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Identification of Psychrophile *Shewanella* sp. KMG427 as an Eicosapentaenoic Acid Producer

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An isolate from holothurians was identified as an eicosapentaenoic acid (EPA)-producing bacterium KMG427, which is characterized by EPA synthesis efficiency, by thin layer and gas chromatographic analyses. The EPA production was maximized to more than 10% of the total fatty acids by incubation at 4°C after cell proliferation at 20°C. The isolated bacterium was categorized as Gram-negative, rod-shaped, aerobic, and motile with a single polar flagellum. According to phylogenetic analysis based on morphological and physiological specificities as an EPA-producing bacterium, the isolate KMG427 was found to belong to the genus *Shewanella*. The 16S rDNA of KMG427 was revealed to have 100% of sequence identity to that of *S. hanedai* CIP 103207^T. Therefore, the isolate might be classified and identified as *Shewanella* sp. KMG427.

Keywords: Eicosapentaenoic acid, *Shewanella* sp. KMG427, 16S rDNA, phylogenetic tree

An eicosapentaenoic acid (EPA) has important physiological and pharmacological roles in anticoagulation of platelets (thrombolysis); anti-atherosclerosis; lowering plasmatriglyceride, blood viscosity, and blood pressure; anti-inflammation; and antitumorigenesis [23]. The lipids in blue-backed fish such as Pacific mackerel are rich in polyunsaturated fatty acids (PUFAs), and have been used as main sources for EPA [3]. Extremely pure EPA has been used in pharmaceuticals with great safety and fewer adverse effects than total fish oils, and the medicinal market has evaluated EPA for its pharmaceutical efficacy and staple commodities. Because of the unpleasant smell, purification difficulty, and supply inconstancy of EPA from fish oil, EPA production requires other sources, such as microorganisms [25]. EPA is produced mainly by lower fungi and marine algae [14, 20], and PUFAs are rarely produced by prokaryotes. However, the bacterial production of EPA appears to be limited to a small number of marine representatives from the genera *Flexibacter*, *Vibrio*, *Shewanella*, and closely related microorganisms [7, 11, 24].

This study was undertaken to isolate a new and high EPA-producing bacterium from the guts of marine animals, and identify it as the genus *Shewanella* along with the morphological, biochemical, and physiological characteristics. Phylogenetic analysis was also performed, based on a 16S rDNA sequence and search of optimal conditions for EPA production.

To isolate EPA-producing bacteria, the intestines of fresh marine animals were excised, diluted with 75% sterilized seawater, and spread onto a modified PYM-G agar plate containing 1% peptone, 0.5% yeast extract, 1% meat extract, 1% glucose, 2% agar, and 50% seawater (adjusted to pH 6.9–7.3) [3]. The plated strains were incubated at 25°C for 4 days. The different types of colonies were isolated and streaked on new plates to obtain a homogeneous culture. A single colony was then isolated and cultured on a modified PYM-G agar plate for 3 days. The production of EPA was measured as described previously [17]. The bacterial growth rates were determined in a temperature gradient incubator (Toyo Inc., Tokyo, Japan) by culturing at various temperatures ranging from 0°C to 40°C. The morphology of the isolated strain was examined by scanning electron microscopy as described previously with slight modification [4]. The biochemical and physiological

