NOTE

***Shewanella olleyana* sp. nov., a marine species isolated from a temperate estuary which produces high levels of polyunsaturated fatty acids**Jennifer H. Skerratt,¹ John P. Bowman² and Peter D. Nichols^{1,3}

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Two polyunsaturated fatty acid (PUFA) producing strains (ACEM 6 and ACEM 9^T) isolated from a temperate, humic-rich river estuary in Tasmania, Australia, were found to be members of the genus *Shewanella*. These strains were able to utilize humic compounds (tannic acid) and derivatives (2,6-anthraquinone disulfonate) as sole carbon sources and as electron acceptors for anaerobic respiration. The major fatty acids were typical of the genus *Shewanella*; however, PUFAs mostly made up of eicosapentaenoic acid were produced at high levels (10.2–23.6% of total fatty acids) and at relatively high incubation temperatures (10.2% at 24 °C). Sequence analysis indicated that ACEM 6 and ACEM 9^T had identical 16S rDNA sequences and were most closely related to *Shewanella japonica* (sequence similarity 97.1%). DNA hybridization and phenotypic characteristics confirmed that the isolates constituted a novel species of the genus *Shewanella*, which is designated *Shewanella olleyana* sp. nov. (type strain ACEM 9^T = ACAM 644^T = LMG 21437^T).

Keywords: *Shewanella*, marine bacteria, polyunsaturated fatty acids, eicosapentaenoic acid, estuarine

Only a limited number of bacterial genera have the ability to produce polyunsaturated fatty acids (PUFAs). Most of these concentrate within the family *Alteromonadaceae* (Bowman, 2001). Of the described PUFA-producing species which reside in the genus *Shewanella* (Russell & Nichols, 1999), most have been isolated from polar regions or are barophilic and largely group amongst cold-adapted, halophilic *Shewanella* species (Kato & Nogi, 2001). The main PUFA produced by *Shewanella* species is eicosapentaenoic acid (EPA), abbreviated to 20:5(n-3). High proportions of EPA in *Shewanella* spp. are predominantly associated with cold adaptation mechanisms and highest production levels occur in the suboptimal region of temperature growth kinetic range (Russell &

Nichols, 1999). To our knowledge, *Flexibacter polymorphus* (Johns & Perry, 1977), *Shewanella pealeana* (Leonardo *et al.*, 1999) and *Shewanella japonica* (Ivanova *et al.*, 2001) are the only other non-barophilic, non-polar bacterial species that produce EPA.

As part of a study examining bacterial–algal interactions (Skerratt, 2001), water samples were collected from the upper reaches of the Huon River estuary, Tasmania, Australia, during February 1998. The salinity was 32‰, the water temperature was 17 °C and the water contained a high concentration of humic substances. Samples were initially cultured on modified marine agar which consisted of 800 ml filtered seawater, 200 ml distilled water, 5 g bacteriological peptone, 1 g yeast extract and 15 g agar. Random colonies with differing morphologies were isolated and purified by the streak plate technique. PUFA-producing abilities were ascertained by screening the fatty acid profiles (see below) of all purified isolates. Two strains able to form EPA were designated ACEM 6 and ACEM 9^T.

The two strains were routinely cultured on ZoBell's

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Abbreviations: AQDS, 2,6-anthraquinone disulfonate; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; PUFA, polyunsaturated fatty acid.

The GenBank accession number for the 16S rDNA sequence of *Shewanella olleyana* is AF295592.

marine agar (prepared with dehydrated seasalts; Sigma) (Bowman *et al.*, 1997) before inoculation of biochemical and growth test media. Biochemical tests were performed with API-NE and API 20E test strips (bioMérieux-Vitek). For carbon and energy source tests, most of the test compounds were used at a concentration of 0.2% (w/v), with the exception of carbohydrates, which were tested at a concentration of 0.5% (w/v). The basal mineral salts medium employed contained per 1000 ml distilled water: 2 g NH_4Cl , 14 g NaCl , 2 ml 1 M NaPO_4 buffer (pH 7.0) and 2 ml SL10 trace element solution (10 ml 7.7 M HCl , 1.5 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 70 mg ZnCl_2 , 100 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 6 mg H_3BO_3 , 190 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 24 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 36 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 990 ml distilled water). The pH was adjusted to 7.0 with 1 M KOH and the medium was solidified with 1.4% agar. Comparison of media with a control which lacked an added carbon source was used to assess carbon substrate use. Neither strain produced clearly interpretable results using the Biolog Microplate system (Biolog Microbial Identification System). Other phenotypic tests used were conducted as described by Bowman *et al.* (1997). Anaerobic growth tests used media formulated by Coates *et al.* (1998) except that the basal medium contained 0.5 M NaCl prepared with 1.0% agar Noble (Difco), incubated in 2.5 l anaerobic jars containing anaerobic GasPaks (Oxoid) and included media controls prepared without an electron acceptor.

