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EXPERIMENTAL ARTICLES

Seasonal Dynamics of Cell Numbers and Biodiversity of Marine Heterotrophic Bacteria Inhabiting Invertebrates and Water Ecosystems of the Peter the Great Bay, Sea of Japan

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Abstract—The annual changes in bacterial numbers and diversity of the heterotrophic microflora in invertebrates and ambient water were studied. During the whole period of observation, bacterial cell numbers were higher in invertebrate specimens than in the ambient water. The highest number of bacterial cells was detected in trepangs and sea urchins, while the lowest number of cells was detected in starfish. Based on the results of phenotypic analysis and analysis of fatty acid composition of bacterial cell lipids, 487 strains (out of the total of 502 isolates) of heterotrophic bacteria were identified to the genus level. Morphological differences between the winter and summer isolates of vibrios and halomonads were analyzed. The seasonal dynamics of the cell numbers of vibrios and halomonads was revealed. The gram-positive microflora was most often present in animals during the winter, fall, and spring periods. The diversity of heterotrophic bacteria was greater in the water column than in animal tissues.

Key words: heterotrophic bacteria, cell numbers, dynamics, associations, hydrobionts.

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Prokaryotes are one of the major constituents of marine coastal ecosystems. Heterotrophic bacteria of marine microbial communities are involved in all the processes occurring in these ecosystems and, as a part of the associated microflora of hydrobionts, play an important role in their life [1, 2]. Hydrobionts, a source of readily available organic compounds, support growth of the associated microflora [3]. The physicochemical parameters of the environment, viruses, and eukaryotic organisms that feed on bacteria, etc., are involved in the complex regulation of bacterial populations [4, 5]. The control of bacterial numbers and activity of natural bacterial populations is a very complex and hitherto poorly understood process. One of the causes of this situation is the inability of marine microorganisms to grow on laboratory media: less than 1% of the total number of bacteria from marine ecosystems are culturable under artificial conditions [6]. Obtaining pure bacterial cultures remains one of the main methods of modern microbial ecology used for isolation, identification, and characterization of the physiological properties of microorganisms, although this method results in underestimation of bacterial numbers [7]. Elucidation of the structure and diversity of microbial communities may allow us to gain some insight into the rules of their functioning. Despite the fact that interactions between microorganisms and marine hydrobionts have received a great deal of attention, the structure, composition, and dynamics of natural marine microbial communities remain poorly understood.

The taxonomic composition of bacterial communities of invertebrates inhabiting the Russian coastal waters of the Sea of Japan is poorly studied, and data on this issue are scarce [8, 9]. Comparative investigation of the taxonomic composition of the heterotrophic microflora of hydrobionts and ambient waters based on cultivation methods is rarely carried out due to certain methodical difficulties, including the need for the phenotypic characterization of a large number of strains. However, this technique allows us to study the physiological properties of each isolate, thus providing a clue to the understanding of its role in marine ecosystems.

The goal of the present work was to carry out a comparative investigation of the cell numbers and community structure of heterotrophic bacteria associated with the most abundant species of marine invertebrates, as well as to study the annual changes in microbial communities inhabiting the water column. The following tasks were set: analysis of the seasonal dynamics of changes in the bacterial numbers; identification and taxonomic description of all the bacterial isolates obtained; and investigation of the distributional patterns of heterotrophic bacteria (free-living and associated with marine invertebrates) as dependent on ambient temperatures.

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Table 1. Numbers of heterotrophic bacteria in hydrobiont tissues and ambient water determined in cultures grown on the Y–K medium and TCBS agar (CFU/g and CFU/ml)

