ISSN 0026-2617, Microbiology, 2017, Vol. 86, No. 3, pp. 317–329. © Pleiades Publishing, Ltd., 2017.
Original Russian Text © I.A. Beleneva, A.V. Skriptsova, V.I. Svetashev, 2017, published in Mikrobiologiya, 2017, Vol. 86, No. 3, pp. 292–306.

EXPERIMENTAL ARTICLES

Characterization of Biofilm-Forming Marine Bacteria and Their Effect on Attachment and Germination of Algal Spores

I. A. Beleneva*, A. V. Skriptsova, and V. I. Svetashev

National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia

**e-mail: beleneva.vl@mail.ru*

Received July 19, 2016

Abstract—In this work, 37 bacterial strains isolated from biofouling of marine organisms and from the Museum of Heterotrophic Bacteria of the National Scientific Center of Marine Biology were studied. The strains were identified based on their phenotypic characteristics and on the fatty acid composition of their cell wall lipids. Members of the genus *Pseudoalteromonas* prevailed both in associated microflora of two dinoflagellate clones and in the biofilms from marine hydrobionts. Associated microflora included also members of the CFB cluster, *Bacillus*, *Sulfitobacter*, *Acinetobacter*, *Shewanella*, and *Psychrobacter*. A considerable portion of strains (48.6%) exhibited antimicrobial activity. Antifouling activity against algal spores was studied using single-species bacterial biofilms and the spores of *Ulva lactuca* и *Undaria pinnatifida*, the algae most common in the Sea of Japan. Strong inhibitory effect on attachment of *Ulva* and *Undaria* spores was observed for 75 and 51% of the strains, respectively. Attached spores were, however, less sensitive to the inhibitory action of biofilms. Species specificity of algal response to bacteria was shown, with a strain having different effect on the spores of different algal species. Biotechnologically promising strains were determined, which exhibited high activity against the spores of macroalgae and could probably be used as producers of antifouling substances and as components of antifouling coatings. No relation was found between antifouling activity of bacteria and the source of their isolation. Our results indicate wide occurrence of bacteria with antifouling activity among associated microflora of marine hydrobionts and demonstrate the extent of complexity and diversity of relations between bacterial biofilms and algal spores.

Keywords: bacterial biofilms, antifouling activity, algal spores

DOI: 10.1134/S0026261717030031

Microorganisms are the first to colonize marine environment, both abiotic surfaces and coatings of various hydrobionts, initiating the biofilm formation. By means of a complicated signal system biofilms regulate behavior of algal spores and the invertebrate larvae by stimulating or preventing their attachment and, therefore, affect the final composition of the fouling communities (Qian et al., 2007; Rao et al., 2007; Mieszkin, 2013). Biofilms are responsible for corrosion of metal surfaces which results in tremendous damage to property. The overall economic losses from corrosion reach from 1 to 5% of the gross national product (GNP) in such countries as the Great Britain, Japan, Australia, and Kuwait (*Techniques*..., 2008). Massive fouling of the sea craft bottoms results in the fuel overexpenditure and economic losses (Yebra et al., 2006). Therefore, biofilm studies and development of novel methods for the fouling prevention are priority problems of sciences and technology. Prohibition for the use of toxic biocides in antifouling preparations stimulates the search for environmentally friendly compounds. Marine microorganisms produce unique substances which are unknown in terrestrial organisms (Fenical and Jensen, 1993); therefore, they are of great interest as promising producers of novel antifouling compounds (Qian et al., 2007; Dobretsov et al., 2013). Bacteria growing in biofilms on the surfaces of marine hydrobionts produce inhibitors preventing development of other organisms on the host surface (Dobretsov et al., 2013). A number of marine bacteria are able to produce substances inhibiting growth of microorganisms, algal spores, and the invertebrate larvae as well as of higher animals (Maki et al., 1988; Egan et al., 2000, 2001).

Among marine bacteria, members of the genus *Pseudoalteromonas* are characterized by the highest antifouling activity (AFA) (Bernbom et al., 2011); a correlation between the AFA level and the colony pigmentation, both positive (Egan et al., 2002) and negative (Huang et al., 2011), has been often revealed. The capability to inhibit biofouling was also found in members of various bacterial taxa (Maki et al., 1988; Fenical et al., 1993; Rao et al., 2007; Ma et al., 2009; Dobretsov et al., 2006, 2013). Antifouling substances belong to various classes, including proteins and

nonprotein compounds (steroids, fatty acids, etc.) (Dobretsov et al., 2013).

In connection with the aforesaid, the development of methods for fouling prevention can also include screening and application of highly active strains, producers of secondary metabolites with antifouling properties.

The goal of the present work was to study antifouling activity of bacteria associated with marine hydrobionts from the Peter the Great Bay (Sea of Japan) and to determine whether there is a relation between antifouling activity, taxonomic position of bacteria, and the source of their isolation.

MATERIALS AND METHODS

Bacterial strains. The study was carried out with isolates from biofilms formed by various marine organisms in the Peter the Great Bay (Sea of Japan) and with strains of marine heterotrophic bacteria from the Culture Collection of the National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia. Strains 1242 and 1444 were used since they were known to possess high antimicrobial activity (Beleneva et al., 2013); strain 1839 was applied as producer of tetrodotoxin (Beleneva et al., 2014). Bacteria were maintained at –85°C in cryotubes containing seawater supplemented with glycerol (30%), peptone (Difco, United States) (1%), and $MgSO_4$ (3–5 g/L).

Bacteria were isolated from biofilms formed on bivalves *Crenomytilus grayanus, Mizuhopecten yessoensis,* and *Saxidomus purpurata,* and from the surfaces of brown alga *Desmarestia viridis,* green alga *Ulva lactuca,* red alga *Mazzaella japonica,* seagrass *Zostera marina,* and starfish *Patiria amurensis.* All tools, glassware, and seawater used for the culture isolation were sterilized by autoclaving. The animal surfaces, algal thalli, and the seagrass leaves were thrice washed with seawater. Biofilms were removed using spatula and a cotton stick, suspended in 5 mL of seawater, and then serial dilutions of suspension were plated onto marine agar to isolate pure cultures of bacteria. Hydrobionts collected by divers were placed into plastic parcels with seawater and transferred to the laboratory within 1 h.

Phenotypic characterization of the isolates and fatty acid analysis of bacterial lipids were carried out according to the earlier described methods (Beleneva et al., 2005).

Antimicrobial activity of the isolates was determined using as test cultures the following bacteria (gram-positive and gram-negative) and yeasts: *Escherichia coli* ATCC 15034, *Bacillus subtilis* VKM B501, *Candida albicans* VKM 455, *Pseudomonas aeruginosa* KMM 433, and *Staphylococcus aureus* ATCC 21027, as well as marine bacteria *Vibrio* sp. 1805 and *Pseudoalteromonas* spp. 2099 and 1813 from the Culture Collection of the National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences. Strains of *E. coli*, *B. subtilis*, *C. albicans*, *P. aeruginosa,* and *S. aureus* were grown on tryptose soy agar (TSA) (Difco, United States); all marine isolates were cultivated on marine agar (MA 2216) (Difco, United States).

