Shewanella canadensis sp. nov. and *Shewanella atlantica* sp. nov., manganese dioxide- and hexahydro-1,3,5-trinitro-1,3,5-triazine-reducing, psychrophilic marine bacteria

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Two strains belonging to the genus *Shewanella*, HAW-EB2^T and HAW-EB5^T, were isolated previously from marine sediment sampled from the Atlantic Ocean, near Halifax harbour in Canada, for their potential to degrade explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). In the present study, strains HAW-EB2^T and HAW-EB5^T were found to display high 16S rRNA gene sequence similarity (90–99.5 %) to species of *Shewanella*, but their *gyrB* sequences were significantly different from each other and from species of *Shewanella* (79–87.6 %). Furthermore, DNA–DNA hybridization showed that the genomic DNA of the two strains was only 22 % related and showed less than 41 % relatedness to closely related species of *Shewanella*. In comparison to other species of *Shewanella*, strains HAW-EB2^T and HAW-EB5^T were also unique in some phenotypic properties such as activities of β -galactosidase and tyrosine arylamidase and the ability to metabolize certain organic acids and sugars. Both strains HAW-EB2^T and HAW-EB5^T utilize malate, valerate, peptone and yeast extract as sole carbon and energy sources. The major membrane fatty acids of the two strains were C_{14:0}, iso-C_{15:0}, C_{16:0}, C_{16:1 ω 7}, C_{18:1 ω 7} and C_{20:5 ω 3} and their major quinones were Q-7, Q-8 and MK-7. On the basis of these results, strain HAW-EB2^T (=NCIMB 14238^T =CCUG 54553^T) is proposed as the type strain of *Shewanella canadensis* sp. nov. and strain HAW-EB5^T (=NCIMB 14239^T =CCUG 54554^T) is proposed as the type strain of *Shewanella atlantica* sp. nov.

Members of the genus *Shewanella* are Gram-negative, oxidase-positive, rod-shaped, aquatic bacteria whose genomic DNA contains 38–54 mol% G + C (Bowman, 2005). At the time of writing, there are 38 species of *Shewanella* with validly published names (<http://www.bacterio.cict.fr/s/shewanella.html>), mostly isolated from marine environments, with a few isolated from freshwater sediment, industrial wastewater, clinical specimen and spoiled foods.

Abbreviations: RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; TMAO, trimethylamine *N*-oxide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains HAW-EB2^T and HAW-EB5^T are AY579749 and AY579752 and those for the *gyrB* gene sequences of strains HAW-EB2^T and HAW-EB5^T are AY842129 and AY842132.

A comparison of the fatty acid compositions of strains HAW-EB2^T and HAW-EB5^T and related species is available as supplementary material with the online version of this paper.

Members of *Shewanella* can grow aerobically or anaerobically by reducing alternative electron acceptors such as trimethylamine *N*-oxide (TMAO) and nitrate (Bowman, 2005). Species of *Shewanella* also require or tolerate NaCl (1–4 %) and low temperatures for growth (Zhao *et al.*, 2005). Some species isolated from cold and deep-sea environments, such as *Shewanella hanedai*, *S. gelidimarina*, *S. violacea* and *S. benthica*, are known for production of polyunsaturated fatty acids (Kato & Nogi, 2001; Russell & Nichols, 1999; Bowman *et al.*, 1997). Species such as *Shewanella oneidensis* and *S. putrefaciens* have been reported to be able to reduce heavy-metal oxides (Myers & Nealson, 1988; Roh *et al.*, 2006) and chlorinated pollutants (Petrovskis *et al.*, 1994) and for their potential to generate electricity (Ringeisen *et al.*, 2006; Park & Zeikus, 2002).

Previously, we reported the isolation of several psychrophilic strains of *Shewanella*, including strains HAW-EB2^T and HAW-EB5^T, for their ability to degrade

hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in sediment from an undersea unexploded ordnance dumping site at Emerald Basin, in the Atlantic Ocean, that is 215 m deep and 50 nautical miles (93 km) from Halifax harbour, Canada (Zhao *et al.*, 2004a, b). In the present study, we provide genetic, chemotaxonomic and phenotypic evidence to propose each of the isolates as the type strain of a novel species of *Shewanella*.

As described previously, strains HAW-EB2^T and HAW-EB5^T are Gram-negative, psychrophilic bacteria with an optimal temperature of 10 °C for growth (Zhao *et al.*, 2004a). The two strains grew well in marine medium 2216 (liquid or agar; Becton Dickinson) or Brewer anaerobic agar (Becton Dickinson) supplemented with either sea salts (Sigma; 4%) or 2% NaCl. The biomass had a pinkish colour, typical of *Shewanella* (Bowman, 2005; Venkateswaran *et al.*, 1999).

