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EXPERIMENTAL ARTICLES

Characterization of the Lipids of Psychrophilic Bacteria *Shewanella frigidimarina* Isolated from Sea Ice of the Sea of Japan

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Abstract—Lipids of ten *Shewanella frigidimarina* strains isolated from sea ice samples of coastal areas of the Sea of Japan and of the type strains of psychrophilic bacteria *S. frigidimarina* ACAM 591^T and *S. hanedai* JCM 20706^T were analyzed. Most of the new isolates contained isoprenoid quinones typical of the genus *Shewanella* (Q-7, Q-8, MK-7, and MMK-7), a high level of branched acids (*i*-13:0 and *i*-15:0), and polyunsaturated fatty acid (20:5 ω3). Phospholipid fractions of marine isolates and the type strain *S. frigidimarina* ACAM 591^T contained not only the main phospholipids (phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol), but also an unknown phosphoaminolipid, which is probably typical of this bacterial species. The isolates exhibited a high level of phylogenetic similarity but were phenotypically heterogeneous. Two strains distinguished by their phenotypic characteristics differed also in the composition of fatty acids, isoprenoid quinones, and phospholipids. The use of chemotaxonomic markers for primary species identification of psychrophilic bacteria of the genus *Shewanella* is discussed.

Keywords: isoprenoid quinones, phospholipids, fatty acids, *Shewanella frigidimarina, Shewanella hanedai.*

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The genus *Shewanella* comprises phylogenetically and ecologically diverse facultatively anaerobic bacteria, which are widespread in marine and freshwater habitats [1]. It consists of both free-living microorganisms and strains associated with various marine organisms. The wide distribution of *Shewanella* results probably from their physiological characteristics, such as a unique respiratory system ensuring the use of a wide range of electron acceptors for anaerobic respiration and the capability of survival within a broad range of sodium chloride concentrations, temperature, and pressure [2].

Bacteria of the genus *Shewanella* are of great interest because of their important role in transformation and mineralization of organic compounds in the environment; moreover, they can be used as a model for investigating molecular mechanisms of bacterial adaptation to extreme conditions [3].

At present, the genus *Shewanella* contains 51 valid species, 29 of which were described in the last five years [4]. Psychrophilic and barophilic bacteria living at low temperature and high pressure are known to produce polyunsaturated fatty acids as a result of their adaptation to extreme environments [5, 6].

According to the literature, the majority of psychrophilic *Shewanella* species inhabit marine ecotopes and polar regions. Psychrophilic and halotolerant bacteria of the species *S. frigidimarina* have been isolated from sea ice, water samples from meromictic lakes, cyanobacterial mats, and sea-ice diatom assemblages collected in the Antarctic and Arctic [7–9], while they have not been found in other ecotopes. Taxonomic studies of the gammaproteobacteria inhabiting sea ice of coastal areas of the Sea of Japan revealed that, among *Shewanella* isolates, which constituted a significant part of the microbial community, those clustering together with *S. frigidimarina* (similarity level of 98.8–99.8%) prevailed [10]. The isolates were phenotypically heterogeneous. Such heterogeneity, which is typical of the microorganisms from extreme habitats, was earlier observed for this bacterial species [7, 8].

The goal of this work was to study the main membranous components of psychrophilic bacteria, namely, fatty acids, phospholipids, and ubiquinones, in *S. frigidimarina* strains isolated from sea ice, as well as in the type strains *S. frigidimarina* ACAM 591^T and *S. hanedai* JCM 20706^T. An attempt to validate the application of these taxonomic markers for tentative species identification of some psychrophilic bacteria of the genus *Shewanella* was made. Earlier, we successfully used these markers for identification of *Shewanella* bacteria at the genus level [11].

