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EXPERIMENTAL ARTICLES

Lipid Composition of Novel *Shewanella* Species Isolated from Far Eastern Seas

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Abstract—A comparative study of the lipid composition of 26 strains (including type strains) of marine *Gammaproteobacteria* belonging to the genera *Shewanella, Alteromonas, Pseudoalteromonas, Marinobacterium, Microbulbifer*, and *Marinobacter* was carried out. The bacteria exhibited genus-specific profiles of ubiquinones, phospholipids, and fatty acids, which can serve as reliable chemotaxonomic markers for tentative identification of new isolates. The studied species of the genus *Shewanella* were distinguished by the presence of two types of isoprenoid quinones, namely, ubiquinones Q-7 and Q-8 and menaquinones MK-7 and MMK-7; five phospholipids typical of this genus, namely, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), lyso-PE, and acyl-PG; and the fatty acids [15:0, 16:0, 16:1 (n-7), 17:1 (n-8), *i*-13:0, and *i*-15:0]. The high level of branched fatty acids (38–45%) and the presence of eicosapentaenoic acid (4%) may serve as criteria for the identification of this genus. Unlike *Shewanella* spp., bacteria of the other genera contained a single type of isoprenoid quinone: Q-8 (*Alteromonas, Pseudoalteromonas, Marinobacterium*, and *Microbulbifer*) or Q-9 (*Marinobacter*). The phospholipid compositions of these bacteria were restricted to three components: two major phospholipids (PE and PG) and a minor phospholipid, bisphosphatidic acid (*Alteromonas* and *Pseudoalteromonas*) or DPG (*Marinobacterium, Microbulbifer*, and *Marinobacter*). The bacteria exhibited genus-specific profiles of fatty acids.

Key words: chemotaxonomy, isoprenoid quinones, phospholipids, fatty acids, marine bacteria, *Shewanella, Alteromonas, Pseudoalteromonas, Marinobacterium, Microbulbifer, Marinobacter.*

The genus *Shewanella* MacDonell et Colwell was created in 1986 [1]; it includes numerous phylogenetically diverse heterotrophic facultatively anaerobic or, more rarely, aerobic microorganisms, which have been frequently isolated from freshwater and marine habitats [2]. At present, the genus *Shewanella* (the type species is *S. putrefaciens*) consists of 25 species, most of which were described in the last five years [3, 4]. The species adapted to high pressure and low temperature are distinguished by their ability to synthesize polyunsaturated fatty acids (eicosapentaenoic acid in particular) [5].

Bacteria of the genus *Shewanella* were originally assigned to the family *Vibrionaceae* [1]; later, they were transferred to the more closely phylogenetically related family *Alteromonadaceae*, which also includes marine gammaproteobacteria of the genera *Alteromonas, Pseudoalteromonas, Marinobacterium, Microbulbifer, Marinobacter*, and others [3]. Finally, based on recent phylogenetic studies, bacteria of this genus were assigned to the family *Shewanellaceae* (class *Gammaproteobacteria*) [6]. The identification of *Shewanella* isolates is rather difficult since their phenotypic properties are quite similar to those of other marine gammaproteobacteria. Earlier, it was shown that the composition of cellular lipids can be used as a criterion for the differentiation of marine bacteria belonging to the genera *Alteromonas, Marinomonas*, and *Pseudoalteromonas* [7]. This work is a continuation of our studies aimed at revealing reliable chemotaxonomic characteristics of marine gamma-proteobacteria isolated from Far Eastern seas; it is devoted to a comparative investigation of isoprenoid quinones, phospholipids, and fatty acids of *Shewanella* isolates and type strains of the closely related genera *Alteromonas, Pseudoalteromonas, Marinobacter, Marinobacterium*, and *Microbulbifer.*

MATERIALS AND METHODS

Objects of investigation. The study was carried out with the following type strains: *Shewanella algae* ATCC 51192^T, *S. frigidimarina* ACAM 591^T, *Pseudoalteromonas tunicata* CCUG 44952^T, *Alteromo-*

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nas macleodii ATCC 27126^T, *A. macleodii* subsp. *fijiensis* CNCM I-1627, *A. infernus* CNCM I-1628, *Marinobacterium georgiense* ATCC 700074^T, *Microbulbifer hydrolyticus* ATCC 700072^T, *Marinobacter aquaeolei* ATCC 700491^T, and *M. hydrocarbonoclasticus* DSM 8798^T. The strains were obtained from the following collections: ACAM (Australian Collection of Antarctic Microorganisms, Australia), ATCC (American Type Culture Collection, United States), CCUG (Culture Collection, University of Göteborg, Sweden), CNCM (Collection Nationale de Cultures de Microorganismes, France), and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany); the cultures were kindly provided by J. Bowman, C. Holstrom, G. Barbier, and H. Stan-Lotter.

