Description of *Shewanella glacialis* sp. nov. and *Shewanella algidipiscicola* sp. nov., isolated from marine fish of the Danish Baltic Sea, and proposal that *Shewanella affinis* is a later heterotypic synonym of *Shewanella colwelliana*

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Two novel species belonging to the genus *Shewanella* are described on the basis of a polyphasic taxonomic approach. A total of 40 strains of Gram-negative, psychrotolerant, H₂S-producing bacteria were isolated from marine fish (cod and plaice) caught in the Baltic Sea off Denmark. Strains belonging to group 1 (seven strains) were a lactate-assimilating variant of *Shewanella morhuae* with a G + C content of 44 mol%. The strains of group 2 (33 strains) utilized lactate, *N*-acetylglucosamine and malate but did not produce DNase or ornithine decarboxylase. Their G + C content was 47 mol%. Phylogenetic analysis of the 16S rRNA gene sequence data placed the two novel species within the genus *Shewanella*. Group 1 showed greatest sequence similarity with *S. morhuae* ATCC BAA-1205^T (99.9 %). However, *gyrB* gene sequence analysis and DNA–DNA hybridization differentiated these isolates from *S. morhuae*, with 95.6 % sequence similarity and less than 57 % DNA relatedness, respectively. Group 2 strains shared more than 99 % 16S rRNA gene sequence similarity with the type strains of *Shewanella colwelliana* and *Shewanella affinis*, but *gyrB* sequence similarity (~85 %) and the results of DNA hybridization (~28 %) indicated that the new isolates represented a novel species. Furthermore, when compared to each other, the type strains of *S. colwelliana* and *S. affinis* had almost identical *gyrB* sequences and significantly high DNA reassociation values (76–83 %), indicating that they belonged to the same species. Based on the conclusions of this study, we propose the novel species *Shewanella glacialis* sp. nov. (type strain T147^T=LMG 23744^T=NBRC 102030^T) for group 1 strains and *Shewanella algidipiscicola* sp. nov. (type strain S13^T=LMG 23746^T=NBRC 102032^T) for group 2 strains, and we propose that *Shewanella affinis* as a later heterotypic synonym of *Shewanella colwelliana*.

The majority of marine fish and seafood spoilage bacteria are reported to be members of the genus *Shewanella* (Shewan *et al.*, 1960; Okuzumi *et al.*, 1981; Gram *et al.*, 1987;

Stenstrom & Molin, 1990; Gram & Huss, 1996; Vogel *et al.*, 1997, 2005). Most *Shewanella* species, particularly *Shewanella putrefaciens* and close relatives, are capable of reducing trimethylamine oxide (TMAO) to trimethylamine and producing hydrogen sulfide (H₂S), both of which are main components of the fishy odour present during low-temperature storage. Recently, it was reported that *Shewanella baltica* rather than *S. putrefaciens* is the dominant spoilage species producing H₂S in iced storage of marine fish caught in the Danish Baltic Sea (Vogel *et al.*, 2005). Initial microbial diversity analysis of the bacterial

Abbreviation: TMAO, trimethylamine oxide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *gyrB* sequences of six of the new isolates are AB205568–AB205571, AB205574 and AB205577 (16S rRNA gene) and AB266200–AB266205 (*gyrB*), as indicated in Figs 1 and 2.

Details of strains and DNA–DNA hybridization results are available as supplementary material in IJSEM Online.

populations present on these fish resulted in the classification of several strains as belonging to the genus *Shewanella* based on biochemical characteristics (Vogel *et al.*, 2005) and resulted in the description of two novel species (Satomi *et al.*, 2006) based on molecular taxonomy. Furthermore, several of the closely related *Shewanella* isolates were physiologically and phylogenetically so distinct that they required further taxonomic analysis (Vogel *et al.*, 2005). Such study will help to understand the microbial species diversity of marine fish and aid in developing countermeasures for fish spoilage. Two groups of H₂S-producing organisms are described herein using a polyphasic taxonomic approach that includes phenotypic characterization, phylogenetic analysis of 16S rRNA and *gyrB* genes and DNA–DNA hybridization.