Antimicrobial activity was determined by the slightly modified method of Penesyan et al. (2009). The cultures of marine isolates (24-h) were inoculated as plaques onto petri dishes with marine agar and incubated for 96 h; then agar surface was covered with 25 mL of warm 0.7% agar supplemented with 0.5 mL of the overnight suspension of the test strain. The upper agar layer contained the following media: (1) for *S. aureus,* Luria–Bertani (LB), g/L: NaCl, 10; trypton, 10; yeast extract, 5; (2) for *C. albicans,* yeast–peptone agar (g/L): peptone, 20; yeast extract, 10; glucose, 20, and (3) for *E. coli*, *B. subtilis*, and *P. aeruginosa,* tryptose soy broth (TSB) (Difco, United States). To prepare cell suspensions, the overnight cultures grown in the relevant media were diluted in saline to the concentration of 10^9 cells/mL using the McFarland standard. Petri dishes with double layers of inoculated agar were incubated at 25°C for 48 h, and zones of the test culture growth suppression around the colonies of marine isolates were examined. The zones exceeding 4 mm in diameter were considered as positive results (Long and Azam, 2001).

Preparation of biofilms. The isolates were maintained on solid VNSS medium (Szewzyk et al., 1991). Directly before the biofilm preparation, bacterial strains were grown in the liquid VNSS medium at 25°C for 1 day; cells were harvested by centrifugation (11000 rpm, 15 min), washed twice, and resuspended in the NNS solution to optical density of 1.0 at 610 nm. In the case of *Ulva lactuca,* 1 mL of the cell suspension was added per well of a 24-well plate; for *Undaria pinnatifida,* 3 mL of the cell suspension was placed onto 36-mm petri dishes. Biofilms formed for 1 day were washed thrice with autoclaved seawater and used in the subsequent studies on the effect of biofilms on algal spores.

Preparation of algal spores. Sporophytes of *Ulva lactuca* (*Ulvales*, *Chlorophyta*) with sporiferous border and *Undaria pinnatifida* (*Laminariales*, *Phaeophyceae*) with sporophylls containing sori of matured sporangia were collected in the morning from 8:30 to 9:00 at the subtidal zone near the Zelyonyi Cape (the Ussuriysk Bay of the Peter the Great Bay, Sea of Japan) and transferred to laboratory in a container with seawater within 30 min. Algae were twice washed with sterile seawater (UV treatment, filtration through 0.1-μm filter, and boiling).

To obtain algal spores, the *Ulva* thalli were placed into vessels with sterile seawater and put against a light source (desk lamp); after 30 min, the released spores possessing positive phototaxis were concentrated near the vessel walls directed to the light source. Spores

were collected with a pipette, placed into petri dishes and concentrated again near the light source. The suspension (0.2 mL) containing 214 ± 38 × 10^3 spores/mL was added into a well of a 24-well plate containing a bacterial biofilm and 1 mL sterile seawater.

To obtain the *Undaria* spores, fragments of the sporophylls (2 × 2 cm) were washed with sterile seawater, placed into weak solution of iodine (in ethanol) in seawater for 30 s, washed again with sterile seawater, dried with filter paper, and air-dried at 16°C for 2 h. Then a drop of sterile seawater was applied to the sporophyll surface; after 20 min, spores were collected, placed into petri dishes with water, and concentrated like similar to the case of the *Ulva* spores. Then 0.1 mL of obtained suspension containing 211 ± 46 × 10^3 spores/mL was placed onto petri dish with bacterial film and 3 mL of sterile seawater.

The plates with spores were incubated in the dark for 4 h for the spore attachment; then the medium was removed, and 1 mL of sterile seawater per well was added. The number of spores attached in a field of vision was enumerated. The germination of the *Undaria* and *Ulva* spores was determined through three and five days, respectively. The number of attached and germinated spores was determined using images made under inverted microscope at a 40× magnification; for each well, 10 images of different fields of vision were made.

RESULTS

Characteristics of the isolates. The study was carried out with 37 strains of heterotrophic bacteria isolated from different marine objects (Tables 1 and 2).

Strains of gram-negative bacteria were predominant (81.1%); among them, the most abundant were *Pseudoalteromonas* spp. (60%); they were rod-shaped motile bacteria with oxidative metabolism; some of them produced yellow or dark brown pigments. Bacteria were oxidase-positive, liquefied gelatin, required sodium ions for growth, and produced no arginine dehydrogenase. Strains were different in their colony morphology, halotolerance, the presence of nitrate reductase and agarase, and in the quantitative composition of fatty acids.

Members of the CFB cluster amounted to 13.3% of gram-negative isolates; they were represented by long thin rods; long flexible filaments also often occurred. As a rule, bacteria produced a yellow or orange pigment; they were characterized by typical gliding motion; some strains were facultative anaerobes. Their fatty acid profile was characterized by considerable amounts of saturated and unsaturated branched acids and minor quantities of hydroxy acids.

Two strains of sulfite bacteria were similar in their phenotype and composition of prevailing fatty acids; they were small thin rods, nonpigmented, oxidase-positive, requiring sodium ions for growth, and producing no nitrate reductase.

Bacteria of the genus *Psychrobacter* were nonmotile coccobacilli, they required sodium ions and grew at NaCl concentration in the medium of up to 8%.

The *Shewanella* strain produced a yellowish pigment; its cells were motile short rods with rounded ends, oxidase- and catalase-positive, possessed no nitrate reductase, showed oxidative metabolism, and did not grow on sodium-free media.

All gram-positive bacteria belonged to the genus *Bacillus;* their identification to the genus level was not complicated due to their typical morphological characteristics; they were gram-positive spore-forming rods with oxidative metabolism. In the fatty acid profile of one tested typical strain, saturated branched fatty acids prevailed.

The rod-shaped cells of strain *Acinetobacter* sp. became coccobacilli in the course of growth; spores were not formed; colonies were nonpigmented. The cells did not oxidize glucose when grown in the Hugh–Leifson medium and showed low activity in the Giss medium. Bacteria produced no oxidase or nitrate reductase, and were resistant to penicillin.

Strains 1242, 1444, and 1839 have been earlier identified as *Pseudomonas aeruginosa*, *Ruegeria* sp., and *Bacillus* sp., respectively (Beleneva et al., 2013, 2014).

Antimicrobial activity of bacterial strains. The activity against at least one test culture was found in 18 out of 37 strains studied (48.6%) (Table 1). The largest number of active strains was isolated from surfaces of marine algae and seagrass (38.8%), as well as from biofilms of the animal fouling (the mollusk shells and the starfish surfaces) (33.3%).

Activity against algal spores. Express analysis of the inhibitory activity of the studied bacterial strains against algal spore attachment and germination was carried out with the use of three test systems: (1) attachment of the *Ulva* spores; (2) attachment of the *Undaria* spores; (3) germination of the *Undaria* spores. These methods allowed the screening of all the isolates to be carried out in several hours.

It was found that over 75% of bacterial isolates considerably inhibited attachment of the *Ulva* spores, seven strains showed a slight inhibitory effect, whereas in the presence of two strains of the genus *Pseudoalteromonas,* the number of the *Ulva* spores attached to the biofilm exceeded that in the control.

The effect of bacteria on the *Undaria* spores was less pronounced: only half of the studied isolates (18 strains) prevented considerably attachment of algal spores, and 31% strains showed an insignificant inhibitory effect.

