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EXPERIMENTAL ARTICLES

Distribution and Characteristics of *Bacillus* Bacteria Associated with Hydrobionts and the Waters of the Peter the Great Bay, Sea of Japan

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Abstract—Bacilli of the species *Bacillus subtilis, B. pumilus, B. mycoides, B. marinus* and *B. licheniformis* (a total of 53 strains) were isolated from 15 invertebrate species and the water of the Vostok Bay, Peter the Great Bay, Sea of Japan. Bacilli were most often isolated from bivalves (22.7%) and sea cucumbers (18.9%); they occurred less frequently in sea urchins and starfish (13.2 and 7.5%, respectively). Most of bacilli strains were isolated from invertebrates inhabiting silted sediments. No *Bacillus* spp. strains were isolated from invertebrates inhabiting stony and sandy environments. The species diversity of bacilli isolated from marine objects under study was low. Almost all bacterial isolates were resistant to lincomycin. Unlike *B. pumilus, B. subtilis* isolates were mostly resistant to benzylpenicillin and ampicillin. Antibiotic sensitivity of *B. licheniformis* strains was variable (two strains were resistant to benzylpenicillin and oxacillin, while one was sensitive). A significant fraction of isolated bacilli contained pigments. Pigmented strains were more often isolated from seawater samples, while colorless ones predominated within hydrobionts. *B. subtilis* colonies had the broadest range of colors. In the *Bacillus* strains obtained, DNase, RNase, phosphatase, elastolytic, chitinase, and agarolytic activity was detected. Bacilli strains with hydrolytic activity occurred in invertebrates more often than in seawater.

Key words: bacilli, associants, invertebrates.

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Gram-positive heterotrophic bacteria are indigenous components of marine bacterial communities and are often isolated from such marine environments as water, sediments, and animals [1–3]; their functional role in marine ecosystems is still poorly understood. Bacilli are spore-forming motile rod-shaped bacteria with fermentative, respiratory, or mixed metabolism. Carotenoid pigments of bacillar spores enhance their resistance to ultraviolet radiation [4]. Spore formation is one of the major factors of ubiquity of bacilli; wind transfer ensures occurrence of these bacteria in practically every environment [5]. However, *Bacillus marinus* is the only valid marine species [6]. Bacilli can utilize plant and animal debris; a broad spectrum of substrates is used, including cellulose, starch, agar, proteins, and carbohydrates [7]. These bacteria play an important role in the process of oil pollution removal in the ocean [8]. They are widespread in marine sponges; as associated microflora, they produce fungicides and various antibiotics [9, 10]. Many marine bacilli produce antibiotics and biologically active compounds [11]; they are therefore used as probiotics in aquaculture [12]. In spite of their importance and ubiquity, marine gram-positive spore-forming bacteria, including bacilli, are more poorly studied than gram-negative microflora. Due to their metabolic plasticity and capacity for spore formation, these allochthonous microorganisms exist in marine environments. High occurrence of bacilli in various marine objects determined by the study of Peter the Great Bay microflora and the absence of exhaustive information concerning ecology of these bacteria in the Russian part of the Sea of Japan suggested the present investigation. Its goal was to determine the distribution and characteristics of the isolated strains of bacilli.

MATERIALS AND METHODS

The work was carried out using the strains of bacilli isolated from hydrobionts and water samples of the Peter the Great Bay in May–October 2005 and 2006. The animals studied included bivalves (*Crenomytilus grayanus*, *Mytilus trossulus*, *Modiolus dificillus*, *Crassostrea gigas*, *Spisula sachalinensis*, and *Mizuhopecten yessoensis*) and echinoderms (sea urchins *Strongylocentrotus nudus*, *S. intermedius*, and *Echinarachnius parma,* starfish *Patiria pectibifera, Asterias amurensis, Distolasterias nipon*, and *Aphelasterias japonica,* and sea cucumbers *Apostichopus japonicus* and *Cucumaria japonica*). The animals were collected on sediments of the following types: i) gravel, anisomerous sand;

