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Carrageenanolytic enzymes from marine bacteria associated with the red alga *Tichocarpus crinitus*

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Abstract

One hundred six bacterial strains (106 strains) were isolated from the Pacific red alga *Tichocarpus crinitus*, collected from the Troitsa Bay of Gulf of Peter the Great (Sea of Japan, Russia), and studied on their ability to degrade different samples of carrageenan as substrates. Some of the studied strains exhibited hydrolytic activity selectively to a total polysaccharide (kappa and lambda) from *Chondrus armatus*, other strains, to κ/β-carrageenan from *T. crinitus*, which did not possess the same effect to a total polysaccharide from *T. crinitus*. The strains with the highest enzyme activity (28 strains) were identified using 16S rRNA gene sequence techniques and classified to the phyla *Bacteroidetes* and *Proteobacteria*. Most (75%) of the studied carrageenase producers belong to phylum *Bacteroidetes* (21 strains). These strains were related with 7 phylotypes of the genera *Aquimarina*, *Cellulophaga*, *Maribacter*, and *Zobellia* (family *Flavobacteriaceae* of the class *Flavobacteriia*). *Cellulophaga* strains were the dominant group (35.7% of total) and closely related to species *C. baltica* and *C. lytica* with 99.8 and 99.9% sequence similarity, respectively. The remaining strains (7 strains) were represented by members of the genera *Altererythrobacter* (the family *Erythrobacteraceae*), *Phaeobacter*, *Sulfitobacter*, *Tateyamaria* (the family *Rhodobacteraceae*), and *Sphingomonas* (the family *Sphingobacteriaceae*) of the class *Alphaproteobacteria* and *Psychrobacter* (the family *Moraxellaceae*) and *Vibrio* (the family *Vibrionaceae*) of the class *Gammaproteobacteria.*

Keywords Marine bacteria · Red alga · Carrageenan · Carrageenase · Hydrolytic activity

Introduction

Carrageenans are a family of natural water-soluble linear sulfated galactans extracted from numerous species of red algae and have wide practical application. Carrageenans are utilized as food supplements due to their excellent physical functional properties, such as thickening, gelling, and stabilizing abilities. Carrageenans are used not only as foodstuff ingredients but also as drugs due to their various biological activities, such as antiviral (Ghosh et al. 2009; Boulho et al. 2017), antifungal (Soares et al. 2016), anticoagulant (Zuniga et al. 2006; Yermak et al. 2012), antitumor (Yuan et al. 2011), and immunomodulatory and immunoadjuvant (Bhattacharyya et al. 2010; Yermak et al. 2012, 2016) activities. This large family of hydrocolloids shares a common linear backbone of D-galactose (G) linked by alternating β-1,4 and α-1,3 glycosidic bonds, giving rise to disaccharide repetition moieties—called carrabiose units—that form the basic building block of the molecule. Carrabiose units are classified according to the occurrence of 3,6-anhydro bridges (DA) in the α-linked galactose residues and the substitution of the free hydroxyl groups with ester sulfate (S), methyl, or pyruvate groups (Rees 1963; Knutsen et al. 1994). The three most industrially exploited carrageenans, namely κ-(DA-G4S), ι-(DA2S-G4S), and λ-(D2S6S-G2S) carrageenans, are distinguished by the presence of one, two, and three ester-sulfate groups per repeating disaccharide unit, respectively. However, carrageenans have very heterogeneous chemical structure: their hybrid structure generally includes several types of carrabiose units whose proportion and distribution along the polysaccharide chain vary with algal kind, growth conditions, and extraction

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procedure. Therefore, the terms κ- and ι-carrageenans are used for polysaccharides which contain κ- and ι-carrabiose moieties, respectively, as main components. For example, κ- and ι-carrageenans contain fractions of their biosynthetic precursors named μ-carrageenan (D6S-G4S) and -carrageenan (D2S6S-G4S), respectively (Bellion et al. 1983; Van de Velde et al. 2001). The large amount of carrabiose structures combined lead to a wide range of possible carrageenan structure (Craigie 1990; Guibet et al. 2007; Usov 2011). Several carrabiose combinations have been demonstrated, such as κ/ι-(DA-G4S/DA2S-G4S) carrageenan hybrid in some species of the Gigartinaceae family and κ/β-(DA-G4S/DA-G) carrageenan found in *Furcellaria* sp. and *Eucheuma gelatinae*. Hybrid κ/β-carrageenans are mainly made up of κ-carrabiose (G4S-DA) and neutral β-carrabiose (G-DA), traditionally extracted from the cell walls of several red algae, including *Furcellaria lumbricalis*, *Eucheuma gelatinae*, *Eucheuma speciosa*, *Endocladia muricata* (Renn et al. 1993), and *Tichocarpus crinitus* (Barabanova et al. 2005; Anastyuk et al. 2011). Earlier we have shown that the gelling polysaccharides isolated from the vegetative forms of *Tichocarpus crinitus* belong to κ/β-carrageenans type (DA-G4S/DA-G) with predominance (80%) of the carrabiose units of κ-type (Barabanova et al. 2005).

