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Shewanella septentrionalis sp. nov. and *Shewanella holmiensis* sp. nov., isolated from Baltic Sea water and sediments

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Abstract

Two bacterial strains, SP1W3^T and SP1S2-7^T, were isolated from samples of water and sediments collected in Vaxholm, a town located on the Stockholm archipelago in the Baltic Sea, in November 2021. The strains were identified as novel genomic species within the genus *Shewanella*, based upon comparative analysis of whole genome sequence data. Strain SP1W3^T (genome size, 5.20 Mbp; G+C content, 46.0 mol%), isolated from water, was determined to be most closely related to *S. hafniensis* ATCC-BAA 1207^T and *S. baltica* NCTC 10735^T, with digital DNA–DNA hybridization (dDDH) values of 61.7% and 60.4%, respectively. Strain SP1S2-7^T (genome size, 4.26 Mbp; G+C content, 41.5 mol%), isolated from sediments, was observed to be most closely related to *S. aestuarii* JCM17801^T, with a pairwise dDDH value of 33.8%. Polyphasic analyses of physiological and phenotypic characteristics, in addition to genomic analyses, confirmed that each of these two strains represent distinct, novel species within the genus *Shewanella*, for which the names *Shewanella septentrionalis* sp. nov. (type strain SP1W3^T=CCUG 76164^T=CECT 30651^T) and *Shewanella holmiensis* sp. nov. (type strain SP1S2-7^T=CCUG 76165^T=CECT 30652^T) are proposed.

INTRODUCTION

The genus *Shewanella* [1] comprises 81 species with a validly published and correct name (https://lpsn.dsmz.de/genus/shewanella, accessed 29 November 2022) of Gram-negative, rod-shaped, facultative anaerobic bacteria ubiquitously distributed in aquatic and sediment environments worldwide [2, 3]. *Shewanella* species are also part of the microbiota of aquatic organisms, have been implicated in food spoilage and are occasional pathogens of aquatic animals, as well as humans [4–7]. The number of *Shewanella* species has steadily increased for more than two decades, in part because of the marked increase in the application of whole-genome sequence (WGS) data for analysing bacteria, including for microbial taxonomy and systematics. Making use of standards for the definition of prokaryotic species based on genomic data [8], evidence of numerous sequenced *Shewanella* isolates needing taxonomic revision was raised [9]. We have recently provided the reference WGS-based taxonomy for the family *Shewanellaceae* upon sequencing all type strains of validly published *Shewanella* species lacking a WGS [10]. This represents a valuable taxonomic resource, since any sequenced member of this family can now be unambiguously assigned to a known species or identified as a potential novel one, thereby facilitating the taxonomic classification of a growing pool of sequenced isolates.

Here, we report the isolation of two *Shewanella* strains, SP1W3^T and SP1S2-7 ^{T}, retrieved from water and sediment samples collected from the Baltic Sea. Based on a polyphasic characterization involving WGS, phylogenomic and phenotypic analyses, strains SP1W3^T (CCUG 76164^T=CECT 30651^T) and SP1S2-7^T (CCUG 76165^T=CECT 30652^T) are defined as representatives

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Keywords: aquatic bacteria; Baltic Sea; novel species; sediments; *Shewanella*.

Abbreviations: dDDH, digital DNA–DNA hybridization; GBDP, Genome BLAST Distance Phylogeny; TYGS, Type (Strain) Genome Server; WGS, whole-genome sequence.

16S rRNA gene sequences were deposited at the NCBI GenBank under the accession numbers: OP339865, *Shewanella septentrionalis* sp. nov. strain SP1W3^T (CCUG 76164^T=CECT 30651^T); OP339862, *Shewanella holmiensis* sp. nov., strain SP1S2-7^T (CCUG 76165^T=CECT 30652^T); whole-genome sequences were deposited at the NCBI GenBank under the accession numbers: JAMTCC000000000, *S. septentrionalis* SP1W3^T; JAMTCD000000000, *S. holmiensis* SP1S2-7^T.

One supplementary figure and two supplementary tables are available with the online version of this article.

of two novel *Shewanella* species for which the names *Shewanella septentrionalis* sp. nov. and *Shewanella holmiensis* sp. nov. are proposed, respectively.