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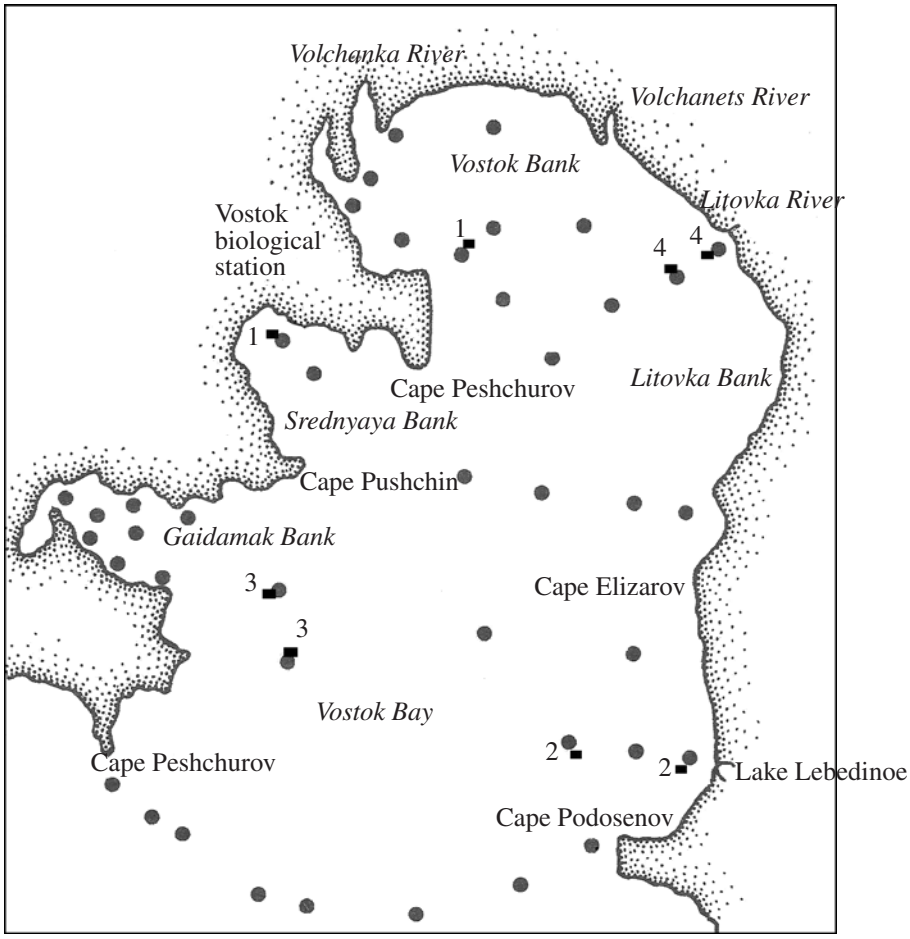

● sampling stations for the gravimetric analysis performed earlier [13]; ■, sites of invertebrates sampling. Types of sediments: gravel, anisomerous sand (1); silted anisomerous sand (2); silted fine and medium sand (3); and silted fine sand (4).

ii) silted anisomerous sand; iii) silted fine and medium sand; and iv) silted fine sand. The sampling sites were as close as possible to the sites where granulometric analysis of sediments was previously carried out (figure) by other researchers [13]. Animal samples were delivered to the laboratory while alive, cleaned of epibioses (if necessary), flamed, and dissected under aseptic conditions. Animal tissues were ground in a glass homogenizer. Serial dilutions of homogenates and water samples (0.1 ml) were plated on solid Youschimizu–Kimura (Y–K) medium and RPA nutrient agar. The plates were incubated at room temperature for three to five days. The strains were maintained in test tubes with semisolid agarized Y–K medium under mineral oil at 8–10°C. The isolates were stored at –75°C in seawater with 20% glycerol. Phenotypic characterization was carried out using the standard microbiological procedures as described previously [14], as well as the API 20E and API 50CH test systems. Other characteristics determined included the following: morphology of vegetative cells; spore shape and location; growth at 5, 30, 50, and 65°C; NaCl requirement (at 0, 2, 5, 10, and 12.5%); growth at pH 5.7–11.5; anaerobic growth and presence of nitrate reductase; hydrolysis of casein, starch, and gelatin; citrate utilization; and production of acid from glucose, arabinose, xylose, and mannitol. DNase, RNase, chitinase, and alkaline phosphatase were determined as described earlier [14]. Elastolytic activity was determined according to [15]. Agarolytic activity was assayed as ability to form perforations of the agar layer when grown on Y–K medium. Antibiotic sensitivity was determined by diffusion in agar using standard 6-mm paper disks containing (per disk) ampicillin (10 μg), benzylpenicillin (10 U), gentamycin (10 μg), lincomycin (15 μg), oxacillin (10 μg), or chloramphenicol (30 μg). After one day of incubation at room temperature, diameter of growth inhibition zones was measured. The isolates were identified according to the manuals [7, 15].