Objects	February, –1.5…1.7°C	March, –1°C	April, 4–5°C	May, 7–8°C	June, 10–13°C	July, 4–18°C	August, 18–21°C	October, 10–15°C	November, 2–3°C
Patiria pectinifera	*1.3	1.0	5.6	2.6	410	3300	1800	850	1.3
	**–	–	0.23	0.18	4.2	230	310	30	0.18
Asterias amurensis	1.0	0.9	5.4	17	320	1700	8300	630	1.8
	–	–	0.12	1.1	3.1	230	300	30	–
Strongylocentrotus intermedius	3.4	2.0	3.1	63	1400	51000	38000	1800	2.7
	–	–	0.36	1.2	16	1400	1000	63	0.2
Strongylocentrotus nudus	18	2.1	14	82	730	12000	24000	1200	6.8
	–	–	0.84	2.3	61	2200	1800	61	–
Apostichopus japonica	35	17	23	310	2800	32000	69000	6100	33
	–	–	0.7	1.6	18	1800	2600	110	0.3
Crenomytilus grayanus	2.2	1.0	3.8	30	630	6600	7100	1200	2.1
	–	–	0.18	1.2	1.2	410	73	1.1	–
Modiolus dificilus	4.2	4.1	4.0	8.4	310	1100	5400	640	3.3
	–	–	0.31	0.7	17	130	130	1.2	–
Seawater (surface)	1.35	1.3	1.6	3.0	1.3	290	350	62	1.0
	–	–	–	0.15	0.13	0.22	2.3	0.31	–
Seawater (sampling depth, 5–7 m)	4.7	4.3	4.8	4.5	5.2	60	110	3.2	1.5
	–	–	–	–	0.11	0.13	1.0	0.26	–

Notes: CFU/g, for animal tissues; CFU/ml, for water samples.
* CFU/g or CFU/ml × 10^3 on the Y–K medium.
** CFU/g or CFU/ml × 10^2 on TCBS agar.

MATERIALS AND METHODS

Animals and water samples were collected monthly, from February to November 2006, by divers near the Avangard Bight of the Vostok Bay, as well as in the Peter the Great Bay. The samples were collected into sterile plastic bags and vials. Water temperature was measured at the sampling sites with a water thermometer. The starfish *Patiria pectinifera* and *Asterias amurensis*, bivalves *Crenomytilus grayanus* and *Modiolus dificilus*, the holothuria *Apostichopus japonica*, sea urchins *Strongylocentrotus nudus* and *S. intermedius*, and seawater samples taken from the surface (0–10 cm) and near-bottom (depth 5–7 m) horizons of the water column were the subjects of this study. The animals were delivered alive to the laboratory within an hour after sampling; bivalves were cleaned to remove epibiotic growth, washed with sterile seawater, flamed, and opened aseptically. Weighed portions (1 g) of animal tissues were disintegrated and homogenized under sterile conditions in a glass homogenizer. Serial dilutions (0.1 ml) of homogenates and water were inoculated on solid nutrient Youschimizu–Kimura medium (Y–K) and TCBS agar. The inoculated petri dishes with the Y–K medium were incubated at room temperature for up to 3 weeks; the dishes with TCBS agar were incubated for 2 days. The cultures were examined daily under a binocular microscope and the colonies were counted. The colonies representing different morphotypes were selected for subculturing and inoculated on petri dishes with relevant media to obtain pure cultures of bacterial isolates. Morphological, cultural, and biochemical characteristics, as well as the fatty acid composition and the content of G+C base pairs in the DNA, were studied as described in [8, 10]. The taxonomic position of the isolated strains was determined using [11] and the searchable online version of *The Prokaryotes* (http://41.150.157.117:8080/proPUB/index.htm).

RESULTS

Enumeration of microflora. To study the population dynamics and microflora composition in hydrobionts and seawater, samples were collected nine times from February to November, 2006. The water temperature during the sampling period ranged from –1.7°C (February) to 21°C (August). The samples were collected once a month, with the exception of September, December, and January, when water temperatures were similar to those of previous months. The number of revealed heterotrophic bacteria (expressed in CFU/g or CFU/ml) was two to three orders of magnitude higher on the Y–K medium than on TCBS agar (Table 1). No growth was detected on TCBS agar during the cold season.