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MATERIALS AND METHODS

Objects of investigation. The study was carried out with the type strains *S. frigidimarina* ACAM 591^T and *S. hanedai* JCM 20706^T obtained from the BCCM/LMG Bacteria Collection (Ghent University, Ghent, Belgium) and the Japan Collection of Microorganisms (JCM) (RIKEN BioResource Center, Japan) and with bacterial strains Pi2, Pi3, Pi5, Pi42, Pi43, Pi2-29, Pi2-35, Pi2-37, Pi2-64, and Pi2-65 isolated from the sea ice samples collected from Peter the Great Bay, Sea of Japan. Phenotypic characterization of the isolates and phylogenetic analysis based on comparing the nucleotide sequences of 16S rRNA genes were performed as described earlier [10].

Methods of investigation. Phenotypic properties of bacteria (Gram reaction, the presence of catalase and oxidase; production of indole and hydrogen sulfide; hydrolysis of gelatin, Tween 80, starch, casein, and DNA) were studied according to the routine methods [12, 13]. Physiological and biochemical characteristics of the isolates grown at 15°C were studied using an API 20NE test system for identification of bacteria (BioMerieux, France). The type of metabolism and acid production from sugars were determined on modified Hugh and Leifson medium [14]. The growth temperature was determined by cultivation of bacteria on marine agar at 4, 7, 10, 15, 20, 25, 28, 30, 35, 37, and 40°C. To examine the sodium ion requirement, bacteria were cultivated on artificial sodium-free seawater medium supplemented with different NaCl concentrations (0–20%) [15]. Antibiotic sensitivity of the isolates was studied by using seawater agar and paper disks impregnated with benzylpenicillin (10 U), oxacillin (10 μg), ampicillin (10 μg), vancomycin (30 μg), lincomycin (15 μg), kanamycin (30 μg), oleandomycin (15 μg), tetracycline (30 μg), streptomycin (30 μg), erythromycin (15 μg), gentamycin (10 μg), carbenicillin (100 μg), rifampicin (5 μg), ofloxacin (5 μg), neomycin (30 μg), nalidixic acid (30 μg), levomycetin (30 μg), polymyxin (300 U), cephazolin (30 μg), and cephalexin (30 μg). Cell morphology and motility were studied by light microscopy using the hanging drop method [12].

Cultivation conditions. Bacteria were grown on marine agar 2216 (Difco, United States) at 15°C for 3 days. The harvested biomass was used for lipid extraction.

Extraction and fractionation of lipids. The lipids were extracted by a routine method [16], wet cells were treated with a chloroform–methanol (2 : 1) mixture, and the combined extract was washed with a 0.5% solution of KCl and dehydrated with sodium sulfate. The fractions of isoprenoid quinones, glyco- and phospholipids were separated by preparative chromatography on a column (0.7 × 14 cm) packed with 100–150 mesh KSK silica gel (Russia) by successive elution with chloroform, acetone, and methanol [16]. After evaporation, the fractions were stored at –15°C.

Analysis of fatty acids. Fatty acid methyl esters (FAMEs) were obtained by two methods: (1) alkaline hydrolysis with a 15% NaOH solution in 50% methanol at 95°C for 30 min with subsequent methylation with a 19% solution of HCl in methanol at 80°C for 10 min according to the MIDI procedure [17] and (2) mild acid hydrolysis with a 3N solution of HCl in methanol at 85°C for 1 h [18]. Analysis of FAME was performed on a "Hewlett-Packard" 6890 GLC-MS chromatograph (United States) equipped with a "Hewlett-Packard" 5973 mass spectrometer (United States) by using a HP 5 MS capillary column (30 m × 0.25 mm, 0.25 μm) at a temperature gradient from 150 to 230°C (5°C/min). FAMEs were identified using the Supelco standards.

Analysis of isoprenoid quinones was carried out on an Agilent 1100 device equipped with an ODS C-18 column (40 × 250 mm, 5 μm) and a UV detector (275 nm); an acetonitrile–isopropanol (65 : 35) mixture was used as the mobile phase (0.5 ml/min, 40°C). Isoprenoid quinones were identified by comparing their retention times with those of the quinones isolated from the type strain *S. frigidimarina* ACAM 591^T [11].