The obtaining of carrageenan sample with low molecular weight is necessary and important to determine the structural peculiarities of natural polymers. Many reports have been published on degradation of carrageenans by different methods such as chemical degradation with different agents. It has been reported that carrageenan can be depolymerized by using an active oxygen species (Zuniga et al. 2006). Hydrolysis with organic acid solution or HCl is also used to prepare carrageenan oligosaccharides (Yu et al. 2002). However, specific cleavage of linkages in the backbones of complex carrageenan polymers without the risk of modification of the native structure requires specific enzymes for the respective structures. Enzymes capable of splitting the carbohydrate chains of carrageenans have been found mainly in marine bacteria (Guibet et al. 2007; Chauhan and Saxena 2016). Carrageenans constitute a crucial carbon source for a number of marine bacteria. These microorganisms, which belong mainly to the classes Gammaproteobacteria, Flavobacteria, or Sphingobacteria, degrade the cell walls of marine red algae by secreting specific glycoside hydrolases, referred to as carrageenases (Guibet et al. 2007). Carrageenases can be obtained from the bacterium *Pseudomonas carrageenovora*, which produces extracellular enzymes specific to different carrageenan, depending on the structure of polysaccharide used in cultivation medium as the only carbon source (Ostgaard et al. 1993). Several genera of marine bacteria which produce carrageenase have been found, such as *Vibrio* (Martin et al. 2016), *Pseudomonas* (Ostgaard et al. 1993), *Alteromonas* (Michel et al. 2001a, b), *Pseudoalteromonas* (Michel et al. 2001a, b), and *Cytophaga* (Sarwar et al. 1983; Potin et al. 1991; Mou et al. 2003). However, some gram-positive bacteria like *Bacillus* sp. (Li et al. 2014a) and *Cellulosimicrobium* have also been reported to produce carrageenase (Youssef et al. 2012; Li et al. 2014; Kang and Kim 2015). In addition, for preparing oligosaccharides, recombinant carrageenases are widely used (Lemoine et al. 2009; Xu et al. 2015; Wang et al. 2015).

To date, about 70 strains of marine bacteria producing carrageenases are known (Mou et al. 2004). Enzymes which degrade carrageenans are called k-, i-, and λ-carrageenases. They all are endohydrolases that cleave the internal β-(1–4) linkages of carrageenans with retention of the anomeric configuration yielding products of the oligocarrageenans (Michel et al. 2006; Chauhan and Saxena 2016). So, it is established that the main products of enzymatic hydrolysis of carrageenans are oligosaccharides with a certain chain length and regular structure, such as k-neo-carratetraose-sulfate and k-neocarabiose-sulfate for k-carrageenase, i-neo-carratetraose-sulfate and i-neocarrahexaose-sulfate for i-carrageenase, and neo-λ-carratetraose and neo-λ-carrageecaose for λ-carrageenase (Guibet et al. 2007; Collén et al. 2009; Lemoine et al. 2009).

Despite the large amount of information about bacterial carrageenases reported to date and presented in a number of different papers, microbial enzymes which hydrolyze carrageenan cause considerable interest at present because enzymatic degraded products of carrageenan are still in infancy compared to other algal polymers such as agar and alginate (Michel et al. 2001a, b, 2006; Chauhan and Saxena 2016).

The purpose of the current study was to search for new sources of microbial enzymes for degrading carrageenans with the following selection among them the most effective in hydrolytic activity to different structural type of carrageenan.

Materials and methods

Substrates

The red algae *Tichocarpus crinitus* (Tichocarpaceae) and *Chondrus armatus* (Gigartinaceae) were harvested from the Gulf of Peter the Great of Japan Sea of the Russian Coast. The selected algae were sterile lacking any reproductive organs. The algae were washed with tap water to remove excess salt. Bleaching of the algae was achieved by maintaining the specimen in pure acetone for 5 days prior to air-drying.

The dried and milled alga (50 g) was suspended in hot water (1.5 L) and the polysaccharides were extracted at 80 °C for 3 h in a boiling water bath; this procedure was repeated three times. The polysaccharide fraction was concentrated, purified using a 200 VivaFlow filtration column (Germany) with a membrane size of 100 kDa. The polysaccharide from *C. armatus* is composed of kappa and lambda types of carrageenans at a ratio 3:1 and is labeled as total carrageenan. The polysaccharide from (*T.*

crinitus) was separated into gelling KCl-insoluble and non-gelling KC1-soluble fractions, as described previously (Yermak et al. 1999). Gelling (KCl-insoluble) sulfated galactans from *T. crinitus* were observed to have hybrid structure kappa/beta carrageenan according to a published protocol (Barabanova et al. 2005). The total polysaccharide from *T. crinitus* is composed of kappa/beta- and iks-type carrageenans (Barabanova et al. 2008).