Twenty-four strains significantly hampered development of the *Undaria* spores. In biofilms formed by strains 2060, 2053, 2074, 1690, 2059, 2064, 2084,

Table 1. Strains used in the study: source of isolation, taxonomic position, and antimicrobial activity

Strain no.	Source of isolation	Taxon	Antimicrobial activity
768	*Crenomytilus grayanus*	*Bacillus* sp.	ND
1242	Brass fouling, Nha Trang, Vietnam	*Pseudomonas aeruginosa*	**B. subtilis* *S. aureus*
1444	Seawater, Nha Trang, Vietnam	*Ruegeria* sp.	*E. coli* *Pseudoalteromonas* sp. 1813
1689	Fouling of the *Mizuhopecten yessoensis* shells	*Pseudoalteromonas* sp.	*C. albicans*
1690		*Pseudoalteromonas* sp.	*B. subtilis*
1693	Fouling of the *Saxidomus purpurata* shells	*Bacillus* sp.	ND
1694		*Pseudoalteromonas* sp.	*C. albicans* *B. subtilis* *Pseudoalteromonas* sp. 1813
1714	Shells of *Crenomytilus grayanus*	*Pseudoalteromonas* sp.	*C. albicans*
1725		*Pseudoalteromonas* sp.	*Pseudoalteromonas* sp. 1813
1839	Nemertea *Cephalotrix simula*	*Bacillus* sp.	ND
2052	Dinoflagellates Clone 22-087	*Acinetobater* sp.	ND
2053		*Psychrobacter* sp.	ND
2054		*Shewanella* sp.	ND
2056	Dinoflagellates Clone 34-087	*Pseudoalteromonas* sp.	*Vibrio* sp. 1805
2057		CFB	*Vibrio* sp. 1805
2059		*Pseudoalteromonas* sp.	ND
2060	Clone 22-087	*Sulfitobacter* sp.	ND
2061		*Sulfitobacter* sp.	ND
2062	Clone 34-087	CFB	ND
2063	*Zostera marina*	*Pseudoalteromonas* sp.	*C. albicans* *S. aureus* *P. aeruginosa*
2064		*Pseudoalteromonas* sp.	ND
2065		*Pseudoalteromonas* sp.	*P. aeruginosa*
2066	*Patiria pectinifera*	*Pseudoalteromonas* sp.	*S. aureus* *Pseudoalteromonas* sp. 1813
2067	Brown alga *Desmarestia viridis*	*Pseudoalteromonas* sp.	*Pseudoalteromonas* sp. 1813
2069		*Pseudoalteromonas* sp.	*B. subtilis* *Vibrio* sp. 1805 *P. aeruginosa*
2070	Red alga *Mazzaella japonica*	*Pseudoalteromonas* sp.	*Vibrio* sp. 1805 *P. aeruginosa*
2071		*Pseudoalteromonas* sp.	*B. subtilis*
2073		*Pseudoalteromonas* sp.	ND
2074		*Pseudoalteromonas* sp.	*C. albicans* *B. subtilis*
2075	*Ulva lactuca*	*Pseudoalteromonas* sp.	ND
2076	Clone 34-087	*Bacillus* sp.	ND

Table 1. (Contd.)

Strain no.	Source of isolation	Taxon	Antimicrobial activity
2077	*Desmarestia viridis*	CFB	ND
2079	*Ulva lactuca*	CFB	ND
2080		*Bacillus* sp.	ND
2082	Clone 34-087	*Bacillus* sp.	ND
2084		*Bacillus* sp.	ND
2099	Nemertea *Malacobdella grossa*	*Pseudoalteromonas* sp.	*E. coli*

* The culture against which antimicrobial activity was revealed; "ND" stands for "not detected."

2075, and 1693, about 30–45% of attached spores formed the germ tubes within 5 h as compared with 45% in control.

No relation was found between the taxonomic position of bacteria, a source of their isolation, and antialgal activity.

Additional studies were carried out with nine strains, which in screening experiments inhibited attachment of both *Ulva lactuca* and *Undaria pinnatifida* spores, germination of *Undaria* spores, and showed antimicrobial activity (Figs. 1–4).

Five out of nine studied strains considerably inhibited attachment of the *Ulva* spores (by more than 50% as compared with the control). On the contrary, strains 2070 and 1839 increased attachment of algal spores 1.5–2 times as compared with the control (Fig. 1a). In the control, the first division of attached zoospores was observed in 5 days; less than 20% of the sporulings were bicellular with dark cell contents; the chloroplast occupied virtually all the cell area (Fig. 3). The sporulings on the biofilms formed by strains 1714, 1725, 2069, and 2070 were considerably larger than those in the control (Figs. 2a and 3); within five days after attachment, sporulings in these variants were 2- to 3-cellular; many of them formed primary rhizoids. In the variant with strain 2057, sporulings

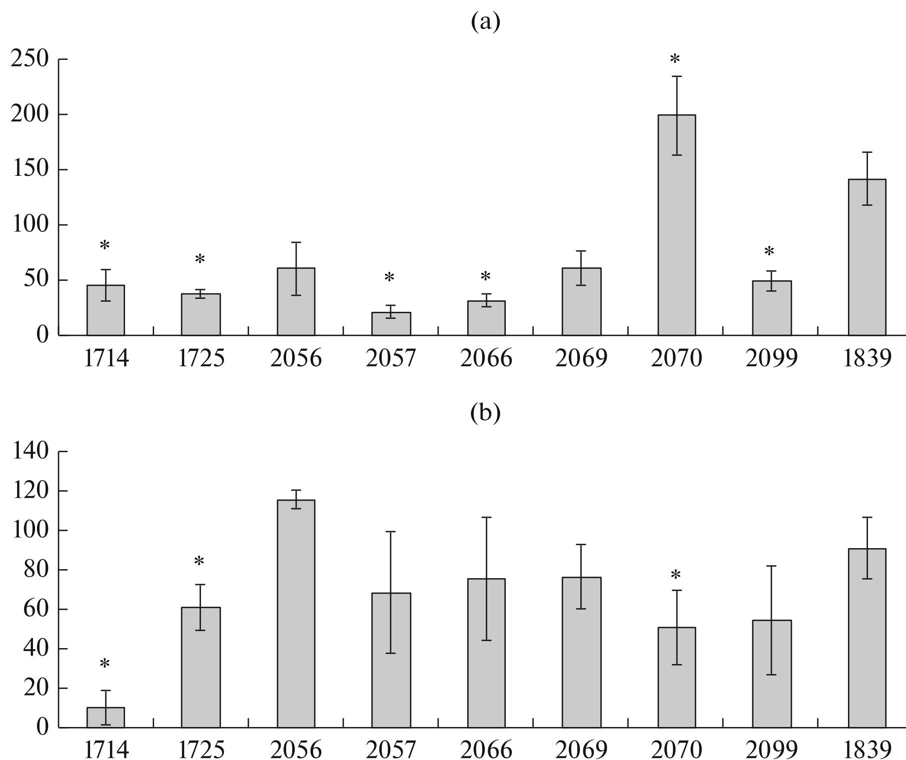

Fig. 1. Attachment of the spores of *Ulva lactuca* (a) and *Undaria pinnatifida* (b) to biofilms. The abscissa shows the strain no.; the ordinate shows the number of attached spores (% of the control); asterisks mark the strains for which the number of attached spores was significantly different from that in the control (the Mann–Whitney criterion was statistically significant at $p < 0.01$).