Cells of strain HAW-EB2^T and HAW-EB5^T harvested after 3 days of aerobic growth in marine broth 2216 were used for transmission (TEM; Hitachi H7500) and scanning (SEM; Hitachi S3000N) electron micrographic imaging analyses under previously described experimental conditions (Zhao *et al.*, 2006). Both strains showed cellular morphology similar to other members of *Shewanella*: straight or slightly curved rods (1–5 µm long and 0.55–0.7 µm in diameter) with a single flagellum in a polar position (Fig. 1).

To determine the phylogenetic affiliation of the two bacteria, genomic DNA (1–5 mg) was purified using Marmur's method (Johnson, 1994) from the present isolates and *Shewanella woodyi* ATCC 51908^T and *S. hanedai* ATCC 33224^T, purchased from the American Type Culture Collection. Genes were amplified using primers generated in this study by the Genetool software (Biotools Inc.) and known sequences published in GenBank, and the experiments were conducted using standard protocols (Sambrook & Russell, 2001). The forward and reverse

primers used to amplify 16S rRNA genes were 5'-AAGCCACGGCTAACTACG and 5'-GTGTGTACAAGN-CCCGGGAA, respectively. The forward and reverse primers used to amplify *gyrB* (encoding the β subunit of DNA topoisomerase II) were 5'-MGYGGTCTGCACG-GCGT and 5'-GTGGCAGAATCGCTTGGT, respectively. The resulting 16S rRNA (1282–1295 bases) and *gyrB* (939–1013 bases) gene PCR products were subsequently sequenced and compared with published sequences using BLAST and aligned with those of closely related species using CLUSTAL_X (1.81). The neighbour-joining method (Saitou & Nei, 1987) included in the MEGA3 package (Kumar *et al.*, 2004), based on pairwise nucleotide distances from Kimura's two-parameter method (complete deletion) (Kimura, 1980), was used to build phylogenetic trees (Figs 2 and 3). The number of bootstrap repetitions was 4000.

Strains HAW-EB2^T and HAW-EB5^T were 98.9% similar to each other in their 16S rRNA gene sequences. Phylogenetic analyses based on 16S rRNA gene sequences showed that both strains were members of *Shewanella*, 90–99.5% similar to members of all 38 recognized species of the genus (Fig. 2). The 16S rRNA genes of strain HAW-EB2^T and strain HAW-EB5^T formed a solid cluster (with a bootstrap value of 82%) with three intermediate-sea species of *Shewanella* (95.7–99.5% similar), including two bioluminescent isolates (*S. woodyi* ATCC 51908^T, *S. hanedai* ATCC 33224^T) and another Halifax isolate, *Shewanella sediminis* DSM 17055^T (Fig. 2). The 16S rRNA genes of both strain HAW-EB2^T and strain HAW-EB5^T displayed the highest similarity to *S. sediminis* DSM 17055^T (HAW-EB2^T, 99.5%; HAW-EB5^T, 98.6%), a bacterium that was previously isolated from this site (Zhao *et al.*, 2005). Two barophilic and deep-sea species of *Shewanella*, *S. benthica* and *S. violacea*, also showed 96.8–97.3% 16S rRNA gene sequence similarity to strains HAW-EB2^T and HAW-EB5^T. However, these two species, isolated from the intestine of a sea animal and sediment of deep Atlantic or Pacific Trenches (Deming *et al.*, 1984; Nogi *et al.*, 1998), appeared

Fig. 1. (a) Scanning electron micrograph of cells of strain HAW-EB2^T. (b, c) Transmission electron micrographs of negatively stained cells of strain HAW-EB2^T (b) and strain HAW-EB5^T (c) showing a single polar flagellum. Bars, 5 µm (a), 0.5 µm (b) and 1.0 µm (c).

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of strains HAW-EB2^T and HAW-EB5^T and type strains of *Shewanella* species. The tree was generated based on pairwise nucleotide distances as described in the text. Bar, 1 substitution per 100 nucleotide positions. Numbers beside nodes are statistical bootstrap values (values lower than 50 % are not shown). Cluster II includes sequences of *Shewanella amazonensis* ATCC 700329^T (GenBank accession no. AF005248), *S. algae* IAM 14159^T (U91546), *S. pacifica* KMM 3597^T (AF500075), *S. japonica* LMG 19691^T (AF145921), *S. olleyana* ACAM 644^T (AF295592), *S. livingstonensis* LMG 19866^T (AJ300834), *S. frigidimarina* ACAM 591^T (U85903), *S. denitrificans* DSM 15013^T (AJ311964), *S. gaetbuli* JCM 11814^T (AY190533), *S. profunda* JCM 12080^T (AY445591), *S. putrefaciens* ATCC 8071^T (X82133), *S. oneidensis* ATCC 700329^T (AF005251), *S. baltica* DSM 9439^T (AJ000214), *S. morhuae* U1417^T (AB205576), *S. hafniensis* P010^T (AB205566) and *S. decolorationis* IAM 15094^T (AJ609571).

to belong to a lineage different from the one including the three intermediate-sea species *S. woodyi*, *S. hanedai* and *S. sediminis*, as shown in Fig. 2.