Fatty acid analysis was completed using a whole-cell methanolysis procedure. Replicate isolates were harvested after 24 h (10 and 24 °C) or 48 h (4 °C) incubation on ZoBell's marine agar. Methylating reagent (5 ml 10:1:1, methanol:chloroform:concentrated HCl) was added and the samples were heated at 90 °C for 60 min to produce fatty acid methyl esters (FAMES). The reaction was cooled and 4 ml distilled water was added followed by 2 ml 4:1 cyclohexane:chloroform. After mixing, the solvent/aqueous phases were left to separate. The solvent layer containing FAMES was transferred to a vial in preparation for chromatographic analysis. Fatty acid profiles were determined using a Hewlett Packard 5890 gas chromatograph (GC) equipped with a 50 m $\times$ 0.32 mm i.d. cross-linked methyl silicone fused silica capillary column (Hewlett Packard HP5 column) and a Fisons GC-mass spectrometer (GC-MS) with conditions as described by Gutierrez *et al.* (1999). Fatty acids were identified prior to GC-MS by comparing retention time data with those obtained for authentic and laboratory standards. The GC data were compiled and analysed with Waters Millennium software. Geometry and position of the double bonds in monounsaturated FAMES were confirmed using dimethyl-disulfide derivatization and analysis using GC-MS (Nichols *et al.*, 1986).

Genomic DNA was extracted from cells and purified using the procedure of Marmur & Doty (1962). The 16S rDNA genes from the Huon Estuary strains were

amplified by PCR using the primers 10f (*Escherichia coli* numbering system: 5'-AGTTTGATCCTGGC-TCA-3') as a forward primer and 1492r (*E. coli* numbering system: 5'-TACGGYTACCTTGTTAC-GACTT-3') as a reverse primer. Conditions used for PCR are described in Bowman *et al.* (1996). PCR products were purified with the Prep-a-Gene kit (Bio-Rad). Sequences of the 16S rDNA insertion were then generated with an Applied Biosystems model 3728A automated sequencer using a fluorescent dye terminator cycle sequencing kit (Applied Biosystems). The 16S rDNA sequences determined for the strains were compared to the GenBank nucleotide database using online BLAST searches (<http://www.ncbi.nlm.nih.gov>). Analyses of the 16S rDNA sequences datasets utilized PHYLIP version 3.57c (Felsenstein, 1993). DNADIST was used to determine sequence similarities using the maximum-likelihood algorithm option. Phylogenetic trees were constructed with the neighbour-joining method by using the program NEIGHBOR. 16S rDNA sequences generated for both ACEM 6 and ACEM 9^T stretched 1459 bp between nucleotide positions 36 and 1490 (*E. coli* equivalent). The GenBank accession number for the 16S rDNA sequence of ACEM 9^T is AF295592.

High molecular mass DNA was extracted using the Marmur & Doty (1962) procedure with nucleic acids spooled following precipitation with 3 M sodium acetate:1 mM disodium EDTA (pH 7.0) and cold propan-2-ol. The DNA G + C content of the extracted DNA was determined by thermal denaturation (Sly *et al.*, 1986) using a GBC 916 spectrophotometer (GBC Instruments). DNA-DNA hybridization was performed by applying the spectrophotometric renaturation kinetics method as adapted by Bowman *et al.* (1998).

The strains demonstrated cellular and colonial morphologies and phenotypic profiles typical of *Shewanella* species. Cells were rod-shaped, Gram-negative, 1–2 μm in length, 0.4–0.5 μm in diameter and were motile, possessing a single polar flagellum. Colonies on agar plates were an opaque tan colour, butyrous in consistency, and circular and convex in shape with an undulate edge. Colonies of 2–4 mm in diameter were formed following 24 h incubation at 20 °C. The agar beneath colonies on ZoBell's marine agar became softened, but not liquefied, with prolonged incubation. Flooding the agar surface with Lugol's iodine solution revealed hydrolysis zones around the growth, suggesting that the strains have a weak agarolytic ability.

