
EXPERIMENTAL
ARTICLES

Comparative Study of Microbial Communities from Cultured and Natural Populations of the Mussel *Mytilus trossulus* in Peter the Great Bay

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Abstract—The 525 strains of heterotrophic bacteria isolated from natural and cultured populations of the mussel *Mytilus trossulus* and the surrounding seawater were identified to a genus level on the basis of phenotypic analysis and the fatty acid composition of cell lipids. Gram-negative isolates were dominated by six genera of the family *Enterobacteriaceae* and by the genera *Pseudoalteromonas*, *Vibrio*, *Photobacterium*, *Cytophaga/Flavobacterium/Bacteroides*, *Pseudomonas*, and *Moraxella*. Gram-positive isolates were mainly represented by the genus *Streptomyces*. The taxonomic compositions of natural and cultured populations of the mussel *M. trossulus* in Peter the Great Bay were similar.

Key words: microbial community, mussel, associates.

The studies of the microflora of bivalves are primarily aimed at identifying pathogenic microorganisms, particularly enterobacteria and vibrios, that may accumulate in mussels because of water filtration and cause diseases in humans eating such mussels [1, 2]. The natural microflora of the oysters *Crassostrea gigas* and *C. virginica* and the surrounding seawater was found to be dominated by the genera *Vibrio*, *Achromobacter*, *Cytophaga/Flavobacterium*, and *Pseudomonas* [3], being different in the oysters and the surrounding water [4]. In contrast, Siguta et al. [5] showed that the bacterial flora of bivalves is the same as in the surrounding seawater. Ortigosa et al. [6] also compared the composition of microbial communities in oysters and the surrounding seawater. At the same time, to the best of our knowledge, there is no data in the literature dealing with the comparative taxonomic composition of the microbial communities of cultured and natural populations of mollusks. The mussel *Mytilus trossulus* has yet to be appropriately studied in this respect, although it is a valuable sea product and a source of biologically active substances.

The aim of this work was to comparatively study the taxonomic composition of microbial communities isolated from cultured populations of the mussel *M. trossulus* and its natural populations inhabiting water areas with different degrees of anthropogenic impact.

MATERIALS AND METHODS

The mussels *Mytilus trossulus* were collected by divers in summer 2000 from a mariculture and natural populations in Trinity Bay with a low anthropogenic load and in the Vanguard and Gaidamak Bays of Peter the Great Bay in the Sea of Japan with a high anthropogenic load. The mussels were immediately placed into plastic bags sterilized with γ radiation. Seawater was sampled into sterile bottles at the same sites where the mussels were collected.

Microorganisms were isolated by plating 0.1 ml of seawater or mussel tissue homogenate onto Y-K agar [7]. The isolates were stored at 8–10°C on the same medium under mineral oil and identified to a genus level based on their morphological, cultural, physiological, and biochemical properties [8, 9].

DNA was isolated by the Marmur method [10] and analyzed for the G+C content from the melting profiles, using a Pye Unicam 1800 spectrophotometer and the formula $G+C$ (mol %) = $T_m - 106.4$ [11].

To analyze the fatty acid composition of isolates, they were grown at 25°C for 24 h on the aforementioned medium. The methyl esters of fatty acids were prepared by a modification of the Carreau and Dubacq method [12]. An aliquot (about 2 μ g) of the biomass was transferred into a flask using a platinum inoculation loop and suspended in 0.3 ml of 1% NaOH in methanol. The suspension was incubated at 60°C for 15 min, mixed with 0.3 ml of 5% HCl in methanol, and incubated at 60°C for the next 15 min. The methyl esters of

fatty acids were extracted twice with 0.2 ml of hexane. The extracts were pooled and analyzed for fatty acid methyl esters using a Shimadzu GC-9A gas chromatograph equipped with (30 m × 0.25 mm ID) capillary columns packed with Supelcowax-10 and SPB-5 (Supelco, United States), a flame-ionization detector, and a Chromatopac C-R3A integrator. The volume of samples was 1–3 µl. The columns were kept at 205 and 210°C, respectively. The carrier gas was helium. Fatty acids were identified by their retention times and carbon numbers using the authentic samples of fatty acids (Supelco, United States). In addition, the methyl esters of fatty acids were hydrogenated in methanol with platinum oxide as a catalyst and the resultant fatty acid derivatives were analyzed by gas chromatography under the same conditions.