ISOLATION AND ECOLOGY

Samples of water and superficial sediments were collected aseptically in Norrhamnsbadet (59.405517°, 18.352361°), a recreational marina in the town of Vaxholm, located in the Stockholm archipelago, on 14 November 2021. Water in the second largest archipelago of the Baltic Sea is brackish, with a salinity lower than 7 p.p.m. and a high input of freshwater [11]. Ice formation is not uncommon during winter. Surface water samples were collected from the shore in sterile high-density polyethylene bottles, while superficial sediments were scooped using sterile 50 ml tubes. Water and sediment samples were transported to the laboratory at room temperature and processed immediately. Thus, a 1 ml aliquot of water was separated and serially diluted (10^{-1}, 10^{-2}) in 0.01 M PBS, pH 7.4. Aliquots (100 µl) of each dilution and undiluted sample were plated on Lyngby's iron agar (Oxoid) supplemented with 0.04% w/v L-cysteine. The bulk sediment sample was first centrifuged at low speed (54 *g*, 4 °C) and supernatant water was decanted. For the extraction of bacteria from sediments, 15 g sediment material were separated, overlaid with 30 ml 0.22 µm-filtered water from the site, and placed on a tilt shaker (100 r.p.m., 60°, 4 °C) for 1 h. The samples were allowed to stand for 1 min in vertical position before separating 1 ml of the overlying water containing the extracted bacteria for serial dilution in PBS (10^{-1} to 10^{-3}) and plating (100 µl) on Lyngby's iron agar supplemented with 0.04% w/v L-cysteine. This medium, while it is not selective for *Shewanella* species, enables the identification of H_2S-producing bacteria, which form 'black' colonies upon deposition of iron sulphide originating from the reduction of thiosulphate or L-cysteine. While not a universal trait of *Shewanella* species, most species of the genus are known to be H_2S producers [12]. Inoculated plates were incubated at 26 °C for 36 h and black deposit-forming colonies were selected and purified by re-streaking three times on the same agar medium. Strains SP1W3^T and SP1S2-7^T were found to grow satisfactorily in Miller's LB medium. Thus, following purification, single colonies were grown in LB broth and aliquots preserved at −80 °C upon supplementation with glycerol at a final concentration of 20% v/v. Isolate SP1W3^T was retrieved from a water sample, whereas SP1S2-7^T was isolated from sediments.

GENOMIC AND GENOTYPIC FEATURES AND PHYLOGENY

Genomic DNA of strains SP1W3^T and SP1S2-7^T was isolated using the DNeasy Blood and Tissue kit (Qiagen), and sequenced as previously described [10]. Briefly, library preparation was carried out with the MGI FS library preparation set, as recommended by the manufacturer, using 50 ng of gDNA. Circularized DNA of equimolar pooled libraries was prepared, using the MGI Easy circularization kit (MGI Tech), and sequenced (2×100 bp paired-ends) on a DNBSEQ-G400 instrument (MGI), according to the manufacturer's instructions. Genome assembly was performed with SPAdes version 3.15.2, as implemented in Bactopia version 1.7.x [13]. Draft genomes were deposited in GenBank under accessions JAMTCC000000000 (SP1W3^T) and JAMTCD000000000 (SP1S2-7^T). The genome size of SP1W3^T was 5199212 bp, with a G+C content of 46.0 mol%, whereas the genome of SP1S2-7^T was 4261322 bp long and had a G+C content of 41.5 mol%. Genome assemblies were submitted to the Type Strain Genome Server (TYGS; www.tygs.dsmz.de) containing a fully updated genome sequence database for *Shewanella* species [10]. In brief, TYGS employs digital DNA–DNA hybridization (dDDH) to reconstruct the phylogenetic relationships of strains, with respect to reference strains and species clusters, based on a 70% dDDH threshold for species delineation [14, 15]. Thus, a phylogenomic reconstruction was inferred with FastME 2.1.6.1 [16] from Genome BLAST Distance Phylogeny (GBDP) distances calculated from genome sequences. The resulting midpoint-rooted tree [17] is shown in Fig. 1a and supports the taxonomic positioning of strains SP1W3^T and SP1S2-7^T as members of two novel *Shewanella* species. The closest phylogenetic relatives of SP1W3^T were observed to be *S. hafniensis* ATCC-BAA 1207^T and *S. baltica* NCTC 10735^T, exhibiting dDDH relatedness of 61.7% and 60.4%, respectively, inferred by distance formula d_4, as determined by the GBDP implemented in TYGS [18]. The closest relative of strain SP1S2-7^T was observed to be *S. aestuarii* JCM17801^T, showing a pairwise dDDH of 33.8%. A core genome alignment was generated using the Panaroo pangenome pipeline [19] using an identity threshold of 80% and a minimum length difference of 75%. A total of 1211 genes were present in over 95% of the genomes included, and the resulting alignment was used as input into the PhyML software [20] to reconstruct a core genome phylogeny using default settings and aLRT branch support estimation. The core genome-based phylogenomic reconstruction (Fig. 1b) presented, essentially, the same topology as that shown in Fig. 1a for whole-genome sequences, thereby reinforcing our taxonomic conclusions.