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characteristics of the EPA-producing strain were examined according to *Bergey's Manual of Systematic Bacteriology* [1], the *Manual of Methods for General Bacteriology* [6], and *The Prokaryotes* [9]. Additional biochemical analyses were performed with API 20E and API 20NE test kits (bioMerieux, Lyon, France). To analyze fatty acid methyl ester (FAME), the cells were cultured for 2 days in a PYM-G broth at 20°C with vigorous shaking (180 rpm). The cells were harvested by centrifugation during the late logarithmic phase at 4°C, washed with 3% NaCl, and then lyophilized at −70°C. The dried cells were placed in a teflon-lined, screw-capped tube containing 1 ml of 1.5% NaCl, and the tube was heated at 100°C for 1 h. After cooling, 2 ml of methanolic HCl was added, and the mixture was heated at 80°C for 10 min. The FAMEs were then extracted with *n*-hexane [5] and analyzed on a cross-linked 5% phenyl silicone capillary column SP-2560 (100 m×0.25 mm×0.2 μm film thickness; Supelco, Bellefonte, U.S.A.) with an HP 5890 gas chromatograph. The column was kept at an initial temperature of 130°C for 1 min and the oven temperature was programmed to increase from 130°C to 300°C by 10°C/min with the final temperature at 300°C for 5 min. The temperature of the injector was maintained at 270°C, and that of the detector was kept at 300°C. The FAME peaks were identified by comparing the retention time and peak areas to those of known amounts of authentic standards (Sigma, St. Louis, U.S.A.) [17]. To prepare chromosomal DNA and analyze 16S rDNA, the strain KMG427 was first cultured overnight in a PYM-G broth with shaking on a rotary shaker at 180 rpm at 18°C. The cells were harvested by centrifugation and chromosomal DNA was extracted as described by Sambrook *et al.* [16]. The full length of the 16S rRNA gene was amplified from the chromosomal DNA by a polymerase chain reaction (PCR) with the primers EC9F (5'-GAGTTTGATCCTGG-CTCAG-3') and EC1542R (5'-AGAAAGGAGGTGATC-CAGCC-3') [26]. The resulting PCR products were visualized on a 0.7% agarose gel. The expected band was excised and purified using a Geneclean Kit (Bio101 Inc., Vista, U.S.A.). The purified PCR products were cloned into a pGEM-T easy vector (Promega Co., Madison, U.S.A.). The clone containing the insert (approximately 1.5 kb) was then sequenced with an ABI Prism 310 Genetic Analyzer (Perkin Elmer, NJ, U.S.A.), using reverse primers (EC536R, 5'-GWATTACCGCGGCKGC-TG-3'; EC926R, 5'-CCGTCAATTCMTTTRAG-3'; and EC1512R, 5'-ACGGHTAACCTTGTTACGACTT-3') and a M13 universal primer. The 1,426 nucleotide sequence was then analyzed in both directions for strain KMG427. The sequences of the genomic region containing the 16S rRNA gene of strain KMG427 was deposited in the GenBank database under Accession No. AF187080. Phylogenetic analysis was carried out as follows: Multiple sequence alignments were performed using the computer program ClustalW. All reference sequences were obtained from the GenBank database. The homologous areas where the 16S rDNA sequence of strain KMG427 was aligned unambiguously with the reference sequences were located at 1,426 nucleotides. A phylogenetic tree was established using the neighbor-joining method in a Phylip Package (3.5c) with a 1,000 bootstrap replicate. A small-subunit (SSU) rRNA sequence of KMG427 was analyzed by other SSU sequences retrieved from the GenBank database. The *Moritella marina* NCIMB 1144^T SSU sequences were used as an outgroup.

Isolation and Identification of the Strain KMG427 Producing EPA

An EPA-producing microorganism was isolated from halothurians and designated as KMG427. Production of EPA by the strain KMG427 was confirmed by gas chromatographic analysis at the retention time of 64.919 min (Fig. 1). Scanning electron microscopy revealed that the strain KMG427 was rod-shaped cells, 0.4–0.6 μm×2–4 μm in size, and with a single polar flagellum; however, some of the cells were long filament types (figure not shown). The strain was also identified as motile, aerobic, Gram-negative, and bioluminescent as described for *Shewanella* sp. [5]. The strain KMG427 grew well between 4°C and 27°C with an optimal temperature between 16°C and 20°C; however, it could not grow at above 30°C. The strain grew well in the presence of up to 5% sodium chloride or seawater.

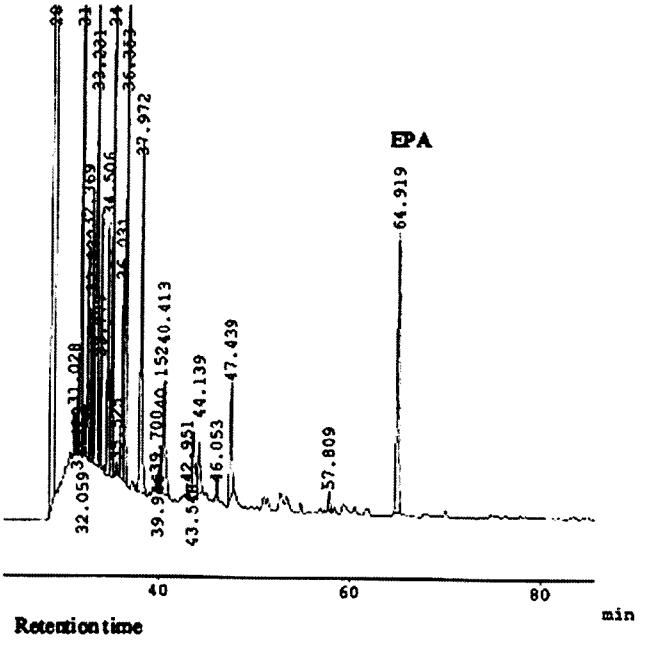

Fig. 1. Capillary gas chromatogram of fatty acids (as methyl esters) from *Shewanella* sp. KMG427.
The cells were grown in a PYM-G medium at 4°C.

Table 1. Biochemical and physiological properties of isolated strain KMG427.