Strains HAW-EB2^T and HAW-EB5^T were 87.6 % similar in the sequences of their *gyrB* genes. This similarity value is lower than the 90 % species cut-off value proposed for *Shewanella* by Venkateswaran *et al.* (1999), thus the two strains appeared to belong to different species of *Shewanella*. The *gyrB* gene sequences of strains HAW-EB2^T and HAW-EB5^T were also found to be less than 90 % similar (79–86.5 %) to known species of *Shewanella* including *S. woodyi*, *S. hanedai*, *S. sediminis*, *S. benthica* and *S. violacea* (Fig. 3; Table 1). Therefore, strain HAW-EB2^T and HAW-EB5^T likely represent novel species of *Shewanella*.

The G + C content of genomic DNA of strains HAW-EB2^T and HAW-EB5^T was determined from the A_{269}/A_{280} ratio at pH 3.0 as described by Johnson (1985a) and was found to be 46.4 and 46.6 mol%, respectively, consistent with reported values (38–54 mol%) for other species of *Shewanella* (Bowman, 2005).

To determine the relatedness between total genomic DNA of strains HAW-EB2^T and HAW-EB5^T and related species of *Shewanella*, we conducted genomic DNA–DNA hybridization tests using the spectrophotometric method as described by Johnson (1985b) and Bowman *et al.* (1998). As shown in Table 1, genomic DNA of strains HAW-EB2^T and HAW-EB5^T showed only 22 % ($n=3$) relatedness to each other and less than 41 % ($n=3$) relatedness to the type strains of three closely related species of *Shewanella*, *S.*

woodyi, *S. hanedai* and *S. sediminis*. Since the relatedness values are below the 70 % species cut-off value recommended for bacteria (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994), strains HAW-EB2^T and HAW-EB5^T likely represent two novel species of *Shewanella*.

To provide chemotaxonomic evidence for the affiliation of the two strains to *Shewanella*, we characterized their quinone content as described by Collins (1985, 1994), Akagawa-Matsushita *et al.* (1992) and Nishijima *et al.* (1997) and analysed their fatty acid compositions using the protocol described by Bowman (2001) and Fay & Richli (1991) under experimental and instrumental conditions described previously (Zhao *et al.*, 2005). Strains HAW-EB2^T and HAW-EB5^T contained ubiquinones (Q-7, Q-8) and menaquinones (MK-7 and/or MMK-7) (details in the species descriptions) that are commonly found in *Shewanella* (Bowman, 2005). Strain HAW-EB2^T appeared not to contain MMK-7, which was found in *S. sediminis* DSM 17055^T (Zhao *et al.*, 2005) and HAW-EB5^T. The major fatty acids of strains HAW-EB5^T and HAW-EB2^T were palmitic acid (C_{16:0}; 19 %), myristic acid (C_{14:0}; 4–12 %), palmitoleic acid (C_{16:1ω7}; 34–39 %) and branched acids iso-C_{13:0} (3–5 %) and iso-C_{15:0} (8–11 %), similar to *S. woodyi*, *S. hanedai*, *S. benthica*, *S. violacea* and *S. gelidimarina* (see Supplementary Table S1 in IJSEM Online). Like *S. gelidimarina*, *S. benthica*, *S. violacea* and *S. hanedai*, the two strains produced eicosapentaenoic acid (C_{20:5ω3}, EPA; 4–6 %).

The two strains were also tested for their tolerance to NaCl on Brewer anaerobic agar containing 0.5, 1.5, 2.0, 2.5, 3.0,

Fig. 3. Phylogenetic tree based on *gyrB* sequences of strains HAW-EB2^T and HAW-EB5^T and type strains of *Shewanella* species. The tree was generated based on pairwise nucleotide distances as described in the text. Bar, 5 substitutions per 100 nucleotide positions. Numbers beside nodes are statistical bootstrap values (values lower than 50 % are not shown).