Determination of 16S rRNA gene sequences is a requisite for the description of novel bacterial species [8]. Thus, partial 16S rRNA sequences of both isolates were obtained by targeted PCR amplification from gDNA, followed by Sanger DNA sequencing, using the primers listed in the supplementary material (Table S1, available in the online version of this article). A partial 16S rRNA sequence of 1403 nt (OP339865) was determined for SP1W3^T (nucleotide positions 93–1495 with respect to *Escherichia coli* 16S rRNA gene sequence numbering); a 1470 nt sequence (OP339862) was determined for SP1S2-7^T (nucleotide positions 28–1496 with respect to *E. coli* 16S rRNA gene sequence numbering). The partial 16S rRNA gene sequences of the type strains shown in Fig. 1 were retrieved and a maximum-likelihood phylogeny was reconstructed with MEGA X [21] using the best-fitting model (HKY+G+I) determined by the software. The topology of this tree (Fig. S1) presented a different branching order than that derived

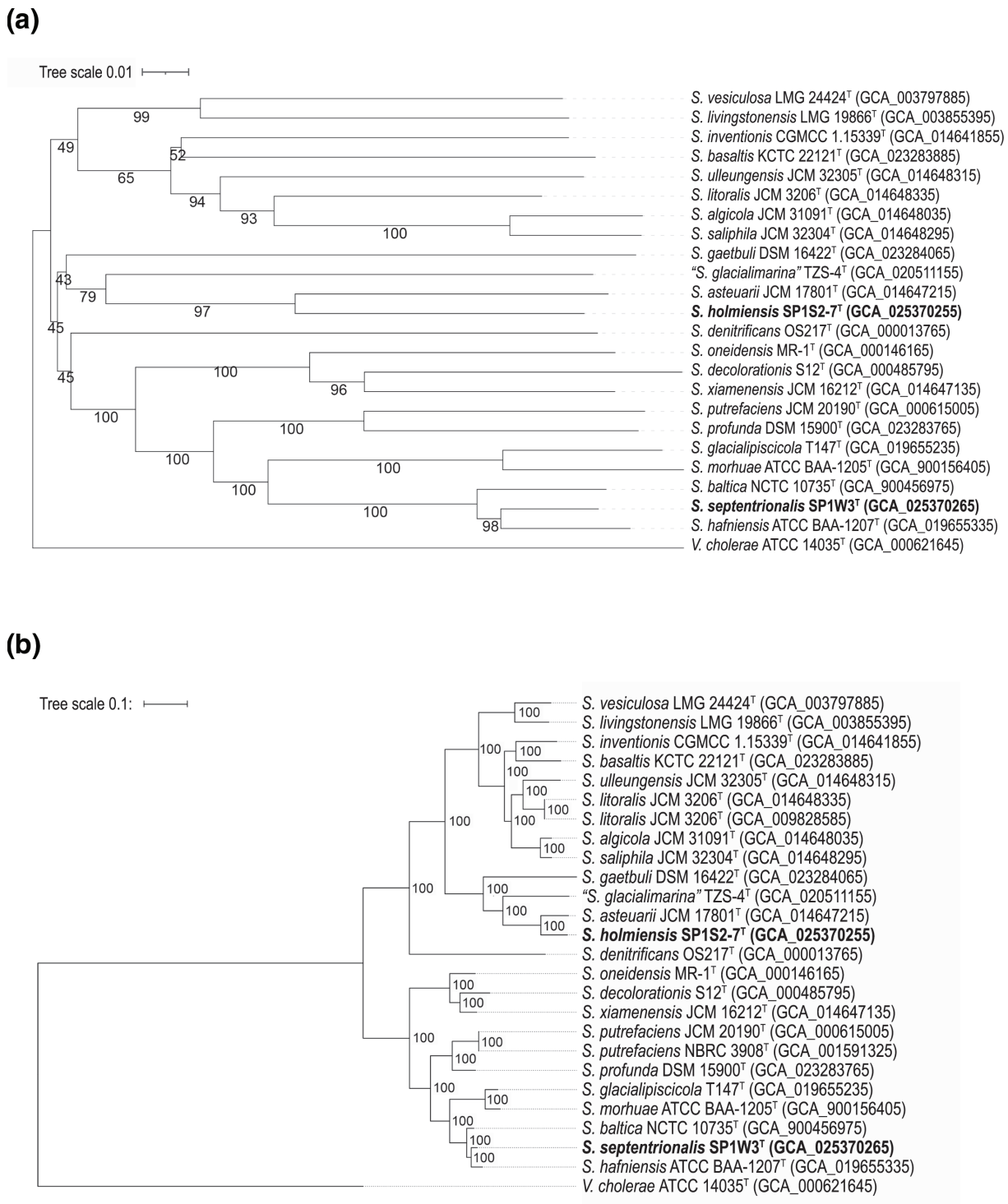