Phospholipid analysis was performed by two-dimensional TLC on Sorbfil (Russia) using different solvent systems: (I) chloroform–methanol–ammonia–benzene (65 : 30 : 6 : 10) and (II) chloroform–methanol–acetone–acetic acid–water–benzene (70 : 30 : 5 : 4 : 1 : 10). Phospholipids were visualized with a nonspecific reagent (a 10% solution of H_2SO_4 in ethanol at 180°C) and with specific reagents, such as ninhydrin- and molybdate-containing reagents, the Dragendorff reagent, and α-naphthol, and identified by comparing their positions with the standards. To analyze the phospholipid amount, chromatograms developed in system I were scanned on an Epson Perfection 3490 photo device, the color intensity of spots was determined using the Digital software package, and the percentage of individual phospholipids was calculated.

RESULTS AND DISCUSSION

The isolates had a high level of phylogenetic similarity between each other and with the type strain *S. frigidimarina* ACAM 591^T but were phenotypically heterogeneous; the strains could be divided into two groups according to their phenotypic features. The first group was composed of halotolerant isolates Pi2, Pi2-29, Pi2-35, Pi2-37, Pi2-64, Pi2-65, Pi42, Pi43, and the type strain *S. frigidimarina* ACAM 591^T; these strains usually exhibited proteolytic activity, reduced nitrates, hydrolyzed esculin, Tween 80, and DNA and utilized glucose, maltose, mannitol, and malate as the sole carbon source (Table 1). The ability of psychrophilic bacteria *S. frigidimarina* to utilize this range of substrates is indicative of their ecologically important

Table 1. Phenotypic properties of the strains isolated from sea ice and the type strain *S. frigidimarina* ACAM 591^T

Properties	Pi2	Pi3	Pi5	Pi2-29	Pi2-35	Pi2-37	Pi42	Pi43	Pi2-64	Pi2-65	ACÀM 591^T
Growth at 30°C	+	–	–	–	+	+	(+)	+	–	–	+
0% NaCl	+	–	–	+	+	+	+	+	+	+	+
8% NaCl	–	–	–	-	+	+	+	+	(+)	–	+
Hydrolysis of: casein	(+)	–	–	+	+	+	+	+	(+)	+	+
gelatin*	–	–	–	+	+	+	+	(+)	+	–	+
PNPG*	–	+	–	–	–	–	–	–	+	–	–
Production of H_2S	+	–	–	+	+	–	–	+	+	+	+
Assimilation:											
D-glucose	–	–	–	+	+	–	–	+	–	–	+
Mannitol	+	–	–	+	+	+	–	+	–	–	+
Maltose	+	–	–	+	+	+	–	+	–	+	+
Malate	+	–	–	+	+	+	–	+	–	+	–
Aerobic acid formation from:											
D-glucose	+	–	–	–	+	+	+	+	–	+	+
Maltose	+	+	(+)	(+)	+	+	+	+	(+)	+	–
Trehalose	–	–	–	–	+	+	+	+	–	–	+
Anaerobic acid formation from:											
D-glucose	+	–	–	–	+	+	+	+	–	+	+
Maltose	+	+	–	–	+	+	+	+	–	+	+
Cellobiose	(+)	–	(+)	–	+	+	+	+	–	–	+
Sucrose	+	(+)	–	–	–	+	–	–	(+)	–	+
Sensitivity to antibiotics:											
Ampicillin	–	+	+	–	–	–	–	–	–	–	–
Benzylpenicillin	–	+	(+)	–	–	–	–	–	–	–	–
Carbenicillin	–	+	+	–	–	–	–	–	–	–	–
Nalidixic acid	+	+	+	+	–	–	–	–	+	+	–
Ofloxacin	+	+	+	+	(+)	(+)	(+)	+	+	–	–
Cephalexin	+	+	+	–	–	–	–	–	+	–	–
Erythromycin	+	+	+	+	(+)	+	–	–	(+)	+	–

Notes: * The data on the hydrolysis of gelatin and the PNPG (4-nitrophenyl-β-D-galactopyranoside) test were obtained using the API 20NE system.