The strains were psychrotolerant and stenohaline. Growth was observed between 2 and 30 °C with best growth at 20–22 °C. Growth on agar media at 2 and 4 °C was slower (2–4 d) and less prolific than that at 10–25 °C (1 d). No growth was observed for either strain above 30 °C. Weak growth was observed for ACEM 9^T at 30 °C in marine broth. Strains required Na^+ for growth and grew between 0.3 and 1.1 M NaCl , with best growth at 0.4–0.6 M NaCl . NaCl concentra-

Table 1. Phenotypic characteristics that differentiate the Huon Estuary strains (ACEM 6 and ACEM 9^T) from psychrotolerant marine *Shewanella* species

+, Test is positive; –, test is negative; d, test results vary amongst strains of the species; ND, data not available. Data for reference species were taken from Bowman *et al.* (1997), Ivanova *et al.* (2001), Leonardo *et al.* (1999), Makemson *et al.* (1997), Weiner *et al.* (1988) and Ziemke *et al.* (1998).

Phenotypic characteristic	ACEM 6, ACEM 9 ^T	<i>Shewanella</i> <i>colwelliana</i>	<i>Shewanella</i> <i>japonica</i>	<i>Shewanella</i> <i>baltica</i>	<i>Shewanella</i> <i>woodyi</i>	<i>Shewanella</i> <i>pealeana</i>	<i>Shewanella</i> <i>frigidimarina</i>
Requires Na ⁺ ions for growth	+	+	–	–	+	+	–
Growth at 35 °C	–	–	+	+	–	–	–
Ornithine decarboxylase	–	–	ND	+	ND	ND	d
NO ₃ → NO ₂ (+O ₂)	d	+	+	ND	ND	ND	+
DNase production	–	+	+	ND	ND	ND	ND
Amylase production	+	+	+	–	d	–	–
Utilization of:							
D-Glucose	–	–	+	+	+	+	+
N-Acetylglucosamine	–	–	+	+	–	ND	–
Maltose	–	–	+	+	–	ND	+
Sucrose	+	–	–	+	–	ND	+
D-Gluconate	+	+	–	+	ND	ND	d
EPA synthesis	+	–	+	–	–	+	+
mol % G + C	44	46	43–44	46	46	45	40–43

tions above the optimal range slightly inhibited PUFA synthesis.

The strains were facultatively anaerobic. Respiratory anaerobic growth was supported on a variety of electron acceptors when sodium lactate was used as the electron donor. Electron acceptors used included Fe(III) (50 mM ferric citrate or 10 mM amorphous ferric oxides), 10 mM sodium nitrate, 20 mM manganese(IV) oxide, 10 mM sodium thiosulfate, 25 mM sodium fumarate and 5 mM humic substance analogue 2,6-anthraquinone disulfonate (AQDS). Sodium nitrite (5 mM), sodium sulfite (10 mM), elemental sulfur (10 mM) and sodium selenate (5 mM) did not support growth. The strains did not produce arginine dihydrolyase, ornithine decarboxylase or lysine decarboxylase and were unable to ferment carbohydrates. ACEM 9^T reduced nitrate to nitrite in the presence of oxygen but ACEM 6 did not.

The Huon Estuary has unusually high levels of humic compounds which provides a nutrient source able to support a high level of microbial production including proteinaceous material, phenolic and hydroxy acid compounds and glycosides (Skerratt, 2001). Strains ACEM 6 and ACEM 9^T demonstrated an ability to assimilate carbon sources that would be derived from the humics, including tannic acid. As mentioned above, the strains could readily utilize AQDS as an electron acceptor for anaerobic respiration. Humic compounds have been shown to play an important role in driving iron reduction and organic matter oxidation in aquatic ecosystems (Coates *et al.*, 1998; King & Garey, 1999; Lovley & Blunt-Harris, 1999). In other respects, the Huon Estuary strains most phenotypically resembled

Shewanella colwelliana (Weiner *et al.*, 1988; Coyne *et al.*, 1989); however this species differed in a number of important traits, including the inability to produce EPA, inability to utilize D-fructose, D-galactose, DL-malate or L-tyrosine or hydrolyse alginate and the ability to form deoxyribonuclease and constitutively form melanin pigments. Other characteristics differentiating the novel strains from other closely related and ecophysiologically similar *Shewanella* species are shown in Table 1.