Table 2. The fatty acid composition of bacterial isolates

Fatty acids	*Pseudoalteromonas*										
	1689	1690	1694	1725	1714	2056	2063	2064	2065	2066	2067
14:0				2.59	2.96	2.15	1.36	3.91	1.34	1.11	2.33
i-15:0		1.04									
15:1			2.27		1.11	3.11	5.48	3.57	4.69	3.61	7.26
15:0	3.32	2.78	5.09	3.28	4.36	5.45	9.80	13.29	6.32	6.10	11.09
i-16:0	1.2				1.16		2.69		1.6		1.44
16:1 (n-7)	23.22	21.61	25.35	32.57	38.57	32.88	33.13	32.16	39.85	41.23	43.42
16:0	15.29	13.56	9.59	27.04	17.33	14.93	19.31	12.57	21.22	19.99	14.26
17:1 (n-8)	25.01	26.71	26.7	6.53	8.79	18.25	6.98	17.94	10.97	14.79	13.23
17:0	9.13	8.68	7.69	3.58	3.66	12.36	4.73	4.65	5.5	6.0	2.72
18:1(n-7)	16.54	16.44	7.57	20.36	15.86	6.44	2.17	9.17	5.04	5.43	1.83
18:0	1.85	2.83					1.60				
19:1	2.28	3.2	1.41								

Fatty acids	*Pseudoalteromonas*						*Bacillus*	CFB			
	2069	2070	2071	2073	2074	2075	2082	2057	2062	2077	2079
13:0				1.63							
14:0	2.67	3.76	1.05	3.61	2.30	4.81	1.15	3.11	2.89	1.79	
i-14:0							5.81				
i-15:1			5.72					20.94	30.75	17.5	21.92
a-15:1			1.09							1.67	2.46
i-15:0							19.55	26.97	24.49	16.66	16.02
a-15:0							65.87	1.9		20.07	20.07
15:1	2.57	5.60		3.45						5.12	6.74
15:0	6.64	10.14	6.08	12.67	3.93	2.32		11.57	6.17	7.16	17.69
i-16:0				1.22		1.28	1.74				
16:1		3.23							21.28		
16:1 (n-7)	33.9	34.82	36.5	29.46	44.66	47.18		18.86	3.93	18.24	5.46
16:0	12.9	14.07	12.08	12.34	35.0	38.64	2.31	5.56		2.64	0.93
i-17:1								2.26	3.15		
a-17:0	1.02						1.58				
17:1 (n-8)	16.8	17.5	15.98	19.18	3.34						
17:0 cyc										3.74	2.41
17:0	4.87	5.65	3.46	5.57	2.46						
18:1 (n-7)	14.7	1.96	13.23	7.74	6.68	4.21					
18:0			1.59		1.63						
19:1			1.2								
a-15:0-2OH										4.69	
a-15:0-3OH										2.09	1.68
16:0-3OH									5.48		

Table 2. (Contd.)

Fatty acids	*Acinetobacter*	*Psychrobacter*	*Shewanella*	*Sulfitobacter*	
	2052	2053	2054	2060	2061
16:1		5.26	3.33		
16:1 (n-7)	29.65		24.5		9.43
16:0	29.90		24.6	3.83	
i-17:0			13.3		
a-17:0	4.83				
17:1 (n-8)	6.51	37.07	2.61		
17:0	4.52		1.58		
18:2				7.74	
18:1 (n-7)	5.96		25.09	78.13	75.46
18:1 (n-9)	11.86	52.37			
18:0	4.73		4.31	1.09	
Methyl18:1				5.56	12.11
19:1	0.30	2.75			

were of smaller size and bicellular; on the films formed by strain 2066, the *Ulva* sporulings were destroyed on the fifth day of the experiment.

Only three out of nine strains (1714, 1725, and 2070) showed negative effects on attachment of the *Undaria pinnatifida* spores (Fig. 1b). Most strains (five out of nine) considerably inhibited development of the *Undaria* gametophytes (Fig. 2b). Strains 1714 and 2056 almost completely suppressed spore germination, whereas on the films formed by the other strains,

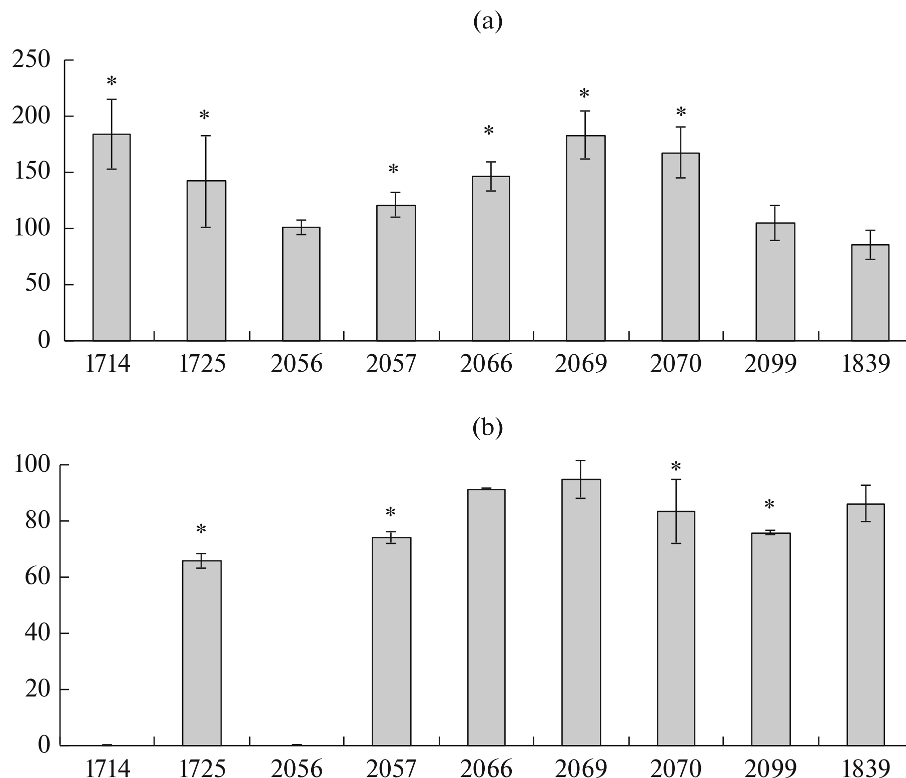

Fig. 2. Relative sizes (% of the control) of the germs of *Ulva lactuca* (a) and *Undaria pinnatifida* (b) grown on biofilms. The abscissa shows the strain no.; asterisks indicate the germ sizes significantly different from those in control (the Mann–Whitney criterion was statistically significant at $p < 0.01$).