Marine isolates assigned to the species *S. affinis* (KMM 3587^T, KMM 3586, KMM 3821, and KMM 3822), *S. pacifica* (KMM 3597^T, KMM 3590, KMM 3601, KMM 3605, KMM 3772, and KMM 3775), *S. fidelis* (KMM 3582^T), *S. japonica* (KMM 3299^T and KMM 3300), *P. ruthenica* (KMM 300^T), *P. elaykovii* (KMM 162^T), and *P. issachenkonii* (KMM 3549^T) were isolated from seawater and various hydrobionts from the Sea of Japan and the Sea of Okhotsk and were obtained from the KMM collection (Collection of Marine Microorganisms, Pacific Institute of Bioorganic Chemistry, Far East Division, Russian Academy of Sciences, Russia).

Cultivation conditions. Bacteria were grown in a medium of the following composition (g/l): peptone (Difco, United States), 5.0; yeast extract (Difco), 2.5; glucose, 1.0; K_2HPO_4, 0.2; $MgSO_4$, 0.05; seawater, 750 ml; distilled water, 250 ml; pH 7.5–7.8. Cultivation was performed in 1-l Erlenmeyer flasks containing 300 ml of the medium on a shaker (150 rpm) at 22°C for 20–24 h. The cells in the late exponential phase of growth were harvested by centrifugation, washed with physiological saline, and lyophilized.

Extraction and fractionation of lipids. Lipids were extracted by a routine method [7]: dry cells were treated three times with a chloroform–methanol (2 : 1) mixture at room temperature for 1 h, and the combined extract was dehydrated with Na_2SO_4 and dried. The fractions of ubiquinones and phospholipids were separated by preparative thin-layer chromatography (TLC) on 250–300 mesh KSK silica gel (Russia); the plates were developed in a hexane–diethyl ether (85 : 15) system. The zones of menaquinones (R_f 0.9), ubiquinones (R_f 0.4), and phospholipids (R_f 0–0.2) were collected; isoprenoid quinones and phospholipids were eluted with dehydrated chloroform and a chloroform–methanol (2 : 1) mixture, respectively; the solutions were stored at –15°C.

Analysis of isoprenoid quinones. The composition of quinones was analyzed on an LC-6A liquid chromatograph (Shimadzu, Japan) equipped with an SPD-2AM spectrometric detector and a Cosmosil $5C_{18}$ reversed-phase column (4.6 × 150 mm); an acetonitrile–isopropyl alcohol (65 : 35 or 50 : 50) mixture was used as the mobile phase (0.5 ml/min, 40°C). Ubiquinones and menaquinones were detected at 275 and 270 nm, respectively. Isoprenoid quinones were identified by comparing their retention times with those of quinones isolated from the type strains *S. algae* ATCC 51192^T and *S. frigidimarina* ACAM 591^T (Q-7, Q-8, MK-7, and MMK-7) and *Alteromonas macleodii* ATCC 27126^T (Q-8).

Analysis of phospholipids. Phospholipids were separated by two-dimensional TLC on Sorbfil (Russia) using different solvent systems: chloroform–methanol–ammonia–benzol (65 : 30 : 6 : 10) in the first direction and chloroform–methanol–acetone–acetic acid–benzol–water (70 : 30 : 5 : 4 : 10 : 1) in the second direction [7]. Phospholipids were visualized with a nonspecific reagent (a 10% solution of H_2SO_4 in ethanol at 180–200°C) and with specific reagents, such as ninhydrin- or molybdate-containing reagents, Dragendorff reagent, and α-naphthol, and identified by comparing their positions with standards. The content of individual phospholipids was calculated as a percentage of their total [7].

