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Short Communication

Isolation and Characterization of a Fucoidan-Degrading Marine Bacterium

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Abstract: Fucoidan, a mixture of sulfated fucose-containing polysaccharides, was prepared from the algal bodies of *Cladosiphon okamuranus* (class Phaeophyceae, order Chordariales, family Chordariaceae) with a yield of 2.0% of the wet weight of the alga. To obtain enzymes that digest the fucoidan, we screened bacteria in the gut contents of the sea cucumber *Stichopus japonicus* for their ability to decrease the fucoidan in their culture media, and successfully isolated one bacterial strain that could decrease it. The bacterial strain was gram-negative and possessed menaquinone 7 as the predominant respiratory quinone, and the GC content of its genomic DNA was 52%. The results of the phylogenetic analysis of its 16S ribosomal DNA sequence indicated that the bacterial strain was a member of the division "Verrucomicrobia." However, as the bacterial strain is phylogenetically and phenotypically distinct from verrucomicrobial species described previously, the strain was assumed to be a new member of the division "Verrucomicrobia." When the bacterial strain was cultivated in an algal fucoidan-containing medium, the strain decreased fucoidan from *C. okamuranus* (44%), *Nemacystus decipiens* (19%), *Laminaria japonica* (31%), *Kjellmaniella crassifolia* (23%), sporophyl of *Undaria pinnatifida* (22%), *Fucus vesiculosus* (42%), and *Ascophyllum nodosum* (61%).

Keywords: fucoidan-degrading marine bacterium, *Cladosiphon okamuranus*, fucoidan, sulfated fucose-containing polysaccharides, Verrucomicrobia.

INTRODUCTION

Cladosiphon okamuranus fucoidan shows medicinally useful activities, such as an antiulcer effect and an inhibitory effect on *Helicobacter pylori* infection (Shibata et al., 1998, 1999). However, its chemical structure was not elucidated yet; only its average structure has been reported (Nagaoka et al., 1999).

Many algal polysaccharides including sulfated fucose-containing polysacchrides (SFCPs) have repeating structural units (Chizhov et al., 1999; Chevolot et al., 2001; Sakai et al., 2003a); therefore, the endoglycosidases that digest SFCPs into their repeating units can be used as tools to analyze their structures (Sakai et al., 2003a). We isolated a marine bacterial strain that decreases *Kjellmaniella crassifolia* fucoidan in its medium (Sakai et al., 2002), and proved that the bacterial strain produces an endo-α-D-mannosidase that digests sulfated fucoglucuronomannan of *K. crassifolia* into 3 or more kinds of trisaccharide (Sakai et al., 2003a). Then the enzyme was purified and

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characterized as sulfated fucoglucuronomannan lyase (Sakai et al., 2003b). However, as the enzyme does not digest *C. okamuranus* fucoidan at all, it cannot be used as a tool for the analysis of its structure. In search for the endoglycosidases that digest the fucoidan, we examined as a potential source sea cucumbers that live on sea mud containing dead algae, hoping to find a bacterial strain that could degrade fucoidan. The novel isolate decreased not only *C. okamuranus* fucoidan but also fucoidans from various kinds of brown algae in its culture media, which indicated that the isolate must be a useful source for the endoglycosidases that digest fucoidans from various algae.

Here we report the properties of a newly isolated bacterial strain. Its phenotypic characteristics and the result of the phylogenetic analysis of its 16S ribosomal DNA sequence indicate that the strain is a new member of the division "Verrucomicrobia."

Materials and Methods

General Methods

Neutral sugars were assayed by phenol-H_2SO_4 reaction (Dubois et al., 1956), using L-fucose as the standard. Ultrafiltration was performed using the Model CH2 Ultrafiltration System equipped with Diaflo Hollow Fiber Cartridge, H1P100-20 (100-kDa cutoff, Millipore Co.). Centrifugation was performed at 11,000 *g* for 30 minutes.

