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EXPERIMENTAL ARTICLES

Screening of Marine Bacteria for Fucoidanases

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Abstract—Twenty-five strains of epiphytic marine bacteria isolated from the brown algae *Fucus evanescens* and *Chorda filum* and fifty-three bacteria isolated from the sea cucumber *Apostichopus japonicus* were screened for fucoidanases using fucoidans prepared from the brown algae *F. evanescens, Laminaria cichorioides*, and *L. japonica.* Eighteen bacterial epiphytes and thirty-eight bacterial isolates from the sea cucumber were found to contain fucoidanases, which were able to hydrolyze either all of the fucoidans studied or some of them. Bacteria of the genera *Cytophaga* and *Alteromonas/Pseudoalteromonas* exhibited the highest fucoidanase activities, which, however, did not exceed the activity of fucoidanases from the already known sources.

Key words: marine bacteria, brown algae, sea cucumbers, fucoidans, fucoidanases

Fucoidans are widespread among marine macrohydrobionts. In brown algae, they primarily play the role of the cell-wall matrix polysaccharides [1]. Fucoidans have also been found in the envelope of sea cucumbers [2]. In sea urchins, they are involved in the sperm–egg adhesion [3]. Fucoidans exhibit a wide range of activities toward different mammalian organs [3–5]. The biological activity of fucoidans is evidently determined by their specific chemical structure: fucoidans represent a family of homo- and heteropolysaccharides composed mainly of fucose residues sulfated at positions 2 and/or 4 and bound by α-1,2- or α-1,3-*O*-glycosidic bonds [6, 7]. In addition to fucose, fucoidans may also contain mannose, xylose, galactose, uronic acids, and rhamnose. The detailed chemical structure of fucoidans remains unknown.

Investigation of fucoidanases may provide insight into the structure and mechanism of the biological activity of fucoidans. Data available in the literature mainly concern the fucoidanases of molluscs [8, 9] and echinoderms [10], whereas information on microbial fucoidanases is scarce [11].

The aim of the present work was to screen marine bacteria for the efficient producers of fucoidanases.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. The isolates of marine bacteria studied in the present work were obtained during scientific offshore expeditions aboard the research vessel *Academician Oparin* in the Sea of Okhotsk near the Kuril islands of Paramushir, Onekotan, and Iturup, as well as at the Marine Experimental Station of the Pacific Institute of Bioorganic Chemistry (PIBOC) in the Peter the Great Bay. At present, these isolates are stored in the PIBOC Collection of Marine Microorganisms (PIBOC CMM).

The isolation and identification of marine bacteria from the brown algae *Fucus evanescens* and *Chorda filum* and the sea cucumber *Apostichopus japonicus* were described elsewhere [12].

Bacterial strains for the primary screening of fucoidanase producers were grown on a shaker (160 rpm) at 28°C for 24 h in a medium containing (g/l sea water) Difco bactopeptone, 5.0; Difco yeast extract, 2.0; fucoidan 1.0; K_2HPO_4, 0.2; and $MgSO_4$, 0.05 (pH 7.5–7.8).

Preparation of bacterial extracts. Bacterial cells (0.5 g) were suspended in 1 ml of 0.05 M phosphate buffer (pH 7.2) and disrupted by sonication in a UZDN-2 ultrasonic disintegrator. The cell homogenate was centrifuged at 10000 *g* for 30 min, and the supernatant was assayed for fucoidanase activity using fucoidans F_1, F_2, and F_3 prepared from the brown algae *F. evanescens, Laminaria cichorioides*, and *L. japonica*, respectively [13].

Isolation and characterization of fucoidans. Ground wet or frozen seaweeds were treated with ethanol and acetone and then extracted with 0.4% HCl at 20–25°C (cold extraction) and with hot water at 60–70°C (hot extraction). The cold- and hot- extracted polysaccharides were subjected to hydrophobic chromatography. The polysaccharides adsorbed on hydrophobic adsorbent were eluted with water and then with a gradient of ethanol in water. Fucoidans that were

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Table 1. Some relevant characteristics of fucoidans used as enzyme substrates

Fucoidan	Carbohydrate content, % of dry wt	Molecular mass, kDa	Carbohydrate composition, %	Fuc/SO_4 mol. ratio	IR(SO_4) maximum, cm^{-1}
F_1 from *F. evanescens*	28	300–500	Fuc, Gal, Xyl, 87 : 1 : 10	1 : 0.8	820
F_2 from *L. cichorioides*	30	60–80	Fuc, Gal, Xyl, GlcUa 82 : 10 : 2 : 5	1 : 1.7	842
F_3 from *L. japonica*	22	28–30	Fuc, Gal, Man, Xyl 42 : 40 : 9 : 2	1 : 0.9	842