16S rDNA sequence analysis indicated that ACEM 6 and ACEM 9^T had identical sequences and that they were most closely related to *S. japonica* (sequence similarity 97.1%) (Fig. 1). The Huon Estuary strains were distinct from *S. colwelliana* ATCC 33888 (similarity 94.3%).

The growth of the Huon Estuary strains at different temperatures (4–24 °C) altered PUFA levels by greater than twofold with total PUFAs ranging from 10.2 (24 °C) up to 23.6% (4 °C). At the higher growth temperatures the proportions of branched-chain and saturated fatty acids also increased while monosaturates declined (Table 1), indicative of the homeostatic adaptation of cellular membrane viscosity (Russell & Nichols, 1999). Compared to the Huon Estuary strains, the closely related *S. japonica* contained significantly lower levels of PUFAs (8.6 and 2.2% at 12 and 28 °C, respectively). PUFA levels produced by both Huon Estuary strains for temperatures between 10 and 25 °C were substantially higher compared to other PUFA-producing *Shewanella* species, and contained the highest proportions of EPA yet reported for a psychrotolerant *Shewanella* species. The PUFA

Fig. 1. Unrooted phylogenetic tree based on 16S rRNA comparisons showing the taxonomic positions of Huon Estuary strain ACEM 9^T within the genus *Shewanella*. The tree was generated from maximum-likelihood distances clustered by the neighbour-joining method.

levels are similar to those reported for polar and deep-sea psychrophilic *Shewanella* species (Bowman *et al.*, 1997; Russell & Nichols, 1999; Kato & Nogi, 2001). Unlike the psychrophiles, the Huon Estuary strains are capable of substantially faster growth and can form equivalent levels of EPA at much higher incubation temperatures. These results are evidence that the presence of EPA in the genus *Shewanella* is not just associated with psychrophily, but is instead a more broadly distributed trait amongst *Shewanella* species that exist in the marine environment and which may be exposed to seasonal cold periods. The proportion of EPA varied inversely with temperature for both of the Huon Estuary strains (Table 1), indicating that temperature remains the primary controlling factor in PUFA synthesis. Production of EPA is an important physiological and descriptive component that allows differentiation between *Shewanella* species, and may also have an important ecological role acting as a nutrient source for estuarine and marine biota requiring essential fatty acids yet unable to synthesize omega-3 fatty acids *de novo*.

Using the thermal denaturation procedure, DNA G+C contents of the two strains averaged 44 mol %. DNA–DNA hybridization values determined between strains ACEM 6 and ACEM 9^T averaged 95% ($n = 4$). Hybridization values between ACEM 9^T and *S. japonica* KMM 3299^T averaged 37% ($n = 5$). Hybridization values between ACEM 9^T and other marine psychrotolerant *Shewanella* species, including *Shewanella frigidimarina* ACAM 591^T, *Shewanella baltica* NCTC 10735^T, *S. colwelliana* ATCC 33888 and *Shewanella woodyi* ATCC 51908^T, were not significant (9–23%, $n = 3–4$).

A polyphasic taxonomic treatment of Huon Estuary strains ACEM 6 and ACEM 9^T unequivocally indi-

cates that they represent a novel species within the genus *Shewanella* which is designated *Shewanella olleyana* sp. nov. The type strain of *Shewanella olleyana* is ACEM 9^T.

Description of *Shewanella olleyana* sp. nov.

Shewanella olleyana (ol.ley'a.na. N.L. fem. adj. *olleyana* named for June Olley for her contributions to food microbiology).

Cells are rod-shaped, Gram-negative, 1–2 µm in length, 0.4–0.5 µm in diameter and have a single polar flagellum. Temperature range for growth is 2–30 °C. Optimum temperature for growth was 20–22 °C. Requires Na⁺ ions for growth (minimum 0.35 M, optimal 0.4–0.6 M, maximum 1.1 M). Oxidase- and catalase-positive. Facultatively anaerobic chemoheterotroph. Can grow anaerobically by respiration using Fe(III), Mn(IV), nitrate, thiosulfate, trimethylamine *N*-oxide and AQDS as electron acceptors and lactate as an electron donor. Carbohydrates are not fermented. Nitrate is reduced to nitrite in the presence of oxygen. Arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase are not produced. Hydrolyses Tween 80, gelatin, starch, alginate and aesculin but not carboxymethylcellulose, chitin, dextran, casein, elastin, DNA or uric acid. Weakly agarolytic. Production of H₂S from L-cysteine is negative (from API 20E test strip results) but H₂S was formed from thiosulfate anaerobically. Negative for tryptophan deaminase and β-galactosidase. Indole is not formed from L-tryptophan. Voges–Proskauer test is negative. In addition to the carbon source utilization data shown in Table 1, D-fructose, D-galactose, *m*-erythritol, glycerol, DL-malate, L-glutamate, L-phenylalanine, L-tyrosine and tannic acid were used as sole carbon and energy