The data presented in Table 1 revealed seasonal fluctuations in the number of bacterial cells in animal tissues: the lowest number of cells (9.0×10^2 cells/ml) was detected in February and March, while the highest cell number (6.9×10^7 cells/ml) was detected in July and August. During the entire period of observations, the population density of heterotrophic bacteria was higher in the samples taken from invertebrates than in water samples. Among the studied animals, the highest number of bacterial cells was observed in trepangs and sea urchins; the lowest number of cells was observed in starfish during both the warm and the cold seasons.

In water samples, the highest number of bacteria (3.5×10^5 and 2.3×10^2 cells/ml on the Y–K medium and TCBS agar, respectively) was observed in August, during the warmest season (Table 1). The lowest number of bacterial cells (1.3×10^3 cells/ml on the Y–K medium) was detected in the water sample collected in March at a temperature of –1.0°C.

Identification and taxonomic description of the isolated strains. A total of 502 strains of heterotrophic bacteria were isolated from seven species of invertebrates, as well as from seawater samples, and taxonomically identified (Table 2). However, we failed to identify 15 strains. Pseudomonads, halomonads, vibrios, and other bacteria isolated in this study were identified based on the characteristics described previously for the microflora of mussels and the bacterial communities of brown and red algae from the Peter the Great Bay [8, 12]; therefore, we considered it unnecessary to list the phenotypic characteristics of the isolates in this paper. Only their specific characteristics are described further. The cell morphologies of the vibrio strains isolated from winter and summer samples differed considerably. In primary isolations, the cells of "winter" strains were large, swollen, and irregularly shaped; only after several transfers on laboratory media at room temperature, they assumed the shape typical of vibrios (short curved rods with rounded ends).

Unlike "summer" strains, the halomonads isolated during the cold season formed very slimy colonies, which indicates that exopolysaccharides were produced. The morphologies of the winter and summer variants of the cultures of other taxonomic groups were similar.

We analyzed the fatty acid composition of bacterial lipids of most isolates (Table 3). The vibrio species were characterized by the predominance of three main fatty acids, 16:0, 16:1(n-7), and 18:1(n-7); the characteristic trait of the studied sulfite-oxidizing bacteria was the high level of *cis*-vaccenic acid 18:1(n-7). In the halomonad strains, the typical fatty acids 18:1(n-7), 16:1(n-7), and 16:0 were found; unlike pseudomonads, these microorganisms did not contain cyclopropanoic acid (*cyclo*-19:0). In agromycetes, *anteiso*-15:0 and *anteiso*-17:0 fatty acids predominated; large amounts of *iso*-15:0 and *iso*-16:0 were detected as well. The presence of high concentrations of branched *iso*-15:0 and *iso*-15:1 is the characteristic trait of the *CFB* cluster; the hydroxy acids *iso*-17:0-3OH and *iso*-15:0-3OH were also detected. The fatty acid profiles of bacilli demonstrated that *iso*-14:0, *iso*-15:0, *iso*-16:0, *anteiso*-15:0, and *anteiso*-16:0 prevailed, which is typical of these microorganisms. According to the combination of these fatty acids, the bacillus strains were divided into two groups obviously belonging to different species.

A predominant majority of the isolates (402 strains, 80% of all the strains isolated) were gram-negative (Table 2). Among them, bacteria of the genera *Vibrio, Pseudomonas, Pseudoalteromonas, Halomonas, Shewanella, Sulfitobacter, Chromobacterium**, and *Xanthomonas**, as well as representatives of the *Cytophaga–Flavobacterium–Bacteroides* phylogenetic cluster were detected. Among gram-positive microflora, members of the genera *Bacillus*, coryneforms, members of the genera *Staphylococcus, Planococcus*, and *Arthrobacter*, actinomycetes, *Marinococcus**, and *Halococcus** were revealed; all these microorganisms are listed in descending order of frequency (the genera marked with an asterisk are not listed in Table 2 due to their scarcity).

