Shewanella sediminis sp. nov., a novel Na⁺-requiring and hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading bacterium from marine sediment

Jian-Shen Zhao, Dominic Manno, Chantale Beaulieu, Louise Paquet and Jalal Hawari

Correspondence

Jalal Hawari

jalal.hawari@cnrc-nrc.gc.ca

Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2

Previously, a psychrophilic rod-shaped marine bacterium (strain HAW-EB3^T) isolated from Halifax Harbour sediment was noted for its ability to degrade hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). In the present study phenotypic, chemotaxonomic and genotypic characterization showed that strain HAW-EB3^T represents a novel species of *Shewanella*. Strain HAW-EB3^T contained lysine decarboxylase, which is absent in other known *Shewanella* species, and distinguished itself from most other species of *Shewanella* by the presence of arginine dehydrolase, ornithine decarboxylase and chitinase, and by its ability to oxidize and ferment *N*-acetyl-D-glucosamine. Strain HAW-EB3^T grew on several carbon sources (*N*-acetyl-D-glucosamine, Tween 40, Tween 80, acetate, succinate, butyrate and serine) and showed distinctive fatty acid and quinone compositions. Both phenotypic and 16S rRNA gene phylogenetic cluster analyses demonstrated that HAW-EB3^T belongs to the Na⁺-requiring group of *Shewanella* species. The HAW-EB3^T 16S rRNA gene sequence displayed ≤97.4 % similarity to all known *Shewanella* species and was most similar to those of two bioluminescent species, *Shewanella hanedai* and *Shewanella woodyi*. However, *gyrB* of strain HAW-EB3^T was significantly different from those of other *Shewanella* species, with similarities less than 85 %. DNA-DNA hybridization showed that its genomic DNA was less than 25 % related to that of *S. hanedai* or *S. woodyi*. Therefore we propose *Shewanella sediminis* sp. nov., with HAW-EB3^T (= NCIMB 14036^T = DSM 17055^T) as the type strain.

In a previous study several *Shewanella* strains capable of degrading hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) were isolated from an unexploded-ordnance-dumping site (Emerald Basin, 215 m deep), 50 nautical miles from Halifax Harbour (Nova Scotia, Canada), Atlantic Ocean (Zhao *et al.*, 2004a, b). In order to understand RDX metabolism and thus *in situ* biodegradation (natural attenuation) of the cyclic nitramine explosives at the Halifax site, phenotypic and genetic characterization of the isolates is necessary.

Shewanella genus was proposed by MacDonell & Colwell (1985) for two bacteria previously classified as *Alteromonas putrefaciens* (Long & Hammer, 1941; Lee *et al.*, 1977) and *Alteromonas hanedai* (Jensen *et al.*, 1980), and one deep-sea barophilic isolate (Deming *et al.*, 1984). *Shewanella* were defined as rod-shaped, Gram-negative, oxidase-positive, motile and chemo-organotrophic aquatic bacteria with 44–47 mol% G + C (MacDonell & Colwell, 1985). Since then many new species have been found, mostly from sea water, sediment and marine organisms, and currently there are 28 recognized *Shewanella* species (*Shewanellaceae*, ‘*Alteromonadales*’, class ‘*Gammaproteobacteria*’) at the time of writing this article (Bozal *et al.*, 2002; Coyne *et al.*, 1989; Bowman *et al.*, 1997; Makemson *et al.*, 1997; Nogi *et al.*, 1998; Leonardo *et al.*, 1999; Brettar *et al.*, 2002; Ivanova *et al.*, 2001, 2003a, b, 2004a, b, c; Skerratt *et al.*, 2002; Satomi *et al.*, 2003; Simidu *et al.*, 1990; Toffin *et al.*, 2004; Venkateswaran *et al.*, 1998b; Yoon *et al.*, 2004a, b; Ziemke *et al.*, 1998).

Some strains of *Shewanella* are known for polyunsaturated fatty acid production (Russell & Nichols, 1999; Bowman *et al.*, 1997; Satomi *et al.*, 2003), metal reduction (Myers &

Published online ahead of print on 17 February 2005 as DOI 10.1099/ijs.0.63604-0.

Abbreviations: MK, menaquinone; MMK, methylmenaquinone; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the *gyrB* gene sequence of strain HAW-EB3^T are AY579750 and AY842130, respectively.

A table of the quinone compositions of strain HAW-EB3^T and other species of *Shewanella* is available as supplementary material in IJSEM Online.