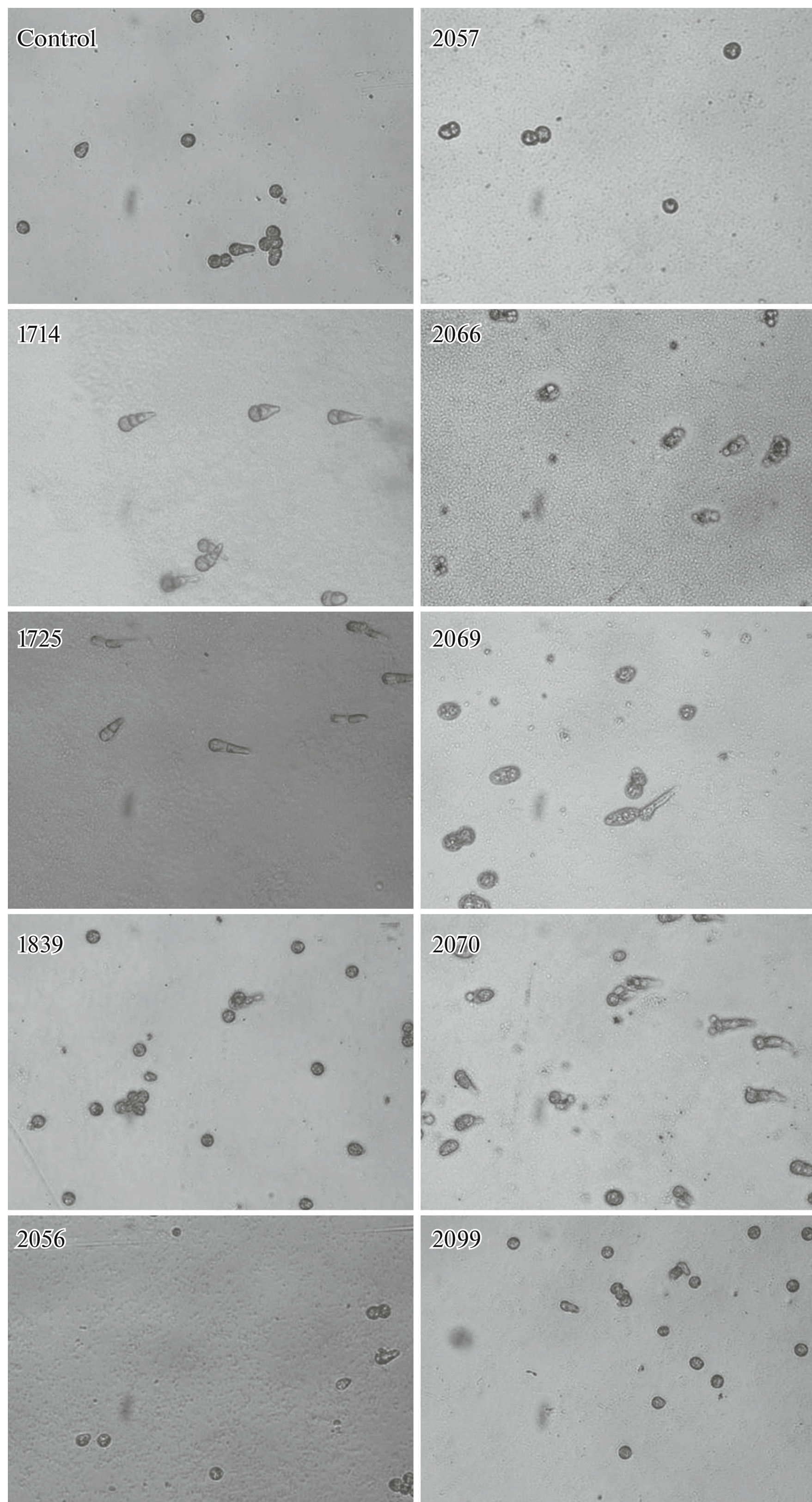

Fig. 3. The *Ulva lactuca* germs on biofilms, four days after the spore attachment (magnification, 40×).

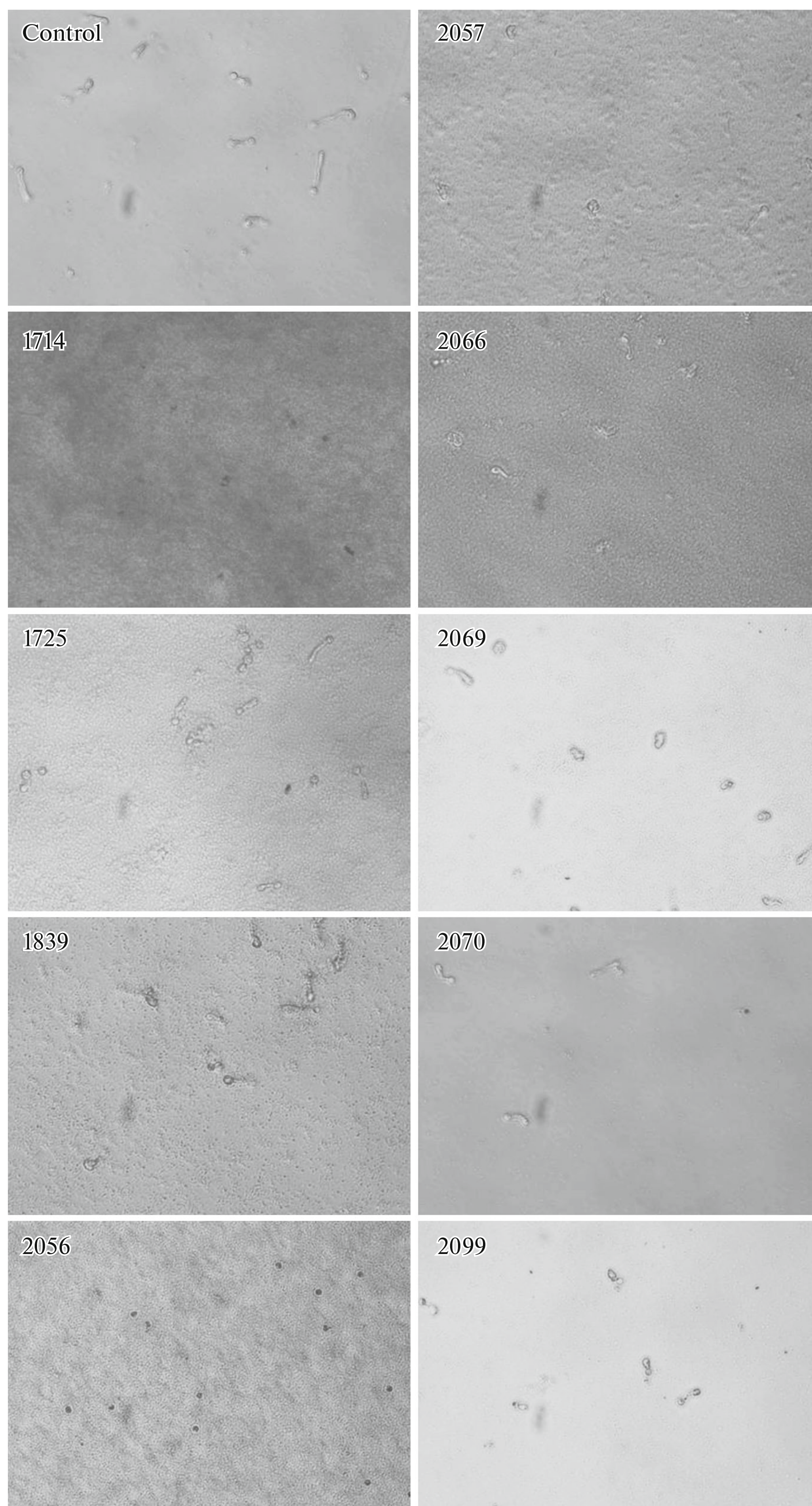

Fig. 4. The *Undaria pinnatifida* germs on biofilms, three days after the spore attachment (magnification, 40×).

virtually all spores germinated (Fig. 4). However, on the films formed by strains 2057 and 2066, the germ cells of *Undaria* were swollen and partly destroyed on the third day. The *Undaria* gametophytes were smaller than those in the control by 20–25% in the films formed by strains 2057, 2070, and 2099 and by 35% on the films formed by strain 1725 (Figs. 2b and 4).

