-2738 were different from those of *S. fidelis*. Although strain SCRC-2738 exhibited very high levels of similarity with *S. schlegeliana* JCM 11561\(^T\), *S. sairae* JCM 11563\(^T\) and *S. marinintestina* JCM 11558\(^T\) in terms of 16S rRNA gene sequence, there were obvious differences in growth characteristics, the fermentation of D-glucose (Table 1), colony colour and nitrate reduction to nitrite (Satomi *et al.*, 2003).

On the basis of the above results, strain SCRC-2738 was designated to belong to a novel species of the genus *Shewanella*, for which the name *Shewanella pneumatophori* sp. nov. is proposed; the type strain is SCRC-2738\(^T\).

### Table 1. Characteristics of *S. pneumatophori* sp. nov. and related species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 °C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>32 °C</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>37 °C</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Growth in NaCl at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 %</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6 %</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Fermentation of D-glucose</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of D-glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EPA production</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Chitin</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DNA</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>ONPG test</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>43</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>43</td>
<td>45</td>
<td>48</td>
<td>44</td>
<td>46</td>
<td>45</td>
<td>39</td>
<td>40–43</td>
<td>43–44</td>
</tr>
</tbody>
</table>
Description of *Shewanella pneumatophori* sp. nov.

*Shewanella pneumatophori* (pneu.ma.to pho’ri. N.L. gen. n. pneumatophori of Pneumatophorus japonicus, the scientific name of the Pacific mackerel).

Cells are rod-shaped (0·4–0·6 μm × 1·4–4·6 μm), Gram-negative and have polar flagella. Colonies are circular with entire margins and are slightly pinkish. Catalase and oxidase reactions are positive. Growth occurs in the presence of 1–8% NaCl. Growth occurs at 4–32°C (optimum, 27°C). The organism is positive for H₂S production, but negative results are obtained in methyl red, Voges–Proskauer and ONPG tests, for nitrate reduction to nitrite and for indole production. Hydrolyses casein, gelatin, DNA and Tweens 20, 40, 60 and 80, but not chitin, starch, aesculin or hippurate. Acid is not produced oxidatively from L-arabinose, D-fructose, D-maltose, D-mannose, melibiose, sucrose, D-xylose, raffinose, *myo*-inositol, mannitol, sorbitol, D-galactose, L-rhamnose, trehalose, lactose or glycerol. Acid is produced from D-glucose, D-fructose and D-galactose under anaerobic conditions. Utilizes D-glucose as sole carbon source, but not L-arabinose, D-fructose, D-maltose, D-mannose, melibiose, sucrose, D-xylose, raffinose, *myo*-inositol, mannitol, sorbitol, D-galactose, L-rhamnose, cellobiose, trehalose, lactose or glycerol. The major isoprenoid quinones are ubiquinone-7 and ubiquinone-8. The major (above 5%) fatty acids are iso-C₁₅ : 0, iso-C₁₅ : 0, C₁₆ : 0, C₁₆ : 1ω₅c and C₂₀ : 5ω₃ (EPA). The DNA G+C content is 42·8 mol%.

The type strain, SCRC-2738T (= JCM 13187T = NCIMB 14060T), was isolated from the intestines of Pacific mackerel (*Pneumatophorus japonicus*).

**References**