Nealson, 1988; Kostka *et al.*, 1996), growth at low temperature (Deming *et al.*, 1984; Jensen *et al.*, 1980; Bozal *et al.*, 2002) and high pressure (Nogi *et al.*, 1998; Deming *et al.*, 1984; Kato *et al.*, 1998), or for the degradation of pollutants such as RDX (Zhao *et al.*, 2004b), petroleum (Semple & Westlake, 1987) and chlorinated solvents (Petrovskis *et al.*, 1994). The objective of the present study is to extensively characterize the RDX-degrading isolate, *Shewanella* sp. HAW-EB3^T, and compare it with the properties of known *Shewanella* species. We found that strain HAW-EB3^T represents a novel species of *Shewanella*, designated *Shewanella sediminis* sp. nov.

Scanning (Hitachi S3000N) or negatively stained transmission (Hitachi H7500) electron micrographs of strain HAW-EB3^T cells were prepared according to previously described protocols (Beveridge *et al.*, 1994; Bozal *et al.*, 2002).

NaCl tolerance was tested on pre-cooled Brewer's anaerobic agar (Becton Dickinson; the reagent contained 0.5% NaCl) that contained 0.5, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0 or 8.0% NaCl, followed by aerobic incubation at 10 °C for 5 days. Bacterial growth was estimated using the following equation: biomass = $\pi \times (D/2)^2 \times \text{c.f.u.}$, where D represents mean diameter of colonies and one unit was equal to 50 µm. A Na⁺-free agar that contained 0.3% bacto beef extract and 0.5% bacto peptone was used to test growth in the absence of Na⁺.

Standard protocols were employed to test Gram reaction, catalase and oxidase activities, spore formation and acid production from various sugars (in Leifson Modified O-F medium) (Smibert & Krieg, 1994). Enzymic hydrolyses of various substrates including casein (skimmed milk, 50%), gelatin (1%), Tween 20, -40 and -80 (1%), olive oil (1%), lecithin (5% egg yolk), pure chitin (0.3%), alginate (1%) and starch (1%) were conducted using marine broth 2216 (Becton Dickinson) as a basal medium as described by Bowman (2001). Production of H₂S from thiosulfate was tested in marine agar 2216 (Becton Dickinson) supplemented with NaS₂O₃ (1%). DNase was determined using BBL DNA test agar (Becton Dickinson) supplemented with sea salts (4%). API Rapid 20E and ID32A (BioMérieux) test kits were used to detect additional enzymic activities as listed in the species description. GN2 microplates (Biolog) were used for metabolism of sugars, organic acids, amino acids, peptides and nucleosides (Table 1). Cells of 0.9 OD₆₀₀, higher than the 0.3 OD₆₀₀ instructed by the manufacturer, were used for GN2 tests because no oxidation of substrates was found at 0.3 OD₆₀₀. Aerobic utilization of substrates (0.1%) as sole carbon and energy sources by washed cells of strain HAW-EB3^T was evaluated in 24-well cell culture clusters containing NH₄Cl (0.1%) to serve as a nitrogen source in basic marine salts medium at pH 7.0 for 2, 4, 6 and 8 weeks. The basic marine salt medium was supplemented with a trace metal solution as described previously (Zhao *et al.*, 2003). The substrates that caused more than 15% increases in OD₆₀₀ (initial OD₆₀₀, 0.1) were recorded as positive because the variation at the low OD₆₀₀ measurement was 5–10%; those exhibiting a change less than 15%

in OD₆₀₀ were considered uncertain or negative. Reduction of electron acceptors was conducted on Brewer's anaerobic agar supplemented with 2% NaCl and one of the substrates MnO₂ (40 mM), ferric citrate (40 mM), amorphous iron oxide (FeOOH, 40 mM), trimethylamine N-oxide (TMAO) (5 mM), nitrate (5 mM), nitrite (5 mM) or elemental sulfur (40 mM, stock solution prepared as described by Moser & Nealson, 1996), followed by incubation in anaerobic jars for 30 days. Clear zones around colonies were used to indicate Fe(III), Mn(IV) and sulfur reduction (Myers & Nealson, 1988). The enhanced growth of HAW-EB3^T in the presence of TMAO, nitrate and nitrite was used as an indicator for dissimilatory reduction. All of the above physiological properties were tested at 10 °C unless noted otherwise. All of the phenotypic tests were run in triplicate.