Molecular weight estimation

The average molecular weight of the carrageenan samples were calculated using the Mark-Houwink equation: $[\eta] = KM^{\alpha}$, where [η] is the intrinsic viscosity and K and α are empirical constants for κ-carrageenans constituting 3×10^{-3} and 0.95 at 25 °C in 0.1 M NaCl, respectively, according to the literature data for this polymer-solvent system (Rochas et al. 1990). The viscosity of the carrageenan solutions (0.1–1.0 mg mL^{-1} in 0.15 M NaCl) was measured in a modified Ubellode viscometer (Pushino, Russia) with a capillary diameter of 0.3 mm at 26 °C, with accuracy within ± 0.1 s. The intrinsic viscosity of the carrageenan samples was calculated by the extrapolation of the dependence ln (η)rel/C to infinite dilution using the least squares method.

The molecular weight of oligosaccharide sample was determined by the reducing sugars method with ferricyanide (Park and Johnson 1949).

Bacterial strain isolation and cultivation

Bacterial strains were isolated from the red alga *Tichocarpus crinitus* (Tichocarpaceae) collected from the Troitsa Bay, Gulf of Peter the Great, Sea of Japan, Russia, by a standard dilution-plating method. The sample of algal fronds (5 g) was homogenized in 10 mL sterile seawater in a glass homogenizer and 0.1 mL homogenate was spread onto marine agar 2216 (MA, Difco) plates. Each of the novel isolates was obtained from a single colony after incubation of the plate at 28 °C for 7 days. After primary isolation and purification, the strains were cultivated at 28 °C on the same medium and stored at – 80 °C in marine broth (Difco) supplemented with 20% (*v*/*v*) glycerol.

For screening of carrageenase activities, the strains were aerobically cultivated in fermentation media composed of (g L^{-1}):

Medium A content: 2.5 g Bacto Peptone (Difco), 2.5 g Meat Peptone Type T (HiMedia Laboratories, India), 2 g Bacto Yeast Extract (Difco), 2 g carrageenan, 0.2 g K_2HPO_4 and 0.05 g $MgSO_4 \cdot 7H_2O$ in 500 mL natural seawater, and 500 mL distilled water

Medium B content: 2.5 g Bacto Peptone (Difco), 2.5 g Meat Peptone Type T (HiMedia Laboratories, India), 2 g Bacto Yeast Extract (Difco), 0.5 g glucose, 1 g carrageenan, 0.2 g K_2HPO_4 and 0.05 g $MgSO_4 \cdot 7H_2O$ in 500 mL natural seawater, and 500 mL distilled water

Medium C content: 1.5 g Bacto Peptone (Difco), 1.5 g Meat Peptone Type T (HiMedia Laboratories, India), 2 g carrageenan, 1 g NaNo3, 15 g NaCl, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.1 g $CaCl_2$ per liter of distilled water

16S rRNA gene sequencing and phylogenetic analysis

DNA was extracted from 0.1–0.2 g of the bacterial cells (wet weight), using the extraction protocol of Sambrook and Russell (2001). PCR was carried out using the universal oligonucleotide primers 11F (5′-GTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) as described by Lane (1991) and the GeneAmp PCR System 9700 (Applied Biosystems Inc.). PCR amplicons were used as templates for sequencing amplification using a BigDye Terminator version 3.1 Cycle sequencing kit (Applied Biosystems). The purified sequencing products were analyzed by electrophoresis on a 50-cm capillary array of an ABI Prism 3130 DNA sequencer and the sequence was assembled with SeqScape version 2.6 (Applied Biosystems). The sequences obtained were aligned with those of representative members of selected bacterial genera by using PHYDIT version 3.2 (http://plaza.snu.ac.kr/~jchun/phydit/).

The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). Pairwise sequence similarities were calculated using EzTaxon-e server (Kim et al. 2012).

Carrageenase activity assay

The water solution of carrageenan (0.2% in medium B and 0.4% for mediums A and C) was diluted by medium twice. After that, the investigated bacterial strains were added to a solution (number of cells per volume polysaccharide solution) and incubated for 4 days at room temperature and pH 7.2. Control sample (polysaccharide solution without bacteria) was incubated simultaneously. At the end of incubation, the solution was centrifuged at 5000×*g* for separation from the cell mass. Carrageenase activity in supernatants was estimated using the reducing-sugar method to determine the presence of low molecular weight products of enzymatic hydrolysis (on the values of absorbance at 700 nm relative to controls) (Park and Johnson 1949).