RESULTS

A total of 53 strains belonging to the genus *Bacillus* was isolated from the water and 15 invertebrate species of the Peter the Great Bay (Table 1). The highest number of the strains (62.2%) was isolated from the sam-

Table 1. Occurrence of *Bacillus* spp. in the water and invertebrates of the Peter the Great Bay

Object	Total number of strains	*B. subtilis* (20 strains)	*B. pumilus* (18 strains)	*B. marinus* (3 strains)	*B. mycoides* (9 strains)	*B. licheniformis* (3 strains)
Bivalves:	12	6	5	–	1	–
Crenomytilus grayanus	1	–	1	–	–	–
Mytilus trossulus	–	–	–	–	–	–
Modiolus dificillus	8	4	3	–	1	–
Crassostrea gigas	3	2	1	–	–	–
Spisula sachalinensis	–	–	–	–	–	–
Mizuhopecten yessoensis	–	–	–	–	–	–
Sea urchins:	7	2	2	–	2	1
Strongylocentrotus nudus	3	–	2	–	1	–
S. intermedius,	4	2	–	–	1	1
Echinarachnius parma	–	–	–	–	–	–
Starfish:	4	1	3	–	–	–
Patiria pectinifera	1		1	–	–	–
Asterias amurensis	3	1	2	–	–	–
Distolasterias nipon	–	–	–	–	–	–
Aphelasterias japonica	–	–	–	–	–	–
Sea cucumbers:	10	5	1	–	3	1
Apostichopus japonicus	8	4	1	–	3	–
Cucumaria japonica	2	1	–	–	–	1
Seawater	20	6	7	3	3	1

ples of marine invertebrates: bivalves (22.6%), sea cucumbers (18.9%), sea urchins (13.2%), and starfish (7.5%). *Bacillus* spp. were confined to the hydrobionts developing under specific ecological conditions. All the strains were isolated from the animals collected of silted sediments (mollusks *M. dificillus* and *C. gigas*, sea urchins *S. nudus* and *S. intermedius*, starfish *P. pectibifera* and *Asterias amurensis*, and both holothurian species). *Bacillus* spp. were not revealed in the animals from rocky and sandy environments (*S. sachalinensis, M. yessoensis, E. parma, D. nipon*, and *A. japonica*). Five species of bacilli were isolates from seawater; *B. marinus* was detected only in water and was not revealed in hydrobionts.

All strains had typical cell and colony morphology, as well as physiological and biochemical characteristics and were therefore assigned to the genus *Bacillus* (Table 2). A significant portion of the strains was pigmented. *B. subtilis* isolates had the most diverse color gamma; white, yellow, orange, and brown pigments were present. Some *B. pumilus* strains developed yellowish coloration in aging cultures, usually after three to five days of incubation at room temperature. One *B. licheniformis* strain was brown-pigmented; no pigment production was detected in *B. mycoides* and *B. marinus.*

All the isolates had protease; gelatin and casein were hydrolyzed efficiently (Table 2). In two species, *B. pumilus* and *B. marinus*, amylases were not detected. Analysis of the distribution of hydrolytic enzymes in the *Bacillus* spp. isolates under study revealed DNase, RNase, phosphatase, elastolytic, chitinase, and agarolytic activity (Table 3). DNases and alkaline phosphatase were the most widespread enzymes, occurring in 24.5 and 13.2% of the strains, respectively. The strains active against various substrates occurred more often in invertebrates than in water. In water samples, strains with RNase, phosphatase, chitinase, and agarolytic activity were not detected.