Seasonal dynamics of the distributional patterns of heterotrophic bacteria. In Table 2, the months in which sampling was performed were organized into four groups using similar values of water temperature as a criterion. Almost a third of the studied isolated (172 strains) were obtained in July and August from animal tissues and water samples (127 and 45 isolates, respectively). During the coldest months, 113 strains were isolated in pure cultures from animal tissues and water samples (86 and 27 strains, respectively), which constitutes about a fifth of the total number of isolated strains. Table 2 shows that vibrios prevail among the gram-negative bacteria isolated from animal tissues. The number and frequency of occurrence of vibrios were highest in the samples collected during the warmest season. At subzero water temperatures, they were not detected in the starfish *P. pectinifera* and the mussel *C. grayanus.* Bacteria belonging to the genera *Pseudomonas* and *Pseudoalteromonas* and to the *CFB* cluster were detected in all the animals and water samples collected during the whole season. Halomonads were found in the samples collected primarily at subzero and low positive temperatures, and they were not detected in 5 out of 7 animal species collected during the summer season (July and August). *Shewanella* species were found in all specimens collected throughout the whole year, and no seasonality of their distribution was observed. The fact that only six *Sulfitobacter* strains were isolated prevents us from determining their distributional patterns. We can only mention that these bacteria were detected in animals collected at positive temperatures. Bacilli prevailed among the gram-positive bacteria found in hydrobionts. They were most often found in the bivalve *M. dificilus*, this fact requires further study. Staphylococci were found to be constant members of the heterotrophic microbial community

Table 2. Taxonomic composition and numbers of bacterial strains isolated from the hydrobionts and water samples collected from the Peter the Great Bay during the year

Objects	Ps.	PsAl.	*Vibrio*	Shewan.	Halomon.	*CFB*	Sulf.	Bacillus	Corynef.	Agromyc.	Arth.	Staph.	Plan.
Patiria pectinifera	*2	1	–	–	1	3	–	–	–	–	–	1	–
	**2	2	–	1	1	3	–	–	–	–	1	1	–
	■4	2	1	1	3	1	1	–	–	–	–	1	–
	●1	1	11	1	–	1	–	1	–	–	–	2	–
Asterias amurensis	1	–	2	1	–	–	–	2	–	–	–	–	–
	4	1	–	–	1	1	–	1	1	–	–	–	–
	3	1	1	–	3	1	–	–	–	–	–	1	–
	2	–	8	1	1	1	–	–	–	–	–	–	2
Strongylocentrotus intermedius	2	1	2	1	2	1	–	3	1	1	–	–	–
	1	2	1	–	2	1	–	1	1	–	–	–	–
	–	2	4	2	4	2	–	–	–	–	–	–	–
	–	–	13	6	–	1	–	–	–	–	–	1	–
Strongylocentrotus nudus	5	2	1	1	3	2	–	1	–	–	–	–	–
	1	3	–	3	1	5	–	1	1	–	1	–	–
	3	3	4	1	2	1	–	–	1	–	–	–	–
	1	–	12	1	–	1	–	–	–	–	–	–	–
Apostichopus japonica	2	1	2	1	1	2	–	2	–	1	–	–	1
	3	1	1	–	1	1	1	1	1	–	1	–	–
	3	2	4	2	2	3	–	–	–	–	–	1	–
	1	–	15	1	–	2	–	–	–	–	–	–	–
Crenomytilus grayanus	2	1	–	–	–	1	–	1	–	1	–	1	–
	–	–	–	1	–	–	–	–	1	–	–	–	–
	2	1	2	–	4	1	–	–	–	–	–	1	–
	1	–	16	2	1	1	1	–	–	–	–	–	–
Modiolus dificilus	1	–	1	2	–	1	–	11	–	–	–	–	2
	3	–	–	1	1	–	1	2	2	–	1	–	–
	1	–	–	–	2	2	–	2	2	–	–	–	–
	–	–	7	5	–	1	–	1	–	–	–	–	–
Water (surface)	1	1	–	–	3	1	1	5	–	–	1	–	1
	5	2	–	–	3	4	–	–	–	–	–	–	1
	–	1	5	–	2	–	–	1	–	–	1	1	2
	1	1	10	1	–	1	–	2	–	1	–	–	1
Water (sampling depth, 5–7 m)	2	1	–	–	2	2	1	4	1	–	1	–	–
	1	1	–	–	4	2	–	3	–	–	–	1	1
	2	1	1	3	3	2	–	–	1	–	–	–	1
	4	–	12	1	–	1	–	2	1	–	1	1	–
TOTAL number of strains	67	35	135	40	53	51	6	47	14	4	8	13	12