Strain HAW-EB3^T, a Gram-negative bacterium, grew well aerobically in marine broth or agar 2216 (Becton Dickinson) or Brewer's anaerobic agar supplemented with either sea salts (Sigma, 4%) or 2% NaCl at 10 °C. The biomass of strain HAW-EB3^T exhibited a slightly orangish or pinkish colour, typical of species of *Shewanella* (Venkateswaran *et al.*, 1999). Strain HAW-EB3^T also grew anaerobically on marine agar 2216 and Brewer's anaerobic agar supplemented with 2% NaCl. Scanning electron micrographs showed that strain HAW-EB3^T was a rod-shaped bacterium (0.5–1 µm in diameter and 1.5–2.0 µm in length) (Fig. 1a). Negatively stained transmission electron micrographs showed the presence of a single polar flagellum (Fig. 1b), consistent with species of *Shewanella* (MacDonell & Colwell, 1985).

The physiological and biochemical properties of strain HAW-EB3^T are presented in the species description below. Generally, physiological and biochemical properties of strain HAW-EB3^T were consistent with *Shewanella* species, most of which are positive for catalase, oxidase, lipase and growth on glucose and/or N-acetyl-D-glucosamine, certain short chain fatty acids and amino acids at temperatures mostly ranging from 4–30 °C and in the presence of 1–4% (w/w) NaCl (Bozal *et al.*, 2002; Brettar *et al.*, 2002; Ivanova *et al.*, 2001, 2003a, b, 2004a, b; Leonardo *et al.*, 1999; Rossello-Mora *et al.*, 1994; Satomi *et al.*, 2003; Skerratt *et al.*, 2002; Toffin *et al.*, 2004; Venkateswaran *et al.*, 1999; Vogel *et al.*, 1997; Yoon *et al.*, 2004a, b; Zhao *et al.*, 2004b; Ziemke *et al.*, 1998). Strain HAW-EB3^T was a psychrophilic bacterium, growing at 4–25 °C with 10 °C as an optimum and no growth observed at 30 °C (Zhao *et al.*, 2004b). In the case of salinity tolerance, strain HAW-EB3^T was a Na⁺-requiring and moderately halophilic bacterium, growing at 1–4% (w/w) NaCl with an optimum of 2%. Like all species of *Shewanella*, strain HAW-EB3^T reduced nitrate and TMAO under anaerobic conditions. In the case of acid substrate profiles, strain HAW-EB3^T utilized acetate, pyruvate, butyrate and valerate, similar to several *Shewanella* species including *Shewanella hanedai*, *Shewanella gelidimarina*, *Shewanella algae* (Simidu *et al.*, 1990) and *Shewanella frigidimarina*.

Table 1. Characterization of HAW-EB3^T and related species of *Shewanella*

+, Positive; –, negative; NAG, *N*-acetylglucosamine; G, glucose; C, chitin; ND, no data. Data sources: Bowman *et al.* (1997); Deming *et al.* (1984); Jensen *et al.* (1980); Kato *et al.* (1998); MacDonell & Colwell (1985); Makemson *et al.* (1997); Nogi *et al.* (1998); Venkateswaran *et al.* (1999); this study.

Characteristic	HAW-EB3 ^T	<i>S. woodyi</i>	<i>S. hanedai</i>	<i>S. violacea</i>	<i>S. benthica</i>	<i>S. gelidimarina</i>
G + C (mol%)	45	39	45	ND	47	48
Bioluminescence	–	+	+	ND	–	–
Pigmentation of biomass	Pinkish	Pinkish	Pinkish	Violet	Red	Tan
Growth factor	–	–	Yeast extract	–	Vitamins	–
Growth at 6% NaCl	–	–	–	–	–	+
Growth at 25 °C	+	+	–	–	–	+
Fermentation (sugar)	+ (NAG)	–	+ (G)	+ (G)	+ (G, C)	+ (NAG, C)
H ₂ S from thiosulfate	+	+	+	–	ND	+
Fe(III) reduction	–	–	–	ND	–	+
Lipase	+	–	+	ND	ND	+
Gelatinase	–	+	+	+	ND	+
Chitinase	+	–	+	ND	ND	+
Lysine decarboxylase	+	–	–	–	–	–
Ornithine decarboxylase	+	–	–	–	–	–
Arginine dehydrolase	+	–	–	–	–	–
Utilization of:						
Glucose	–	–	+	+	+	–
Cellobiose	–	+	–	+	ND	–
<i>N</i> -Acetylglucosamine	+	–	+	ND	+	+
Ribose	–	–	+	ND	ND	ND
Galactose	–	+	+	+	ND	–
Propionate	+	+	+	ND	ND	–
Pyruvate	+	–	+	ND	ND	+
Lactate	+	–	–	ND	ND	+
Malate	+	–	–	ND	ND	–
Succinate	+	+	–	ND	ND	–
Valerate	+	–	+	ND	ND	+
Serine	+	–	+	ND	ND	+
Glutamate	–	–	+	ND	ND	+
Putrescine	–	+	+	ND	ND	–