Fig. 1. Genome sequence-based phylogeny showing the taxonomic relationships of strains SP1W3^T and SP1S2-7^T with respect to other *Shewanella* species. (a) WGS-based phylogeny. The tree was inferred with FastME 2.1.6.1 [16] from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d_5 [18]. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications. The tree was rooted at the midpoint [17]. (b) Maximum-likelihood (PhyML) tree based on core genome alignment. aLRT branch support values are shown. Type strains listed in the LPSN were used as references in both reconstructions, irrespective of their nomenclatural status. Note that the species "*S. glacialimarina*" is not validly published under the International Code of Nomenclature of Prokaryotes.

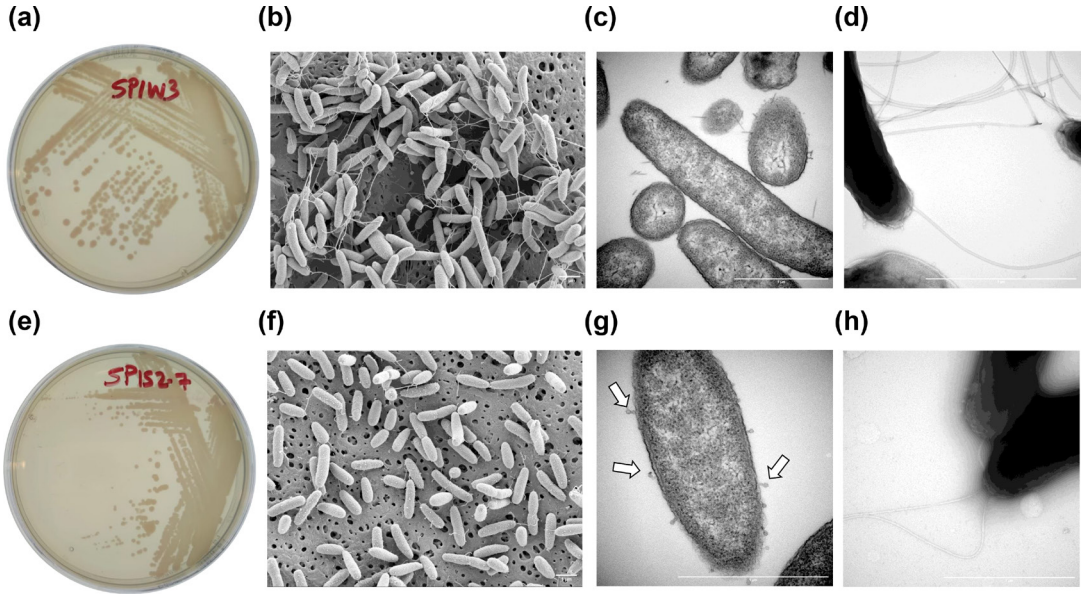

Fig. 2. Macroscopic and microscopic features of strains SP1W3^T and SP1S2-7^T. Shown is the appearance of streaked cells on LB agar (a, e), the appearance of cells visualized by SEM (b, f), single cell structure, as visualized by TEM (c, g), and presence of single, polar flagella, as evidenced by nsTEM (d, h). The arrows in panel g highlight vesicle-like protuberances observed on the surface of SP1S2-7^T cells.