A total of 40 strains of novel H₂S-producing bacteria were isolated from cod and plaice caught between August 1995 and September 2001 from the Baltic Sea off Denmark. Bacterial isolation was carried out as reported by Vogel *et al.* (2005). Briefly, tissue samples were taken from the belly flap area of iced fish, homogenized, serially diluted in sterile peptone saline and pour-plated in iron agar (Oxoid CM964). Plates were then incubated at 25 °C for 3 days and black colonies, indicative of H₂S-producing bacteria, were picked, purified and stored for further characterization. Of 40 strains tested for phenotypic analysis, representative strains were selected for molecular taxonomy to determine their phylogenetic affiliation. The bacterial strains analysed in detail in this study are shown in Supplementary Table S1, available in IJSEM Online. In addition to these newly described strains, type strains of closely related species were purchased from several established culture collections and were used as reference strains. All isolates were maintained in trypticase soy agar (TSA; Becton Dickinson) stab at room temperature for short-term analysis and in a medium containing skimmed-milk powder and glycerol at –80 °C for long-term storage. Liquid cultures were grown in trypticase soy broth (TSB; Becton Dickinson) incubated at 25 °C for 2–7 days. Representative strains have been deposited in the BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium (LMG), and the National Institute of Technology and Evaluation, Biological Resource Center (NBRC), Japan (Supplementary Table S1).

All strains were tested at 25 °C for the following key characteristics: Gram reaction (Gregerson, 1978), motility and cell shape [phase-contrast microscopy after growth in veal infusion broth (Difco), for 24 h], cytochrome oxidase (BBL DrySlide oxidase; Becton Dickinson), catalase reaction (3 % H₂O₂), reduction of TMAO in TMAO medium (Gram *et al.*, 1987) and production of H₂S from thiosulfate (Gram *et al.*, 1987). Fermentation of glucose was tested in O-F medium (Merck) at 25 °C. Growth at various temperatures (4, 37 and 42 °C) and in 6 % NaCl (Vogel *et al.*, 1997) and assimilation of several carbon and energy sources, citrate, gluconate, glucose, lactate and sucrose (Ziemke *et al.*, 1998), were used to differentiate the *Shewanella* isolates further.

API 20NE kits (bioMérieux) were also employed for assimilation tests according to the manufacturer's instructions. Type strains of *S. putrefaciens*, *Shewanella affinis*, *S. baltica*, *Shewanella colwelliana*, *Shewanella hafniensis* and *Shewanella morhuae* were included in each trial and served as controls. The genomic G + C content was determined by HPLC (Vogel *et al.*, 1997). The ability to degrade gelatin (Frazier, 1926), DNA (Difco DNase test agar with methyl green) and ornithine (Difco) was also tested.

All of the new isolates were Gram-negative, motile rods, with positive oxidase and catalase reactions. They were unable to ferment glucose or produce indole but reduced nitrate and TMAO and produced H₂S. Based on these traits, the strains were tentatively classified as members of the genus *Shewanella* according to established procedures (Stenstrom & Molin, 1990). However, these characteristics were not sufficient to allow for a complete identification at the species level among the psychrotolerant shewanellae (Ziemke *et al.*, 1997). Under optimum conditions, cells of these new isolates were 0.5–0.7 µm in diameter and 1.0–1.2 µm long. Colonies were round, undulate, beige and non-luminescent and had irregular margins on TSA plates incubated at 25 °C for 1 day. All strains were able to grow between 4 and 30 °C.

The first group (group 1; group C5 of Vogel *et al.*, 2005) was composed of seven strains, including strain T147^T, and showed similar phenotypic traits to psychrotolerant *S. morhuae* strains, but differed from *S. morhuae* in that they assimilated lactate (Table 1). These strains were also distinguished from *S. putrefaciens*, *S. baltica* and *S. hafniensis* by their carbohydrate assimilation patterns, ability to grow at 37 °C and production of gelatinase. Furthermore, the G + C content of these strains was 44 mol%, which differs from that of *S. putrefaciens*, *S. baltica* and *S. hafniensis*. These results suggest that group 1 was phenotypically similar to, but nevertheless distinguishable from, known authentic *Shewanella* species. A second group (group 2; groups C1 and C2 of Vogel *et al.*, 2005) encompassing 33 strains and represented by strain S13^T grew well at 4 °C, but assimilated only three of the carbohydrates tested (Table 1). This group was phenotypically different from *S. colwelliana* ATCC 39565^T and *S. affinis* ATCC BAA-642^T, which were phylogenetically closest to the new isolates based on 16S rRNA gene sequence analysis (Vogel *et al.*, 2005). Strains of group 2 grew in 6 % NaCl but did not degrade DNA or ornithine. The G + C content was 47 mol%, indicating that they were different from *S. colwelliana* (46 mol%) and *S. affinis* (45 mol%). Although the phenotypic traits of group 2 strains were somewhat similar to those of *S. putrefaciens*, they showed a number of phenotypic differences (Table 1): they liquefied gelatin and grew in 6 % NaCl, arabinose was not utilized as a sole carbon source and they did not produce DNase or ornithine decarboxylase.