Table 2. Specific fucoidanase activity* of marine bacteria isolated from the brown alga *F. evanescens*

Taxonomic position of strain	Strain	Fucoidan used as enzyme substrate		
		F_1 from *F. evanescens*	F_2 from *L. cichorioides*	F_3 from *L. japonica*
Cytophaga sp.	1F2	0	0	3510
Cytophaga sp.	2F3	0	0	2980
Cytophaga sp.	2F5	0	0	2190
Cytophaga sp.	2F7	0	4000	4000
Cytophaga sp.	2F9B	660	4980	1010
Cytophaga sp.	2F13	1140	12550	2140
Cytophaga sp.	2F16	600	4120	1520
Cytophaga sp.	12F2	0	6220	0
Cytophaga sp.	12F5	0	0	17680
Cytophaga sp.	12F9	0	8590	8590
Flexibacter/Cytophaga	12F8	3920	9650	4130
Flexibacter sp.	12F6	0	0	6710
Pseudoalteromonas sp.	2F10	710	12090	37360
Pseudoalteromonas sp.	12F1	220	8600	0
Pseudoalteromonas sp.	12F3	370	8680	0
Pseudoalteromonas sp.	1F5	1490	19650	8940
Pseudoalteromonas sp.	20-92	5290	3640	–
Pseudoalteromonas sp.	20-101-1**	2130	2630	–

* Specific activity was expressed as enzymatic units per 1 g of wet bacterial biomass.

** This strain was isolated from the brown alga *Chorda filum.*

eluted with water were precipitated with 80% ethanol and reprecipitated with 60% ethanol.

The molecular mass of fucoidans was determined by gel filtration on Sephadex G-50 and Sepharose CL-4B columns (100 × 1 cm; water, 15 ml/h) using 10-, 20-, 40-, and 80-kDa dextrans as molecular weight markers. Detection of polysaccharides in fractions was performed colorimetrically with the phenol–sulfuric acid reagent [14].

The carbohydrate composition of fucoidans was determined after hydrolyzing them in 4 N HCl at 100°C for 2 h. The hydrolysates were analyzed on a Biotronik carbohydrate analyzer (0.63 × 30 cm Durrum X4-20 column; 60°C; bicinchoninic acid reagent; Shimadzu CR 2AX detector) using the authentic samples of fucose (Fuc), glucose (Glu), galactose (Gal), mannose (Man), xylose (Xyl), and rhamnose (Rha) purchased from Serva (Germany) as reference standards.

Sulfate groups were determined turbidimetrically after the hydrolysis of fucoidans in 4 N HCl and the addition of a $BaCl_2$ suspension in gelatin. The IR spectra of fucoidans were recorded on a Carl Zeiss IR-75 spectrophotometer. Some relevant characteristics of the fucoidans used in this study are presented in Table 1.

Assay of the fucoidanase activity of bacterial cell extracts. A solution (0.4 ml) containing 4 mg of fucoidan in 0.05 M phosphate buffer (pH 7.2) was mixed with 0.1 ml of bacterial cell extract and incubated at 20°C for 20 h. Fucoidanase activity was estimated from the increase in the reducing sugar content of the reaction mixture. The concentration of reducing sugars was determined by the Nelson–Somogyi method [15]. One unit of fucoidanase activity was defined as the amount of enzyme releasing 1 nmole of fucose from fucoidan in 20 h.

Table 3. Fucoidanase activity of marine bacteria isolated from the sea cucumber *A. japonicus*