Fig. 1. Micrographs of strain HAW-EB3^T: (a) scanning electron micrograph of cells; (b) transmission electron micrograph of a negatively stained cell showing a single polar flagellum.

Most *Shewanella* species are reported to utilize glucose and/or *N*-acetyl-D-glucosamine, fructose, sucrose or galactose as aerobic substrate(s). In comparison to known *Shewanella* species regarding utilization of sugars, strain HAW-EB3^T only utilized *N*-acetyl-D-glucosamine among the seven sugars tested (glucose, galactose, lactose, fructose, sucrose, mannose and *N*-acetyl-D-glucosamine), similar to *S. gelidimarina* (Bowman *et al.*, 1997). More than half (16 species) of all known *Shewanella* species (28 species in total) including strain HAW-EB3^T are fermentative. In the case of strain HAW-EB3^T, *N*-acetyl-D-glucosamine was fermented, as observed for *S. gelidimarina*. Most other fermentative species (*Shewanella benthica*, *S. hanedai*, *S. frigidimarina*, *Shewanella livingstonensis*, *Shewanella violacea*, *Shewanella affinis*, *Shewanella felis*, *Shewanella japonica*, *Shewanella pacifica* and *Shewanella waksmanii*) fermented glucose, a few (*Shewanella marinintestina*, *Shewanella schlegeliana* and *Shewanella sairae*) fermented ribose.

In contrast to reports that most species of *Shewanella* are negative for chitinase, strain HAW-EB3^T produced this enzyme, similar to *S. hanedai* and *S. gelidimarina*. More importantly, strain HAW-EB3^T contained lysine decarboxylase, which is absent in all reported species of *Shewanella*. Strain HAW-EB3^T also exhibited activities of arginine

dehydrogenase and ornithine decarboxylase, which are only found in a few species (Venkateswaran *et al.*, 1999; Ivanova *et al.*, 2004a, b; Bozal *et al.*, 2002). Strain HAW-EB3^T also did not exhibit gelatinase activity as found in most *Shewanella* species.

Thirty-two characteristics of strain HAW-EB3^T and 28 other known species of *Shewanella* were used for further phenotypic cluster analyses using methods described by Eisen *et al.* (1998) (Cluster and Maple Tree View software, version 2.11; Pearson correlation similarity, uncentred, agglomerative hierarchical clustering, complete linkage). The 32 characteristics were: fermentation; growth factor requirement; growth in the presence of 0%, 1%, 2%, 4%, 6%, 8% and 10% (w/v) NaCl; reduction of Fe(III) or TMAO; H₂S formation; growth at 4, 21, 30, 35, 37 and 42 °C; lipase; gelatinase; chitinase; alginase; amylase; utilization of glucose, fructose, sucrose, *N*-acetyl-D-glucosamine and citrate; alginase dehydrogenase; ornithine decarboxylase and lysine decarboxylase. Data matrix values were prepared as follows: 4, positive; 2, weakly positive; 1, dependent or varying data; -2, negative; -, missing data. All species of *Shewanella* separated into two clusters (Fig. 2). The first cluster (I) was composed of Na⁺-requiring and psychrophilic species, including strain HAW-EB3^T, two barophilic

