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EXPERIMENTAL ARTICLES

Characterization, Identification, and Screening for Tetrodotoxin Production by Bacteria Associated with the Ribbon Worm (Nemertea) *Cephalotrix simula* (Ivata, 1952)

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Abstract—The taxonomic composition of bacteria associated with the proboscis worm *Cephalothrix simula* was studied and screening of the tetrodotoxin (TTX)-producing bacteria was carried out using confocal laser scanning microscopy and polyclonal antibodies. Bacterial isolates were identified using the 16S rRNA gene sequencing and phenotypic characteristics. A *Bacillus s*pecies was found to be responsible for tetrodotoxin production in proboscis worm *C. simula*. *Vibrio* spp. dominated the associated microflora (68.18% of the total number of isolates). The analysis of sensitivity of 16 strains to antibiotics of various classes revealed multiple resistance to three or more antibiotics in all the studied isolates. Poor growth of most of the isolates on all the laboratory media was an indirect confirmation of the symbiotic relationships between the micro- and macroorganisms.

Keywords: marine heterotrophic bacteria, associations, nemerteans, tetrodotoxin production

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Nemerteans (or ribbon worms) are primarily marine invertebrates; they are widespread in the littoral zone worldwide, and inhabit various sediments; many of them are predators. To capture prey, these worms use a special organ, the proboscis; moreover, many of the known 1200 species are able to produce tetrodotoxin (TTX), which provides both their defense against enemies and hunting [1].

TTX in nemerteans is of great interest because of the broad occurrence of these animals in marine ecosystems. TTX has been also revealed in a number of other hydrobionts, such as cephalopods, gastropods, bivalves [2], arthropods [3], echinoderms [4], and in various marine worms [5].

The origin of TTX in marine organisms remains controversial. The endogenous theory suggests that neurotoxin is produced directly in the animal organism. However, the fact that toxicity of fugu fish varies considerably between individuals and depends on the ecotopes, from which it was retrieved, cannot be explained by the endogenous theory of TTX origin [6]. The exogenous hypothesis states that TTX is accumulated along a food chain, in which bacteria form the first link; moreover, symbiotic or parasitic bacteria may also produce toxin. This viewpoint is confirmed by numerous studies on isolation of TTX-producing bacteria from poisonous marine animals [7, 8].

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The bacteria producing TTX were also isolated from ribbon worms [9]. However, these studies only characterized only TTX-producing strains and paid no attention to the diversity and properties of bacterial flora as a whole in the organs and tissues of poisonous nemerteans. Moreover, it remains unclear what the share of toxin-producing bacteria is in the total number of the animal-associated bacteria, whether it is a constant value, and to what bacterial taxa TTX producers most frequently belong.

The goal of the present work was to study the properties and taxonomic composition of cultivated bacteria associated with *C. simula* and conduct screening for the TTX producers.

MATERIALS AND METHODS

Nemerteans were collected in Avangard Bay of Peter the Great Bay in June 2012. Each sample was washed thrice with sterile seawater to remove microorganisms from the surface of animals. The combined sample containing five worms was homogenized and diluted with physiological solution to 1 mL; 0.1 mL of this native homogenate was spread over TCBS agar in a petri dish. To isolate individual colonies, series of culture dilutions were inoculated onto solid Youschimizu–Kimura (Y–K) medium [10] with the following composition (g/L): peptone, 5.0; yeast extract, 2.0; glucose, 1.0; K_2HPO_4, 0.2; $MgSO_4 \times 7H_2O$, 0.1;

agar, 12.0; distilled water, 500 mL; seawater, 500 mL; pH 7.8–8.0. Inoculated dishes with TCBS agar and Y–K medium were incubated at 23°C for two and six days, respectively. Pure cultures were obtained after three successive transfers of the isolates onto the relevant medium. In further studies, vibrios were cultivated on alkaline yeast agar, GRM agar (Obolensk, Russia), Caso agar (Merck, Germany), and Marine agar 2216 (United States). Other strains were grown on the Y–K medium and Marine agar 2216.

The tests for Gram reaction, motility, cytochrome oxidase, utilization of carbon sources, formation of acids from carbohydrates, antibiotic resistance, and Na^+ demand, as well as for ability to grow at different temperatures, pH, and salinity, were carried out as described earlier [11]. The ability to hydrolyze gelatin and starch was studied according to [12]. Additional biochemical analyses were performed with the use of test systems SIB (Mikrogen, Russia) for vibrios and API 20NE (bioMérieus, France) for other isolates. The isolates were identified by using their biochemical characteristics and results of the 16S rRNA gene sequencing.