The 16S rRNA and *gyrB* genes were amplified by PCR with universal primer sets as described by Weisburg *et al.* (1991) and Yamamoto & Harayama (1995) and subsequently sequenced. To extract DNA, cells were cultured in TSB and

Table 1. Phenotypic characteristics of the new isolates and the type strains of *Shewanella* species

Strains: 1, *Shewanella* sp. T147^T group (seven strains); 2, *S. morhuae* ATCC BAA-1205^T; 3, *S. hafniensis* ATCC BAA-1207^T; 4, *S. putrefaciens* ATCC 8071^T; 5, *S. baltica* NCTC 10335^T; 6, *Shewanella* sp. S13^T group (33 strains); 7, *S. affinis* ATCC BAA-642^T; 8, *S. colwelliana* ATCC 39565^T. All strains are Gram-negative, rod-shaped, motile and produce H₂S. All strains are positive for cytochrome oxidase and catalase, hydrolyse aesculin, reduce TMAO and reduce nitrate to nitrite. All strains are non-fermentative, do not produce arginine dihydrolase, β -galactosidase or indole and do not hydrolyse urea. None of the strains grows at 42 °C or assimilates mannose, mannitol, adipic acid or phenylacetic acid. For the new isolates, numbers refer to percentages of strains that are positive.

Characteristic	Reference/medium/ID kit	1	2	3	4	5	6	7	8
G + C content (mol%)	Vogel <i>et al.</i> (1997)	44	44	47	45	46	47	45	46
Growth at:									
4 °C	50 % marine agar	100	+	+	+	+	100	–	+
37 °C	50 % marine agar	0	–	–	+	–	70	–	–
Growth in 6 % NaCl	Vogel <i>et al.</i> (1997)	0	–	+	–	+	100	+	–
Gelatinase	Gelatin agar	100	+	+	+	+	0	+	+
DNase	DNase test agar	100	+	+	+	+	0	+	+
Ornithine decarboxylase	Decarboxylase agar	100	+	+	+	+	0	+	+
Assimilation of:									
Lactate	Vogel <i>et al.</i> (2005)	100	–	+	+	+	100	–	–
Sucrose	Vogel <i>et al.</i> (2005)	0	–	–	–	+	0	–	–
Gluconate	API 20NE	100	+	+	–	+	0	–	+
Glucose	API 20NE	0	–	+	–	+	0	–	–
Arabinose	API 20NE	57	–	–	+	–	0	–	–
N-Acetylglucosamine	API 20NE	100	+	+	+	+	100	–	–
Maltose	API 20NE	0	–	+	–	+	0	–	–
Capric acid	API 20NE	0	–	+	–	+	3	–	–
Malate	API 20NE	100	+	+	+	+	100	–	–
Citrate	API 20NE	0	–	+	–	+	0	–	–

collected by centrifugation. Cell pellets were suspended in TE buffer (pH 8.0) and treated with SDS (final concentration 5 mg ml^{–1}) for lysis. Extraction of chromosomal DNA and subsequent purification steps were carried out according to standard methods (Johnson, 1981; Sambrook *et al.*, 1989). The identity of a given PCR product was verified by bidirectional sequencing analysis. The phylogenetic relationships of organisms covered in this study were determined by comparison of individual 16S rRNA gene or *gyrB* sequences with existing sequences in public databases using the BLAST algorithm (Altschul *et al.*, 1990). The multiple alignment calculation of nucleotide substitution rates (K_{nuc} values; Kimura, 1980) and the construction of phylogenetic trees by the neighbour-joining method (Saitou & Nei, 1987) were performed using the CLUSTAL W program (Thompson *et al.*, 1994). Alignment gaps, primer regions for PCR amplification and unidentified base positions were not taken into consideration for the calculations. The topological robustness of the phylogenetic trees was evaluated by a bootstrap analysis through 1000 replications.

