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Shewanella oshoroensis sp. nov.: A Mesophilic Eicosapentaenoic Acid and Hentriacontanonaene-producing Bacterium

[1]Shinji Sugihara, [1]Ahmad Iskandar Bin Haji Mohd Taha, [1]Taro Motoigi, [2]Akio Ueno, [2]Satoru Shimizu, [3]Mamiko Sato, [4]Takako Kaneko, [5]Kentaro Watanabe, [6]Isao Yumoto, and [1,7]Hidetoshi Okuyama

[1]Course in Environmental Molecular Biology and Microbial Ecology, Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan
[2]Subsurface Microbiology Research Group, Horonobe Research Institute for the Subsurface Environment (H-RISE), Horonobe, Hokkaido 098-3221, Japan
[3]Laboratory of Electron Microscopy, [4]Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Bunkyo-ku, Tokyo 112-8681, Japan
[5]Bioscience Group, National Institute of Polar Research, Tachikawa, Tokyo 190-8518, Japan
[6]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Toyohira-ku, Sapporo 062-8517, Japan
[7]Laboratory of Environmental Molecular Biology, Faculty of Environmental Earth Science, Hokkaido University, Kitaku-ku, Sapporo 060-0810, Japan

Corresponding Author: Hidetoshi Okuyama, Laboratory of Environmental Molecular Biology, Faculty of Environmental Earth Science, Hokkaido University, Kitaku-ku, Sapporo 060-0810, Japan

ABSTRACT

A mesophilic marine bacterial strain (strain osh08^T) that produced eicosapentaenoic acid and hentriacontanonaene, was identified by the molecular and ordinary microbiological methods. This strain had the high sequence similarity of its 16S rRNA gene to those of *Shewanella basaltis* JCM 14937^T (EU143361; 99.8%) and *Shewanella hafniensis* NBRC 100975^T (AB205566; 98.4%). But, strain osh08^T was phenotypically different from *S. basaltis* J83^T and *S. hafniensis* NBRC 100975^T in that it was able to ferment glucose, produce β-glucosidase and β-galactosidase and utilize D-mannose and unable to utilize malate. However, all three strains used iron(III) and manganese(IV) as terminal electron acceptors under anaerobic conditions. The DNA-DNA relatedness between strain osh08^T and *S. basaltis* J83^T and that between strain osh08^T and *S. hafniensis* NBRC 100975^T were 4.7% and 11.8%, respectively. The G+C content of strain osh08^T was 43.4 mol%. Strain osh08^T had, in addition to a polar flagellum, many pilus-like structures around the cell that were not observed in the cells of *S. basaltis* J83^T. Thus strain osh08^T was identified as a new species of *Shewanella*. The name *Shewanella oshoroensis* sp. nov. was proposed and strain osh08^T (=NBRC 107685^T) is the type strain.

Key words: Long chain hydrocarbon, marine mesophile, polyunsaturated fatty acid-producing bacteria, *Shewanella oshoroensis*

INTRODUCTION

Previously a mesophilic bacterial strain that produced eicosapentaenoic acid (EPA) was isolated from an alga sample collected on the Hokkaido coast, a cool-temperate region of

Japan (Sugihara *et al.*, 2010). This strain has been tentatively identified as *Shewanella* sp. strain osh08^T based on the high similarity of its 16S rRNA gene sequence with those of *Shewanella basaltis* J83^T (EU143361; 99.8%) and *Shewanella hafniensis* NBRC 100975^T (AB205566; 98.4%). *Shewanella* sp. strain osh08^T produced, in addition to EPA, a very long-chain polyunsaturated hydrocarbon, hentriacontanonaene (C31:9). C31:9 has been detected only from psychrophilic bacteria from polar marine (Nichols *et al.*, 1995) and deep sea environments (Tamaoka *et al.*, 1998). The configuration and positions of the double bonds of this compound were determined to be all *cis* at positions 3, 6, 9, 12, 15, 19, 22, 25 and 28 (Sugihara *et al.*, 2010). As C31:9 have been detected only in EPA- or docosahexaenoic acid (DHA)-producing bacteria and the EPA-deficient mutant did not produce C31:9, it is speculated that the *pfa* genes responsible for the biosynthesis of EPA and DHA are involved in the synthesis of C31:9 (Sugihara *et al.*, 2010). Recently it was shown that C31:9 can be generated by head-to-head condensation of two hexadecatetraenoic fatty acid molecules (Sukovich *et al.*, 2010), although no biochemical evidence has been available. Strain osh08^T is the first reported EPA and C31:9-producing bacterium isolated from non-psychrophilic environments. In this study, strain osh08^T was taxonomically characterized as a new *Shewanella* sp. and the name *Shewanella oshoroensis* sp. nov. was proposed.