Screening of TTX-producing strains was carried out using confocal laser scanning microscopy. The strains were grown on Y–K medium for 2 days; then the cells were washed off the agar surface with sterile seawater and separated on a MiniSpin centrifuge (Eppendorf) at 3000 *g* for 10 min. The pellet was suspended in 4% solution of paraformaldehyde in phosphate buffer (PB) of the following composition (mM): NaCl, 137; KCl, 2.7; Na_2HPO_4, 6.4; KH_2HPO_4, 1.2; pH 7.4. Then it was fixed at room temperature for 1 h, washed with PB, dehydrated with increasing concentrations of alcohols and acetone, and embedded in LR White water-soluble resin (EMS). Semithin sections (0.9 μm thick) were made on an Ultracat E microtome (Reichert-Jung, Germany) and placed onto the slides. The semithin sections were permeabilized with 1% Triton X-100 (Aldrich) in PB for 1 h, washed with PB and incubated for 1 h in the blocking solution containing 1% BSA and 10% normal goat serum (Invitrogen, United States) in PB. To reveal TTX, the sections were incubated for 2 days at 4°C in a solution of primary antibodies against TTX (rabbit polyclonal antibodies diluted 1 : 25) (Abnova, Taiwan) in PB containing 1% BSA, washed with PB, and incubated in a solution of secondary antispecies antibodies labeled with Alexa 488 (Invitrogen, United States) in concentration of 1 : 800 in PB for 2 h at room temperature. The sections were washed with PB and embedded in Moviol (Aldrich). The specimens were examined under an LSM-510 Meta confocal microscope (Carl Zeiss, Germany); the images were processed using the CLSM-510 Meta program.

Antimicrobial activity of the isolates against type strains of the test cultures (*Escherichia coli* ATCC 15034, *Bacillus subtilis* VKM B501, *Candida albicans* KMM 455, *Pseudomonas aeruginosa* KMM 433, and *Staphylococcus aureus* ATCC 21027) was determined as described earlier [11].

The Bacterial strains were maintained in the Collection of Marine Heterotrophic Bacteria, Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences, in cryotubes containing seawater with glycerol (30%), peptone (Difco, United States) (1%), and $MgSO_4$ (3–5 g/L) at –85°C.

DNA isolation, amplification, sequencing, and analysis. Total DNA was extracted from 1 mL of a bacterial culture by the standard method [13]; the procedures for 16S rRNA gene amplification, sequencing, and primary data processing were described earlier [14]. Species affiliation of the isolates was established by comparing their nucleotide sequences with those available in the NCBI/GenBank database, as well as on the basis of biochemical tests. The obtained sequences of the 16S rRNA gene fragments were deposited in GenBank under accession nos. KF444392–KF444416. A phylogenetic tree was constructed using the 16S rRNA gene sequences of the closest bacterial species, which were retrieved from the GenBank database (accession nos. AB571866, AB681203, DQ492722, EF599163, EU091332, FJ188310, FJ943260, HE584792, JF811908, JQ670709, and KC160911). The nucleotide sequences were aligned using the ClustalW program. Construction of the phylogenetic tree and bootstrap analysis were carried out as described earlier [11].

RESULTS

Twenty-two strains of heterotrophic bacteria were isolated from ribbon worms *C. simula*: eleven on TCBS and Y–K media each (Table 1). The isolates were identified by comparing their 16S rRNA gene sequences with those available in the NCBI/GenBank database. Based on phylogenetic data and phenotypic characteristics, the isolates were divided into three phylogenetic groups: *Gammaproteobacteria* (81.82%), *Firmicutes* (13.64%), and *Bacteroidetes* (4.54%). Gram-negative bacteria prevailed (86.36% of the total number of isolates); they were represented mainly by vibrios (15 strains), *Pseudoalteromonas* spp. (2 strains), *Kistimonas* sp. (1 strain), and *Salegentibacter* sp. (1 strain). All three gram-positive isolates belonged to the genus *Bacillus*. Based on the analysis of 16S rRNA gene sequences, all vibrios were divided into four groups: (1) three strains were closest to the isolate *Vibrio* sp. 04Ya001; (2) three strains were close to *V. atlanticus* LMG 24300; (3) five strains were closely related to *V. splendidus*; (4) four strains with low identity were close to marine isolate *Vibrio* sp., clone KWE30-5 (Fig. 1). On the whole, phenotypic properties of the isolates corresponded to the description of the genus *Vibrio* and were characterized by facultative anaerobic metabolism, presence of cytochrome oxidase, nitrate reductase, Na^+ demand for growth, and sensitivity to the 0-129 vibriostatic agent