RESULTS AND DISCUSSION

All of the 525 microbial strains isolated from natural and cultured populations of the mussel *M. trossulus* and the surrounding seawater were identified to a genus level based on the analysis of their morphological, physiological, and biochemical characteristics, although it is known that some marine isolates are difficult to identify [13]. About 85% of the isolates were gram-negative bacteria. Motile, gram-negative, oxidase-positive, sensitive to the vibriostatic agent O-129, curved rods with respiratory or fermentative metabolism and characteristic G+C content of DNA were ascribed to the genus *Vibrio*.

The bacteria that were oxidase-positive, had polar flagella when grown in liquid media, and did not require Na⁺ ions for growth were assigned to the family *Enterobacteriaceae*.

The bacteria with an oxidative metabolism that did not contain arginine dihydrolase and showed the G+C content of DNA within the range 40.7–48.1 mol % were referred to pseudoalteromonads. Pseudomonads were differentiated from pseudoalteromonads based on their ability to grow on Na⁺-free media, the presence of arginine dihydrolase, and high biochemical activity.

The bacteria that exhibited high halotolerance, the absence of glucose fermentation, the ability to accumulate poly-β-hydroxybutyrate, and had peritrichous flagella were assigned to the genus *Deleya*.

Gliding gram-negative bacteria were assigned to the phylogenetic cluster *Cytophaga/Flavobacterium/Bacteroides*.

The pleiomorphic cells (either filamentous or arranged in four-cell aggregates bounded by a capsule) that were unable to produce acids from carbohydrates on a Hiss medium, were sensitive to penicillin at a dose of 1 U/ml, and had the G+C content of DNA equal to 43.3–46.6 mol % were assigned to the genus *Moraxella*.

In addition to phenotypic properties, some of the bacterial isolates were analyzed for the fatty acid com-

position of cell lipids, which is considered a valuable chemotaxonomic marker [14]. In general, the results of this analysis confirmed the preliminary classification of the isolates.

Overall, 35 fatty acids (from C₁₂ through C₁₉), including saturated, monoenoic, straight-chain, branched-chain (*iso* and *anteiso*), and alicyclic fatty acids, were identified (Table 3). The fatty acid composition of strains 25 and 80 was typical of bacteria of the genus *Pseudoalteromonas* [15]. The major fatty acids of both strains were 16 : 0, 16 : 1(*n*-7), and 17 : 1(*n*-8), the fatty acids 14 : 0, 15 : 1(*n*-8), 18 : 0, and 18 : 1(*n*-7) being minor. The absence of cyclopropane and hydroxy fatty acids is a characteristic feature of pseudoalteromonads.

Three major fatty acids, namely, 16 : 0, 16 : 1(*n*-7), and 18 : 1(*n*-7), comprised 90% of all fatty acids of strain 80M, dominant being 18 : 1(*n*-7) (40% of the total fatty acids). Based on the results of biochemical analysis, this isolate was assigned to the genus *Deleya*.

The major fatty acids of strains 41M and 48M were 16 : 1, 16 : 1(*n*-7), and 18 : 1(*n*-7). The high content of the latter fatty acid (about 40%) is a specific feature of vibrios [16]. The minor fatty acids of these isolates were represented by odd-carbon, branched, saturated, monoenoic, and cyclopropane fatty acids. The fatty acid profiles of the two isolates were very similar, indicating a close taxonomic relationship between them.

The major fatty acids (16 : 0, 16 : 1(*n*-7), 18 : 1(*n*-7), and 17 : 0-cyclo) of three other isolates (B-67M, B-77M, and B-1M) were dominated by the saturated acid 16 : 0, which comprised from 22 to 38.6% of the total fatty acids. The high content of cyclopropane fatty acids (up to 22%) and the *cis*-vaccenic fatty acid 18 : 1(*n*-7) (up to 34%) is a characteristic feature of the genus *Pseudomonas* [17]. According to their fatty acid profiles, the three isolates we assigned to this genus.