Taxonomic position of strain	Strain	Fucoidan used as enzyme substrate	
		F_1 from *F. evanescens*	F_2 from *L. cichorioides*
Acinetobacter sp.	M-3HL-8/1	0	240
Alteromonas/Pseudoalteromonas	M-3HB-8/1	1150	230
Alteromonas/Pseudoalteromonas	M-3HB-8/2	40	1270
Alteromonas/Pseudoalteromonas	M-3HB-9	420	2940
Alteromonas/Pseudoalteromonas	M-3HB-15/3	1540	5300
Alteromonas/Pseudoalteromonas	M-3HB-17/1	330	1260
Alteromonas/Pseudoalteromonas	M-3HB-18	1010	3150
Alteromonas/Pseudoalteromonas	M-3HB-19	0	1530
Alteromonas/Pseudoalteromonas	M-3HL-6/2	3180	1060
Alteromonas/Pseudoalteromonas	M-3HL-14/2	2420	0
Alteromonas/Pseudoalteromonas	M-3HL-14/3	1520	450
Alteromonas/Pseudoalteromonas	M-3HL-18/1	0	2000
Alteromonas/Pseudoalteromonas	M-3HL-21/1	220	60
Bacillus sp.	M-3HL-1/2	5790	2320
Coryneforms	M-3HB-14/2	370	2280
Coryneforms	M-3HB-25/2	930	2190
Coryneforms	M-3HB-27/1	2010	1590
Coryneforms	M-3HB-27/2	0	810
Coryneforms	M-3HL-5/2	1520	0
Cytophaga sp.	M-3HB-28/1	170	120
Flavobacterium sp.	M-3HB-26	0	1040
Halomonas sp.	M-3HL-13/2	990	610
Halomonas sp.	M-3HL-17/3	330	910
Pseudoalteromonas sp.	M-3HB-2	2230	1480
Pseudomonas/Halomonas	M-3HB-14/1	1230	4960
Pseudomonas/Halomonas	M-3HB-15/2	0	1310
Pseudomonas sp.	M-3HL-7/1	3060	2350
Pseudomonas sp.	M-3HL-9	1470	680
Micrococcus sp.	M-3HB-15/1	720	1980
Micrococcus sp.	M-3HB-25/3	0	1540
Vibrio sp.	M-3HB-3	450	1230
Vibrio sp.	M-3HB-11/2	970	1700
Vibrio sp.	M-3HL-6/3	0	120
Unknown	M-3HL-2	3360	3950
Unknown	M-3HL-7/2	3350	340
Unknown	M-3HB-15/4	660	950
Unknown	M-3HB-16/1	0	340
Unknown	M-3HB-16/2	440	630

RESULTS AND DISCUSSION

Marine microorganisms are promising sources of valuable biologically active compounds, including rare enzymes [16, 17]. Some marine bacteria are able to degrade the insoluble polysaccharides chitin and agar, as well as the cell-wall polysaccharides of seaweeds [18] and other organisms. When damaged during storms, brown seaweeds become the subject of attack by epiphytic bacteria, which possess the necessary

Table 4. Taxonomic composition of fucoidan-degrading marine bacteria

Taxon	Number of strains isolated from					
	sea cucumber		sea alga		total	
	all	active	all	active	all	active
Acinetobacter	1	1	–	–	1	1
Alteromonas/Pseudoalteromonas	23	13	8	6	31	19
Bacillus	1	1	–	–	1	1
Coryneforms	6	4	–	–	6	4
Cytophaga/Flexibacter	2	1	12	12	14	13
Flavobacterium	1	1	–	–	1	1
Micrococcus	4	3	1	0	5	3
Pseudomonas/Halomonas	6	6	–	–	6	6
Vibrio	4	3	2	0	6	3
Unidentified	6	5	2	0	8	2
Total	53	38	25	18	78	56

enzymatic systems capable of degrading algal cell-wall polysaccharides to mono- and oligosaccharides. These saccharides are utilized not only by epiphytic bacteria themselves, but also by other marine microorganisms. Glycosidases are often produced by symbiotrophic bacteria. For instance, bacteria of the genus *Vibrio* associated with the brown alga *Laminaria longicruris* are able to hydrolyze laminarin [19], whereas bacteria isolated from the red algae *Polisiphonia lanosa, Hypnea charoides*, and *Gracilaria gracilis* are able to hydrolyze agar [20, 21].

These data suggest that the seaweeds under study can also be a source of nutrients for bacterial epiphytes. On the other hand, the envelope of sea cucumbers contains fucoidans [2]; therefore, the associated bacteria may possess enzymes capable of degradating these polysaccharides. For these reasons, screening for fucoidanases was primarily carried out among the marine bacteria isolated from the brown alga *F. evanescens* (Table 2) and from the coelomic liquid and homogenates of the sea cucumber *A. japonicus* (Table 3), which is abundant in the Peter the Great Bay of the Sea of Japan.