4.0, 6.0 or 8.0 % (w/v) NaCl (aerobic growth conditions). Growth in the absence of Na⁺ was tested in a Na⁺-free agar that contained 0.3 % Bacto beef extract and 0.5 % Bacto peptone. The two strains required a minimum of 1 % (w/v) NaCl for growth and both showed optimal growth in the presence of 1.5–3 % NaCl, similar to many species of *Shewanella*. Strain HAW-EB5^T appeared to be more halo-tolerant than HAW-EB2^T because, at 4 % (w/v) NaCl, strain HAW-EB5^T showed good growth whereas strain HAW-EB2^T did not grow.

To compare the phenotypic properties of strains HAW-EB2^T and HAW-EB5^T with known species of *Shewanella* further, we used previously described protocols (Bowman, 2001; Smibert & Krieg, 1994) to test the following biochemical and physiological properties: spore formation, H₂S formation from thiosulfate, acid production from various sugars (1 % w/v in Leifson modified O-F medium), activities of catalase, oxidase and DNase (in BBL DNA agar, supplemented with 4 % w/v sea salts; Becton Dickinson), hydrolysis of casein (50 % w/v skimmed milk), gelatin (1 %, w/v), Tweens 20, 40 and 80 (1 % w/v), olive oil (1 % w/v), lecithin (5 % w/v egg yolk), pure chitin (0.3 % w/v), alginate (1 % w/v) and starch (1 % w/v) using

marine broth 2216 as a basal medium. Aerobic utilization of substrates (0.1 % w/v) as sole carbon and energy sources was tested in basic marine salts medium (pH 7.0) containing 0.1 % (w/v) NH₄Cl as the nitrogen source (Zhao *et al.*, 2005). Reduction of electron acceptors was tested on Brewer anaerobic agar supplemented with 2 % (w/v) NaCl (in anaerobic jars) and one of the following compounds: manganese dioxide (MnO₂, 40 mM), ferric citrate (40 mM), amorphous iron oxide (FeOOH, 40 mM), TMAO (5 mM), nitrate (5 mM), nitrite (5 mM) and elemental sulfur (40 mM). Clear zones around colonies were used to indicate reduction of Fe(III), Mn(IV) and sulfur (Myers & Nealson, 1988). Enhanced growth of strains in the presence of electron acceptors was used as an indicator of dissimilatory reduction. Bacterial growth on agar was estimated by multiplying the average area of colonies and the total number of colony-forming units (Zhao *et al.*, 2005). Additional enzyme activities and utilization of substrates were tested by API Rapid 20E and ID32A (bioMérieux) test kits and GN2 microplates (Biolog) (using a cell suspension in sea-salts medium with an OD₆₀₀ of 0.9; 10 days incubation). Results were determined by colour change as instructed by the manufacturers: no colour change, negative; faint colour change,

Table 1. Similarity of 16S rRNA and *gyrB* gene sequences and total genomic DNA–DNA relatedness between the novel isolates and type strains of related species of *Shewanella*

Values above the diagonal are percentages of 16S rRNA/*gyrB* gene sequence similarity; values below the diagonal are percentages of DNA–DNA relatedness. ND, Not determined.

Strain	1	2	3	4	5
1. HAW-EB2 ^T	—	98.9/87.6	99.5/85.5	97.8/83.0	97.8/83.0
2. HAW-EB5 ^T	22	—	98.6/86.5	97.8/84.5	97.1/80.6
3. <i>S. sediminis</i> HAW-EB3 ^T	41	40	—	ND	ND
4. <i>S. woodyi</i> ATCC 51908 ^T	9	14	ND	—	ND
5. <i>S. hanedai</i> ATCC 33224 ^T	20	15	ND	ND	—

negative for API Rapid 20E and ID32A and weakly positive for Biolog tests; strong colour change, positive. For comparison, *S. sediminis* HAW-EB3^T, *S. woodyi* ATCC 51908^T, *S. hanedai* ATCC 33224^T and *S. benthica* ATCC 43991 were tested for reduction of MnO₂, H₂S production from thiosulfate, activity of catalase and other enzymes using ID32A and metabolism of Biolog (GN2) substrates under the conditions used for strains HAW-EB2^T and HAW-EB5^T. All of the above physiological properties were tested in triplicate at 10 °C unless noted otherwise.

The two strains, like all species of *Shewanella*, were positive for oxidase activity and for reduction of TMAO and nitrate. Cluster analyses of phenotypic properties, conducted as described previously (Zhao *et al.*, 2005), of the two strains and all species of *Shewanella* showed that strains HAW-EB5^T and HAW-EB2^T were close to the intermediate-sea species *S. sediminis* and *S. woodyi*, consistent with the earlier observation made from 16S rRNA and *gyrB* gene sequencing. Like *S. sediminis*, *S. woodyi* and *S. benthica*, the two strains reduced MnO₂, a common sediment component in the marine environment. As shown in Table 2, strains HAW-EB5^T and HAW-EB2^T exhibited significant differences from each other and from related species (*S. sediminis*, *S. woodyi*, *S. hanedai*, *S. violacea* and *S. benthica*) in several phenotypic properties such as bioluminescence, requirements for temperature, NaCl and pressure and metabolism of certain sugars and acids.