MATERIALS AND METHODS

Sampling, bacterial strains and culture conditions: Seaweed samples (fragments of red alga), which were used to isolate *Shewanella* sp. strain osh08^T, were collected on the mid-latitude seashore of Oshoro, Hokkaido, Japan (43.19 °N, 141.00 °E), as described previously (Sugihara *et al.*, 2010). Samples were subjected to isolation and purification procedures immediately after collection using ZoBell agar medium (Z medium: 0.1% peptone, 0.1% yeast extract, 0.01% $Fe_3(PO_4)_2$ and 1.5% agar) in 50% (v/v) seawater (ZoBell, 1946). Filtered natural seawater was utilized to prepare media (Sugihara *et al.*, 2010). Plates were then incubated at 20°C for several days. Bacterial colonies were directly treated with acetyl chloride in methanol for methanolysis, as described below. *Shewanella* sp. strain osh08^T was the sole EPA-producing bacterium isolated in this study. The following type strains were used: *Shewanella hafniensis* NBRC100975^T (Satomi *et al.*, 2006), *Shewanella basaltis* J83^T (Chang *et al.*, 2008) and *Shewanella oneidensis* MR-1^T (ATCC 700550^{TM}) (Venkateswaran *et al.*, 1999). Strain osh08^T and these reference strains were cultivated for characterization at the indicated temperatures in Luria-Bertani (LB) medium containing 3.0% NaCl.

Physiological and biochemical characterization of strain osh08^T: Routine biochemical tests were performed as described in Cowan and Steel's manual (Barrow and Feltham, 1993). Reduction of Mn(IV) under anaerobic conditions was ascertained by clear zone formation and visible growth of cells during cultivation on agar plates using the published method of Myers and Nealson (1988), with a slight modification and reduction of Fe(III) by the same method as described above but Mn(IV) was substituted with iron, using not only strain osh08^T but also *S. hafniensis* NBRC100975^T, *S. basaltis* J83^T and *S. oneidensis* MR-1^T (ATCC 700550^{TM}) for comparison. Ferric citrate (100 mM) and manganese dioxide (100 mM) were used as electron acceptors. Sodium acetate (15 mM) was added to both media as electron donor. Agar plate preparation and inoculation of cells were carried out under anaerobic conditions (10% hydrogen, 10% carbon dioxide and 80% nitrogen) in an anaerobic chamber (Te-Her Anaerobox ANX-1W, Hirasawa Works, Tokyo). Plates were then incubated anaerobically at 30°C for more than two weeks. For the determination of other

Table 1: Phenotypic characteristics of strain osh08^{T} and related type strains[1]

	Strains						
	1	2	3	4	5	6	7
Optimum temperature for growth (°C)	30	25	30[2]	30	20-25	4-20	30
Growth at 37 °C	+	–	+	+	–	–	+
40°C	+	–	–	–	–	–	+
in 0% NaCl	+	+	+[2]	–	+	+	+
7% NaCl	+	+	–	+	–	–	–
at pH 10.0	+	+[2]	+	NA	NA	–	+[2]
DNA G+C (mol%)	43.4	47	NA	42	46.8-48.1	41	45
Nitrite reduction	–	–	–	–	+	–	+
Fermentation of glucose	+	–	–	–	–	+	–
Production of indole	–	–	–	NA	NA	NA	–
Production of gelatinase	+	+	–	+	+	+	+
Arginine dihydrolase	–	–	–	NA	NA	–	–
β-glucosidase	+	–	–	–	+	–	+[2]
β-galactosidase	+	–	–	–	–	+	+[2]
Urease	–	–	–	NA	NA	–	–
Utilization of glucose	+	+	+	–	+	+	+[2]
L-arabinose	–	+	–	–	NA	–	–
D-mannose	+	–	–	–	NA	+	+[2]
D-mannitol	–	–	+	–	–	+	–
N-acetylglucosamine	–	+	–	+	+	+	+[2]
Maltose	+	+	+	–	+	+	–[3]
Gluconate	–	+	–	NA	NA	–	–[2]
Caprate	–	+	–	NA	NA	–	–[2]
Adipate	–	–	–	NA	NA	–	–[2]
Malate	–	+	+	–	–	–	–
Citrate	–	+	–	–	–	–	–
Reduction of iron oxide	+	+[2]	+[2]	NA	–	+	+[3]
Manganese dioxide	+	+[2]	+[2]	NA	NA	NA	+[3]