Fig. 2. Phenotypic cluster analysis of *Shewanella* species. Cluster I: all species are Na⁺-requiring psychrophiles and do not grow at 35 °C. Cluster II: most species do not require Na⁺ for growth (except *S. pacifica*, *Shewanella gaetbuli*, *Shewanella aquimarina* and *Shewanella olleyana*), tolerate 6% NaCl (except *S. japonica*, *Shewanella oneidensis* and *Shewanella amazonensis*) and grow at 35 °C (except *S. pacifica*, *S. felis*, *Shewanella denitrificans*, *S. affinis*, *S. livingstonensis* and *S. frigidimarina*). All *Shewanella* species grow at 4 °C except *Shewanella colwelliana* of cluster I, and *S. affinis*, *S. aquimarina*, *S. algae* and *S. amazonensis* of cluster II. The phenotypic properties (see text for details) used for cluster analysis of known species of *Shewanella* are from the following references: Bowman *et al.* (1997); Bozal *et al.* (2002); Brettar *et al.* (2002); Deming *et al.* (1984); Ivanova *et al.* (2001, 2003a, b, 2004a, b); Jensen *et al.* (1980); Lee *et al.* (1977); Leonardo *et al.* (1999); MacDonell & Colwell (1985); Makemson *et al.* (1997); Myers & Nealson (1988); Nogi *et al.* (1998); Nozue *et al.* (1992); Rossello-Mora *et al.* (1994); Satomi *et al.* (2003); Simidu *et al.* (1990); Skerratt *et al.* (2002); Toffin *et al.* (2004); Venkateswaran *et al.* (1998b, 1999); Vogel *et al.* (1997); Weiner *et al.* (1985, 1988); Yoon *et al.* (2004a, b); Zhao *et al.* (2004b); Ziemke *et al.* (1998). The phenotypic properties of strain HAW-EB3^T are from the present study.

species (*S. benthica* and *S. violacea*), two bioluminescent species (*Shewanella woodyi* and *S. hanedai*), three marine intestinal inhabitants (*S. marinintestina*, *S. schlegeliana* and *S. sairae*) and one iron-reducer (*S. gelidimarina*). The second cluster (II) contained mainly non- Na^+ -requiring and 6 % NaCl-tolerant species with several exceptions (see legend to Fig. 2). The cluster analysis indicated that HAW-EB3^T appeared most similar to *S. gelidimarina* and *S. woodyi* of cluster I (Fig. 2). However, strain HAW-EB3^T was significantly different from *S. gelidimarina* and *S. woodyi* and other related species of *Shewanella* in many phenotypic characteristics as listed in Table 1.

Using a protocol described previously by Bowman (2001) and Fay & Richli (1991), cellular fatty acids were extracted from cells (pre-grown in marine broth 2216 at 10 °C for 5 days) and analysed from their methyl esters or 2-alkenyl-4,4-dimethylloxazoline-derivatives on a GC-MS system composed of a HP5-MS capillary column (50 m) and a MS detector (6890 MSD 5973, Agilent Technologies). A standard mixture of bacterial fatty acid methyl esters (CP Mix; Matreya) was used as a reference. Fatty acids found in HAW-EB3^T were similar to those of *Shewanella* species in cluster I (Fig. 2; Table 2; Russell & Nichols, 1999). Like *S. gelidimarina*, *S. benthica* and *S. hanedai* (Bowman *et al.*,

1997; Satomi *et al.*, 2003; Brettar *et al.*, 2002), strain HAW-EB3^T produced unsaturated palmitoleic acid C_{16:1}ω7 (33 %) and saturated palmitoleic acid C_{16:0} (17 %) as major acids. HAW-EB3^T also produced eicosapentaenoic acid (C_{20:5}ω3) as found in most species of *Shewanella* in cluster I (Fig. 2).

In the present study we analysed the quinone compositions of strain HAW-EB3^T and two related *Shewanella* species for comparison: *S. woodyi* ATCC 51908^T (grown in marine broth 2216 at 21 °C; Makemson *et al.*, 1997) and *S. hanedai* ATCC 33224^T (grown in *Photobacterium* broth at 10 °C; Jensen *et al.*, 1980) purchased from the American Type Culture Collection. Briefly, quinones extracted using the method described by Collins (1985), were identified by their UV spectra (Collins, 1994) of chromatographic peaks obtained on a Waters HPLC-UV system (Discovery C₁₈ column, 25 cm × 4.6 mm × 5 μm, Supelco; 70 % methanol/30 % acetonitrile at rate of 1.5 ml min⁻¹; 40 °C; 2996 photodiode array UV detector) and by their mass spectra (Nishijima *et al.*, 1997; Akagawa-Matsushita *et al.*, 1992) obtained on a LC/UV-MS system (Platform LC fronted with Agilent 1100 Micromass). Menaquinone (MK)-4 (6H) (vitamin K1), MK-4 (vitamin K2), ubiquinone (Q)-6 and -10 (Aldrich) were used as standards. Strain HAW-EB3^T

Table 2. Fatty acid compositions of *Shewanella* species

Each fatty acid methyl ester of strain HAW-EB3^T was quantified based on the peak area of its total ion chromatogram MS spectra scanned between 45 and 500 Da relative to those of all fatty acid methyl esters found.