Fatty acid analysis was performed as described earlier [8]. Fatty acid methyl esters were analyzed by gas–liquid chromatography on a GC-9A chromatograph (Shimadzu, Japan) equipped with SPB-5 and Supelcowax-10 (Supelco, United States) capillary columns (30 m × 0.25 mm) at 230 and 200°C, respectively. The carrier gas (helium) linear flow rate was 25 cm/s. Identification was performed using fatty acid standards.

RESULTS AND DISCUSSION

Earlier, we revealed that bacteria of the genus *Shewanella* comprise about 11% of the total amount of cultivated microorganisms from the Far Eastern seas [9]. The lipid composition of new marine isolates, both free-living and associated with various hydrobionts, was studied; these isolates, based on their phenotypic, genotypic, and phylogenetic features, were assigned to the new species *S. japonica* [10], *S. fidelis* [11], *S. pacifica* [12], and *S. affinis* [13]. In order to confirm the taxonomic significance of lipid markers, in this work, we studied the cellular lipids of the type strains of species phenotypically similar to marine proteobacteria: *Alteromonas macleodii, A. macleodii* subsp. *fijiensis*, *A. infernus, Marinobacter aquaeolei, M. hydrocarbonoclasticus, Marinobacterium georgiense, Microbulbifer hydrolyticus, Pseudoalteromonas tunicata, P. ruthenica, P. elyakovii*, and *P. issachenkonii.*

The occurrence of isoprenoid quinones (ubiquinones and menaquinones), which are involved in the bacterial respiratory chain and termed "respiratory quinones," is widely used as a taxonomic criterion [14]. For the analysis of isoprenoid quinones, we developed a routine procedure, which includes separation of individual quinones by preparative TLC and their identification by high-performance liquid chromatography [15].

Table 1. Composition of isoprenoid quinones in some *Shewanella* species and phylogenetically similar marine gamma-proteobacteria (% of total quinones)

Strains	Q-7	Q-8	Q-9	MK-7	MMK-7	Q-7/MK-7
Shewanella pacifica KMM 3597^T	35.7	58.3	1.4	3.7	0.3	39.7
S. pacifica KMM 3590	21.0	50.7	1.1	20.1	4.5	45.6
S. pacifica KMM 3605	28.3	50.3	1.1	16.8	1.0	46.1
S. *pacifica* KMM 3772	25.4	54.9	1.1	15.4	2.1	42.9
S. *pacifica* KMM 3601	29.0	58.8	1.3	7.8	1.4	38.2
S. *pacifica* KMM 3775	41.2	53.9		2.4	1.1	44.7
S. affinis KMM 3587^T	62.4	26.3				62.4
S. affinis KMM 3821	41.8	43.3	1.1	15.5		57.3
S. affinis KMM 3822	47.0	36.7	0.7	13.1		60.1
S. affinis KMM 3586	54.7	33.8	2.4	5.4	0.3	60.4
S. fidelis KMM 3582^T	67.1	16.6		7.6	2.9	77.6
S. japonica KMM 3299^T	4.8	34.5	56.2			
S. japonica KMM 3300	2.7	33.1	62.8			
S. algae	30.2	45.0	1.1	18.0	2.3	50.5
S. frigidimarina	36.2	46.3	0.8	8.8	5.3	50.3
Pseudoalteromonas elyakovii	1.7	96.4	1.2			
P. issachenkonii	2.8	96.2	0.5			
P. ruthenica	2.2	86.0				
P. tunicata	3.0	93.9	2.1			
Alteromonas macleodii	0.9	95.3	2.9			
"*A. infernus*"	0.9	95.3	2.9			
"*A. macleodii* subsp. *fijiensis*"	0.9	97.8	0.7			
Marinobacterium georgiense	1.0	96.6	1.7			
Microbulbifer hydrolyticus		65.9				
Marinobacter aquaeolei		4.1	94.9			
M. hydrocarbonoclasticus		3.2	95.8			

Note: Q-7, Q-8, and Q-9 signify ubiquinones with seven, eight, and nine isoprenoid units, respectively; MK-7, menaquinone with seven isoprenoid units; and MMK-7, 8-methylmenaquinone with seven isoprenoid units.