Results

Diversity of carrageenan-degrading bacteria

A total of 332 bacterial strains were isolated from the red alga *T. crinitus*, and 106 strains were studied on their ability to degrade carrageenans. Twenty-eight strains with high enzyme activity were selected, identified using the 16S rRNA gene sequence techniques, and classified to the phyla *Bacteroidetes* and *Proteobacteria* (Table 1). Among the carrageenase producers, members of the phylum *Bacteroidetes* were most abundant (21 strains or 75% of total). All strains were affiliated with 7 phylotypes of the genera *Aquimarina*, *Cellulophaga*, *Maribacter*, and *Zobellia* belonging to a single family *Flavobacteriaceae* of the class *Flavobacteriia*. *Cellulophaga* strains were the dominant group (35.7% of total) and closely related to species *C. baltica* (7 strains) and *C. lytica* (3) with 99.8 and 99.9% sequence similarity, respectively. Six maribacters were the most diverse phylogenetic group and related to species *M. aestuarii*, *M. arcticus*, and *M. vaceletii* (Table 1). The remaining 7 strains (25% of total) were represented by members of the genera *Altererythrobacter* (the family *Erythrobacteraceae*), *Phaeobacter*, *Sulfitobacter*, *Tateyamaria* (the family *Rhodobacteraceae*), and *Sphingomonas* (the family *Sphingobacteriaceae*) of the class *Alphaproteobacteria* and *Psychrobacter* (the family *Moraxellaceae*) and *Vibrio* (the family *Vibrionaceae*) of the class *Gammaproteobacteria* (Table 1).

Screening of the isolates

Three different media were used for incubation-isolated strains with substrates for screening enzymatic activity (chapter Materials and methods). According to series of trial comparative experiments, all of the following tests were conducted with *Medium B content* (Materials and methods chapter) as the most optimal.

After the first screening, the strains that possessed enzymatic activity have been retested again in three experiments with each of the used substrates—carrageenans isolated from red algae *T. crinitus* and *C. armatus* (chapter Material and methods). There were the following samples of carrageenans: total polysaccharides from *C. armatus* and also total polysaccharides from *T. crinitus* and their gelling fraction having the chemical structure of κ/β-carrageenan, which was determined earlier (Yermak et al. 1999).

Chemical composition and structures of individual fractions (disaccharide repeating unit) of used carrageenan samples and their viscosity average molecular weights are listed in Table 2.

The total carrageenan from *C. armatus* contains gelling fraction belonged the κ-carrageenan structure and non-gelling fraction having the λ-carrageenan structure (Yermak et al. 1999). Non-gelling fraction of polysaccharide from *T. crinitus* has a chemical structure which was determined first by us earlier in PIBOC FEB RAS and named as x-carrageenan (Barabanova et al. 2005; 2008).

All carrageenan samples presented in used fraction differ by number and position of sulfated groups and by the presence (κ, κ/β, and x) or absence (λ) of a 3,6-anhydrogalactose unit (Table 2).

Neighbor-joining phylogenetic tree is based on the 16S rRNA gene sequences showing the position of strain *Amylibacter ulvae* 6Alg 255^T and representative members of the family *Rhodobacteraceae* within the class *Alphaproteobacteria*. The asterisks indicate branches that were also recovered using maximum likelihood tree. Bootstrap values of > 60% based on 1000 replicates are indicated at branch points.

The data on the quantitative determination of used strain enzymatic activity to selected substrates are listed in Table 3. The data presented here were obtained as a result of a series of experiments. Taxonomic positions of the active strains are shown in Figs. 1, 2, and 3.

As can be seen from Table 3, some strains exhibited hydrolytic activity selectively to a total polysaccharide from *C. armatus*, another strains, to κ/β-carrageenan from *T. crinitus*, wherein some of those did not possess the same effect to a total polysaccharide from *T. crinitus* used as a substrate. Taxonomic position strains specific to a different type of substrate are shown in Figs. 1, 2, and 3.

So, 8 strains specific to a total polysaccharides from *C. armatus* (Fig. 1) were referred to members of the phylum *Bacteroidetes*, such as *Cellulophaga*, *Maribacter*, *Zobellia*, and *Aquimarina*, and 4 strains were affiliated with the two classes of the phylum *Proteobacteria*: A*lphaproteobacteria* (*Phaeobacter*, *Sphingomonas*, and *Sulfitobacter*) and *Gammaproteobacteria* (*Vibrio*).

The strains specific to κ/β-carrageenan from *T. crinitus* (Fig. 2) are referred as in the case of total polysaccharides from *C. armatus* to the phylum *Bacteroidetes*, genera *Aquimarina*, and *Cellulophaga*.