Fatty acid	Fatty acid composition (%)					
	HAW-EB3 ^T	<i>S. woodyi</i>	<i>S. hanedai</i>	<i>S. benthica</i>	<i>S. violacea</i>	<i>S. gelidimarina</i>
12:0 3-OH	7	2		0.2–1.4		
12:0		4.5	0.1–0.4		4	0.3–0.7
i13:0	3	10	5.1–5.8	2.8–8.9	8	5.4–15.8
13:0		1	0–0.6	0–0.3		Trace–1.3
13:0 3-OH	8	5.3–6.6				
14:0	4	4.7–6.4	7.6–10	11–12	6	3.8–4.4
i15:0	8	14–20	4.6–8.2	4.4–6.2	14	8.3–11.6
a15:0		0.4–0.7	0.2			
15:0	1	4–4.8	2.9–5	0.4–0.9	7	1.6–6.7
15:1			0–0.6			1.1–2.9
16:0	17	21–26	12–21	11–12	16	6.4–11.1
16:1ω9			1.1			0–0.7
16:1ω7	33	16–17	19–33	24–44	19	27.4–37
17:0			0.6–1	0.5–8.1	2	0.4–0.5
17:1		1.5–2.1	0.8–2.4	1.6–7.8	2	2.7–5.1
18:0	2		0–0.5	0–0.5		0.1–0.2
18:1ω9			0.8	0–0.2		0.3–0.9
18:1ω7	7	1.0	1.4–3.7	0–1.7	5	1.0–8.4
20:4ω3			1.4			Trace–0.3
20:5ω3	3		19–23	16–18	15	11.7–16.0
Reference	This study	Makemson <i>et al.</i> (1997)	Bowman <i>et al.</i> (1997)	Bowman <i>et al.</i> (1997)	Nogi <i>et al.</i> (1998)	Bowman <i>et al.</i> (1997)

produced (A_{273} , relative to the sum of total quinones) Q-7 (33.7%), Q-8 (13.5%), MK-7 (48.6%) and methylmenaquinone (MMK)-7 (4.2%), consistent with quinone profiles previously reported for *Shewanella* species (Akagawa-Matsushita *et al.*, 1992; Venkateswaran *et al.*, 1999; Bozal *et al.*, 2002) and those of *S. woodyi* (Q-7, 53.3%; Q-8, 34.5%; MK-7, 9.2%; MMK-7, 2.9%) and *S. hanedai* (Q-7, 11%; Q-8, 24%; MK-7, 56%; MMK-7, 9%) obtained in the present study (Supplementary Table, available in IJSEM Online).

DNA extraction, amplification and sequencing were conducted according to standard molecular biology methods (Sambrook & Russell, 2001). The universal primers described previously by Yoon *et al.* (1998) were used for amplification of the 16S rRNA gene. The 16S rRNA gene (1288 bases) sequence was compared to published sequences using BLAST. The gene sequences of the isolate and those of closely related species were aligned using CLUSTAL_X (1.81). The neighbour-joining method (in the MEGA2 package; Kumar *et al.*, 2001), based on the pair-wise

nucleotide distance of the Kimura two-parameter, was used to build the phylogenetic tree. The number of bootstrap repetitions was 4000.

Phylogenetic analysis of the 16S rRNA gene sequences of strain HAW-EB3^T and known species of *Shewanella* clearly showed that strain HAW-EB3^T belonged to the *Shewanella* genus (Fig. 3). The 16S rRNA gene sequence cluster analysis separated all species of *Shewanella* into two clusters (I' and II'; Fig. 3) similar to those obtained by phenotypic analysis (Fig. 2). Cluster I' contained mainly Na⁺-requiring species (13 out of 16) whereas cluster II' contained mainly non-Na⁺-requiring species (10 out of 13). The isolate HAW-EB3^T 16S rRNA gene sequence displayed ≤97.4% similarity to those of the known *Shewanella* species and was found to best match those in cluster I'.