Designations: +, –, and (+) stand for positive, negative, and slight reaction or growth, respectively. All bacteria hydrolyzed esculin, DNA, and Tween-80; reduced nitrates and nitrites; did not produce indole, arginine dihydrolase, urease, and amylase; did not assimilate arabinose, mannose, N-acetyl glucosamine, gluconate, caprate, adipate, citrate, and phenyl acetate; were resistant to tetracycline, cephazolin, oxacillin, lincomycin, and vancomycin; and were sensitive to streptomycin, polymyxin, neomycin, levomycetin, kanamycin, and gentamycin.

Table 2. Fatty acid composition of the lipids from *S. frigidimarina* strains isolated from sea ice (% of the sum)

Fatty acids	Pi2-37		Pi2-64		Pi42		Pi2-29	Pi2-35	Pi2-65	Pi43	Pi2	Pi3*	Pi5*
	HCl	NaOH	HCl	NaOH	HCl	NaOH	HCl	HCl	HCl	HCl	HCl	HCl	HCl
12:0	6.0	4.6	1.5	4.3	4.5	4.4	4.7	5.8	4.9	4.4	3.7	6.5	5.0
i-13:0	7.7	5.8	3.1	7.7	9.0	8.8	8.4	8.9	7.3	7.3	6.0	24.2	13.0
14:0	2.1	4.2	2.3	5.0	3.7	5.6	3.1	2.5	4.1	4.9	4.6	3.4	4.1
i-15:0	14.9	14.9	9.8	10.7	10.1	10.9	11.8	13.2	13.5	10.1	12.4	5.4	12.8
15:0	4.0	3.8	2.8	3.7	3.4	4.9	3.6	3.9	5.2	5.8	5.0	5.1	5.0
16:1ω7	42.2	42.7	47.7	42.7	47.7	49.3	45.6	46.1	35.8	40.2	41.2	39.6	25.2
16:0	8.5	10.0	7.1	8.3	7.5	9.1	8.8	7.6	9.7	6.8	9.7	4.1	4.8
17:1	2.8	3.8	4.3	3.0	1.9	3.7	3.7	4.2	4.8	4.1	4.7	2.4	8.7
18:1ω7	4.6	3.3	7.3	3.6	2.7	1.7	3.4	2.7	6.5	2.3	5.6	–	3.7
20:5ω3	4.5	1.3	7.9	2.3	5.1	1.3	6.1	2.4	8.2	3.8	7.2	–	–

* In strains Pi3 and Pi5, additional fatty acids were revealed: 13:0 (2.4 and 3.0%, respectively), *i*-14:0 (3.1 and 5.1%, respectively), and 13:0 3OH (3.4 and 9.2%, respectively). In other strains, these acids were present in trace amounts (less than 1%).

role in the degradation of organic substances of coastal ecosystems of the Sea of Japan at low temperatures. The second group was formed by isolates Pi3 and Pi5, which differed considerably from the other strains in their properties, such as halophilicity and inability to grow at 30°C, to produce hydrogen sulfide; to hydrolyze gelatin and casein, to assimilate glucose, mannitol, maltose, and malate, and had a specific profile of antibiotic resistance (Table 1).

Lipid components of the cell membranes (fatty acids, phospholipids, and ubiquinones) are widely used in taxonomy of bacteria as additional phenotypic criteria. The role of ubiquinones and fatty acids in anaerobic respiration and adaptation of bacteria of the genus *Shewanella* to extreme conditions is well studied [6, 19–21], whereas information on phospholipids of these bacteria is scarce [11, 22, 23].