by WGS-based phylogenetic analysis, which is not unusual when comparing divergent species of a genus, although the same closest relatives of SP1W3^T and SP1S2-7^T were observed by 16S rRNA gene sequence-based phylogeny and in the WGS-based reconstruction. It should be noted that 16S rRNA gene sequence-based phylogenetic inferences do not have enough taxonomic resolution for unambiguously discriminating between some *Shewanella* species, as we have previously documented [9, 10, 22]. Overall, based on the WGS phylogenomic analysis, as well as 16S rRNA gene sequence analysis, strains SP1W3^T and SP1S2-7^T clearly represent novel genotypic species within the genus *Shewanella*.

PHENOTYPIC FEATURES

Both strains were observed to grow satisfactorily in Marine Broth or on Marine Agar (Difco), as well as on LB agar medium and on blood agar medium. On LB agar, both strains formed smooth, circular, convex colonies with entire margins, displaying a characteristic orange-pink ('salmon') colour typical of *Shewanella* species, attributed to high cytochrome content (Fig. 2a, e). Gram-staining of bacterial suspensions in PBS (pH 7.4, 0.01 M, Medicago), using Millipore's Gram Staining Kit, revealed Gram-negative bacilli upon microscopic examination. Following fixation with 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and critical point drying (Leica EM CPD 030), microscopic examination of cells grown on LB medium, by scanning electron microscopy (SEM, Zeiss Ultra 55), revealed bacilli, 1.2–4.5 µm long, 0.4–0.8 µm wide (SP1W3^T), and 1.2–2.7 µm long, 0.4–0.6 µm wide (SP1S2-7^T) (Fig. 2b, f). Cells of both strains presented the archetypal cell envelope architecture, as evidenced by transmission electron microscopy (TEM) (Fig. 2c, g). A polar flagellum was observed in the cells of both strains, as evidenced by negative staining TEM (Fig. 2d, h). Of note, vesicle-like protrusions of unknown origin or function were observed in the cells of strain SP1S2-7^T (Fig. 2g).

Strains SP1W3^T and SP1S2-7^T were able to grow at a temperature range of 4–30 °C (tested at 4 °C, 23 °C, 28 °C, 30 °C, 37 °C and 42 °C), with optimal growth between 23–28 °C. Both strains were unable to grow at 37 °C or higher temperatures. They were able to grow anaerobically (tested at 28 °C in 2.5 l anaerobic jars with induced anaerobiosis, using AnaeroGen sachets, Oxoid, Ltd., Thermo Fisher Scientific Inc.). Growth of the strains at different salinities was tested on LB agar supplemented with NaCl (1.0%, 2.5%, 5.0%, 7.5%, 10.0%) and NaCl-free medium. Growth was observed at the NaCl range of 0–2.5% for SP1W3^T, whereas SP1S2-7^T was found to be slightly halophilic, tolerating up to 2.5% NaCl in our tests but being unable to grow in its absence, which is characteristic for *Shewanella*, with some species being able to grow without NaCl but other species requiring Na^+ for growth [1]. To assess the ability of the strains to grow at different pH values, the pH of LB broth was adjusted using 50 mM of the following buffers: acetic acid-acetate (pH 4.5 and 5.5); MES (pH 6.5); HEPES (pH 7.5); Tris-HCl (pH 8.5); and carbonate-bicarbonate (pH 9.5 and 10.5). Growth was monitored, at 28 °C, as OD_{600} in a 96-well plate, using a SpectraMax i3x microplate reader, with shaking between absorbance measurements. The resulting growth curves are presented in Fig. 3. The optimal pH of 7.5 was observed for the growth of both strains. However, while strain SP1W3^T was able to sustain satisfactory growth at pH 6.5 and 8.5 (Fig. 3a), strain SP1S2-7^T exhibited a marked deficit in growth at the lower pH (Fig. 3b).