DISCUSSION

Endospore-forming rods are known to be widespread in marine bottom sediments [1, 2, 15]. Information concerning the distribution of bacilli in various types of sediments is not available in the literature. We

Table 2. Phenotypical characteristics of marine *Bacillus* isolates

Characteristics		*B. subtilis*	*B. pumilus*	*B. marinus*	*B. mycoides*	*B. licheniformis*
Oxidase		V (8)*	V (5)	–	–	V(1)
Catalase		+	+	+	+	+
Oxidation/fermentation on Hugh and Leifson's medium		–/–, –/+trace (2)	–/–	–/–	+/+	+/+
Voges–Proskauer test		+	+	–	+	+
Growth at pH	5.7–7.5	+	+	+	+	+
	9.5–11.5	+	+	V (1)	V (7)	–
Growth at temperature	5°C	–	–	+	–	–
	30°C	+	+	–	+	+
	50°C	+	V (11)	–	V (7)	+
	65°C	–	–	–	–	–
Growth with NaCl	0%	+	+	–	+	+
	2%	+	+	+	+	+
	5%	+	+	+	+	+
	10%	+	+	–	–	+
	12.5%	+	+	–	–	V (1)
Hydrolysis of:	starch	+	–	–	+	+
	gelatin	+	+	+	+	+
	casein	+	+	+	+	+
Nitrate reduction		+	–	–	+	+
Indole production		–	–	–	–	–
Acid from:	glucose	+	+	+	+	+
	arabinose	+	+	–	–	+
	xylose	+	+	+	–	+
	mannitol	+	+	–	–	+
Utilization of:	*D*-glucose	+	+	+	+	+
	D-xylose	+	+	–	+	+
	D-arabinose	–	+	–	+	–
	D-mannitol	+	+	–	+	+
	lactose	–	V (4)	–	–	V (2)
	citrate	V (5)	+	–	–	+
Sensitivity to antibiotics:						
Ampicillin		20–30**	34–46	24–30	0–18 (8)	16–20
Benzylpenicillin		0–32 (17)***	28–45	25–36	28–35	0–16 (2)
Gentamycin		18–26	15–17	22–25	18–20	15–18
Lincomycin		Res	Res	Res	0–15 (8)	Res
Oxacillin		0–28 (14)	30–44	16–18	16–18	0–16 (2)
Chloramphenicol		21–36	18–22	20–22	18–20	Res
G + C base content, mol %		41.4–42.9	43.6–46.2	37.1–38.9	33.9–35.2	44.3–47.5

Notes: "+", indicate the presence of a feature; "–", its absence.

* V(x) stands for a variable feature; in parentheses, the number of strains exhibiting this feature is indicated.

** The range of growth inhibition zone diameters (mm) for different microorganisms.

*** (x) stands for the number of strains resistant to this antibiotic.

Table 3. Occurrence of hydrolytic enzymes among *Bacillus* isolates

Species	Strains isolated	Number of producers					
		DNase	RNase	Alkaline phosphatase	Elastase	Chitinase	Agarase
B. subtilis	14*/6**	4/2	3/0	3/0	1/0	1/0	1/0
B. pumilus	11/7	2/1	0	3/0	4/1	0	0
B. marinus	3	2	0	0	0	0	0
B. mycoides	6/3	1/0	0	1/0	0	0	0
B. licheniformis	2/1	0/1	0	0	0	0	0

Notes: * indicates the number of strains isolated from hydrobionts.
** indicates the number of strains isolated from seawater.

revealed a correlation between occurrence of bacilli in marine invertebrates and the type of sediments in their environment. Silted anisomerous sand and silted fine sand are preferable to these bacteria over sandy or rocky soil, including gravel. The content of organic matter in sediments probably plays an important role in this preference. In the Vostok Bay in the summer, C_{org} content of silted and sandy sediments was 19.4 and 6.0 mg/cm^3, respectively [16]; thus, silted sediments are significantly richer in C_{org} than sandy ones. This pattern was confirmed by recent studies of C_{org} content in the sediments of the Vostok Bay (T.S. Tarasova, unpublished results). Fine bottom sediments enriched with organic matter are probably an environment favorable for growth of bacilli. Along with silt particles, bacteria get into the organism of bottom invertebrates; it proves a favorable ecological niche for bacilli due to the wide range of their metabolic capacities.