[1]Strains 1: osh08^{T} (present study), 2: *S. hafniensis* NBRC 100975^{T}; Satomi *et al.* (2006), 3: *S. basaltis* J83^{T}; Chang *et al.* (2008), 4: *S. gaetbuli* LMG 19866$_{T}$; Yoon *et al.* (2004), 5: *S. denitrificans* OS217$_{T}$; Brettar *et al.* (2002), 6: *S. livingstonensis* LMG 19866$_{T}$; Bozal *et al.* (2002) 7: *S. oneidensis* MR-1^{T}; Venkateswaran *et al.* (1999), [2]Result obtained in the present study, [3]The same result was obtained in this study, +: Positive, -: Negative, NA: No data available, All strains grew at 4°C and were positive for cytochrome oxidase and catalase and for reduction of nitrate to nitrite

phenotypic characteristics, API 20NE tests were performed in triplicate using the methods recommended by the manufacturer (bioMérieux Japan, Tokyo, Japan). Some phenotypic characteristics unavailable for *S. oneidensis* MR-1^{T} (ATCC 700550^{TM}) were also examined in this study using API 20NE tests (Table 1).

The growth of *S. hafniensis* NBRC100975^{T} and *S. oneidensis* MR-1^{T} (ATCC 700550^{TM}) in medium at pH 10 and that of *S. basaltis* J83^{T} in medium containing no NaCl, which had not been previously reported, were determined as described previously (Sugihara *et al.*, 2010). Optimum temperature for growth of *S. basaltis* J83^{T} was evaluated visibly by the extent of growth on LB agar plates containing 3% NaCl incubated at 20, 25, 30 and 35°C for 24 h.

Electron microscopy: Cells of strain osh08^{T}, which were grown on liquid Z medium as described above, were suspended in liquid Z medium. A small drop of the suspension was placed on a carbon-

coated copper grid and negatively stained with 2% (w/v) uranyl acetate as described (Haschemeyer and Myers, 1972) for observation with a transmission electron microscope (JEM1200EXS, JEOL, Tokyo).

DNA procedures: Genomic DNA was extracted from strain osh08^T and various type strains by the method of Marmur (1961). High-performance liquid chromatography (HPLC) was used to determine the DNA G+C content (Tamaoka and Komagata, 1984). Briefly, the DNA preparations were denatured at 100°C for 10 min, digested with nuclease P1 (Yamasa Shoyu, Tokyo) and subjected to HPLC using a Cosmosil 5C18 column (type: Waters, 4.6×150 mm, Nakalai Tesque, Kyoto, Japan). An equimolar mixture of four deoxyribonucleotides (Yamasa Shoyu) was used as the standard. The nucleotides were detected with a spectrophotometer detector set at 260 nm.

Levels of DNA relatedness were determined fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labeled DNA probes and microplates.

Amplification, cloning and sequencing of the 16S rRNA gene were performed as described previously (Sugihara *et al.*, 2010). The sequence-comparative searches were performed using the NCBI (http://www.ncbi.nlm.nih.gov/) databases. Multiple alignments of the sequence were performed, nucleotide substitution rates (K_{nuc} values) were calculated and a neighbour-joining phylogenetic tree was constructed using the CLUSTALW program of DDBJ (http://clustalw.ddbj.nig.ac.jp/top-j.html). The accuracy of the phylogenetic tree branches was confirmed by a bootstrap method (Felsenstein, 1985). The strain osh08^T 16S rRNA gene sequence was previously deposited in DDBJ/GenBank/EMBL with the accession number AB447987 (Sugihara *et al.*, 2010).