Table 2. Cellular fatty acid composition of ACEM 6 and ACEM 9^T over a range of temperatures

Fatty acids are designated as total number of carbon atoms: number of double bonds followed by the position of the double bond from the aliphatic end of the molecule. The prefixes i, a, br and cy indicate iso, anteiso, branched and cyclopropyl-containing fatty acids, respectively. SFA, saturated fatty acids; BCFA, branched-chain fatty acids; MUFA, monounsaturated fatty acids. Values are given as mean percentages of total fatty acid content between ACEM 6 and ACEM 9^T and values in parentheses are the standard deviation. Fatty acids present at trace levels up to 0.5% of total fatty acid include: cy19:0, 22:0, 13:1, 14:1, 15:1, 22:1, 20:1, 20:3(n-6) and 20:4(n-3).

Fatty acid	Fatty acid composition (%):		
	4 °C	10 °C	24 °C
12:0	2.0 (0.2)	2.4	3.8 (0.6)
13:0	0.7	0.6 (0.2)	1.3 (0.8)
14:0	3.4 (0.8)	4.5 (0.4)	3.2 (0.2)
15:0	2.6 (0.2)	2.0 (0.7)	3.7 (2.4)
16:0	10.8 (0.8)	14.6 (0.2)	15.2 (3.9)
17:0	1.2 (0.4)	1.0 (0.6)	2.0 (0.2)
18:0	0.7	1.1 (0.2)	1.5 (1.2)
Sum SFA	21.4 (1.6)	26.5 (1.5)	30.7 (2.2)
i13:0	7.3	5.8 (0.3)	8.0 (2.5)
i14:0	0.9	1.0 (0.2)	2.5
i15:0	8.7 (0.6)	9.3 (0.2)	17.8 (3.5)
a15:0	2.0 (1.2)	1.9 (1.1)	2.8
i16:0	0.2	0.2	0.5
i17:0	0.6	0.7	1.9 (0.7)
Sum BCFA	19.7 (2.0)	18.9 (0.7)	33.5 (4.9)
16:1(n-7)	23.0 (2.2)	24.9 (4.7)	16.2 (5.9)
17:1(n-8)	1.5	1.8 (0.8)	3.4 (1.2)
17:1(n-6)	0.3 (0.2)	0.6 (0.4)	0.3
18:1(n-9)	0.6	1.3 (0.2)	2.2 (1.5)
18:1(n-7)	8.7 (2.4)	7.1 (0.7)	2.4
Sum MUFA	35.0 (4.4)	36.9 (2.5)	25.2 (6.5)
18:4(n-3)	1.1	0.7 (0.2)	0.2
20:5(n-3)	19.2 (0.8)	14.2 (0.9)	8.8 (3.7)
20:4(n-3)	2.3 (0.3)	1.1 (0.3)	0.5
22:6(n-3)	0.5	0.3	0.2
Sum PUFA	23.6 (0.5)	16.8 (0.6)	10.2 (3.9)

sources. The utilization of D-melibiose, DL- β -hydroxybutyrate, fumarate and succinate was variable between strains while L-arabinose, lactose, D-mannose, D-mannitol, D-sorbitol, caproate, citrate and phenylacetate were not utilized. Forms tan-coloured, butyrous, regular, convex, undulate, opaque colonies 2–4 mm in diameter following 24 h incubation at 20 °C. Major fatty acids are i13:0, i15:0, 16:0, 16:1(n-7), 18:1(n-7) and 20:5(n-3) (Table 2). Based on 16S rDNA nucleotide sequence analysis, the species belongs to the family *Alteromonadaceae*, order 'Alteromonadales' and class 'Gammaproteobacteria'. The type strain is ACEM 9^T (= ACAM 644^T = LMG 21437^T) isolated

from saline waters of the Huon River estuary, Tasmania, Australia.

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