According to 16S rRNA gene sequence, two bioluminescent marine bacteria *S. hanedai* and *S. woodyi* of cluster I' were phylogenetically most related to strain HAW-EB3^T, with similarities of 96.4% and 97.4%, respectively. As reported

Fig. 3. Phylogenetic tree of 16S rRNA gene of type strains of *Shewanella* species and strain HAW-EB3^T. The phylogenetic tree was generated based on the pair-wise nucleotide distance of the Kimura two-parameter using the neighbour-joining method (pair-wise deletion) included in the MEGA2 software package (Kumar *et al.*, 2001). The bar indicates the difference of 1 nucleotide per 100. The numbers beside the nodes are the statistical bootstrap values (values lower than 50 are not shown).

by Stackebrandt & Goebel (1994), strains sharing less than 97.5 % 16S rRNA gene similarity are unlikely to belong to the same species. Therefore strain HAW-EB3^T was not likely to be a strain belonging to *S. hanedai* or *S. woodyi*, but rather possibly represented a new species.

The sequence of *gyrB* (coding the β -unit of DNA topoisomerase II) has been used to differentiate species similar in their 16S rRNA genes sequences (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994). To further differentiate strain HAW-EB3^T from related *Shewanella* species, we also sequenced *gyrB* (1007 bases) of strain HAW-EB3^T using previously described universal primers (Yamamoto & Harayama, 1995; Venkateswaran *et al.*, 1998a), and compared its sequence with those of other *Shewanella* species (Fig. 4). The *gyrB* sequence similarity between HAW-EB3^T and other species of *Shewanella* (*S. hanedai*, 82.7 %; *S. woodyi*, 85.4 %; *S. gelidimarina*, 79.6 %; *S. benthica*, 82.6 %) (Fig. 4) was found to be lower than the 90 % species cut-off value proposed by Venkateswaran *et al.* (1999) for *Shewanella*. We also found that the similarity of *gyrB* between species of *Shewanella* ranged from 60 % to 90 %, with the exception of *S. marinintestina* and *S. sairae*, which were 92.2 % similar (Fig. 4). The sequence data on *gyrB* further supported that HAW-EB3^T represented a new species of *Shewanella*.

Marmur's procedure (Johnson, 1994) was used to prepare and purify genomic DNA (1–5 mg) for DNA–DNA hybridization and G+C content measurement. DNA–DNA hybridization was determined based on the spectrophotometric measurement of renaturation rates as described by Johnson (1985b) and Bowman *et al.* (1998). As defined by Wayne *et al.* (1987), a species only includes those strains with approximately 70 % or greater genomic DNA–DNA relatedness. In the present study we found that the genomic DNA of strain HAW-EB3^T was less than 70 % related to that of *S. hanedai* (with 25 % DNA relatedness) and *S. woodyi* (with 17.8 % DNA relatedness) ($n=5$), confirming that it represents a new species of *Shewanella*. The genetic finding

was also consistent with the significant phenotypic difference between strain HAW-EB3^T and the two known bioluminescent species, *S. hanedai* and *S. woodyi* (Table 1).

According to the UV absorbance (A_{269}/A_{280}) at pH 3.0 and the thermal melting profile (T_m) of genomic DNA as described by Johnson (1985a), the G+C content of HAW-EB3^T was found to be 45 mol%, consistent with the reported values for other species of *Shewanella* (39–53 %) (Table 1).

Based on the previous discussions of phenotypic, chemotaxonomic (fatty acid and quinone compositions) and genetic data (Gillis *et al.*, 2001), we propose that HAW-EB3^T represents a new species of *Shewanella*, designated *Shewanella sediminis* sp. nov.

Description of *Shewanella sediminis* sp. nov.

Shewanella sediminis (se.di.mi'nis. L. gen. n. *sediminis* of sediment, the source of the type strain).

Gram-negative, motile, non-spore-forming rods with mean length of 1.7 μ m and mean diameter of 0.7 μ m. Single flagellum is found at polar position. Biomass is slightly pinkish and non-bioluminescent. Growth at temperatures ranging from 4 to 25 °C, with 10 °C as optimum. No growth at 30 °C. Na⁺ is required for growth. Growth at 1–4 % NaCl, with 2 % NaCl as optimum, but no growth at 5 % NaCl. Anaerobic growth on Brewer's anaerobic agar supplemented with 2 % NaCl. TMAO, MnO₂, nitrate, nitrite, thiosulfate and RDX are reduced. Negative for reduction of Fe(III) and elemental sulfur. Casein, chitin and DNA are hydrolysed, but not gelatin, alginate, starch and agar. Positive for catalase, oxidase, nitroreductase, arginine dehydrolase, ornithine decarboxylase, lysine decarboxylase, *N*-acetyl- β -D-glucosaminidase, β -galactosidase, β -galactosidase-6-phosphate, alkaline phosphatase, proline arylamidase, leucine glycine arylamidase, alanine arylamidase, and glutamyl glutamic acid arylamidase. Weakly positive for urease. Negative for β -glucosidase and *p*-nitrophenylalanine deaminase by API rapid test, and for