Approximately 1.4 kbp nucleotide sequences of 16S rRNA genes and 1.1 kbp nucleotide sequences of the *gyrB* genes were used for phylogenetic analyses. A phylogenetic tree based on the 16S rRNA gene (Fig. 1) indicated that the new isolates clustered with members of the genus *Shewanella*. Based on 16S rRNA gene sequences, the nearest neighbour of

the group 1 (T147^T) strains was *S. morhuae* ATCC BAA-1205^T, with sequence similarity of 99.9%. The sequences of the group 2 (S13^T) strains shared more than 99% similarity with *S. colwelliana* ATCC 39565^T (99.4%) and *S. affinis* ATCC BAA-642^T (99.6%). In the case of the genus *Shewanella*, Venkateswaran *et al.* (1999) and Satomi *et al.* (2003) have reported that analysis of *gyrB* sequences is useful, since the resolution of the 16S rRNA gene sequence is insufficient to evaluate their phylogenetic position. Fig. 2 shows the phylogenetic tree constructed based on *gyrB* sequences. The group 1 strains clustered with *S. morhuae*, sharing 95.6% sequence similarity, and the group 2 strains were positioned monophyletically, apart from both *S. affinis* (85.6%) and *S. colwelliana* (85.6%), clearly delineating them as a distinct species. The *gyrB* sequences of *S. affinis* and *S. colwelliana* were almost identical; there is only one base difference, and the deduced amino acid sequences were identical between the two type strains, indicating that they were probably members of the same species. The taxonomic status of the group 1 strains was still unresolved, since they shared 99.2 and 95.6% similarity with *S. morhuae* based on 16S rRNA gene and *gyrB* sequences, respectively. Such similarity values are not sufficient to distinguish closely related species according to established procedures (Stackebrandt & Goebel, 1994; Satomi *et al.*, 2003; Venkateswaran *et al.*, 1999). Therefore, DNA–DNA hybridization was carried out to confirm the novelty of these isolates.

Fig. 1. Phylogenetic tree of the genus *Shewanella* based on 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method and genetic distances were computed by Kimura's model. The scale bar indicates a genetic distance of 0.01. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 40 % are shown. *Pseudoalteromonas tetraodonis* IAM 14160^T was included as an out-group. GenBank accession numbers are given in parentheses.

DNA–DNA hybridization was performed by microplate hybridization methods (Ezaki *et al.*, 1989) with photobiotin labelling and colorimetric detection, using 1,2-phenylenediamine (Sigma) as the substrate and streptavidin–peroxidase conjugate (Boehringer Mannheim) as the colorimetric substrate (Satomi *et al.*, 1997). Supplementary Table S2 shows the results of DNA–DNA hybridization between strains T147^T and S13^T and the type strains of closely related *Shewanella* species. Within each of the groups themselves, DNA relatedness values were significantly higher, with the group 1 and group 2 strains exhibiting relatedness values of 85 and 81–91 %, respectively. Strains T147^T and U718 showed DNA–DNA hybridization values of 53–57 % with *S. morhuae* strains and less than 22 % with *S. putrefaciens* ATCC 8071^T and *S. baltica* NCTC 10735^T. Similarly, the group 2 strains exhibited ~24 and ~28 % DNA relatedness with *S. affinis* ATCC BAA-642^T and *S. colwelliana* ATCC 39565^T, respectively. The DNA–DNA hybridization results strongly support the claim that isolates of group 1 (T147^T) and group 2 (S13^T) represent novel species within the genus *Shewanella* (Wayne *et al.*, 1987).

Interestingly, *S. affinis* ATCC BAA-642^T and *S. colwelliana* ATCC 39565^T had a DNA–DNA hybridization value of 76–83 %. This supported the results of *gyrB* and 16S rRNA gene sequence analysis, though some phenotypic differences exist between the two strains, such as growth at 4 °C and in 6 % NaCl and gluconate assimilation (Table 1). However, Ivanova *et al.* (2004) reported that DNA relatedness values were only 45–52 % between *S. affinis* ATCC BAA-642^T and *S. colwelliana* ATCC 39565^T. The *gyrB* and 16S rRNA gene sequence analyses and DNA–DNA hybridization newly performed in this study show no evidence to separate the two species genetically. Based on these results, *S. affinis* ATCC BAA-642^T and *S. colwelliana* ATCC 39565^T received from the ATCC in March 2005 should be considered members of the same species.