Notes: * number of strains isolated in February and March; ** number of strains isolated in April and November; ■ number of strains isolated in May, June, and October; ● number of strains isolated in July and August. Ps., *Pseudomonas,* PsAl., *Pseudoalteromonas*; Shewan., *Shewanella*; Halomon., *Halomonas*; Sulf., *Sulfitobacter*; Corynef., coryneform bacteria; Argomyc., *Argomyces*; Arth., *Arthrobacter*; Staph., – *Staphylococcus*, Plan., – *Planococcus*.

Table 3. Composition of the major fatty acids in the studied bacterial strains

Fatty acids	*Vibrio*	*Sulfltobacter*	*Halomonas*	*Agromyces*	*CFB*	*Bacillus*	
12:0	4.2*	2.9	5.0		0.5		
13:0-*i*	1.1			1.3			
14:0-*i*				0.5		4.4	20.6
14:0	6.4		1.1		2.2		
15:0-*i*	0.7			6.2	39.7	27.0	7.0
15:0-*a*				49.7	4.5	9.8	41.0
15:1-*i*					9.2		
15:0	1.6		0.5	0.9	4.4	0.3	
16:0-*i*	1.1			8.6		28.7	16.6
16:0-*ai*						18.3	11.0
16:0	22.8	1.4	21.5	0.9	2.3	0.9	0.4
16:l(n-7)	39.6	7.5	19.0	1.0	19.2	1.4	1.0
17:0-*i*	1.8		0.4	1.8		2.6	
17:0-*a*	0.4	0.9	1.5	30.2		4.7	1.5
17:l-*a*					1.4	1.8	0.9
17:0	1.2		3.4	0.5			
17:l(n-8)	1.6	4.3	0.9	0.3			
18:0	0.6	4.0	1.8				
18:l(n-7)	15.4	77.9	38.5				
12:0-3OH					3.5		
15:0-*i*3OH					2.7		
16:0-3OH					2.0		
17:0-*i*3OH					4.1		

Note: * average fatty acid concentrations in the studied strains.

associated with the starfish *P. pectinifera*, which was confirmed by the samples taken throughout the year; however, they were occasionally isolated from other animal species. Planococci were occasionally found in animals collected during the coldest and warmest months of the year. It should be noted that gram-positive microorganisms were more often detected in animals collected at low ambient temperatures. For instance, the highest diversity of heterotrophic bacteria was observed in trepangs caught at low temperatures; no gram-positive bacteria were detected in animals caught in July and August. The virtual absence of gram-positive bacteria was also observed in sea urchins and bivalves collected during the warm season; however, these bacteria were often detected at moderate and low temperatures.

Vibrios prevailed among gram-negative microorganisms isolated from the water samples collected during the warm season, and these bacteria were not detected in winter samples (Table 2). Unlike vibrios, halomonads were found in water samples collected at low temperatures; they were not detected in the samples collected during the warm season. Representatives of *Pseudomonas, Pseudoalteromonas*, and *Shewanella*, as well as of the *CFB* cluster, were detected in all water samples collected during the year; no seasonality of their distribution was observed. Two strains of *Sulfitobacter* spp. were isolated from water samples collected during the winter period. Bacilli and planococci were predominant among gram-positive bacteria isolated from water samples. Gram-positive microorganisms were detected in all water samples collected throughout the year; *Marinococcus* spp. and *Halococcus* spp. strains were detected in water samples, but not in animal tissues.