In the case of total polysaccharides from *T. crinitus* (Fig. 3), the strains having the most hydrolytic activity are referred to the phylum *Bacteroidetes* (genera *Aquimarina*, *Cellulophaga*, and *Maribacter*), and also to the phylum *Proteobacteria*, class *Alphaproteobacteria*: *Altererythrobacter* and *Tateyamaria*, and the representative of the class *Gammaproteobacteria*, the genus *Psychrobacter*.

Therefore, the carrageenan-specific enzymes were revealed in marine bacteria from two phylogenetic groups: the phyla *Bacteroidetes* and *Proteobacteria*.

Discussion

This study describes the taxonomic diversity of carrageenan-degrading bacteria isolated from the red alga *T. crinitus* collected from the Troitsa Bay, Gulf of Peter the Great, Sea of Japan,

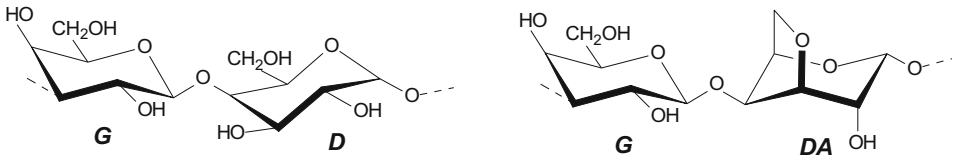
Table 1 Similarity of 16S rRNA gene sequences of selected bacterial isolates from the red alga *T. crinitus* to the sequences from the type strains of the recognized species in NCBI database

Phylogenetic group	Genus	Strain number	Nearest validly published phylogenetic neighbor	16S rRNA gene similarity (%)	Substrate (carrageenan)		
					C. armatus total	*T. crinitus* total	*T. crinitus* κ/β
Bacteroidetes							
Flavobacteriia							
Flavobacteriaceae							
	Aquimarina	9Alg 79	*Aquimarina aggregata*	98.2		+*	+
		9Alg 80	*Aquimarina aggregata*	98.2	+		+
		9Alg 103	*Aquimarina aggregata*	98.2		+	
		9Alg 196	*Aquimarina aggregata*	98.2			+
	Cellulophaga	9Alg 17	*Cellulophaga baltica*	99.8			+
		9Alg 50	*Cellulophaga baltica*	99.8	+		
		9Alg 57	*Cellulophaga baltica*	99.8	+		+
		9Alg 104	*Cellulophaga baltica*	99.8		+	
		9Alg 173	*Cellulophaga baltica*	99.8			+
		9Alg 188	*Cellulophaga baltica*	99.8			+
		9Alg 211	*Cellulophaga baltica*	98.2			+
		9Alg 1	*Cellulophaga lytica*	99.9	+		+
		9Alg 4	*Cellulophaga lytica*	99.9	+		+
		9Alg 241	*Cellulophaga lytica*	99.9	+		
	Maribacter	9Alg 94	*Maribacter aestuarii*	97.7	+		
		9Alg 96	*Maribacter arcticus*	96.2		+	
		9Alg 97	*Maribacter vaceletii*	99.4		+	
		9Alg 109	*Maribacter vaceletii*	99.4		+	
		9Alg 115	*Maribacter vaceletii*	99.4		+	
		9Alg 116	*Maribacter vaceletii*	99.4		+	
	Zobellia	9Alg 68	*Zobellia russellii*	99.9	+		
Proteobacteria							
Alphaproteobacteria							
Erythrobacteriaceae							
	Altererythrobacter	9Alg 117	*Altererythrobacter ishigakiensis*	98.0		+	
Rhodobacteraceae	*Phaeobacter*	9Alg 58	*Phaeobacter gallaciensis*	99.8	+		
	Sulfitobacter	9Alg 83	*Sulfitobacter marinus*	99.8	+		
	Tateyamaria	9Alg 99	*Tateyamaria omphalii*	98.2		+	
Sphingomonadaceae	*Sphingomonas*	9Alg 62	*Sphingomonas hankookensis*	99.1	+		
Gammaproteobacteria							
Moraxellaceae							
	Psychrobacter	9Alg 101	*Psychrobacter piscatorii*	99.7		+	
Vibrionaceae	*Vibrio*	9Alg 45	*Vibrio jasicida*	99.9	+		

*+ the strain possesses enzymatic activity to the mentioned substrate

Table 2 Chemical composition, structures of individual fractions (disaccharide repeating unit) of used carrageenan samples and their molecular weight

Algal species	Polysaccharide composition	Structure of disaccharide repeating unit		Molecular weight (total)
		3-linked	4-linked	
C. armatus	λ-carrageenan	G2S	D2S, 6S	250g mol^{-1}
	κ-carrageenan	G4S	DA	
T. crinitus	κ/β-carrageenan	G4S/G	DA/DA	400 (350*) g mol^{-1}
	x-carrageenan	G2S, 4S	DA	