DISCUSSION

Identification of bacterial isolates. Fatty acids of the cell wall lipids from *Pseudoalteromonas* spp. are characterized by low content of branched and hydroxy acids and predomination of 15:0, 16:0, 16:1 (n-7), 17:1 (n-8), and 18:1 (n-7) acids (Ivanova et al., 2000). Different proportions of the mentioned acids in the studied strains indicate that they belong to different species within the genus *Pseudoalteromonas*.

Four strains of the *Cytophaga-Flavobacterium-Bacteroides* cluster had specific cell morphology forming very thin, long rods. Strains of these bacteria were identified on the basis of high content of branched *iso*- and *anteiso*-acids as well as hydroxy acids. Hydroxy acids are known to be a distinct taxonomic criterion of this bacterial group (Fautz, 1979).

Nonfermenting and nonmotile gram-negative coccobacilli with low fermentative activity, catalase-positive, and unable to produce oxidase and nitrate reductase were identified as *Acinetobacter* sp., mainly on the basis of their phenotypic characteristics. In their fatty acid profile, palmitoleic (16:1 n-7), palmitic (16:0), and oleic (18:1 n-9) acids prevailed, the latter being typical of this genus; however, 3-hydroxydodecanoic (12:0-3OH) acid, which is of common occurrence in *Acinetobacter,* was absent. At the same time, Kampfer (1993) pointed out that not all *Acinetobacter* species contained this acid, and, on the whole, fatty acid analysis was not a sufficiently informative and suitable method for identification of these bacteria.

Sulfite bacteria were characterized by their specific fatty acid profile and differed from other bacteria by extremely high level of *cis*-vaccenic acid (18:1 n-7) and the presence of 18:2 acid, which is unusual for bacteria and acts as a characteristic feature of this genus (Pukall, 1999). The α-*Proteobacteria* are characterized by high content of 18:1 acid (from 40 to 70%). Based on predominance of the mentioned fatty acids, we identified two strains of *Sulfitobacter* spp.

According to Bowman et al. (1997), bacteria of the genus *Psychrobacter* were characterized by high contents of 18:1 (over 50%) as well as 17:1 and 16:1 acids (more than 80% in the sum); based on these results, we identified strain 2053 as a member of this genus.

Identification of the *Shewanella* strain was carried out on the basis of its phenotypic properties and fatty acid composition of its lipids. The strain was characterized by a complex of morphological and biochemical features typical of the genus *Shewanella* except for the absence of nitrate reductase. However, species lacking this enzyme have been described (Lee et al., 2006). In the fatty acid profile of the *Shewanella* sp. strain, 16:0, i-17:0, 16:1 (n-7), and 18:1 (n-7) acids prevailed. These acids are typical of the genus *Shewanella*. Hydroxy acids, which are widespread in *Shewanella,* were not revealed in our strain; however, this feature had also been observed in other species of this genus (Lee et al., 2006).

As mentioned above, identification of bacilli was not complicated due to their characteristic phenotypic properties. Therefore, fatty acids were analyzed only in one strain; predominance of the *iso*- и *anteiso*-methyl-branched acids confirmed identification of isolate 2082 as a *Bacillus* strain.

This study showed that members of the genus *Pseudoalteromonas* were the most abundant (20 strains) in bacterial biofilms formed on the surfaces of marine invertebrates and macrophytes, that is in agreement with literature data (Egan et al., 2001). Members of this genus are known to possess a wide range of antifouling activities preventing adhesion and growth of bacteria as well as attachment and development of algal spores; moreover, they exhibit an algacidal effect (Egan et al., 2000). We performed screening of the isolates to select the most active strains possessing antimicrobial activity and exhibiting activity against algal spores.

Antimicrobial activity of bacterial strains. It has been earlier assumed that bacteria producing antimicrobial substances may be able to prevent biofouling (Rao et al., 2007). Therefore, before performing experiments on the effect of the single-species bacterial films on algal spores, we studied antimicrobial activities of the isolates against both the type test strains and the true marine bacteria *Vibrio* sp. and *Pseudoalteromonas* sp.

Almost half of the studied isolates exhibited antimicrobial activity, that was in agreement with the results of our previous studies concerning antagonism of marine bacteria isolated from various climatic zones (Beleneva et al., 2013). Among the studied isolates, strains belonging to the genus *Pseudoalteromonas* were characterized by the widest spectra of antibacterial activity, whereas bacteria of the genera *Bacillus*, *Sulfobacter*, *Acinetobacter*, *Psychrobacter*, and *Shewanella* showed no antimicrobial activity. Earlier, the highest antimicrobial activity has been also found in isolates belonging to the genera *Pseudoalteromonas* and *Ruegeria* (Dobretsov et al., 2013).

The results obtained by Long and Azam (2001) clearly demonstrate that antagonistic interrelations between different bacteria in marine environment are widespread. This finding stimulates selection of producers of novel antibiotics and antifouling substances from various regions of the World Ocean.

Activity against algal spores. In the studies of antifouling activity, single-species films rather than natu-

ral biofilms are often used; thus, the potential of each bacterial strain may be revealed and comparison of the strains is simplified. The strains with strong inhibitory or stimulatory effects on fouling can be subsequently used both for identification of their biologically active compounds (Dobretsov et al., 2006, 2013) and for their production.

Screening of a large number of strains for their antifouling activity is a rather labor-consuming method; therefore, we carried out a series of preliminary experiments with 35 bacterial strains from 9 genera, in which no relation between antialgal activity and taxonomic position of bacteria was revealed. Earlier, no taxonomic specificity was revealed among strains inhibiting attachment of algal spores: active strains were distributed among several bacterial taxa (Patel et al., 2003). However, according to other studies, *Pseudoalteromonas* strains possessed the highest antifouling activity among a large number of bacteria tested (Bernbom et al., 2011).

For the main experiments with algal spores, we selected nine strains which (1) possessed antimicrobial activity against the test cultures; (2) in preliminary experiments showed activities against algal spore attachment and germination; (3) formed associations with other hydrobionts, that is important since bacteria attached to various surfaces were shown to exhibit more pronounced antifouling activity comparing with planktonic bacteria (Long and Azam, 2001; Ma et al., 2009). Most of selected strains belonged to the genus *Pseudoalteromonas*. Strain 1839 (*Bacillus* sp.) was taken as a producer of tetrodotoxin (Beleneva et al., 2014).

Our studies revealed two strains (2057 and 2066), which were the most active against algal spores. These strains not only suppressed spore attachment, but also caused mass death of the germinated spores of both algal species. It was shown that different strains of the same bacterial genus can differ considerably in their action on algal spores, showing sometimes directly opposed effects on different species as well as on spore attachment and germination. For instance, four out of nine studied strains inhibited attachment of the *Ulva* spores, but stimulated their germination. On the contrary, most strains did not affect attachment of the *Undaria* spores, but showed negative effect on their germination. The same strains (e.g., 2057 and 2066) could inhibit attachment of the *Ulva* spores, but showed no effect on attachment of the *Undaria* spores, or stimulated attachment of the former and inhibited attachment of the latter (strain 2070).

Different reactions of the *Ulva* and *Undaria* spores to bacterial films are not unusual; experiments carried out with unicellular marine algae showed that the bacteria–algae interactions were species-specific (Lovejoy et al., 1998). Thus, a *Pseudoalteromonas* sp. strain rapidly lysed dinoflagellates *Gymnodinoids* and *Raphidophytes,* but showed no effect on other unicellular organisms (diatoms and cyanobacteria). The *Ulva* spores were found to be more sensitive to the inhibitory effect of bacteria than the spores of red alga *Polysiphonia* sp. (Egan et al., 2000). The authors explained such variability in the spore response by the presence of different (or modified) bacterial antifouling compounds or by different physiology of the target organisms. Moreover, it was shown that some antialgal metabolites of bacteria were specific only to the certain algal species or the genera.