Table 1. Taxonomic position of bacterial strains associated with *C. simula*

Strain no.	GenBank accession no.	Closely related organism	Identity, %
1796	KF444392	*Vibrio* sp. 04Ya001 (AB571866)	99.8
1797	KF444393	*Vibrio* sp. 04Ya001 (AB571866)	100
1798	KF444394	*Vibrio* sp. 04Ya001 (AB571866)	99.9
1799	KF444395	*V. atlanticus* LMG 24300 (EF599163)	99.0
1800	KF444396	*V. atlanticus* LMG 24300 (EF599163)	99.0
1801	KF444397	*V. atlanticus* LMG 24300 (EF599163)	99.0
1802	KF444398	*V. splendidus* AP57 (HE584792)	99.5
1803	ND	*Bacillus circulans*	ND
1804	KF444399	*Kistimonas* sp. A36 (JF811908)	98.2
1805	KF444400	*V. splendidus* PB1-10rrnH (EU091332)	99.0
1806	KF444401	*Vibrio* sp. clone KWE30-5 (JQ670709)	98.0
1807	KF444402	*V. splendidus* PB1-10rrnH (EU091332)	99.9
1808	KF444403	*Vibrio* sp. clone KWE30-5 (JQ670709)	97.9
1809	KF444404	*Bacillus* sp. P307 (FJ943260)	100
1810	ND	*Pseudoalteromonas* sp.	ND
1811	KF444405	*Vibrio* sp. clone KWE30-5 (JQ670709)	98.0
1812	KF444406	*Salegentibacter mishustinae*(AB681203)	99.8
1813	KF444407	*Pseudoalteromonas* sp. SS12.19 (KC160911)	100
1814	KF444408	*V. splendidus* PB1-10rrnH (EU091332)	99.2
1815	KF444409	*V. splendidus* PB1-10rrnH (EU091332)	99.9
1816	KF444410	*Vibrio* clone KWE30-5 (JQ670709)	98.0
1839	KF444412	*Bacillus* sp. CU040510-015 (FJ188310)	99.8

"ND" stands for "not determined".

(Oxoid) (150 μg). A common property of the isolates within each group was their attitude to saccharose; however, individual strains differed in their capacity for indole synthesis, acid formation from mannose, as well as in their resistance to antibiotics and salinity.

The strain 1813 showed 100% similarity in the 16S rRNA gene sequences with the strain *Pseudoalteromonas* sp. KC160911 isolated from Antarctic littoral sediments. The isolate obtained from the ribbon worms possessed cytochrome oxidase, nitrate reductase, and gelatinase; it hydrolyzed esculin, utilized glucose, *N*-acetyl glucosamine, and malate, grew at NaCl concentrations from 3 to 10%, exhibited neither arginine dihydrolase nor urease activity, and was incapable of glucose fermentation. The second representative of the genus *Pseudoalteromonas*, strain 1810, excreted yellow-brown pigment into the medium, hydrolyzed esculin and gelatin, exhibited neither urease, nitrate reductase, nor arginine dihydrolase activity; it grew at 3–6% NaCl.

The strain *Kistimonas* sp. 1804 formed yellow, semitransparent, slightly convex colonies on marine agar; the cells were motile, hydrolyzed gelatin and starch, exhibited neither urease nor nitrate reductase, required Na^+ ions for growth.

The strain *Salegentibacter* sp. 1812, which was the only member of the CFB cluster of the *Bacteria* domain, possessed oxidative metabolism, required Na^+ ions for growth, formed yellow colonies, showed positive tests for oxidase, catalase, and amylase, hydrolyzed esculin, and did not produce nitrate reductase, hydrogen sulfide, indole, or gelatinase.