Analysis of fatty acids and polyunsaturated hydrocarbons: The cellular fatty acid composition of the isolates was determined as described previously (Sugihara *et al.*, 2010) with a minor modification. Briefly, small aliquots of wet cells were subjected to methanolysis using 1 mL of 10% (v/v) acetyl chloride in methanol at 100°C for 1 h. A 10 μg aliquot of heneicosanoic acid (21:0; Sigma-Aldrich, Tokyo) was used as an internal standard. Fatty acid methyl esters were extracted with hexane together with hydrocarbon and this was designated the fatty acid and hydrocarbon fraction. In this study, the amount of hydrocarbons was estimated using 21:0 as the internal standard, because the difference in the amount of hydrocarbons estimated using either 21:0 or hydrocarbon (n-tetracosane) as standards was negligible.

Fatty acid methyl esters and hydrocarbons were analysed by gas-liquid chromatography (GLC) as described by Sugihara *et al.* (2010) on a gas chromatograph (model GC-353B; GL Sciences, Tokyo) equipped with a capillary column BPX70 (25 m long×0.22 mm I.D., 0.25 mm film thickness, SGE Japan, Yokohama, Japan) and flame ionization detection with nitrogen as the carrier gas. These compounds were identified by ion-trap mode gas chromatography-mass spectrometry (GC/MS) on a Varian system (model CP-3800 gas chromatograph and a Saturn 2200 ion-trap mass spectrometer, Varian Technologies Japan, Inc., Tokyo, Japan) under the same conditions as described previously (Orikasa *et al.*, 2006). Data were analysed using a Saturn™ Software Workstation Version 5.52. In GC/MS analysis, two modes of electron impact ionization and chemical ionization were utilized. In chemical ionization-gas chromatography-mass spectrometry, acetonitrile was used as an ionization reagent.

RESULTS AND DISCUSSION

Morphological characteristics: Strain osh08^T was Gram-negative (Sugihara *et al*., 2010) and had a single unsheathed flagellum (Fig. 1a). Interestingly, the strain had pilus-like structures 40-500 nm in length on the cell surface (Fig. 1b). Although *S. basaltis* J83^T is the species closest to strain osh08^T in its 16S rRNA gene sequence (Fig. 2), it has no pilus-like structures (not shown). The typical form of strain osh08^T cells was rods 0.3-0.6 mm wide by 0.8-1.5 mm long.

Strain osh08^T formed opaque, pink-brown, glossy, convex and rounded colonies with an entire margin. The size of the colonies was less than 1 mm in diameter after incubation for 18 h at 30°C. A clear zone approximately 1 mm wide around the colony was observed only for strain osh08^T. This clear zone may be generated by digestion of peptides in the medium by secreted peptidase.

Physiological and biochemical characteristics: Strain osh08^T grew in a temperature range of 4 to 40°C with an optimum temperature of 30°C (Sugihara *et al*., 2010). It grew in media containing no NaCl and in media with 7% NaCl and growth was observed at pH 10. The physiological and biochemical characteristics of strain osh08^T were compared with those of six type strains of *Shewanella* species (Table 1).

When strain osh08^T was compared with the six reference strains, characteristics common to all strains were growth at 4°C, positive oxidase and catalase reactions and nitrate reduction. Strain osh08^T, *S. basaltis* J83^T and *S. hafniensis* NBRC 100975^T had common features of no nitrite reduction; no indole, urease, or arginine dihydrolase production; positive utilization of glucose and maltose and no utilization of adipate. Of these four strains, fermentation of glucose, production of β-galactosidase and β-glucosidase and utilization of D-mannose were detected only for strain osh08^T (Table 1). When strain osh08^T was compared with the sole non-marine mesophilic