*Molecular weight of κ/β-carrageenan

The letter code nomenclature of disaccharide repeating unit is according to Knutsen et al. (1994)

Russia. Based on the 16S rRNA data, the 106 algal isolates were affiliated to 33 genera of the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* within the domain Bacteria. Our results are correlated with known literature data. Thus, it have been shown that carrageenan-degrading enzymes, namely carrageenases, have been produced by marine bacteria associated with brown and red algae belonging mainly to the two distantly related lineages, *Proteobacteria* and *Bacteroidetes*, although most of these enzymes are included in the former group (Michel et al. 2006; Chauhan and Saxena 2016). Among marine bacteria, the sources of carrageenases are mainly Gram-negative bacteria affiliated with the genera *Cellulophaga* (Michel et al. 2006; Martin et al. 2015, 2016; Chauhan and Saxena 2016), *Pedobacter* (Sun et al. 2014), *Maribacter*, *Algibacter*, *Cytophaga* (Sarwar et al. 1987; Potin et al. 1991; Michel et al. 2006), *Tamlana* (Feixue et al. 2010), and *Zobellia* (Groisillier et al. 2015; Martin et al. 2016) within the class *Flavobacteria* from one side, and *Pseudoalteromonas* (Michel et al. 2006; Ohta and Hatada 2006; Guibet et al. 2007; Shangyong et al. 2013), *Pseudomonas* (Ostgaard et al. 1993; Khambhaty et al. 2007; Ziayoddin et al. 2012), *Alteromonas* (Michel et al. 2001a, 2001b), *Microbulbifer* (Khambhaty et al. 2007; Hatada et al. 2011; Tayco et al. 2013), *Vibrio* (Zhu and Ning 2016), *Cobetia*, *Shewanella* (Wang et al. 2015), *Colwellia*, *Marinomonas*, *Catenovulum* (Li et al. 2015), and *Paraglaceciola* within the class *Gammaproteobacteria* (Michel et al. 2001a, 2001b; Guibet et al. 2007; Martin et al. 2015; Chauhan and Saxena 2016).

According to our data, the strains phylogenetically related to species *Cellulophaga lytica* (5 strains) and *Cellulophaga baltica* (14) of the family *Flavobacteriaceae* showed the highest carrageenase activity. A number of algal polysaccharide-degrading enzymes, including carrageenases were previously found in *Cellulophaga* sp. (Michel et al. 2006; Martin et al. 2015; Chauhan and Saxena 2016). In addition, the significant carrageenase activity of flavobacteria of genera *Maribacter* (4) and *Zobellia* (1) and proteobacteria *Sphingomonas*, and *Vibrio* in agreement with the results obtained by Martin et al. (2015, 2016) and Zhu and Ning (2016). Thus, a new κ-carrageenase was isolated from marine

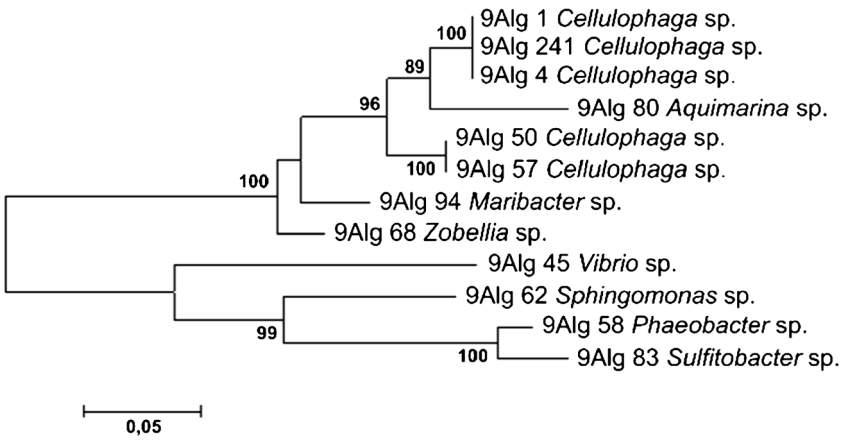


Fig. 1 Maximum likelihood phylogenetic tree based on the 16S rDNA genes showing the position of the strains degrading carrageenan from *C. armatus* (total). Bootstrap values of > 65% based on 1000 replicates are indicated at branch points. Bar, 0.01 substitutions per nucleotide position

Table 3 The quantitative determination of used bacterial strains enzymatic activity to selected substrates