According to the 16S rRNA gene sequencing analysis, the strain 1809 showed 100% similarity with the strain *Bacillus* sp. P307 (FJ 943260) isolated from a deep-water sponge, which in turn resembled the species *B. circulans* and *B. nealsonii*; at the same time, the strain 1809 differed from these species in a number of biochemical characteristics: it was unable to produce acid from carbohydrates or to hydrolyze gelatin. Phenotypic properties of the strains 1809 and 1803 were similar except for gelatin hydrolysis. Bacteria formed terminal ellipsoidal spores, hydrolyzed starch and esculin, grew at 0–8% NaCl, produced neither arginine dihydrolase, ornithine- and lysine decarboxylases, indole, nor hydrogen sulfide, and did not form acid from glucose, arabinose, or mannitol.

Comparing the 16S rRNA gene sequences of the strain 1839 with those available in the NCBI/GenBank revealed that this strain had 99.8% similarity

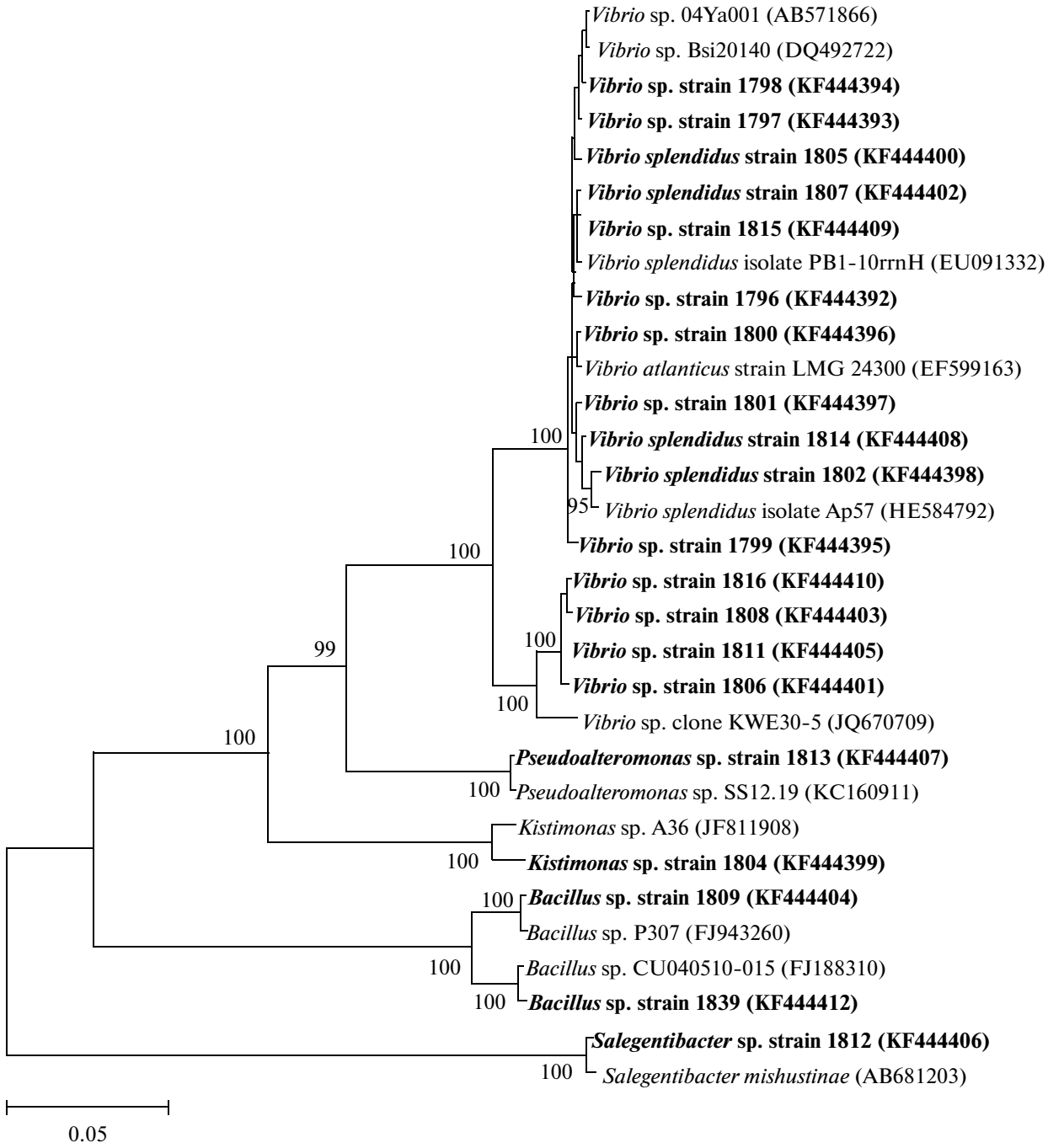

Fig. 1. Phylogenetic tree constructed with the use of NJ algorithm on the basis of comparative analysis of the 16S rRNA gene sequences. Bootstrap values higher than 70% are given at the branching points. Strains isolated from the ribbon worm *C. simula* are highlighted in bold.