All of the genetic (Figs 2 and 3; Table 1), chemotaxonomic (Supplementary Table S1) and phenotypic (Table 2) data presented above demonstrate that strains HAW-EB2^T and HAW-EB5^T represent novel species of *Shewanella*, named *Shewanella canadensis* sp. nov. and *Shewanella atlantica* sp. nov., respectively.

Description of *Shewanella canadensis* sp. nov.

Shewanella canadensis (ca.na.den'sis. N.L. fem. adj. *canadensis* from Canada, the country nearest to the sediment sampling site, Emerald Basin, where the type strain was isolated).

Cells are Gram-negative, non-spore-forming, straight or slightly curved rods, 1.5–3.5 µm long and 0.65–0.75 µm in diameter. Motile by a single flagellum in a polar position. Biomass is dark orange or slightly pinkish and non-bioluminescent. Psychrophilic growth at temperatures of 4–25 °C (inclusive; optimum growth at 10 °C); no growth at 30 °C. Able to grow at 1–3 % (w/v) NaCl. Requires at least 1 % (w/v) NaCl for growth. Little growth at 4 % (w/v) NaCl (biomass obtained at 4 % NaCl is 3 % of optimum). No growth at 6 % (w/v) NaCl. TMAO, MnO₂, nitrate, nitrite and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are reduced. Negative for reduction of Fe(III) and elemental sulfur. Weak production of H₂S from thiosulfide. Positive for oxidase, nitroreductase (reduction of nitrate, nitrite and nitro group of RDX), caseinase, chitinase, gelatinase and DNase. Weakly positive for catalase. Negative for alginase,

amylase, agarase and lipase (hydrolysis of Tweens 20, 40 and 80). In API ID32A and API 20E tests, positive for urease, ornithine decarboxylase, lysine decarboxylase, *N*-acetyl-β-D-glucosaminidase, β-galactosidase, β-galactosidase-6-phosphate, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucine glycine arylamidase, tyrosine arylamidase, alanine arylamidase, leucine arylamidase and glutamyl glutamic acid arylamidase. In Biolog GN2 microplate tests, positive for metabolism of Tween 80, *N*-acetyl-D-glucosamine, acetate, β-hydroxybutyrate, DL-lactate, propionate, succinate, L-alanine, L-serine, L-threonine, L-leucine, L-aspartate, L-proline, L-alanyl glycine, glycyl L-aspartate, glycyl L-glutamate, inosine, uridine and thymidine; weakly positive for Tween 40 and methylpyruvate. *N*-Acetyl-D-glucosamine is only oxidized to acid under oxic conditions. Galactose, lactose, fructose, sucrose, mannose and glucose are not oxidized or fermented to acids. Tween 40, malate, succinate, valerate, peptone and yeast extract are used as sole carbon and energy sources. Fatty acids C_{12:0} 3-OH (1 %), iso-C_{13:0} (5 %), C_{14:0} (12 %), C_{14:1} (1 %), C_{15:0} (1 %), iso-C_{15:0} (8 %), C_{16:0} (19 %), C_{16:1ω7} (39 %), C_{18:0} (1 %), C_{18:1ω7} (4 %), C_{20:1} (1 %), C_{20:5ω3} (4 %) and C_{21:0} (1 %) are produced. Quinone composition is Q-7 (39.1 %), Q-8 (15.9 %), MK-7 (44.9 %) and MMK-7 (trace). The molar DNA G + C content is 46.4 ± 0.3 mol%.

The type strain is HAW-EB2^T (=NCIMB 14238^T =CCUG 54553^T).

Description of *Shewanella atlantica* sp. nov.

Shewanella atlantica (at.lan'ti.ca. L. fem. adj. *atlantica* of the Atlantic Ocean, where the type strain was isolated).