As can be seen from Table 1, the composition of respiratory quinones proved to be specific for particular bacterial genera, which is consistent with the occurrence of two types of quinones in facultative anaerobes and one type in aerobic gram-negative bacteria [14]. Most of the studied *Shewanella* strains contained two types of quinones: the major ubiquinones Q-7 and Q-8, which amounted in total to 72–98%, and menaquinones MK-7 and MMK-7 (8-methylmenaquinone-7), which conforms to data in the literature [2, 16–18]. However, no menaquinones were revealed in strains KMM 3587^T, KMM 3299^T, and KMM 3300; the absence of menaquinones in some strains of the genus *Shewanella* was also reported earlier [16, 17, 19]. The presence of only one quinone type proved to be typical of other bacterial genera: for instance, Q-9 was revealed in bacteria of the genus *Marinobacter* and Q-8 was characteristic of bacteria belonging to the genera *Marinobacterium, Microbulbifer, Alteromonas*, and *Pseudoalteromonas*.

Table 1 demonstrates certain regularities in the proportions of quinones with the same chain length (Q-7, MK-7, and MMK-7) in bacteria; while their sum remained relatively constant, an increase in the amount of Q-7 was accompanied by a decrease in the content of menaquinones (strains KMM 3775, KMM 3597^T, and KMM 3586) and, conversely, an increase in the level of menaquinones was attended by a decrease in the amount of Q-7 (strains KMM 3590, KMM 3605, and KMM 3772). The ratio between the major isoprenoid quinones may be a species-specific criterion. For instance, bacteria *S. pacifica* were characterized by an increased content of Q-8 (50–59%), *S. affinis* contained large amounts of Q-7 and MK-7/MMK-7 (57–62%), and *S. fidelis* contained an unusually low level of Q-8 (17%). Two strains of *S. japonica* (KMM 3299^T and

Table 2. Phospholipid composition of some *Shewanella* species and phylogenetically similar marine gammaproteobacteria (%, mean of three measurements)

Microbial species	PE	PG	DPG	PL-A	bis-PA	lyso-PE
S. frigidimarina	61.0	30.0	3.9	4.3		2.0
S. algae	54.0	34.2	2.9	5.9		1.6
S. fidelis	63.3	10.7	14.4	4.5		7.1
*S. japonica**	74.7	9.9	10.1	2.9		2.4
*S. affinis***	61.8	22.3	8.9	1.9		8.4
*S. pacifica****	57.7	25.0	7.1	4.2		7.5
Marinobacterium georgiense	51.2	35.5	12.1			traces
Microbulbifer hydrolyticus	58.6	36.4	5.2			traces
Marinobacter aquaeolei and *M. hydrocarbonoclasticus*	60.1	31.2	5.8			traces
"*Alteromonas infernu*" Ë "*A. macleodii* subsp. *fijiensis*"	56.2	38.6			2.1	traces
*Pseudoalteromonas*****	67.5	23.2			4.5	traces

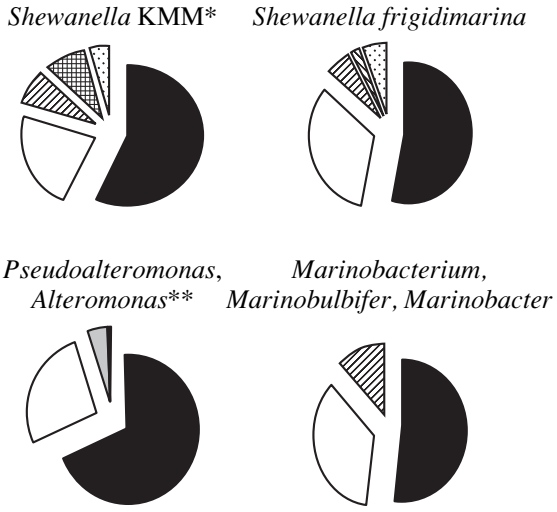
* Averaged for two isolates.
** Averaged for three isolates.
*** Averaged for six isolates.
**** Data from [7].

KMM 3300) were distinguished by the presence of quinones Q-8 and Q-9 (Table 1); at present, this species is considered to be the only representative of the genus *Shewanella* that is characterized by such a quinone profile. The species specificity of the bacterial quinone composition correlates with the results of phylogenetic studies [10–13].

Thus, marine bacteria of the genus *Shewanella* are characterized by a distinctive, frequently species-specific composition of isoprenoid quinones. The absence of menaquinones and the occurrence of quinones with different chain lengths (Q-7, Q-8, and Q-9) in some strains appear to indicate the heterogeneity of this genus.