Strain B-90 showed a very high content (more than 70%) of branched fatty acids, including the rare fatty acid 17 : 1-*iso*, whose content reached 16.2%. The major branched fatty acid was 15 : 0-*iso* (almost 30% of the total). Such fatty acid composition is typical of the species *Desulfovibrio desulfuricans* [18], which allowed us to assign strain B-90 to the genus *Desulfovibrio* based merely on the fatty acid profile.

The microflora of cultured populations of the mussel *M. trossulus* was mainly represented by the genera *Pseudoalteromonas*, *Vibrio*, *Photobacterium*, *Cytophaga/Flavobacterium/Bacteroides*, and *Pseudomonas* (Tables 1, 2). The fraction of bacteria from the family *Enterobacteriaceae* in the microflora of the cultured *M. trossulus* populations amounted to 29% of the total number of the isolates. These data are in agreement with the results of investigation of the microflora of bivalves collected off the Japan coast, which was represented by the genera *Vibrio*, *Aeromonas*, *Pseudomonas*, *Moraxella*, and *Micrococcus* and by bacteria of the family *Enterobacteriaceae* [5]. According to the data of

Table 1. The taxonomic composition of microbial communities isolated from Trinity Bay

Genus or family	Number of strains isolated from		
	cultured mussels	natural mussel population	water
<i>Enterobacteriaceae</i>	12 (12.8)	6 (12.8)	7 (21.2)
<i>Pseudoalteromonas</i>	8 (8.5)	6 (12.8)	6 (18.2)
<i>Pseudomonas</i>	6 (6.4)	4 (8.5)	6 (18.2)
<i>Vibrio</i>	8 (8.5)	10 (21.3)	6 (18.2)
<i>Photobacterium</i>	7 (7.4)	5 (10.6)	4 (12.1)
<i>Aeromonas</i>	0	0	0
<i>Moraxella</i>	9 (9.6)	9 (19.1)	0
<i>Cytophaga/Flavobacterium/Bacteroides</i>	24 (25.5)	2 (4.3)	0
<i>Deleya</i>	2 (2.1)	0	2 (6.1)
<i>Bacillus</i>	8 (8.5)	2 (4.3)	0
<i>Micrococcus</i>	4 (4.3)	2 (4.3)	0
<i>Streptomyces</i>	6 (6.4)	1 (2.1)	2 (6.1)
<i>Desulfovibrio</i>	0	0	0
Total	94 (100)	47 (100)	33 (100)

Note: Parenthesized is a percent of the total number of the respective isolates.

Ivanova and Mikhailov [19], the microflora of the mussel *Crenomytilus grayanus* is represented by the genera and generic groups *Pseudomonas/Alteromonas*, *Vibrio/Photobacterium*, *Aeromonas*, *Acinetobacter/Moraxella*, *Fla-*

vobacterium, *Enterobacter*, and *Bacillus*, as well as by actinomycetes and yeasts.

The taxonomic compositions of the microbial communities isolated from the mussel *M. trossulus* populations cultured in distant Trinity and Vanguard Bays differed insignificantly. For instance, the mussels collected in Trinity Bay did not contain *Aeromonas* spp., whereas the mussels collected in Peter the Great Bay contained no *Micrococcus* spp. Of interest is the fact that the waters surrounding these mussels did not contain aeromonads and micrococci, respectively. This suggests the existence of a relationship between the microfloras of a hydrobiont and the surrounding water. Some microbial genera, however, did not show such a relationship. For instance, the water of Trinity Bay did not contain bacteria of the genera *Moraxella*, *Cytophaga/Flavobacterium/Bacteroides*, *Bacillus*, and *Micrococcus*, whereas these bacteria were found in the mussels collected in this bay. Similar data were obtained by other researchers [4, 5]. This phenomenon can be accounted for by the accumulation of minor bacterial species occurring in the surrounding water by water-filtering mussels. As a result, being scarce in the surrounding water, some free-living bacteria cannot be detected there by conventional microbiological methods. At the same time, the same bacteria may actively multiply in mussels due to a large amount of easily metabolizable organic substances and hence can easily be detected in these mussels.