Cells are Gram-negative, non-spore-forming, straight or slightly curved rods, 2–5 µm long and 0.5–0.6 µm in diameter. Motile by a single flagellum in a polar or subpolar position. Biomass is slightly pinkish and non-bioluminescent. Psychrophilic growth at temperatures of 4–25 °C (optimum growth at 10 °C); no growth at 30 °C. Na⁺ is required for growth; grows at 1–4 % (w/v) NaCl (growth at 4 % NaCl is 32 % of optimum). No growth at 6 % NaCl. TMAO, MnO₂, nitrate, nitrite, thiosulfate and RDX are reduced. H₂S is produced from thiosulfate. Negative for reduction of Fe(III) and elemental sulfur. Positive for oxidase, nitroreductase, chitinase, lipase (hydrolysis of Tweens 20, 40 and 80) and DNase. Weakly positive for catalase and caseinase. Negative for gelatinase, alginase and amylase. In API ID32A and API 20E tests, positive for urease, ornithine decarboxylase, lysine decarboxylase, *N*-acetyl-β-D-glucosaminidase, β-galactosidase, glutamate decarboxylase, β-galactosidase-6-phosphate, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucine glycine arylamidase, alanine arylamidase, glycine arylamidase, leucine arylamidase, serine arylamidase and glutamyl glutamic acid arylamidase. In Biolog GN2 microplate tests, positive for metabolism of Tweens 40 and 80, *N*-acetyl-D-glucosamine, acetate, lactate,

Table 2. Characteristics that differentiate strains HAW-EB2^T and HAW-EB5^T and related species of *Shewanella*

Unless otherwise noted, properties of *S. woodyi*, *S. hanedai* and *S. benthica* were obtained in the present study using strains ATCC 51908^T, ATCC 33224^T and ATCC 43991^T, respectively, and under the same experimental conditions used for HAW-EB2^T and HAW-EB5^T. Other sources of data for reference species are indicated. +, Positive; –, negative; w, weak; ND, no data. In the present study, all strains were found to be positive for reduction of MnO₂, activities of *N*-acetyl-β-D-glucosaminidase, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucine glycine arylamidase, alanine arylamidase, glutamyl glutamic acid arylamidase and arabinosidase (the latter was weak for *S. benthica* ATCC 43991^T) and metabolism of Tweens 40 and 80, DL-lactate, L-alanine, L-serine, L-threonine, glycyl L-aspartate, glycyl L-glutamate, L-proline, L-leucine, L-alanyl glycine, inosine, uridine and thymidine. All strains were negative for metabolism of other substrates of GN2 microplates and activities of enzymes tested by API Rapid ID 32A.

Characteristic	HAW-EB2 ^T	HAW-EB5 ^T	<i>S. sediminis</i> HAW-EB3 ^T	<i>S. woodyi</i>	<i>S. hanedai</i>	<i>S. violacea</i>	<i>S. benthica</i>
DNA G + C content (mol%)	46.4	46.6	45 ^{a*}	46 ^b	44–47 ^b	47 ^b	46–47 ^b
Bioluminescence	–	–	–	+	+	– ^b	–
Barophilic growth	–	–	– ^a	– ^b	– ^b	+ ^e	+ ^e
Growth at 4% NaCl†	– (3%)	+ (30%)	w (17%) ^a	+ ^c	+ ^b	ND	ND
H ₂ S from thiosulfate	w (or –)	Strong	Strong	–	Very strong	– ^b	Strong
Activities of:							
Catalase	w (or –)	w (or –)	+	Strong	Strong	+ ^e	Very strong
Lipase	–	+	+	+ ^b	+ ^b	ND	+ ^b
Gelatinase	+	–	– ^a	+ ^c	+ ^d	+ ^e	+ ^e
β-Galactosidase	+	+	+	–	–	ND	+
α- and β-Glucosidases	–	–	–	+	– (+ for β)	ND	–
Tyrosine arylamidase	+	–	–	–	+	ND	+
Glutamic decarboxylase	w	+	w	+	+	ND	–
Growth substrates							
Butyrate, pyruvate	–	–	+	ND	+ ^d	ND	ND
Fructose	–	+	–	ND	– ^d	ND	ND
Utilization of Biolog substrates‡							
Cellobiose, glucose, galactose, trehalose, maltose, glutamate, gentiobiose	– (w; Glc)	– (w; Glc)	– (w; Glc, Glu)	+	+ (–; Tre, Mal)	ND	w (–; Mal, Gen, Tre)
Asparagine	–	–	+	+	–	ND	–
Methylpyruvate	w	+	+	+	–	ND	+
Propionate	+	w	+	+	w	ND	–
α-Hydroxybutyrate	–	w	+	–	–	ND	–
β-Hydroxybutyrate	+	–	+	–	–	ND	–
Succinate	+	+	–	w	–	ND	–
Bromosuccinate	–	+	–	–	–	ND	–
Sampling depth (m)§	215	215	215	200–400	100–200	5110	5920

*Data taken from the following studies: *a*, Zhao *et al.* (2005); *b*, Bowman (2005); *c*, Makemson *et al.* (1997); *d*, Jensen *et al.* (1980); *e*, Nogi *et al.* (1998).