with *Bacillus* sp. CU040510-015 (*B. asahii*). However, it differed in a number of phenotypic characteristics, such as the presence of cytochrome oxidase, gelatinase, and NaCl demand. The strain *Bacillus* sp. 1839 formed whitish, opaque, flat colonies on marine agar, produced large spores (spore-forming cells looked like rackets), exhibited nitrate reductase, gelatinase, and amylase activities, had no cytochrome oxidase, did not produce indole, hydrogen sulfide, or acid from glucose, mannitol, and arabinose, and did not grow on McConkey medium or at over 3% NaCl. Confocal laser scanning microscopy conbined with polyclonal antibodies revealed characteristic cell fluorescence that indicated TTX production by the strain 1839 (Fig. 2).

The isolates associated with the ribbon worm *C. simula* showed antimicrobial activity against four

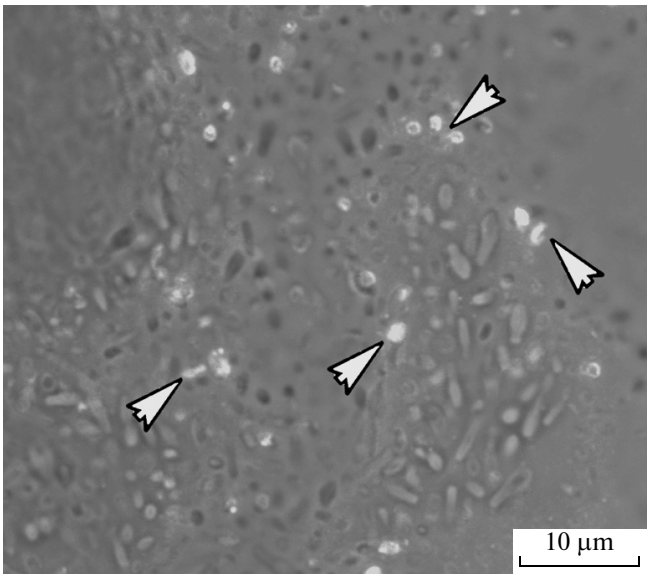

Fig. 2. Cells of the strain *Bacillus* sp. 1839 (semithin section, confocal laser scanning microscopy). Arrows indicate tetrodotoxin-positive cells.

out of five type cultures tested (Table 2); none of the strains exhibited activity against *Staphylococcus aureus*. The most active representatives of *Vibrio* spp. suppressed growth of two (strains 1806 and 1811) and three (strain 1816) test organisms.

All the studied isolates showed resistance to three or more antibiotics irrespective of their taxonomic position. Only two strains (1801 and 1806) were characterized by similar phenotypes of resistance to 13 out of 16 antibiotics tested.

Vibrios showed high resistance to macrolides (oleandomycin and erythromycin), rifampicin, and lincomycin (86.6 to 100% of the strains were resistant to these antibiotics). Resistance to β-lactams (ampicillin, penicillin, carbenicillin, and oxacillin) was found in 40.0, 53.3, 66.6, and 100% of *Vibrio* sp. strains, respectively. Aminoglycosides (streptomycin and gentamycin) inhibited growth of 26.6 and 60% of strains, respectively. Resistance to chloramphenicol and polymyxin was found in only 6.66% of *Vibrio* sp. strains.

Three strains of *Bacillus* spp. were characterized by similar phenotypes of antibiotic resistance; they were sensitive to aminoglycosides, β-lactams, tetracycline, chloramphenicol, rifampicin, moderately resistant to macrolides, and resistant to lincomycin and fluoroquinolones.

Two strains of *Pseudoalteromonas* spp. were resistant to β-lactams (except for ampicillin) and cephalosporins of the I and III generations, moderately resistant to aminoglycosides and erythromycin, and were sensitive to rifampicin.

The strain *Kistimonas* sp. 1804 was resistant to 8 out of 16 antimicrobial agents tested; the strain *Salegentibacter* sp. 1810 was resistant to antibiotics: aminoglycosides, polymyxin, and oxacillin.