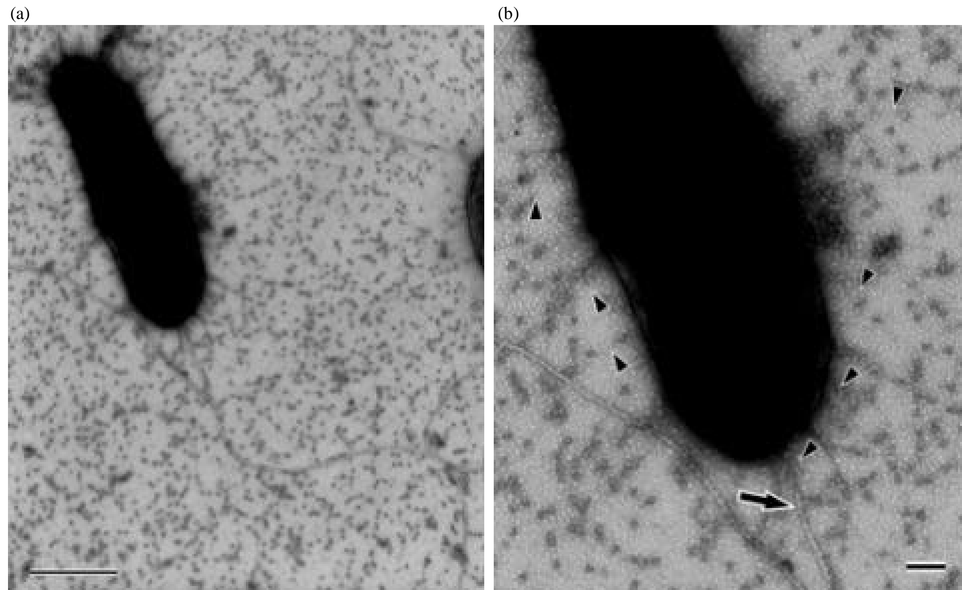

Fig. 1(a-b): Electron micrographs of (a) Negatively stained cells of strain osh08^T, In (b) A cell of strain osh08^T with a flagellum (arrow) and pilus-like structures (arrowheads), Bar indicates 500 nm in (a) and 100 nm in (b)

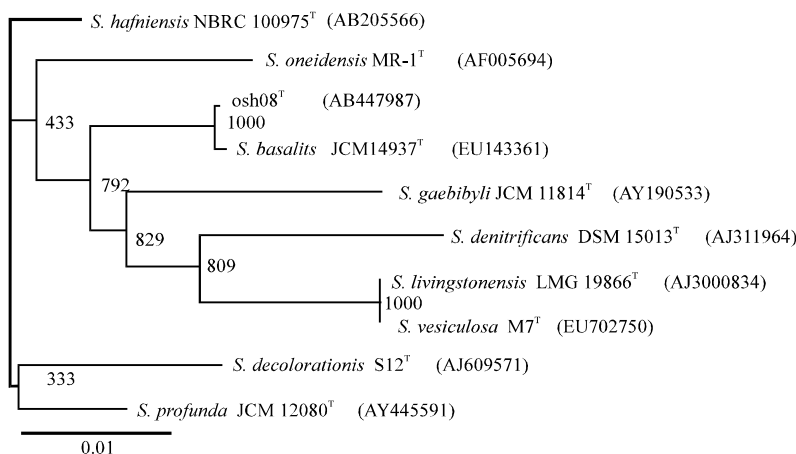

Fig. 2: Phylogenetic trees derived from the 16S rRNA gene sequence data of strain osh08^T and related *Shewanella* type strains using the neighbour-joining method for calculation. Numbers indicate bootstrap values of greater than 500. Bar, 0.01 nucleotide substitution rate (K_{nuc}) unit

bacterium *S. oneidensis* MR-1^T (Venkateswaran *et al*., 1999), there were many characteristics common to these two strains, such as an optimum growth temperature of 30°C, growth at 4°C and 40°C and growth in medium with no NaCl and at pH 10. Strain osh08^T was positive for reduction of iron oxide and manganese oxide under anaerobic conditions in the presence of 15 mM sodium acetate as electron donor (Table 1), as was *S. oneidensis* MR-1^T (Venkateswaran *et al*., 1999). The reduction of these metal ions was also observed in this study for *S. basaltis* J83^T and *S. hafniensis* NBRC 100975^T (Table 1). Both strain osh08^T and *S. oneidensis* MR-1^T contained C31:9 in addition to EPA (Sugihara *et al*., 2010).