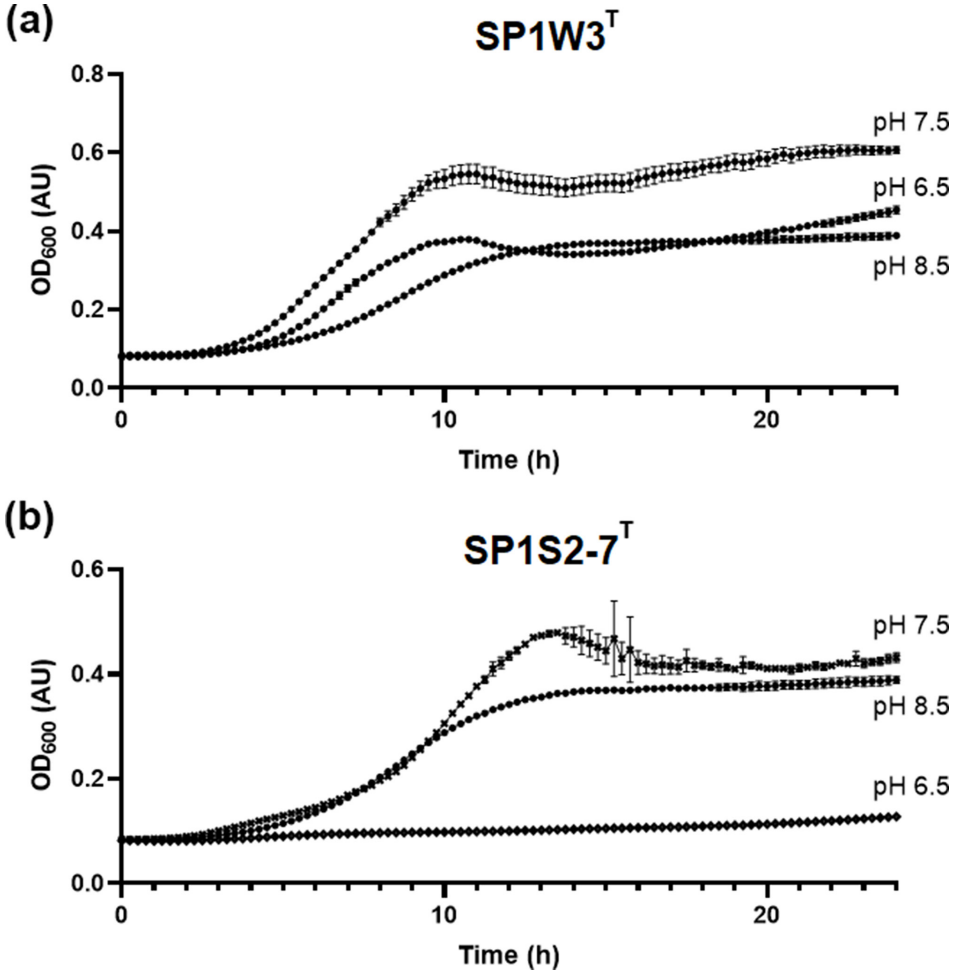

Fig. 3. Growth curves of strains SP1W3^T and SP1S2-7^T at different pH. Shown are the curves in which detectable growth in buffered LB medium, 28 °C, at the indicated pH values, was recorded.

A comprehensive phenotypic characterization of the designated type strains of the two proposed species, with comparison to the type strains of other *Shewanella* species, including the closest genotypic relatives, was performed (Table S2), using the CCUG NFX worksheet (https://ccug.se/documents/worksheets/nfx.pdf) for Gram-negative non-enteric bacilli, which is applicable for *Shewanella* species. The protocol includes in-house panels of metabolic tests, as well as commercial, bioMérieux (www.biomerieux-diagnostics.com) API biochemical test strips, API 20 NE and API ZYM, applied as described by the manufacturer. Both strains SP1W3^T and SP1S2-7^T are oxidase-positive and catalase-negative, reduce nitrate, which is characteristic for all *Shewanella* species [1], and nitrite, produce H_2S but not acid on Triple Sugar Iron (TSI) medium, again, typical of most *Shewanella* species [1], and are resistant to penicillin. Strains SP1W3^T and SP1S2-7^T grow, but are not haemolytic on horse blood agar medium, and do not grow on Drigalski agar medium. Strain SP1S2-7^T is resistant to the antiseptic cetrimide, although strain SP1W3^T is sensitive to this quaternary ammonium compound. Acid production from carbohydrates (glucose, lactose, maltose, adonitol, fructose, and xytlose) is negative. Both strains are able to hydrolyse Tween 80 and were positive for DNAse activity and aesculin hydrolysis (Table S2). Distinctively, SP1W3^T tested positive for ornithine decarboxylase and gelatine hydrolysis.