In spite of the extensive taxonomic investigations of the bacteria belonging to the genus *Shewanella*, information concerning the phospholipid composition of these bacteria is scarce and contradictory; for instance, diphosphatidylglycerol (DPG) was not revealed earlier in any strains of *S. putrefaciens* studied [17]. Comparative data on the phospholipid composition of the new isolates and type strains of the genus *Shewanella* (*S. frigidimarina* and *S. algae*) are presented in Table 2 and the figure. It was revealed that all the strains studied had a similar composition of phospholipids, which were dominated by phosphatidylethanolamine (PE) (54–62%) and phosphatidylglycerol (PG) (22–34%); the minor components were represented by lyso-PE; an unidentified phospholipid A (PL-A), which seems to be acyl-PG; and DPG (3–9%). The occurrence of DPG in all of the strains studied is consistent with its important role in the structure of cellular membranes of facultative anaerobes [20]. Strains of the species *S. japonica* and *S. fidelis* were distinguished by a relatively low amount of PG (10–11%) and high contents of DPG (10–14%) and PE (63–75%).

Some strains of *S. pacifica* and *S. affinis* contained larger amounts of lyso-PE (11–15%) than the type strains of *S. frigidimarina* and *S. algae* (2%), which can be due to a high phospholipase activity of recently isolated strains. It is known that long-term maintenance of



Phospholipid composition of marine gammaproteobacteria of the genera *Shewanella, Alteromonas, Pseudoalteromonas, Marinobacterium, Microbulbifer*, and *Marinobacter*: ■, PE; □, PG; ▨, DPG; ▦, lyso-PE; ◻, acyl-PG; ◼, bis-PA. The *Shewanella* KMM data are average values for the species *S. affinis* and *S. pacifica.* The *Pseudomonas/Alteromonas* data were taken from [7].

bacterial strains under laboratory conditions results in the loss of activity of some enzymes.

The phospholipid composition of the other bacterial genera was relatively simple; the major components were PE and PG (51–68 and 23–39%, respectively) (Table 2, figure). However, the minor phospholipids were specific for certain genera; for instance, DPG (5–12%) was revealed in *Marinobacterium georgiense, Microbulbifer hydrolyticus, Marinobacter aquaeolei*, and *M. hydrocarbonoclasticus*, whereas bisphosphatidic acid (bis-PA) occurred (in the amount of 2–5%) in bacteria of the genera *Alteromonas* and *Pseudoalteromonas*. According to our earlier results [7], bacteria of the latter genera were distinguished by a lack of DPG and the presence of bis-PA (diacyl-PG).

All the strains studied were characterized by a specific composition of fatty acids, which implies the applicability of this chemotaxonomic criterion to the differentiation of marine proteobacteria at the genus level. The phylogenetically related genera *Alteromonas* and *Pseudoalteromonas* had a rather simple composition of saturated and unsaturated acids with the predominance of 16:0, 16:1 (n-7), 17:1 (n-8), and 18:1 (n-7) (72–80%) and contained no branched fatty acids. Bacteria of the genera *Marinobacterium* and *Microbulbifer* were distinguished by the synthesis of branched saturated and unsaturated fatty acids; the genus *Marinobacterium* was characterized by the predominance of 16:0, 18:1 (n-7), and *i*-15:0 acids, whereas in *Microbulbifer* 18:1 (n-7), *i*-15:0, and *i*-17:1 (n-9) acids prevailed. Bacteria of the genus *Shewanella* produced not only fatty acids typical of gram-negative bacteria, such as 15:0, 16:0, 16:1 (n-7), and 17:1 (n-8), but also polyunsaturated (20:5 (n-3)) and branched (*i*-13:0 and *i*-15:0) fatty acids. The high level of branched acids (38–45%) and the presence of eicosapentaenoic acid may serve as a taxonomic criterion of this genus.

Thus, the application of lipid markers provides for rapid and reliable differentiation of certain marine proteobacteria. Bacteria of the genus *Shewanella* are distinguished among phylogenetically related proteobacteria by the presence of two types of ubiquinones, a specific composition of phospholipids, a high level of branched fatty acids, and the ability to synthesize eicosapentaenoic acid.

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