DISCUSSION

While molecular genetic methods, especially 16S rRNA gene sequencing, are necessary for identification of phenotypically close bacterial species, they provide no information concerning their physiological characteristics and in some cases are inapplicable for identification of closely related microorganisms at the species level [15]. A polyphasic approach combining the standard bacteriological methods and molecular genetic techniques makes it possible to determine the taxonomic status of isolates and to gain insight into their physiology, biochemical potential, and role in marine ecosystems. The studied bacterial isolates (except for the strains 1803 and 1810) were identified on the basis of 16S rRNA gene sequencing. However,

Table 2. Antimicrobial activity of bacteria associated with *C. simula*

Type test culture	Active strain no.	Taxonomic position of the active strain
Escherichia coli ATCC 15034	1810	*Pseudoalteromonas* sp.
Pseudomonas aeruginosa KMM 433	1796	*Vibrio* sp.
	1806	*Vibrio* sp.
	1809	*Bacillus* sp.
	1811	*Vibrio* sp.
Candida albicans KMM 455	1816	*Vibrio* sp.
Bacillus subtilis BKM B501	1802	*V. splendidus*
	1806	*Vibrio* sp.
	1811	*Vibrio* sp.
	1814	*V. splendidus*
	1816	*Vibrio* sp.

the shortcomings of these methods hindered identification of all the isolates to the species level. Taxonomic position of the isolates was confirmed by comparing their phenotypic characteristics with those of respective taxa available in the literature.

Predominance of the isolates belonging to the *Gammaproteobacteria* among the microflora associated with *C. simula* agreed well with the data on bacterial diversity in marine ecosystems [16]. Vibrios are widespread in various ecological niches of marine environments and form a major portion of associative microflora in many organisms from zooplankton to fishes [17].

It should be noted that vibrios grew poorly on the laboratory media used in the present work, which are usually favorable for the growth of these bacteria, possibly because of deficiency of additional growth factors. In our opinion, this peculiarity of the vibrios isolated from ribbon worms is an indirect confirmation of symbiotic interrelations between micro- and macroorganisms.

Vibrio spp. were the first bacteria in which TTX synthesis was found [7]. At present, the list of bacterial TTX producers includes *Pseudomonas* spp., *Bacillus* spp., *Aeromonas, Actinomyces, Serratia, Shewanella, Microbacterium, Raoultella terrigena*, and a number of other bacteria [8].

Since vibrios prevailed among bacteria associated with *C. simula*, it could be expected that they are TTX producers; however, TTX synthesis was found only in one out of 22 isolates, in the strain *Bacillus* sp. 1839, which also poorly grew on laboratory media. It is the first report describing TTX-producing bacilli isolated from marine invertebrates. TTX-producing *Bacillus* spp. were previously revealed only in fugu fish [18, 19].

Antimicrobial activity was found in 8 out of 22 tested strains (36.36%). According to Long and Azam [20], microbial antagonism is widespread in marine environments that agrees with our earlier data [11]; it can be considered as an adaptation mechanism to provide organisms with advantages in their struggle for food supply and living space.

It should be emphasized that all the strains isolated from *C. simula* were characterized by unusually high multiple-antibiotic resistance. We could not find information in available literature on the antibiotic resistance of bacterial strains isolated from the toxic marine animals; this property is probably a specific feature of the microflora associated with TTX-containing ribbon worms. On the other hand, all of the strains were isolated from the animals inhabiting a wastewater-polluted bay; it is known that in marine environments, the determinants of antibiotic resistance are transferred between enterobacteria, vibrios, and other bacteria at a high rate [21]. The multiple antibiotic resistance, revealed in our isolates, appeared to be a result of this vertical transfer. Moreover, the fact that the genes encoding antibiotic resistance are widespread among environmental bacteria was known long before the first medical application of antibiotics [22]. It is obvious that the problem of multiple drug resistance of associatied microflora requires further investigations.

This study revealed for the first time that TTX in the ribbon worm *C. simula* is produced by a representative of the genus *Bacillus*. The associated microflora was not characterized by a high taxonomic diversity, although some peculiarities of its members made it possible to suggest symbiotic interrelations between bacteria and the host organism. In general, interrelations between marine invertebrates and microorganisms are poorly known and the study of such characteristic features of the microflora associated with *C. simula* as toxin synthesis, antibiotic resistance, and microbial antagonism can provide deeper knowledge of the mechanisms of these interrelations.

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