Number of bacterial strains	Substrate (carrageenan)								
	Total (*C. armatus*) 250 g mol^{-1}			Total (*T. crinitus*) 400 g mol^{-1}			κ/β-carrageenan (*T. crinitus*) 350 g mol^{-1}		
	A_{700}	Molecular weight (g mol^{-1})	Decrease of molecular weight (%)	A_{700}	Molecular weight (g mol^{-1})	Decrease of molecular weight (%)	A_{700}	Molecular weight (g mol^{-1})	Decrease of molecular weight (%)
9Alg 211							0.559	2.9	99.2
9Alg 188							0.435	4.2	98.8
9Alg 173							0.372	5.3	98.5
9Alg 241	0.360	5.6	97.8						
9Alg 17							0.403	4.7	98.7
9Alg 4	0.349	5.9	97.7				0.366	5.4	98.4
9Alg 1	0.361	5.6	97.8				0.388	5.0	98.6
9Alg 57	0.362	5.5	97.8				0.408	4.6	98.7
9Alg 68	0.373	5.3	97.9						
9Alg 50	0.358	5.6	97.7						
9Alg 83	0.310	7.2	97.1						
9Alg 94	0.338	6.2	97.5						
9Alg 45	0.375	5.2	97.9						
9Alg *80*	0.426	4.3	98.3				0.468	3.8	98.9
9Alg 62	0.403	4.7	98.1						
9Alg 96				0.382	5.1	98.7			
9Alg 99				0.352	5.8	98.5			
9Alg 109				0.388	4.9	98.7			
9Alg 116				0.422	4.4	98.9			
9Alg 117				0.367	5.4	98.6			
9Alg 115				0.357	5.7	98.6			
9Alg *79/2*				0.715	2.1	99.5	0.484	3.6	99.0
9Alg 97				0.389	4.9	98.8			
9Alg 103				0.619	2.6	99.3			
9Alg 101				0.325	6.6	98.3			
9Alg 104				0.521	3.2	99.1			
9Alg *103/2*							0.378	5.2	98.5

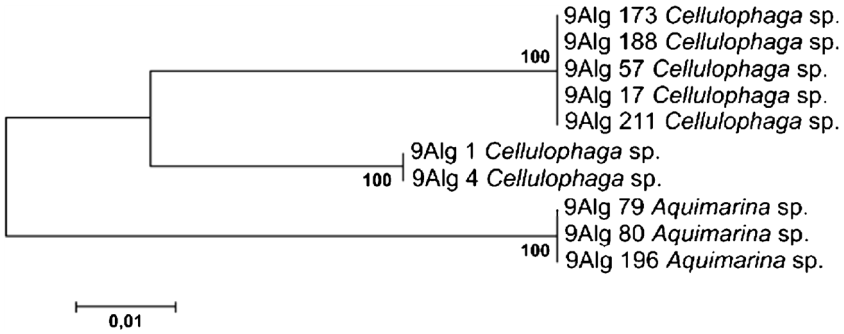


Fig. 2 Maximum likelihood phylogenetic tree based on the 16S rDNA genes showing the position of the strains degrading carrageenan from *T. crinitus* (*κ*/*β*). Bootstrap values of > 65% based on 1000 replicates are indicated at branch points. Bar, 0.01 substitutions per nucleotide position

bacterium *Vibrio* sp. strain NJ-2 associated with rotten red algae (Zhu and Ning 2016). In addition, from marine bacterium *Vibrio alginolyticus* was isolated multifunctional enzyme Amy63, which has hydrolytic activity toward different polysaccharides including agars and carrageenans (Liu et al. 2016). Also the enzymes with significant hydrolytic activity toward agar and various sulfated marine polysaccharides such as porphyran, fucoidan, and carrageenan were isolated from *Sphingomonas* sp. AS6330 collected from the Southeastern coast of Korea (Kim et al. 2004). Carrageenase activity also was identified for enzymes isolated from marine bacteria *Zobellia galactanivorans* and *Zobellia* sp. ZM-2 (Liu et al. 2013; Groisillier et al. 2015). We also have shown in the present study that some of the mentioned genera of marine bacteria associated with red alga *T. crinitus* collected from the Russian Coast of Japan Sea possessed a carrageenase activity.

It should be noted that the ability to degrade carrageenans by the strains belonging to the genera *Aquimarina* (the class *Flavobacteriia*), *Psychrobacter* (the class *Gammaproteobacteria*), and alphaproteobacteria *Altererythrobacter*, *Phaeobacter*, *Sulfitobacter*, and *Tateyamaria* were first found in the present study.