Although over 200 species of bacilli are presently known, their species diversity in the marine objects under study was low. This pattern of distribution has been reported by other researchers [2, 6, 17]. Only halotolerant and halophilic *Bacillus* species are isolated from marine environments; widespread species sensitive to high NaCl concentrations including, among others, *B. alvei, B. macerans, B. circulans,* and *B. polymyxa*, are not found in marine objects [15].

B. marinus, presently reclassified as *Marinibacillus marinus*, [18] was not isolated from hydrobionts samples under study. This species has previously been isolated from seawater and marine sediments [19, 20]; this pattern probably reflects its ecology.

Pigmented strains of bacilli were more often isolated from water samples than from hydrobionts. Pigments are known to protect bacteria from ultraviolet radiation [4]. The protective function of bacterial pigments is important in insolated water layers but probably useless inside invertebrate organisms. Moreover, the ecological conditions of the water column and invertebrate interior differ significantly in nutrient availability. Inhibition of pigment formation in rich nutrient medium was reported for *Pseudoalteromonas tunicata* [21]. An organic-enriched interior of marine invertebrates probably suppresses expression of pigment formation in bacilli, resulting in colorless bacterial isolates.

Antibiotic sensitivity can serve as additional taxonomic characteristics for classification of environmental isolates [22]. The resistance of our isolates to lincomycin correlates with the data obtained by other authors [23]. *B. subtilis* strains isolated from marine objects were usually resistant to benzylpenicillin and oxacillin. The differences in reaction to the penicillin type antibiotics between *B. subtilis* and *B. pumilus* can be used for their identification [15, 23]. Except for one strain, *B. mycoides* isolates were resistant to ampicillin; this is probably characteristic of this species [15]. *B. licheniformis* strains varied in their antibiotic sensitivity; two were resistant to benzylpenicillin and oxacillin, while one strain was sensitive to them. Since we had few isolates of *B. marinus* and *B. licheniformis* (three of each species), the data concerning their relation to antibiotics are preliminary. In general, the strains under investigation were highly resistant to a wide range of antibiotics. Antibiotic sensitivity of bacilli is known to reflect their adaptation to environmental conditions [22]. Bacteria capable of neutralizing the effect of antibiotics produced by other microorganisms gain certain selective advantages. Their populations are highly competitive in the struggle for survival and distribution.

The biological function of hydrolytic enzymes is to provide energy, carbon, nitrogen, and phosphorus for microbial growth. Specific organic materials (chitin, agar, carrageenan, phucoidan, etc.) are utilized by marine microorganisms due to the presence of these enzymes [24]. Occurrence of hydrolytic enzymes primarily in bacilli associated with marine invertebrates

(Table 3) is confirmed by literature data. For example, producers of physiologically active compounds were found among associated microflora more often than among free-living microorganisms [24–26]. The strains of bacilli isolated from the *Modiolus dificillus* stomach had the broadest spectrum of hydrolytic enzymes. They exhibited DNase, RNase, chitinase, elastolytic, and phosphatase activity. Since high-molecular polymers are decomposed to simple fragments, this activity of associated microflora may be beneficial both for bacteria and for their hydrobiont host, possibly facilitating nutrition of the macroorganism.

Our data demonstrate that *Bacillus* species (*B. subtilis, B. mycoides*, and *B. licheniformis*) are widespread in the water and invertebrates of the Peter the Great Bay, while *B. marinus* occurs only in the water. The distribution of bacilli in marine invertebrates correlates with the type of sediments in the animal's environments. Bacterial isolates were resistant to a number of antibiotics and had a broad range of hydrolytic enzymes. Most of the producers were revealed among associated bacteria. Marine invertebrates are possibly a favorable environment for these bacteria. Highly specific relations, which have developed between bacteria and macroorganisms in the course of evolution, provide optimal conditions for both sides.

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