Characteristics	KMG427	*S. hanedai*[a]	Characteristics	KMG427	*S. hanedai*[a]
Cell shape	Straight rods	Straight rods	Utilization of		
Luminescence	+	+	Sucrose	–	–
Growth at			Maltose	–	–
4°C	+	+	Melibiose	–	–
30°C	–	–	N-Acetylglucosamine	+	+
37°C	–	–	Citrate	+	–
Growth in NaCl (%)			Mannitol	–	–
0%	–	–	Sorbitol	–	–
3%	+	+	Malate	+	+
6%	–	–	Arabinose	–	–
NO^{-3} to NO^{-2}	+	+	Rhamnose	–	–
Production of			Glucose	–	–
Indole	–	–	Caprate	+	+
Gelatinase	+	+	Inositol	–	–
Chitinase	+	+	Phenylacetate	–	–
β-Glucosidase	–	–	Mannose	–	–
Urease	–	–	Adipate	–	–
Arginine dehydrolase	–	–	Amygdalin	–	–
Lysine decarboxylase	–	–			
Ornithine decarboxylase	–	+-			
Tryptophan desaminase	–	–			
H_2S production	–	–			

+, Growth or positive; –, no growth or negative.
[a]Data of *S. hanedai* were obtained from *Bergey's Manual of Systematic Bacteriology*, Volume 1 [1] and Reference [12].

The strain KMG427 revealed the presence of oxidase, catalase, gelatinase, chitinase, β-glucosidase, and ornithine decarboxylase; however, it did not produce urease, arginine dihydrolase, lysine decarboxylase, tryptophan desaminase, and β-galactosidase. The colony was reddish brown on a PYM-G agar plate. The strain KMG427 also showed physiological characteristics quite similar to those of *S. hanedai*, as summarized in Table 1. An almost complete 16S rRNA gene sequence of the strain KMG427 was determined, comprising 1,426 nucleotides. Based on the 16S rRNA gene sequence analysis, the strain KMG427 was identical to be related to recognized species of the genus *Shewanella*, with *Shewanella hanedai* CIP 103207^T (99% sequence similarity) as the closest relative (Fig. 2). The sequence similarity of the KMG427 rDNA with others from the genus *Shewanella* ranged between 94% and 99%. Therefore, based on the phenotypic study and 16S rDNA sequence analysis, the bacterium KMG427 should be assigned as a species of the genus *Shewanella*, and designated as *Shewanella* sp. KMG427 (Fig. 2).

Fatty Acid Biosynthesis and Optimal Condition of EPA Production

The total fatty acids composition of KMG427 is shown in Table 2. The major fatty acids of the bacterium were iso-$C_{13:0}$ (17.15%), $C_{14:0}$ (7.69%), iso(OH)-$C_{13:0}$ (7.72%), iso-$C_{15:0}$ (8.42%), $C_{15:0}$ (9.33%), $C_{16:0}$ (9.36%), $C_{16:1\omega7}$ (10.93%), and $C_{20:5\omega3}$ (11.22%). The rest of them were saturated fatty acids [$C_{12:0}$ (3.65%), $C_{13:0}$ (2.99%), and $C_{17:0}$ (0.7%)], branched fatty acids [iso-$C_{14:0}$ (0.35%) and iso-$C_{18:0}$ (0.43%)], and monounsaturated fatty acids [$C_{15:1\omega8}$ (1.88%), $C_{15:1\omega6}$ (0.64%), $C_{16:1\omega9}$ (0.77%), $C_{17:1\omega8}$ (2.74%), $C_{17:1\omega6}$ (0.25%), $C_{18:1\omega9}$ (0.34%), and $C_{18:1\omega7}$ (0.38%)]. The EPA content in the strain KMG427 was analyzed by culturing the cells at various temperatures (4°C, 12°C, 20°C, and 25°C), pH and salinity. EPA production was the lowest at 25°C (2% of total fatty acid), whereas it gradually increased by lowering the culture temperature (6% to 11.5% of total fatty acid; see Fig. 3). EPA biosynthesis in *Shewanella* sp. KMG427 seemed to be affected by temperature similar to the pattern in the other bacteria for the purpose of adapting itself to changed physiological environment. One of the physiological roles of EPA may be to lower the phase transition temperature of the membrane phospholipid bilayer to increase flexibility of the cell membrane, as in the marine *Flexibacter* sp. [11]. It has also been suggested that a large amount of EPA in deep-sea bacteria appears to be useful in maintaining membrane fluidity and function with high hydrostatic pressure and low temperature [2, 7, 10, 12, 15, 18, 22]. An increase in the salinity of the medium from 1% to 5% yielded a larger amount of cell EPA. The optimum initial pH for the production of EPA was in the range of 8–9 (data not shown).