Moreover, it is important to notice that carrageenase activity firstly was revealed in genera *Aquimarina* (*Flavobacteriia*), *Psychrobacter* (*Gammaproteobacteria*), and *Altererythrobacter*, *Phaeobacter*, *Sulfitobacter*, and *Tateyamaria* (*Alphaproteobacteria*). The strain of *Aquimarina* possessed a hydrolytic activity against all used substrates (total polysaccharide from *C. armatus*, total polysaccharide from *T. crinitus*, and k/β-carrageenan from *T. crinitus*). It can be assumed that this strain produces λ, k-, and β-carrageenase. *Tateyamaria* and *Altererythrobacter* strains have activity toward total polysaccharide from *T. crinitus*; most likely these strains produce different types of carrageenanolytic enzymes. It is known that usually the bacterial strains produce a group of enzymes with a similar mechanism of action (Lemoine et al. 2009); however, at the same time, the enzyme degrading-related group of polysaccharides may refer to different families of hydrolases (Barbeyron et al. 1998; Michel et al. 2006; Rebuffet et al. 2010). For example k-carrageenases (E.C. 3.2.1.83) belong to the GH16 glycoside hydrolase family, a polyspecific family which includes about eight different enzymatic activities, together with β-agarases (http://afmb.cnrs–75 mrs.fr/CAZY/) (Coutinho and Henrissat 1999). In addition, phylogenetic analysis has demonstrated that the GH16 family of enzymes evolved from a common ancestor and that k-carrageenases were formed from the β-agarase branch (Barbeyron et al. 1998). At the same time, ι-carrageenases are included in the unrelated family GH82 (Michel et al. 2006; Rebuffet et al. 2010), while λ-carrageenases constitute a new GH family, although it has not been yet classified in the carbohydrate-active enzymes database (Barbeyron et al. 1998).

According to our results, four strains are selected as the most effective ones to hydrolysis of carrageenan. There are 9Alg 211, 9Alg 188, 9Alg 17, 9Alg 79/2, and 9Alg 103/2 because they possessed the highest hydrolytic action for k/β-carrageenan. While 9Alg 211, 9Alg 188, and 9Alg 17 belonged to the genus

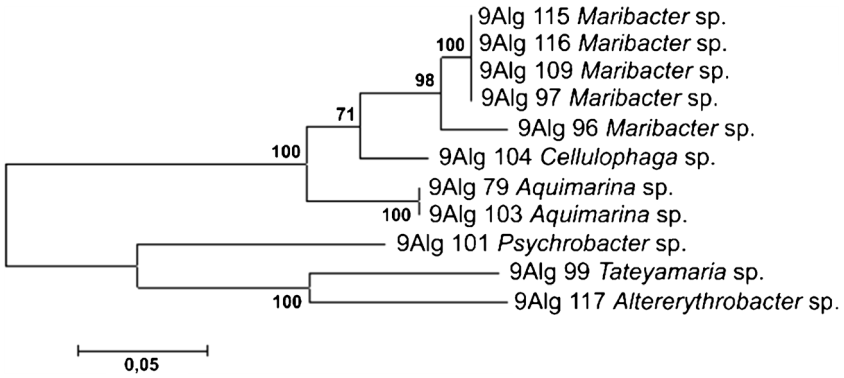


Fig. 3 Maximum likelihood phylogenetic tree based on the 16S rDNA genes showing the position of the strains degrading carrageenan from *T. crinitus* (*total*). Bootstrap values of > 65% based on 1000 replicates are indicated at branch points. Bar, 0.01 substitutions per nucleotide position

Cellulophaga, strains 9Alg 79/2 and 9Alg 103/2 were the representatives of the genus *Aquimarina* which firstly was found as a perspective source of carrageenanolytic enzymes. Strain 9Alg 79/2, in addition, demonstrated a high activity to the total polysaccharide from *T. crinitus*. This fact let us to purpose that this strain produces k-carrageenase and may be β-carrageenase and also carrageenase with hydrolytic action toward a non-gelling fraction (iks–carrageenan) of polysaccharide from *T. crinitus*.

In conclusion, among 106 isolated bacterial strains, 28 from them possessed the significant ability to degrade carrageenans. As was shown by the 16S rRNA gene sequence techniques, the studied strains were referred to the phyla *Bacteroidetes* and *Proteobacteria*. The most strains (21 strains) with enzyme activity belonged to the phylum *Bacteroidetes*. All strains were affiliated with 7 phylotypes of the genera *Aquimarina*, *Cellulophaga*, *Maribacter*, and *Zobellia* belonging to a single family *Flavobacteriaceae* of the class *Flavobacteriia*. The strains related to the phyla *Proteobacteria* were represented by members of the genera *Altererythrobacter* (the family *Erythrobacteraceae*), *Phaeobacter*, *Sulfitobacter*, *Tateyamaria* (the family *Rhodobacteraceae*), and *Sphingomonas* (the family *Sphingobacteriaceae*) of the class *Alphaproteobacteria* and *Psychrobacter* (the family *Moraxellaceae*) and *Vibrio* (the family *Vibrionaceae*) of the class *Gammaproteobacteria*. This is the first investigation of carrageenan-degrading bacteria associated with red alga, and the results obtained herein suggest that they represent a potential source of carrageenases for a possible biotechnological employment (application).

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