Fatty acid composition. The use of the MIDI identification method [17] for the fatty acid analysis allowed us to compare the obtained results with the literature data. We found that while alkaline hydrolysis of lipids had almost no effect on the content of saturated and monounsaturated fatty acids, the amount of eicosapentaenoic acid (20:5ω3) decreased considerably (Table 2); therefore, to characterize the fatty acid composition of the studied strains, we used the method of mild acid hydrolysis [18].

Most isolates had a fatty acid composition typical of the genus *Shewanella* (Table 2); in addition to the major acids (12:0, 15:0, 16:0, 16:1ω7, and 17:1), they contained 20:5ω3 acid and branched fatty acids (*i*-13:0 and *i*-15:0), which are usually used for the identification of psychrophilic bacteria of this genus [3, 7]. Two strains (Pi3 and Pi5) were characterized by the absence of 20:5ω3 acid and by higher content of *iso*-acids (26–30%) as compared with the other strains (13–23%) (Table 3).

Analysis of the literature data and our results suggested the use of the ratio of 20:5ω3 acid and *iso*-acids as additional species characteristics of psychrophilic bacteria of the genus *Shewanella*; all the isolates (except for strains Pi3 and Pi5) and the type strain *S. frigidimarina* ACAM 591^T had similar proportions of fatty acids. The same fatty acid profile had been earlier revealed in other *S. frigidimarina* strains isolated from Antarctic samples [7, 8]. In all the strains, 16:1ω7 acid prevailed, whereas the amounts of *iso*-acids and 20:5ω3 acid varied slightly (Table 3). The absence of eicosapentaenoic acid in two strains of *S. frigidimarina* reported by Bozal et al. [8] may be possibly explained by degradation of this acid in the course of alkaline hydrolysis applied in the MIDI system used.

Comparative study of two type strains of psychrophilic bacteria showed that, while they had the same qualitative composition of fatty acids, *S. hanedai* JCM 20706^T differed from *S. frigidimarina* ACAM 591^T in higher content of 20:5ω3 acid. The fatty acid composition of isolates Pi3 and Pi5 was different from both

Table 3. Fatty acid composition of the lipids from bacteria *S. frigidimarina* and *S. hanedai* (% of the sum)

Fatty acids	*S. frigidimarina*					*S. hanedai*	
	$n = 8$ [7]**	$n = 8$*	Pi3*	Pi5*	ACAM 591^T*	JCM 20706^T*	$n = 2$ [7]**
12:0	0–1.8	1.5–6.0	5.0	6.5	3.1	1.8	0.1–0.4
i-13:0	3.1–8.3	3.1–9.0	13.0	24.2	6.0	11.3	5.1–7.7
14:0	2.7–4.1	2.1–4.9	3.4	4.1	5.7	10.6	7.6–10.1
i-15:0	5.6–10.7	9.8–14.9	5.4	12.8	7.1	11.2	7.9–8.2
15:0	1.7–2.9	2.8–5.8	5.0	5.1	3.9	5.6	2.9–3.5
16:1ω7	36.6–55.1	35.8–47.7	25.2	39.6	57.0	23.3	25.0–33.1
16:0	4.5–16.9	6.8–12.9	4.1	4.8	6.6	13.2	12.2–15.0
17:1	1.7–3.7	1.9–4.8	2.4	8.7	3.3	1.8	0.5–1.5
18:1ω7	3.1–6.6	2.3–7.3	0	3.7	3.3	1.3	2.2–3.7
20:5ω3	1.7–6.8	2.4–8.2	–	–	3.7	19.8	19.2–22.2

Notes: * Results were obtained by the authors.

** $n = 8$ for *S. frigidimarina* strains ACAM 591^T, ACAM 122, ACAM 584, ACAM 589, ACAM 590, ACAM 593, ACAM 598, and ACAM 600; $n = 2$ for *S. hanedai* strains ACAM 540^T and ACAM 585 [7].