To summarize the results obtained, it should be noted that seasonality of bacterial distribution in hydrobionts and ambient water was demonstrated for representatives of the genera *Vibrio* and *Halomonas* and for gram-positive bacteria in general. The diversity of microorganisms detected in water samples collected during the year was greater than that observed in animals. This finding was supported by the fact that representatives of all taxa of heterotrophic bacteria listed in Table 2 were detected in the water samples, while representatives of some or other taxa were absent from the microflora of each animal species studied. We failed to confirm the specificity of the bacterial community composition typical of a certain species of invertebrates.

DISCUSSION

The maximum number of heterotrophic bacteria isolated from hydrobionts and water samples coincided with maximum water temperatures in the Peter the Great Bay. This can be attributed to a strictly positive correlation between the population density of bacteria and water temperatures in estuaries and coastal oceanic ecosystems [13, 14].

High numbers of heterotrophic bacteria in hydrobionts (as compared to water samples) indicates a high accumulating capacity of invertebrates. It seems likely that some microorganisms remain viable and reproduce in the host's intestine as the associative microflora.

Phenotypic analysis of the obtained isolates performed to determine their taxonomic position revealed certain morphological differences between summer and winter bacterial isolates. Changes in the cell morphology of vibrios caused by low temperatures, as well as the fact that the cells of the halomonad strains isolated during the winter period were surrounded by a capsule-like layer, suggest that these microorganisms are well adapted to the physical conditions of the environment. An increase in the average volume of the cells may be regarded as one of the forms of adaptation of microorganisms to low temperatures [15].

The results of the comparison of the fatty acid profiles of the studied isolates and the previously described specific characteristics of the fatty acid composition of vibrios, pseudomonads, and some other bacteria [8, 10] allowed us to determine the taxonomic position of these isolates.

The predominance of gram-negative bacteria in some species of marine invertebrates may be considered a characteristic feature of the symbiotic relationships between animals and microorganisms. A decrease in the frequency of occurrence of gram-positive bacteria in marine hydrobionts during summer (as compared to the winter period) suggests that gram-positive microflora is replaced by gram-negative bacteria (vibrios) due to their active growth during this period.

The observed seasonality of the halomonad distribution can be attributed to their inability to compete with vibrios whose population reaches its height in the warm season. However, our observations indicate that halomonads get a certain advantage in the winter season. In particular, the presence of the capsule-like layer may promote survival and growth of the halomonad population during the cold season.

The number of *Sulfitobacter* spp., *Chromobacterium* spp., and *Xanthomonas* spp. strains was insufficient for analyzing their seasonal dynamics.

Further study is required to investigate the confinement of *Staphylococcus* spp. to the starfish *P. pectinifera*, which was observed during the whole period of observation and aroused considerable scientific interest. There is no published information on the heterotrophic microflora composition in starfish.

The results of our study allowed us to determine annual changes in the microflora counts and composition in invertebrates and ambient water. The number of heterotrophic microorganisms associated with hydrobionts and water was subject to seasonal fluctuations and reached their peaks in the warm season. We believe that the abundance of bacteria in animal tissues as compared to the water samples can be attributed to the amount of available nutrients and, possibly, attractants in the host organism which provides a favorable ecological niche for microorganisms. It is our opinion that the ability of the trepang *A. japonica* and the studied species of sea urchins to synthesize some specific physiologically active compounds is one of the causes of the abundance of bacterial species associated with these animals as compared to the numbers of bacteria in other invertebrates. The predominance of aerobic gram-negative bacteria in hydrobionts in the winter season is probably due to the unique adaptive capabilities of these microorganisms. The presence of vibrios in host organisms collected during the winter season and their absence in water samples allows us to suggest that the studied hydrobionts represent a natural reservoir for proliferation and distribution of vibrios in the Peter the Great Bay. The highest diversity of heterotrophic bacteria in seawater was revealed due to the presence of minor species of bacteria that were not detected in hydrobionts. It seems likely that only ecologically adaptive microorganisms with certain physiological and biochemical properties are able to survive in the host's intestine, be included in the associative microflora, and maintain their numbers despite seasonal fluctuations in ambient temperature.

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