†Relative amount of biomass obtained at 4% NaCl shown in parentheses in comparison with that obtained at 2% NaCl.

‡Gen, Gentiobiose; Glc, glucose; Glu, glutamate; Mal, maltose; Tre, trehalose.

§Sampling sites: strains HAW-EB2^T, HAW-EB5^T and *S. sediminis* HAW-EB3^T, Emerald Basin sediment, Atlantic Ocean off Halifax; *S. woodyi* strains, detritus, squid ink and seawater of Alboran Sea; *S. hanedai* strains, sediment and water of Antarctic, Arctic and Pacific Oceans; *S. violacea* strains, sediment of Ryukyu Trench; *S. benthica* ATCC 43991^T, intestine of *Scopelocheirus shellengi*, Puerto Rico Trench.

methylpyruvate, L-alanine, L-aspartate, L-serine, L-leucine, L-proline, L-threonine, L-alanyl glycine, glycyl L-aspartate, glycyl L-glutamate, inosine, uridine and thymidine; weakly positive for glycogen, α-hydroxybutyrate, α-ketobutyrate, succinate and bromosuccinate. Acetate, malate, valerate, fructose, peptone and yeast extract are used as sole carbon and energy sources for growth. *N*-Acetyl-D-glucosamine is oxidized weakly and fermented to acid. Fructose and

sucrose are fermented very slowly to acids. Galactose, lactose, mannose and glucose are not oxidized or fermented to acids. Fatty acids C_{12:0} 3-OH (3%), iso-C_{13:0} (3%), C_{13:0} 3-OH (5%), C_{14:0} (4%), C_{14:1} (1%), anteiso-C_{15:0} (1%), C_{15:0} (1%), iso-C_{15:0} (11%), C_{16:0} (19%), C_{16:1ω7} (34%), iso-C_{17:0} (1%), C_{18:0} (2%), C_{18:1ω7} (7%), C_{20:1} (1%), C_{20:5ω3} (6%) and C_{21:0} (1%) are produced. Quinone composition is Q-7 (13.5%), Q-8

(10.3 %), MK-7 (67.9 %) and MMK-7 (8.3 %). The molar DNA G + C content is 46.4 ± 0.1 mol%.

The type strain is HAW-EB5^T (=NCIMB 14239^T =CCUG 54554^T).