Table 4. Composition of isoprenoid quinones and phospholipids of marine isolates and the type strain *S. frigidimarina* ACAM 591^T (% of the sum)

Quinones and phospholipids	ACAM 591^T	Pi2-35	Pi42	Pi43	Pi2-64	Pi2-65	Pi2-37	Pi2	Pi2-29	Pi3	Pi5
Q-7	38.4	35.4	34.9	37.5	38.8	25.0	29.5	43.8	48.0	64.8	60.7
Q-8	46.8	57.7	57.5	57.1	46.7	37.0	60.2	49.1	51.9	32.3	38.1
MK-7	2.9	3.3	3.2	5.4	6.6	37.0	10.4	–	–	3.0	1.2
MMK-7	11.9	3.6	4.2	0.8	5.2	–	–	–	–	–	–
DPG	3.2	2.6	5.1	5.1	4.1	4.3	6.1	–	–	4.4	1.8
PG	21.0	24.2	15.7	22.9	14.7	14.3	13.3	28.6	34.3	27.5	29.8
PE	29.8	20.3	25.5	26.7	21.8	22.3	14.7	33.7	37.8	51.4	49.9
PAL	45.9	52.9	53.7	45.2	59.4	59.2	65.5	37.8	27.5	16.6	18.3

type strains in the absence of 20:5ω3 acid and the highest content of *i*-13:0 acid.

Isoprenoid quinones (ubiquinones and menaquinones) involved in the bacterial respiratory chain are largely used in taxonomy of bacteria [24]. Analysis of "respiratory quinones" confirmed the phenotypic heterogeneity of *S. frigidimarina* isolates: most isolates were characterized by quinone composition typical of the genus *Shewanella* and contained quinones Q-7 and Q-8 and menaquinones MMK-7 (8-methylmenaquinone-7) and MK-7 (Table 4). However, isolates Pi3 and Pi5 differed from the other studied strains in the proportion of Q-7 and Q-8 quinones; this parameter was earlier considered as a species-specific characteristic of the genus *Shewanella* [11, 22].

Phospholipid profile of the isolates was typical of the genus *Shewanella* [11, 22, 23]. The major phospholipids were represented by phosphatidylethanolamine (PE) and phosphatidylglycerol (PG); diphosphatidylglycerol (DPG) was a minor component. The phospholipid composition of *S. frigidimarina* isolates differed from that of the type strain *S. hanedai* JCM 20706^T and from the earlier studied mesophilic bacteria of the genus *Shewanella* in the presence of an unknown phosphoaminolipid (PAL), which showed positive reactions with ninhydrin and molybdate reagents. We revealed that the isolates and the type strain *S. frigidimarina* ACAM 591^T were able to synthesize this lipid only at low temperatures (10–15°C), but not at 25°C. This lipid seems to be a taxonomic

marker of this species and plays an important role in the adaptation of bacteria to low temperatures.

The studied *S. frigidimarina* isolates differed considerably in the content of the major phospholipids; with respect to the PE/PAL ratio, all the isolates could be divided into two groups: Pi2-35, Pi2-37, Pi2-64, Pi2-65, Pi42, and Pi43, as well as the type strain *S. frigidimarina* ACAM 591^T, were characterized by predominance of PAL; the second group was formed by isolates Pi3 and Pi5 in which PE prevailed (Table 4).

Thus, the division of *S. frigidimarina* isolates into two groups based on the content of fatty acids, ubiquinones, and phospholipids correlated well with their phenotypic characteristics. These results allowed us to assume that isolates Pi3 and Pi5 can be assigned to separate genospecies of halophilic and psychrophilic bacteria of the genus *Shewanella* inhabiting sea ice of coastal areas of the Sea of Japan. Complete and detailed identification of strains Pi3 and Pi5 at the species level requires additional phylogenetic studies with the use of DNA/DNA hybridization.

The application of complex analysis of lipid components allowed us to confirm the phenotypic heterogeneity of *S. frigidimarina* isolates and to use such markers as branched fatty acids, eicosapentaenoic acid, isoprenoid quinones, and phospholipids as additional taxonomic criteria for species identification of the psychrophilic bacteria belonging to the genus *Shewanella*.

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