The microbial communities isolated from the natural *M. trossulus* populations of Trinity and Peter the Great Bays, differing in the degree of anthropogenic load, showed a taxonomic difference. For instance, the

Table 2. The taxonomic composition of microbial communities isolated from the Vanguard and Gaidamak Bays of Peter the Great Bay

Genus or family	Number of strains isolated from			
	mussels cultured in Vanguard Bay	natural mussel population in Gaidamak Bay	water of Vanguard Bay	water of Gaidamak Bay
<i>Enterobacteriaceae</i>	9 (10.1)	45 (51.1)	12 (16)	36 (33.6)
<i>Pseudoalteromonas</i>	18 (20.2)	4 (4.5)	15 (20)	4 (3.7)
<i>Pseudomonas</i>	12 (13.5)	2 (2.3)	5 (6.6)	8 (7.5)
<i>Vibrio</i>	7 (7.9)	11 (12.6)	6 (8)	10 (9.3)
<i>Photobacterium</i>	7 (7.9)	5 (5.7)	2 (2.6)	2 (1.9)
<i>Aeromonas</i>	6 (6.7)	8 (9)	18 (24)	32 (29.9)
<i>Moraxella</i>	6 (6.7)	0	0	0
<i>Cytophaga/Flavobacterium/Bacteroides</i>	5 (5.6)	3 (3.4)	0	0
<i>Deleya</i>	2 (2.2)	2 (2.3)	5 (6.6)	3 (2.8)
<i>Desulfovibrio</i>	1 (1.1)	0	0	0
<i>Bacillus</i>	4 (4.5)	0	3 (4)	7 (6.5)
<i>Micrococcus</i>	0	0	0	0
<i>Streptomyces</i>	12 (13.5)	8 (9)	9 (12)	5 (4.7)
Total	89 (100)	88 (100)	75 (100)	107 (100)

Note: Parenthesized is a percent of the total number of the respective isolates.

Table 3. The fatty acid composition of bacteria associated with the mussel *M. trossulus*

Fatty acid	<i>Pseudoalteromonas</i>		<i>Vibrio</i>		<i>Pseudomonas</i>			<i>Halomonas</i>	<i>De-sulfovibrio</i>
	B-25	B-80	B-41	B-48M	B-67M	B-77M	B-81M	B-80M	B-90
12:0	0.2	0.2	0.6	1.3	0.3	2.4		4.5	
12:1	0.3	0.2							
13:0- <i>i</i>			0.8	0.3					0.7
13:0	0.6	0.5	0.3	0.3		1.2			
13:1	0.5	1.							
14:0- <i>i</i>	0.1	0.1	0.3	0.2					
14:0	1.4	0.7	5	4.7	1.6		2.5	0.8	0.5
14:1 (<i>n</i> -7)	0.2	0.3	0.3	0.2		0.6		0.1	
15:0- <i>i</i>		0.1	0.9	0.5				0.1	29.8
15:0- <i>a</i>	0.1	0.2	0.3	0.3				1.6	0.5
15:1- <i>i</i>									1.8
15:1- <i>a</i>									0.5
15:0	9.5	7.1	4.6	4.4	0.2	0.5	1.2	0.1	0.5
15:1 (<i>n</i> -8)	5.4	3.3	0.4	0.6					0.3
15:1 (<i>n</i> -6)	1.4	0.7	0.4	0.2	0.1		0.1		0.2
16:0- <i>i</i>	1.2	1.3	2.4	1.5				0.3	0.7
16:0	9.3	7.2	16.1	14.6	38.6	22	26.5	18.6	4.5
16:1 (<i>n</i> -7)	24.7	16.7	36.2	38.9	34.6	25.3	17.1	27.9	7.2
16:1 (<i>n</i> -5)	0.2		0.2	0.2	0.2	0.3	0.3	0.1	
17:0- <i>i</i>	0.2	0.3	2.1	0.8	0.1	0.4		0.5	19.5
17:1- <i>i</i>	0.4		0.6	0.5	0.4	0.4	0.2		
17:0- <i>a</i>		0.4						1.1	0.7
17:1- <i>iso</i>									16.2
17:1- <i>a</i>									0.4
17:0	8	9.8	5.2	3.4	0.5	0.5	2.3	0.1	1.4
17:1 (<i>n</i> -8)	28.9	37.7	4.8	7.3		0.4	0.7	0.1	1.2
17:0- <i>cy</i>	2.7	3.8	1.3	1.5	8.1	3.4	12.2	0.1	2.5
18:0- <i>i</i>	0.4	0.3	0.3	0.2					
18:0- <i>ai</i>	0.4	0.5			0.1		0.1		
18:0	0.4	0.5	0.7	1.3	1.8	1.5	4.3	0.8	2.3
18:1 (<i>n</i> -9)	0.5	0.4	0.5	0.4	0.1		0.1		1.5
18:1 (<i>n</i> -7)	2.1	4.4	15	15.1	12	39.7	30.1	42.9	6.3
19:0									
19:1	0.6	1.5	0.1	0.2	0.7	1.1	0.1	0.1	0.6
19:0- <i>cy</i>							1.9		