Analysis of 16S rRNA gene sequences: As described previously (Sugihara *et al*., 2010), the 1,534 bp nucleic acid sequence of the 16S rRNA gene of strain osh08^T was determined (AB447987) and showed 99.8 and 98.4% similarity to those of *S. basaltis* JCM 14937^T (EU143361) and *S. hafniensis* NBRC 100975^T (AB205566), respectively. Figure 2 shows the phylogenetic tree derived from the 16S rRNA gene sequences of strain osh08^T and of various *Shewanella* type strains.

DNA base composition and DNA-DNA hybridisation: The DNA G+C content of strain *Shewanella* sp. strain osh08^T was 43.4 mol% (Table 1). The level of DNA relatedness between strain osh08^T and *S. basaltis* J83^T and that between strain osh08^T and *S. hafniensis* NBTC 100975^T were 4.7 and 11.8%, respectively.

Fatty acids and hydrocarbons: The detailed fatty acid and hydrocarbon compositions of osh08^T were described previously by Sugihara *et al*. (2010). The major Δ9-hexadecenoic acid, 16:1(Δ9), accounted for 28.8% of the total fatty acid and hydrocarbon fraction and this strain contained isomers of monounsaturated fatty acids with a double bond at different positions, such as Δ7-pentadecenoic acid [15:1(Δ7)], Δ9-pentadecenoic acid [15:1(Δ9)], Δ7-hexadecenoic acid [16:1(Δ7)], 16:1(Δ9), Δ9-heptadecenoic acid [17:1(Δ9)], Δ11-heptadecenoic acid [17:1(Δ11)], Δ9-octadecenoic

acid [18:1(Δ9)] and Δ11-octadecenoic acid [18:1(Δ11)] (Sugihara *et al.*, 2010). These isomers of monounsaturated fatty acids are found in most *Shewanella* species (Satomi *et al.*, 2003, 2006). Normal and iso-branched odd-carbon-number fatty acids were also observed in strain osh08^T. Iso-pentadecanoic (i15:0) and pentadecanoic (15:0) acids accounted for approximately 10% of the total fatty acid and hydrocarbon fraction and both EPA and C31:9 were detected at approximately 1 and 3%, respectively, in this strain (Sugihara *et al.*, 2010), as well as in *S. basaltis* J83^T and *S. hafniensis* NBTC 100975^T.

Description of *Shewanella oshoroensis* sp. nov.: *Shewanella oshoroensis* (o.sho.ro.en'sis. L. adj. *oshoroensis*, from Oshoro, a harbour village of Hokkaido, Japan from which the sample alga was collected).

Cells are Gram-negative rods, 0.3-0.6 mm wide by 0.8-1.5 mm long, facultatively anaerobic, motile by a single unsheathed flagellum. Cells have pilus-like structures of 40-500 nm in length on the surface and are mesophilic with an optimum temperature for growth at 30°C. Growth occurs in medium with no NaCl and in that containing 7.0% NaCl and in that at pH 10. Catalase and oxidase reactions are positive, gelatinase and β-galactosidase and β-glucosidase are produced, but urease and arginine dihydrolase are not produced. Fermentation of glucose was detected. Iron oxide and manganese oxide were reduced under anaerobic conditions. The G+C content of the DNA is 43.4 mol%. Other physiological characteristics are shown in Table 1. Major fatty acids are 16:1(Δ9), 16:0, 17:1(Δ9), i15:0 and 15:0. Small amounts of EPA and C31:9 are detected. The type strain is strain osh08^T (=NBRC 107685^T).

CONCLUSION

In this study the mesophilic EPA and C31:9-producing bacterium was identified as a novel *Shewanella* species and named as *Shewanella oshoroensis* sp. nov. *S. oshoroensis* strain osh08^T (NBRC 107685^T) is a promising strain for the production of C31:9 at moderate temperatures, such as at 25°C (Sugihara *et al.*, 2010).

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