The effect of bacteria on algal spores is known to be determined mainly by various metabolites excreted into the medium (Lovejoy et al., 1998; Egan et al., 2000, 2001). These metabolites can either act as repellents or exhibit algicidal activity. Proteins, amino acids, antibiotics, pigments, and fatty acids were shown to have an algicidal effect (Li et al., 2014); among the latter, α-linolenic (18:3 n-3), palmitic (16:0), palmitoleic (16:1 n-7), linoleic (18:2 n-6), and oleic (18:1 n-9) acids were the most active. The genus *Pseudoalteromonas* was characterized by high content of 16:0 and 16:1 (n-7) acids (over 50% of the total fatty acids). The content of 16:1 (n-7) in the studied strains varied from 32 to 41% and was the largest in strain 2066, which lysed the germ cells of both algal species studied. It has been earlier found that palmitic acid damaged the plasmalemma and such intracellular organelles as mitochondria and chloroplasts, impairing their functions and finally leading to cell death (Li et al., 2014). However, strain 2057 (CFB) containing low amounts of palmitic acid (about 20%) showed a similar effect on the germinating *Undaria* spores. Apparently, apart from fatty acids, some other components of *Pseudoalteromonas* possess algicidic activity. Their identification requires further investigations.

As mentioned above, most of bacterial isolates showed contradictory effects on attachment and germination of the *Ulva* spores inhibiting the former and stimulating the latter. This finding is in agreement with the literature data. In particular, it was revealed that the inhibitory effect of *Pseudoalteromonas tunicata* was sharply decreased if the *Ulva* spores have been already precipitated (Egan et al., 2001). The authors explained this phenomenon either by the presence of an algicidal factor affecting the spore cytoplasmic membrane (after attachment to a substrate, the spores rapidly form a protective cell wall and become invulnerable) or by the fact that the inhibitory compound showed a negative effect on spore attachment, but did not possessed algicidal activity.

Various strains of the same bacterial genus can differ considerably in their effects on attachment of algal spores. In our study, most of the *Pseudoalteromonas* strains inhibited attachment of the *Ulva* spores; however, strain 2059 showed a stimulatory effect, which is not an unusual phenomenon. According to Patel et al. (2003), out of 14 *Pseudoalteromonas* strains isolated from the stone surfaces and *Ulva linza,* 4 strains

strongly inhibited spore attachment, 7 strains had no effect, and 2 strains slightly stimulated spore attachment. Different strains of *Pseudoalteromonas* spp. and *Photobacterium* spp. either suppressed or stimulated attachment of the *U. australis* spores (Bernbom et al., 2011). The strain specificity in production of inhibiting or stimulating compounds was revealed in the study of the effect of bacterial films on attachment of the *Balanus amphitrite* larvae (Maki et al., 1988). These results demonstrate strain specificity of bacteria in their ability to produce metabolites stimulating or inhibiting attachment and development of algal spores. It was found that some bacteria, including *Pseudoalteromonas* spp. (Huang et al., 2008), were able to produce acyl homoserine lactones (AHLs), which play the role of attractants for the *Ulva* spores (Tait et al., 2005). However, not all species of the genus *Pseudoalteromonas* were able to synthesize AHLs; this ability was revealed only in 6 out of 17 strains (Huang et al., 2008). On the other hand, the stimulatory effect of bacteria may be explained by complicated interrelations between spores and biofilms, particularly, by intensive formation of free energy on the surfaces of bacterial films, which promotes spore adhesion (Dillon et al., 1989). Further experiments are necessary to elucidate the mechanisms responsible for the stimulatory or inhibitory effects of biofilms on attachment of algal spores observed in our experiments.

Thus, our studies showed that bacteria with antifouling activity are widespread among the associated microflora of dinoflagellates, as well as in biofilms formed on the surfaces of marine hydrobionts in the Peter the Great Bay (Sea of Japan). No relation was revealed between antifouling activity of bacteria and the source of their isolation. Most of the active strains belonged to the genus *Pseudoalteromonas*. Strain 2066, which exhibited high antibacterial activity and showed the strongest effect on the both macroalgal species studied, causing mass death of germinated spores, appeared to be the most promising producer of substances for the production of antifouling coating.

The understanding of continual biological interrelations between biofilms and various marine organisms and selection of producers of biologically active metabolites will make a great contribution to the development of biotechnology, specifically, of the means of biological control and production of novel antifouling coatings, since marine bacteria represent a virtually inexhaustible source of physiologically active compounds.

Although the study of the mechanisms responsible for the biofilm–algal spore interrelations was not a goal of our work, the obtained results indicate their complexity and variety. Undoubtedly, wide occurrence of the fouling producers in marine environment, as well as negative consequences of this process had a strong evolutionary impact on marine organisms and stimulated production of various protective compounds (Rao et al., 2007). Occurrence in biofilms of the microorganisms producing biologically active compounds may protect algae from various fouling forms.

Until now, the role of microorganisms in protection of the host hydrobionts remains unclear. It is quite obvious that some compounds produced by microorganisms can fulfill several functions. Thus, indole, which is involved in biofilm formation by the cholera vibrio, shows high activity against bacterial pathogens (Dobretsov et al., 2013). Many compounds inhibit fouling process under laboratory conditions; however, it is unclear whether they are produced by microorganisms in situ in amounts sufficient for protection of marine organisms.

ACKNOWLEDGMENTS

This work was partly supported by the Far Eastern Branch, Russian Academy of Sciences (grant no. 15-I-6-059 and grant with registration no. A16-116101110096-3).

REFERENCES

Beleneva, I.A., Dautova, T.N., and Zhukova, N.V., Characterization of communities of heterotrophic bacteria associated with healthy and diseased corals in Nha Trang Bay (Vietnam), *Microbiology* (Moscow), 2005, vol. 74, no. 5, pp. 579–587.

Beleneva, I.A., Magarlamov, T.Yu., and Kukhlevsky, A.D., Characterization, identification, and screening for tetrodotoxin production by bacteria associated with the ribbon worm (Nemertea) *Cephalotrix simula* (Ivata, 1952), *Microbiology* (Moscow), 2014, vol. 83, no. 3, pp. 220–226.

Beleneva, I.A., Kukhlevsky, A.D., and Kharchenko, U.V., Antimicrobial activity of heterotrophic bacterial strains of marine origin, *Jundishapur J. Microbiol.*, 2013, vol. 6, pp. 166–175.

Bernbom, N., Ng, Y.Y., Kjelleberg, S., Harder, T., and Gram, L., Marine bacteria from Danish coastal waters show antifouling activity against the marine fouling bacterium *Pseudoalteromonas* sp. strain S91 and zoospores of the green alga *Ulva australis* independent of bacteriocidal activity, *Appl. Environ. Microbiol.*, 2011, vol. 77, pp. 8557–8567.

Bowman, J.P., Nichols, D.S., and McMeekin, T.A., *Psychrobacter glacincola* sp. nov., a halotolerant, psychrophilic bacterium isolated from Antarctic sea ice, *Syst. Appl. Microbiol.*, 1997, vol. 20, pp. 209–215.