Twenty-three of the twenty-five strains of heterotrophic bacteria isolated from the brown algae *F. evanescens* and *Chorda filum* were identified to the genus level (Table 3). Similar to other marine bacteria [22], the majority of the epiphytes isolated turned out to be gram-negative aerobic rods.

All of the strains studied, except 20-92 and 20-101/1, were analyzed for the presence of fucoidanases using the hot-extracted fucoidans F_1, F_2, and F_3 of the brown algae *F. evanescens, L. cichorioides*, and *L. japonica*. Eighteen of the twenty-five bacteria associated with the brown seaweeds were able to hydrolyze fucoidans (Table 4). All of the bacteria of the genus *Cytophaga* under study exhibited fucoidanase activity (Table 2). Five of the twelve bacterial strains of this genus produced fucoidanases that were able to hydrolyze only fucoidan F_3. The fucoidanase of *Cytophaga* sp. 12F2 was specific for fucoidan F_2, and the fucoidanases of *Cytophaga* sp. 2F7 and 12F9 were specific for fucoidans F_2 and F_3. The fucoidanases of *Cytophaga* spp. 2F9B, 2F13, 2F16, and 12F8 exhibited the widest substrate specificity: they were able to degrade all three fucoidans under study.

Six of the eight bacterial epiphytes of the genus *Pseudoalteromonas* also possessed fucoidanase activity (Table 2). The fucoidanase of *Pseudoalteromonas* sp. 12F1 was able to hydrolyze only fucoidan F_3, whereas the fucoidanases of other strains of this species could hydrolyze all three fucoidans (it should be noted that the fucoidanase activity of these strains was higher than that of the fucoidan-degrading *Cytophaga* sp. None of the bacterial strains belonging to the genera *Vibrio* and *Micrococcus* could degrade fucoidans. Fifty-three bacterial strains associated with the sea cucumber *A. japonicus* were tested for fucoidanase activity using two fucoidans, F_1 and F_2 (Table 3). This bacterial group was dominated by gram-negative aerobic rodlike bacteria of the genera *Alteromonas/Pseudoalteromonas*. Some fucoidanase-producing bacteria of this group belonged to coryneforms and to the genera *Vibrio, Micrococcus*, and *Halomonas* (Tables 3 and 4). The thirty-eight bacterial associants of *L. japonica* possessed fucoidanase activity, of which twenty-eight strains, belonging mainly to *Alteromonas/Pseudoalteromonas*, could hydrolyze both fucoidans F_1 and F_2, nine bacterial strains could hydrolyze fucoidan F_2, and two strains, *Cytophaga* sp.

M3HL5/2 and *Alteromonas/Pseudoalteromonas* sp. M3HL14/2, could hydrolyze fucoidan F_1. Strains M3HB2, M3HB9, M3HB14/1, M3HB15/3, and M3HL6/2 were the best producers of fucoidanases among the bacterial associants of the sea cucumber *A. japonicus*.

It should be noted that the fucoidanase activity of the most efficient fucoidan-degrading bacterial strains studied by us was only two–four times lower than that of *Vibrio* sp. 5, the proposed producer of fucoidanases [11]. However, the efficiency of this fucoidanase producer is much less than the efficiency of the known superproducers of laminarinases, amylases, cellulases, and other glycosidases.

The different activities of fucoidanases toward the three fucoidans studied are most likely due to the different structures of these substrates (see Table 1). It can be seen that fucoidans F_1, F_2, and F_3 considerably differ in molecular mass, carbohydrate composition, and the content of sulfate groups. Some of the fucoidanases studied were highly specific for particular fucoidans (Table 2). The relatively great number of bacterial strains that are able to hydrolyze fucoidan F_3 can be explained by the fact that this polysaccharide contains, in addition to fucose (42%), a large amount of galactose (40%). As a result, fucoidan F_3 can be hydrolyzed not only by fucoidanases but also by galactanases and galactosidases present in bacterial extracts.

The high percentage of fucoidan-degrading bacteria among the 78 bacterial strains studied (72% of strains isolated from brown seaweeds and 70.4% of strains isolated from sea cucumbers) indicates the nutritional importance of fucoidans for the bacterial assassinates of macrohydrobionts. The fucoidan-degrading ability may be part of the adaptive response of microorganisms colonizing seaweeds and various marine animals to the environment, through which these microorganisms satisfy their requirements for carbon, sulfur, and energy. The abundance of fucoidan-degrading marine bacteria and their low fucoidanase activity may be due to the specific role of fucoidans and fucoidanases in the metabolism of marine organisms.

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