EPA ($C_{20:5\omega3}$) synthesized in the strain KMG427 could easily be purified, because EPA was the sole PUFA in this strain. PUFAs such as EPA are known to be effective in

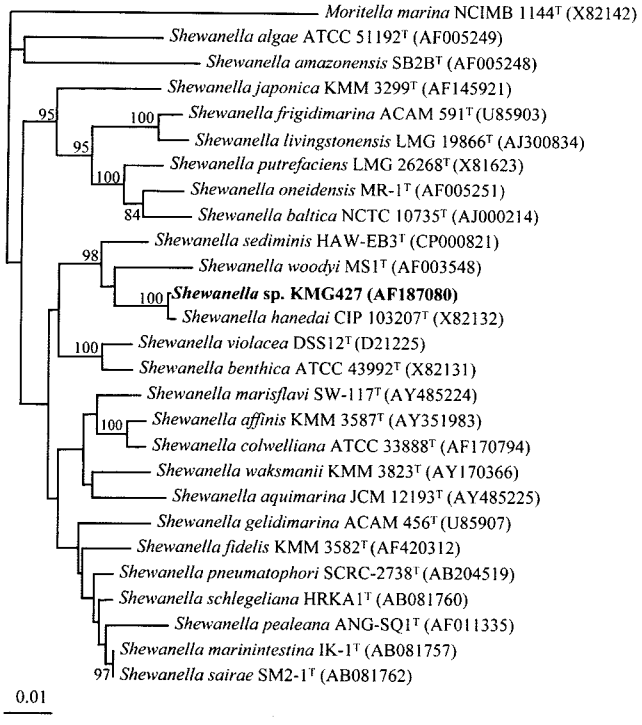

Fig. 2. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of *Shewanella* sp. KMG427 and representatives of related taxa.
GenBank/EMBL/DDBJ accession numbers are shown in parentheses. Numbers at branch points indicate levels of bootstrap support, based on 1,000 resamplings. *Moritella marina* NCIMB 1144^T (X82142) was used as an outgroup. Bar, 0.01 expected nucleotide substitutions per position.

the prevention and cure of thrombosis, atherosclerosis, hyperglyceridemia, and high blood pressure [8, 13, 21]. Such PUFAs are abundant in fish oils and have been used as principle sources of EPA in industry and in the area of human health. However, the purification of EPA has been quite difficult until now, because of the highly complicated

Table 2. Fatty acid composition of total membrane lipid extracted from the strain KMG427.

Fatty acid(s)[a]	KMG427	Fatty acid(s)[a]	KMG427
12:0	3.65	16:1ω9c	0.77
13:0(i)	17.15	16:1ω7c	10.93
13:0	2.99	16:0	9.36
14:0(i)	0.35	17:0(i)	0.20
14:0	7.69	17:1ω8c	2.74
13:0(i 30H)	7.72	17:1ω6c	0.25
15:1(i)	0.48	17:0	0.70
15:0(i)	8.42	18:0(i)	0.43
15:1ω8c	1.88	18:1ω9c	0.34
15:1ω6c	0.64	18:1ω7c	0.38
15:0	9.33	20:5ω3 (EPA)	11.22

[a]Fatty acids are abbreviated so that the number of carbon atoms precedes the colon and the number of double bonds follows the colon.
The prefixes iso(i) represent a branched-chain structure.

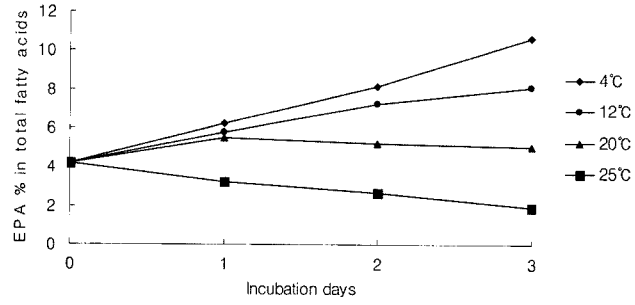

Fig. 3. EPA production by *Shewanella* sp. KMG427 at four different temperatures.
Shewanella sp. KMG427 was grown in a PYM-G medium at 20°C for 2 days, and then incubated at 4°C, 12°C, 20°C, or 25°C for 3 days. TFA, total fatty acid; EPA, eicosapentaenoic acid.

fatty acid composition of the raw materials such as fish oil. As a result, the supply of ω-3 fatty acids from conventional sources is inadequate, and other alternate sources need to be identified. EPA-producing microalgae such as *Chlorella* may be used as an alternative source to fish oils; however, the cultivation of microalgae requires strictly controlled growth conditions [19], which can result in considerable expense. Therefore, the EPA-producing bacterium KMG427 is a good candidate for EPA production.

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