Note: The data are expressed as a percent of the total amount of fatty acids.

mussels and seawater collected in Peter the Great Bay with a severe anthropogenic load exhibited a high content of bacteria of the family *Enterobacteriaceae* (*Escherichia*, *Enterobacter*, *Klebsiella*, *Proteus*, *Serratia*, and *Salmonella*) (51% of the total isolates), as well as bacteria of the genera *Vibrio* and *Aeromonas* (12.5 and 9%, respectively).

The gram-positive bacteria isolated from the cultured and natural populations of *M. trossulus* were dominated by bacteria of the genus *Streptomyces*, which produce extracellular enzymes and are commonly isolated from the bottom sediments of marine and freshwater ecosystems, where they decompose complex organic compounds [20]. Ivanova and

Mikhailov also reported the occurrence of actinomycetes in the mussel *C. grayanus* [19].

The microbial community isolated from the natural mussel population inhabiting Trinity Bay was dominated by vibrios (21.3%), enterobacteria (13%), pseudoalteromonads (13%), and *Moraxella* (19%) (Table 1).

In general, the microfloras of the cultured and natural populations of the mussel *M. trossulus* were dominated by microorganisms with high metabolic activity. The mussel interior favors the growth of anaerobic vibrios, which may utilize a number of carbohydrates in the intestinal tract of mussels and nitrate as the terminal electron acceptor. Nitrate-utilizing pseudomonads also are well adapted to anaerobic conditions in the mussel interior. Many pseudoalteromonads produce biologically active substances, including antibiotics, toxins, and extracellular enzymes [21], which enhance their competitiveness with respect to bacteria lacking such a capability. Bacteria of the group *Cytophaga/Flavobacterium/Bacteroides* possess both oxidative and fermentative types of metabolism and are able to utilize a diversity of substrates (carbohydrates, polysaccharides, proteins, etc.) [21]. As a result, like the aforementioned microorganisms, bacteria of this group are well adapted to life in the mussel interior. This may explain why most of the mussels collected in different regions of the world ocean contain the same groups of microorganisms [4, 5, 13]. The hydrolytic enzymes secreted by associative bacteria are useful not only to the producer but also to the host animal, since the latter gains a possibility of utilizing the hydrolysis products of food components [22]. The occurrence of several taxonomic groups of bacteria in the mussel *M. trossulus* may provide for a continuous supply of the necessary nutrients to this animal under varying conditions.

The composition of the microbial communities of hydrobionts is largely determined by the condition of the surrounding water, which is due to their water-filtering activity [21]. Unlike Trinity Bay, Peter the Great Bay suffers from an anthropogenic impact coming mostly from the marine port in Gaidamak Bay, which discharges domestic and industrial sewage into the bay water. The high content of bacteria from the family *Enterobacteriaceae* and the genus *Aeromonas* in the microbial communities isolated from the mussels and the surrounding water is a reflection of adverse ecological conditions in Peter the Great Bay. In contrast, the microbial communities isolated from the cultured and natural *M. trossulus* populations and the surrounding water in Trinity Bay contain few enterobacteria and no aeromonads, indicating more sound ecological conditions in this bay.

Thus, the taxonomic compositions of the microbial communities isolated from the natural and cultured populations of the mussel *M. trossulus* are similar, which suggests a similarity of symbiotic digestive processes in these populations. The investigation of the

range of hydrolytic enzymes produced by the bacterial isolates is in progress in our laboratory.

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