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References

- Akagawa-Matsushita, M., Itoh, T., Katayama, Y., Kuraishi, H. & Yamasato, K. (1992). Isoprenoid quinone composition of some marine *Alteromonas*, *Marinomonas*, *Deleya*, *Pseudomonas* and *Shewanella* species. *J Gen Microbiol* **138**, 2275–2281.
- Bowman, J. P. (2001). Methods for psychrophilic bacteria. *Methods Microbiol* **30**, 591–615.
- Bowman, J. P. (2005). Genus XIII. *Shewanella* MacDonell and Colwell 1986, 355^{VP}. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, part B, pp. 480–491. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer-Verlag.
- Bowman, J. P., McCammon, S. A., Nichols, D. S., Skerratt, J. H., Rea, S. M., Nichols, P. D. & McMeekin, T. A. (1997). *Shewanella gelidimarina* sp. nov. and *Shewanella frigidimarina* sp. nov., novel Antarctic species with the ability to produce eicosapentaenoic acid (20:5 ω 3) and grow anaerobically by dissimilatory Fe(III) reduction. *Int J Syst Bacteriol* **47**, 1040–1047.
- Bowman, J. P., McCammon, S. A., Lewis, T., Skerratt, J. H., Nichols, D. S. & McMeekin, T. A. (1998). *Psychroflexus torquis* gen. nov., sp. nov., a psychrophilic species from Antarctic sea ice, and reclassification of *Flavobacterium gondwanense* (Dobson et al. 1993) as *Psychroflexus gondwanense* gen. nov., comb. nov. *Microbiology* **144**, 1601–1609.
- Collins, M. D. (1985). Analysis of isoprenoid quinones. *Methods Microbiol* **18**, 329–366.
- Collins, M. D. (1994). Isoprenoid quinones. In *Chemical Methods in Prokaryotic Systematics*, pp. 265–309. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.
- Deming, J. W., Hada, H., Colwell, R. R., Luehrsens, K. R. & Fox, G. E. (1984). The ribonucleotide sequence of 5S rRNA from two strains of deep-sea barophilic bacteria. *J Gen Microbiol* **130**, 1911–1920.
- Fay, L. & Richli, U. (1991). Location of double bonds in polyunsaturated fatty acids by gas chromatography-mass spectrometry after 4,4-dimethyloxazoline derivatization. *J Chromatogr* **541**, 89–98.
- Jensen, M. J., Tebo, B. M., Baumann, P., Mandel, M. & Nealson, K. H. (1980). Characterization of *Alteromonas hanedai* (sp. nov.), a nonfermentative luminous species of marine origin. *Curr Microbiol* **3**, 311–315.
- Johnson, J. L. (1985a). Determination of DNA base composition. *Methods Microbiol* **18**, 23–24.
- Johnson, J. L. (1985b). DNA reassociation and RNA hybridisation of bacterial nucleic acids. *Methods Microbiol* **18**, 33–74.
- Johnson, J. L. (1994). Similarity analysis of DNAs. In *Methods for General and Molecular Bacteriology*, pp. 655–682. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Kato, C. & Nogi, Y. (2001). Correlation between phylogenetic structure and function: examples from deep sea *Shewanella*. *FEMS Microbiol Ecol* **35**, 223–230.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Makemson, J. C., Fulayfil, N. R., Landry, W., Van Ert, L. M., Wimpee, C. F., Widder, E. A. & Case, J. F. (1997). *Shewanella woodyi* sp. nov., an exclusively respiratory luminous bacterium isolated from the Alboran Sea. *Int J Syst Bacteriol* **47**, 1034–1039.
- Myers, C. R. & Nealson, K. H. (1988). Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science* **240**, 1319–1321.
- Nishijima, M., Araki-Sakai, M. & Sano, H. (1997). Identification of isoprenoid quinones by frit-FAB liquid chromatography-mass spectrometry for the chemotaxonomy of microorganisms. *J Microbiol Methods* **28**, 113–122.
- Nogi, Y., Kato, C. & Horikoshi, K. (1998). Taxonomic studies of deep-sea barophilic *Shewanella* strains and description of *Shewanella violacea* sp. nov. *Arch Microbiol* **170**, 331–338.
- Park, D. H. & Zeikus, J. (2002). Impact of electrode composition on electricity generation in a single-compartment fuel cell using *Shewanella putrefaciens*. *Appl Microbiol Biotechnol* **59**, 58–61.
- Petrovskis, E. A., Vogel, T. M. & Adriaens, P. (1994). Effects of electron acceptors and donors on transformation of tetrachloromethane by *Shewanella putrefaciens* MR-1. *FEMS Microbiol Lett* **121**, 357–364.
- Ringeisen, B. R., Henderson, E., Wu, P. K., Pietron, J., Ray, R., Little, B., Biffinger, J. C. & Jones-Meehan, J. M. (2006). High power density from a miniature microbial fuel cell using *Shewanella oneidensis* DSP10. *Environ Sci Technol* **40**, 2629–2634.
- Roh, Y., Gao, H., Vali, H., Kennedy, D. W., Yang, Z. K., Gao, W., Dohnalkova, A. C., Stapleton, R. D., Moon, J. W. & other authors (2006). Metal reduction and iron biomineralization by a psychrotolerant Fe(III)-reducing bacterium, *Shewanella* sp. strain PV-4. *Appl Environ Microbiol* **72**, 3236–3244.
- Russell, N. J. & Nichols, D. S. (1999). Polyunsaturated fatty acids in marine bacteria – a dogma rewritten. *Microbiology* **145**, 767–779.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Venkateswaran, K., Moser, D. P., Dollhopf, M. E., Lies, D. P., Saffarini, D. A., MacGregor, B. J., Ringelberg, D. B., White, D. C., Nishijima, M. & other authors (1999). Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *Int J Syst Bacteriol* **49**, 705–724.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Zhao, J.-S., Spain, J., Thiboutot, S., Ampleman, G., Greer, C. & Hawari, J. (2004a). Phylogeny of cyclic nitramine-degrading psychrophilic bacteria in marine sediment and their potential role

in the natural attenuation of explosives. *FEMS Microbiol Ecol* **49**, 349–357.

Zhao, J.-S., Greer, C. W., Thiboutot, S., Ampleman, G. & Hawari, J. (2004b). Biodegradation of nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine in cold marine sediment under anaerobic and oligotrophic conditions. *Can J Microbiol* **50**, 91–96.

Zhao, J.-S., Manno, D., Beaulieu, C., Paquet, L. & Hawari, J. (2005). *Shewanella sediminis* sp. nov, a novel Na⁺-requiring and hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading bacterium from marine sediment. *Int J Syst Evol Microbiol* **55**, 1511–1520.

Zhao, J.-S., Manno, D. & Hawari, J. (2006). *Shewanella halifaxensis* sp. nov, a novel obligately respiratory and denitrifying psychrophile. *Int J Syst Evol Microbiol* **56**, 205–212.