Strain SP1S2-7^T exhibited a low response for metabolizing carbon/energy sources, with negative results for assimilation of all substrates tested, including the substrates of the in-house test panel, as well as the substrates used according to the instructions of the manufacturer of the commercial API 20 NE test panel (Table S2). Strain SP1W3^T demonstrated assimilation of some substrates, exhibiting a profile that is characteristic of some other species of *Shewanella* (Table S2).

In respect to the enzymatic activities of the commercial API ZYM panel, SP1W3^T and SP1S2-7^T were positive for esterase (C4), ester lipase (C8), phosphoamidase, and *N*-acetyl-β-glucosaminidase. In addition, strain SP1W3^T is positive for alkaline phosphatase, leucine arylamidase, trypsin, and chymotrypsin; strain SP1S2-7^T is positive for lipase activity (Table S2). The phenotypic features that differentiate SP1W3^T and SP1S2-7^T in comparison to the type strains of other *Shewanella* species are summarized in Table 1.

Table 1. Phenotypic features that differentiate strains SP1W3^T or SP1S2-7^T from other *Shewanella* species that are closely related phylogenetically

Species: 1, *S. septentrionalis* sp. nov. SP1W3^T (CCUG 76164^T); 2, *S. holmiensis* sp. nov. SP1S2-7^T (CCUG 76165^T); 3, *S. aestuarii* JCM 17801^T; 4, *S. atlantica* CCUG 54554^T; 5, *S. baltica* CCUG 39356T; 6, *S. canadensis* CCUG 54553^T; 7, *S. chilikensis* CCUG 57101^T; 8, *S. glacialipiscicola* LMG 23744^T; 9, *S. hafniensis* KCTC 22180^T; 10, *S. putrefaciens* CCUG 13452D^T. ++, Very positive; +, positive; –, negative; 0, test not done; R, resistant; I, intermediate; S, sensitive.

Phenotypic feature	1	2	3	4	5	6	7	8	9	10
Growth media:										
Blood agar (30 °C)	++	++	++	–	++	–	++	++	++	++
Blood agar (37 °C)	–	–	–	–	–	–	++	–	–	++
Drigalski agar	–	–	–	–	+	–	++	–	++	++
NaCl 0.5 % in agar medium	++	–	++	–	++	–	++	–	++	++
NaCl 1.5 % in agar medium	++	++	++	–	++	–	++	++	++	++
NaCl 3.0 % in agar medium	++	–	–	–	++	–	++	–	+	+
NaCl 4.5 % in agar medium	–	–	–	–	–	–	++	–	–	+
NaCl 6.0 % in agar medium	–	–	–	–	–	–	++	–	–	–
Sensitivity or resistance to:										
Cetrimide	S	R/I	S	S	S	S	S	S	S	R
Penicillin	R	R/I	S	I/S	R	R/I	R	R	R	R
Hydrolysis of:										
Tween 80	++	++	++	–	++	–	–	+	++	++
Gelatin, Kohn's test	++	–	–	–	++	–	++	+	++	–
Assimilation of:										
N-Acetyl-glucosamine	++	–	–	–	++	–	–	–	++	++
Citrate	++	–	–	–	++	–	++	–	–	–
Gluconate	++	–	–	–	++	–	–	++	++	–
Lactate	++	–	+	–	–	–	++	–	++	++
Lactate+methionine	++	–	–	–	++	–	++	–	++	++
Malate	++	–	–	–	++	–	++	++	++	++
Maltose	++	–	–	–	++	–	–	–	++	–
Sucrose	++	–	–	–	++	–	–	–	–	–
Enzyme activity:										
Catalase activity	–	–	–	–	–	–	+	++	+	–
NO_2^- reduction	+	+	+	++	0	–	0	+	+	++
DNAse	++	++	–	–	+	–	–	+	++	+
Trypsin	++	–	–	–	–	–	+	++	+	++
Chymotrypsin	+	–	–	+	–	–	++	–	–	++
Alkaline phosphatase	++	–	+	++	++	++	++	++	++	++
Esterase (C4)	++	++	–	–	+	–	–	–	–	+
Esterase lipase (C8)	++	++	–	–	+	–	–	–	–	+
Lipase (C-14)	–	+	–	–	–	–	–	–	–	–
Leucine arylamidase	++	–	–	–	+	–	++	+	++	++

Continued

Table 1. Continued

Phenotypic feature	1	2	3	4	5	6	7	8	9	10
N-Acetyl-β-glucosaminidase	++	++	++	++	++	–	++	–	++	–
Ornithine decarboxylase	++	–	–	0	0	–	0	++	–	0
Phosphoamidase	++	++	–	+	–	–	–	–	+	–

DESCRIPTION OF *SHEWANELLA SEPTENTRIONALIS* SP. NOV.