Based on the findings of the polyphasic taxonomic study, it was determined that some of the strains isolated from marine fish from the Danish Baltic Sea represent two novel species within the genus *Shewanella*, *Shewanella glacialis*

Fig. 2. Phylogenetic tree of the genus *Shewanella* based on *gyrB* gene sequences. The tree was constructed using the neighbour-joining method and genetic distances were computed by Kimura's model. The scale bar indicates a genetic distance of 0.1. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 40% are shown. *Alteromonas macleodii* ATCC 27126^T was included as an outgroup. GenBank accession numbers are given in parentheses.

sp. nov. (represented by strain T147^T) and *Shewanella algidipiscicola* sp. nov. (represented by strain S13^T). In addition, *Shewanella affinis* Ivanova *et al.* 2004 should be considered as a later heterotypic synonym of *Shewanella colwelliana* (Weiner *et al.* 1988) Coyne *et al.* 1990.

Description of *Shewanella glacialipiscicola* sp. nov.

Shewanella glacialipiscicola (gla'ci.a.li.pis'ci.co'la. L. adj. *glacialis* icy; L. n. *piscis* fish; L. suff. *-cola* dweller; N.L. n. *glacialipiscicola* iced fish dweller).

Mesophilic, aerobic and chemoheterotrophic. Cells are Gram-negative rods, 0.5–0.7 µm in diameter and 1.0–1.2 µm long, motile by means of polar flagella. None of the strains are capable of growing at concentrations of 6% (w/v) NaCl. The temperature range for growth is 4–30 °C, with 25 °C being optimal. Growth does not occur at temperatures above 37 °C. Colonies are round, undulate, white–dull and non-luminescent and have irregular margins on marine agar plates incubated at 25 °C for 24 h. Cells are positive for oxidase and catalase reactions. They are unable to ferment glucose but reduce TMAO and produce H₂S. Cells hydrolyse gelatin and aesculin, reduce nitrate and are positive for the production of DNase and ornithine decarboxylase, but do not produce acetoin, arginine dihydrolase, indole, urease or β-galactosidase. Cells utilize lactate, gluconate, *N*-acetylglucosamine and malate readily as energy sources.

D-Glucose, citrate, sucrose, mannose, mannitol, adipic acid and phenylacetate are not utilized as sole carbon sources, but more than half of the strains tested assimilate arabinose. Cells do not grow on minimal media, indicating the presence of a required growth factor. The DNA G + C content is 44 mol%.

The type strain is T147^T (= LMG 23744^T = NBRC 102030^T), isolated from cod from Danish waters of the Baltic Sea. Strain U718 (= LMG 23745 = NBRC 102031) is a reference strain.

Description of *Shewanella algidipiscicola* sp. nov.

Shewanella algidipiscicola (al.gi'di.pis'ci.co'la. L. adj. *algidus* cold; L. n. *piscis* fish; L. suff. *-cola* dweller; N.L. n. *algidipiscicola* cold-fish dweller).

Mesophilic, aerobic and chemoheterotrophic. Cells are Gram-negative rods, 0.5–0.7 µm in diameter and 1.0–1.2 µm long, motile by means of polar flagella. Capable of growing at 6% (w/v) NaCl. The temperature range for growth is 4–37 °C, with 25 °C being optimal. Growth does not occur at temperatures above 42 °C. Colonies are round, undulate, white–dull and non-luminescent and have irregular margins on TSA plates incubated at 25 °C for 24 h. Cells are positive for oxidase and catalase reactions. They are unable to ferment glucose or hydrolyse gelatin, but reduce TMAO and produce H₂S. Cells reduce nitrate and are positive for the production of DNase and

ornithine decarboxylase, but do not produce acetoin, arginine dihydrolase, indole, urease or β -galactosidase. Cells are unable to use most of the carbon substrates tested, although lactate, *N*-acetylglucosamine and malate are readily utilized as energy sources. D-Glucose, gluconate, maltose, citrate, sucrose, mannose, mannitol, adipic acid and phenylacetate are not utilized as sole carbon sources, but several strains assimilate arabinose and capric acid. Cells do not grow on minimal media, indicating the presence of a required growth factor. The DNA G+C content is 47 mol%.

The type strain is S13^T (=LMG 23746^T=NBRC 102032^T), isolated from plaice from Danish waters of the Baltic Sea. Strains Q14 (=LMG 23477=NBRC 102033), Q213 and W01 are reference strains.

Acknowledgements

The technical assistance of Anemone Bundvad is acknowledged. Shariff Osman is acknowledged for critically reviewing the manuscript.

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