Fig. 4. Phylogenetic tree of *gyrB* of *Shewanella* species and strain HAW-EB3^T. The phylogenetic tree was generated based on the pair-wise nucleotide distance of the Kimura two-parameter using the neighbour-joining method (pair-wise deletion) included in the MEGA2 software package. The bar indicates the difference of 5 nucleotides per 100. The numbers beside the nodes are the statistical bootstrap values (values lower than 50 are not shown).

α -galactosidase, α -glucosidase, β -glucosidase, α -arabinosidase, β -glucuronidase, mannose and raffinose fermentation, indole production, arginine arylamidase, phenyl arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase and glycine arylamidase by Rapid ID32 A test. H_2S is produced from thiosulfate. By Biolog GN2 microplate test, positive for utilization of *N*-acetyl-D-glucosamine, acetate, methyl pyruvate, propionate, DL-lactate, L-alanine, L-aspartate, glycyl-L-aspartic acid, glycine-L-glutamic acid, L-alanine-glycine, inosine, uridine and thymidine; weakly positive for Tween 80, β -hydroxy butyrate, L-leucine and L-proline; negative for α -cyclodextrin, dextrin, glycogen, adonitol, L-arabinose, D-arabitol, i-erythritol, D-fucose, m-inositol, lactulose, D-melibiose, methyl β -D-glucoside, D-raffinose, L-rhamnose, D-trehalose, turanose, xylitol, succinic acid mono methyl ester, *cis*-aconitic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α - or γ -hydroxybutyric acid, *p*-hydroxy phenylacetic acid, itaconic acid, α -keto glutaric acid, α -ketovaleric acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, hydroxy-L-proline, L-ornithine, L-pyroglutamic acid, D-serine, DL-carnitine, γ -aminobutyric acid, urocanic acid, phenylethylamine, 2-aminoethanol, 2,3-butanediol, DL- α -glycerolphosphate, α -D-glucose 1-phosphate and D-glucose 6-phosphate. *N*-acetyl-D-glucosamine is oxidized and fermented to acid. Galactose, lactose, fructose, sucrose, mannose and glucose are not oxidized or fermented to acids. *N*-Acetyl-D-glucosamine, Tween 40, Tween 80, acetate, succinate, butyrate, valerate, pyruvate, serine, proline, peptone and yeast extract are sole carbon and energy sources. Tween 20, malate, propionate and glutamic acid are weak carbon sources. Galactose, lactose, fructose, sucrose, mannose, glucose, citrate, lactate, formate, phenol, glycine, leucine and threonine are not utilized. Fatty acids $C_{12:0}$ 3-OH (7%), $C_{13:0}$ 3-OH (8%), $C_{14:0}$ (4%), $C_{15:0}$ (8%), $C_{16:0}$ (17%), $C_{16:1\omega7}$ (33%), $C_{18:0}$ (2%), $C_{18:1\omega7}$ (7%) and $C_{20:5\omega3}$ (3%) are produced. Quinone composition is Q-7 (33%), Q-8 (13%), MK-7 (49%) and MMK-7 (4%). The G+C content is 45 mol%.

The type strain, HAW-EB3^T (=NCIMB 14036^T=DSM 17055^T), was isolated from a site 215 m deep and 50 nautical miles from Halifax Harbour, in the Atlantic Ocean.

Acknowledgements

Financial support from the USA Navy Office of Naval Research (ONR) (Award N000140310269) is gratefully acknowledged. The authors would like to thank Sonia Thiboutot, Guy Ampleman and D. Faucher (Defense Research and Development Canada, Valcartier, Quebec, Canada) and K. Penny (Canadian Navy) for sampling sediment. Dr Edwin Wang is acknowledged for discussion of phenotypic cluster analyses. Mass spectral analysis of bacterial quinones was conducted by Annamaria Halasz. TEM and SEM electron micrographs were prepared by C. Leggiadro and D. O'Neil of the Institute of Marine Biosciences, National Research Council, Halifax, Nova Scotia, Canada.

The authors would also like to thank Dr Jean Euzéby (École Nationale Vétérinaire, France) and Professor Jiri Tucker (McGill University, Canada) for advice on Latin nominations.

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