Shewanella septentrionalis (sep.ten.tri.o.na'lis. L. fem. adj. *septentrionalis*, pertaining to the North).

Cells stain Gram-negative and are 1.2–4.5 μm long and 0.4–0.8 μm wide, with a single polar flagellum. Strains are facultative anaerobic. Growth is satisfactory on Miller's LB agar, where it forms smooth, convex, circular colonies with entire margins and salmon coloration. On Lyngby's iron agar, cells form black deposits because of their ability to produce H_2S. Proficient growth is also observed on Marine Agar. Growth occurs from 4 to 30 °C (optimal, 23–28 °C) and at pH 6.5–8.5 (optimal, pH 7.5). Strains grow without NaCl and are slightly halotolerant, able to grow at concentrations as high as 3 %. Strains are positive for oxidase, nitrate and nitrite reduction, ornithine decarboxylase, DNAse, and hydrolysis of aesculin, Tween 80 and gelatin. Strains assimilate *N*-acetyl-glucosamine, maltose, gluconate, malate, citrate, sucrose, lactate and lactate+methionine. In the commercial API 20 NE strip, strains are positive for nitrate reduction, aesculin, gelatinase, *N*-acetylglucosamine, maltose, D-gluconate, L-malate and citrate tests. In the commercial API ZYM strip, strains are positive for alkaline phosphatase, esterase (C4), ester lipase (C8), leucine arylamidase, trypsin, chymotrypsin, phophoamidase and *N*-acetyl-β-glucosamidase tests.

The type strain is SP1W3^T (CCUG 76164^T=CECT 30651^T), isolated from Baltic water collected in Vaxholm, Sweden. The genome size is 5199212 bp, with a G+C content of 46.0 mol%.

DESCRIPTION OF *SHEWANELLA HOLMIENSIS* SP. NOV.

Shewanella holmiensis (hol.mi.en'sis. M.L. fem. adj. *holmiensis*, from Holmia, the Latin name for Stockholm).

Cells are Gram-stain-negative and are 1.2–2.7 μm long and 0.4–0.6 μm wide, with a single polar flagellum. Growth is satisfactory on Miller's LB agar, where it forms smooth, convex, circular colonies with entire margins and salmon coloration. On Lyngby's iron agar, cells form black deposits because of their ability to produce H_2S. Proficient growth is also observed on Marine Agar. Growth occurs from 4 to 30 °C (optimal, 23–28 °C) and at pH 6.5–8.5 (optimal, pH 7.5). Strains are slightly halophilic, growing in the range of 1.0–2.5% NaCl; they do not grow in the absence of NaCl. Strains are oxidase positive but catalase negative, positive for DNAse, reduce nitrate and nitrite, and hydrolyse Tween 80 and aesculin.

Assimilation was not observed in any of the substrates used for phenotypic profiling. In the commercial API ZYM strip, strains are positive for esterase, ester lipase, lipase, phophoamidase and *N*-acetyl-β-glucosamidase tests.

The type strain is SP1S2-7^T (CCUG 76165^T=CECT 30652^T), isolated from sediments collected in Vaxholm, Sweden. The genome size is 4261322 bp, with a G+C content of 41.5 mol%.

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Author contribution
Conceptualization: A.J.M.-R.; methodology: A.J.M.-R., E.J., E.M.; software: K.T.; validation: A.J.M.-R., E.M.; formal analysis: A.J.M.-R.; investigation: A.J.M.-R.; resources: A.J.M.- R.,S.J.M., E.M., Å.S.; data curation: A.J.M.-R.; writing – original draft preparation: A.J.M.-R.; writing – review and editing: A.J.M.-R., E.M., Å.S.; visualization: A.J.M.-R., K.T.; supervision: A.J.M.-R.; project administration: A.J.M.-R.; funding: A.J.M.-R., Å.S.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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