Dillon, P.S., Maki, J.S., and Mitchell, R., Adhesion of *Enteromorpha* swarmers to microbial films, *Microb. Ecol.*, 1989, vol. 17, pp. 39–47.

Dobretsov, S.V., Dahms, H.U., and Qian, P.Y., Inhibition of biofouling by marine microorganisms and their metabolites, *Biofouling*, 2006, vol. 22, pp. 43–54.

Dobretsov, S., Abed, R.M.M., and Teplitski M., Minireview: inhibition of biofouling by marine microorganisms, *Biofouling*, 2013, vol. 29, pp. 423–441.

Egan, S., Thomas, T., Holmström, C., and Kjelleberg, S., Phylogenetic relationship and antifouling activity of bacte-

rial epiphytes from the marine alga *Ulva lactuca, Environ. Microbiol.*, 2000, vol. 2, pp. 343–347.

Egan, S., Holmström, C., and Kjelleberg, S., *Pseudoalteromonas ulvae* sp. nov. bacterium with antifouling activity isolated from the surface of a marine alga, *Int. J. Syst. Evol. Microbiol.*, 2001, vol. 51, pp. 1499–1504.

Egan, S., James, S., and Kjelleberg, S., Identification and characterization of a putative transcriptional regulator controlling the expression of fouling inhibitors in *Pseudoalteromonas tunicate, Appl. Environ. Microbiol.*, 2002, vol. 68, pp. 372–378.

Fautz, E., Rosenfelder, G., and Grotjahn, L., *iso*-Branched 2- and 3-hydroxy fatty acids as characteristic lipid constituents of some gliding bacteria, *J. Bacteriol.*, 1979, vol. 140, pp. 852–858.

Fenical, W. and Jensen, P.R., Enzyme inhibitors and other bioactive compounds from marine actinomycetes, in *Marine Biotechnology*, Attaway, D. and Zaborsky, O., Eds., New York: Plenum, 1993, vol. 1, pp. 419–457.

Huang, Y.L., Ki, J.S., Case, R.J., and Qian, P.Y., Diversity and acyl-homoserine lactone production among subtidal biofilm-forming bacteria, *Aquat. Microb. Ecol.*, 2008, vol. 52, pp. 185–193.

Huang, Y.L., Li, M., Yu, Z.L., and Qian, P.Y., Correlation between pigmentation and larval settlement deterrence by *Pseudoalteromonas* sp. sf57, *Biofouling*, 2011, vol. 27, pp. 287–293.

Ivanova, E.P., Zhukova, N.V., Svetashev, V.I., Gorshkova, N.M., Kurilenko, V.V., Frolova, G.M., and Mikhailov, V.V., Evaluation of phospholipid and fatty acid compositions as chemotaxonomic markers of *Alteromonas*-like proteobacteria, *Curr. Microbiol.*, 2000, vol. 41, pp. 341–345.

Kampfer, P., Grouping of *Acinetobacter* genomic species by cellular fatty acids composition, *Med. Microbiol. Lett.*, 1993, vol. 2, pp. 394–400.

Lee, O.O., Lau, S.C.K., Tsoi, M.M.Y., Li X., Plakhotnikova, I., Dobretsov, S., Wu, M.C.S., Wong, P.-K., Weinbauer, M., and Qian, P.-Y., *Shewanella irciniae* sp. nov., a novel member of the family *Shewanellaceae*, isolated from the marine sponge *Ircinia dendroides* in the Bay of Villefranche, Mediterranean Sea, *Int. J. Syst. Evol. Microbiol.*, 2006, vol. 56, pp. 2871–2877.

Li, D., Zhang, H., Fu, L., An, H., Zhang, B., Li, Y., Chen, Z., Zheng, W., Yi, L., and Zheng, T., A novel algicide: evidence of the effect of a fatty acid compound from the marine bacterium, *Vibrio* sp. BS02 on the harmful Dinoflagellate, *Alexandrium tamarense, PLoS One*, 2014, vol. 9, no. 3. e91201.

Long, R.A. and Azam, F., Antagonistic interactions among marine pelagic bacteria, *Appl. Environ. Microbiol.*, 2001, vol. 67, pp. 4975–4983.

Lovejoy, C., Bowman, J., and Hallegraeff, G., Algicidal effects of a novel marine *Pseudoalteromonas* isolate (class *Proteobacteria*, γ-subdivision), *App. Environ. Microbiol.*, 1998, vol. 64, pp. 2806–2813.

Ma, Y.X., Liu, P.L., Yu, S., Li, D.T., and Cao, S.M., Inhibition of common fouling organisms in mariculture by epiphytic bacteria from the surfaces of seaweeds and invertebrates, *Acta Ecol. Sin.*, 2009, vol. 29, pp. 222–226.

Maki, J.S., Rittschof, D., Costlow, J.D., and Mitchell, R., Inhibition of attachment of larval barnacles, *Balanus amphitrite*, by bacterial surface films, *Mar. Biol.*, 1988, vol. 97. 199–206.

Mieszkin, S., Callow, M.E., and Callow, J.A., Interactions between microbial biofilms and marine fouling algae: a mini review, *Biofouling*, 2013, vol. 29, pp. 1097–1113.

Patel, P., Callow, M.E., Joint, I., and Callow, J.A., Specificity in the settlement – modifying response of bacterial biofilms towards zoospores of the marine alga *Enteromorpha, Environ. Microbiol.*, 2003, vol. 5, pp. 338–349.

Pukall, R., Buntefuß, D., Frühling, A., Rohde, M., Kroppenstedt, R. M. Burghardt, J., Lebaron, P., Bernard, L., and Stackebrandt, E. *Sulfitobacter mediterraneus* sp. nov., a new sulfite-oxidizing member of the α-*Proteobacteria, Int. J. Syst. Bacteriol.*, 1999, vol. 49, pp. 513–519.

Qian, P.-Y., Lau, S.C.K., Dahms, H-U., Dobretsov, S., and Harder, T., Marine biofilm as mediator of colonization by marine macroorganisms: implications for antifouling and aquaculture, *Marine Biotechnol.*, 2007, vol. 9, pp. 399–410.

Rao, D., Webb, J.S, Holmström, C., Case, R., Low, A., Steinberg, P., and Kjelleberg, S., Low densities of epiphytic bacteria from the marine alga *Ulva australis* inhibit settlement of fouling organisms, *Appl. Envir. Microbiol.*, 2007, vol. 73, pp. 7844–7852.

Szewzyk, U., Holmström, C., Wrangstadh, M., Samuelsson, M.-O., Maki, J.S., and Kjelleberg, S., Relevance of the exopolysaccharide of marine *Pseudomonas* sp. strain S9 for the attachment of *Ciona intestinalis* larvae, *Mar. Ecol. Prog. Ser.*, 1991, vol. 75, pp. 259–265.

Tait, K., Joint, I., Daykin, M., Milton, D.L., Williams, P., and Cámara, M., Disruption of quorum sensing in seawater abolishes attraction of zoospores of the green alga *Ulva* to bacterial biofilms, *Environ. Microbiol.*, 2005, vol. 7, pp. 229–240.

Techniques for Corrosion Monitoring, Yang, L., Ed., Elsevier, 2008.

Yebra, D.M., Kiil, S., Weinell, C.E., and Dam-Johansen, K., Presence and effects of marine microbial biofilms on biocide-based antifouling paints, *Biofouling*, 2006, vol. 22, pp. 33–41.

Translated by E. Dedyukhina