Preparation of Algal Fucoidan

Unless indicated otherwise, all experiments were performed at room temperature. Cultured brown alga *Cladosiphon okamuranus* (class Phaeophyceae, order Chordariales, family Chordariaceae) was harvested in Okinawa, Japan, in February 1997, and immediately, about 700 kg of the algal bodies was salted with 100 kg of NaCl and preserved at 5°C. The salted alga (625 g) was homogenized with 8.4 L of 30 mM sodium phosphate buffer (pH 6.0), and the homogenate was incubated at 90°C for 2 hours with occasional stirring. The heat-treated homogenate was cooled to 30°C in an ice bath, 9 g of active carbon was added to it, and the mixture was stirred for 1 hour and centrifuged at 25°C to remove the added active carbon. The resulting supernatant was concentrated to 2 L, and its solvent was exchanged with 20 mM NaCl (pH adjusted to 8.0 with 1 N NaOH) by Ultrafiltration (100-kDa cutoff). The retained solution thus obtained (2.2 L), which contained fucoidan, was centrifuged at 25°C, and the supernatant was adjusted to pH 8.0 with 1 N NaOH and lyophilized to obtain dried fibrous fucoidan. All fucoidan from other algae were prepared as described previously (Sakai et al., 2002).

The neutral sugar composition of *C. okamuranus* fucoidan was analyzed by high-performance liquid chromatograph (HPLC) (Suzuki et al., 1991), and the absolute configuration of its major constituent monosaccharide, fucose, was determined by using the method described previously (Sakai et al., 2002). L-Fucose in the fucoidan was also assayed by cysteine-H_2SO_4 reaction (Dische and Shettles, 1948), using L-fucose as the standard. Uronic acid in the fucoidan was assayed by the carbazole reaction (Bitter and Muir, 1962), using D-glucuronic acid as the standard, and the uronic acid was identified by using the method described previously (Sakai et al., 2002). The amount of acetyl residues in this fucoidan was assayed using F-Kit acetic acid (Boehringer Mannheim). Briefly, 3 mg of the fucoidan was dissolved in 400 μl of 1 M NaOH and left for 17 hours at 25°C to hydrolyze *O*-acetyl residue in the fucoidan, and the solution was adjusted to pH 7.0 with 6 M HCl and used as the sample solution for the acetic acid assay. The amount of sulfate residues in the fucoidan was assayed by the $BaCl_2$-gelatin method (Dodgson and Price, 1962), using Na_2SO_4 as the standard. The average relative molecular mass of the fucoidan was estimated by HPLC using a size-fractionation column (OHpak SB-806HQ, 8.0 × 300 mm, Showa Denko Co.), and the fucoidan was eluted with 50 mM NaCl containing 5 mM sodium azide done at the flow rate of 1.0 ml/min at 25°C. Pullulans (P-82, Showa Denko) were used as the relative molecular mass standards, and a refractive index detector was used to detect these polysaccharides.

Isolation and Preliminary Characterization of Fucoidan-Degrading Bacterial Strain from Sea Cucumbers

Unless indicated otherwise, all water and all fucoidan used for the media of the microorganisms were artificial seawater (pH adjusted to 8.0 with 0.5 N NaOH) made from Jamarin S (Jamarin Laboratory) and *C. okamuranus* fucoidan prepared as described above, respectively; and the ingredients of the media were as follows: medium A, 0.02% fucoidan, 0.01% peptone, and 0.005% yeast extract; medium B, 0.02% fucoidan, 0.1% peptone, and 0.005% yeast

extract; medium C, 0.2% fucoidan, 0.01% peptone, and 0.005% yeast extract; medium D, 0.2% fucoidan, 0.2% peptone, and 0.01% yeast extract; these media were sterilized at 120°C for 20 minutes; and all incubations for cultivation of the microorganisms were done at 25°C with shaking.

Sea cucumbers *Sticopus japonicus* were harvested in Mutsu Bay, Aomori, Japan, in January 1998, and the gut contents of 2 sea cucumbers were collected separately as sources of the fucoidan-degrading microorganisms. Samples of 100 mg of the gut contents were inoculated into 5 ml of media A and B and cultivated for 7 days. Then, neutral sugars in these cultures were assayed by phenol-H_2SO_4 reaction to estimate the amount of the fucoidan decreased by the microorganisms during the cultivation, which showed that the fucoidan in media A and B was decreased by more than half. Therefore, 30 μl each of the cultivated media A and B was transferred into a test tube containing 5 ml of medium C, and the test tubes were incubated for 7 days. Neutral sugars in these cultures were assayed by phenol-H_2SO_4 reaction, which showed that the fucoidan in all these cultures was decreased by the microorganisms. Therefore, 30 μl of the cultivated medium (medium C) was transferred into a test tube containing 5 ml of medium D, and the test tubes were incubated for 7 days. Then, each of these cultures was diluted appropriately with sterilized artificial seawater and spread onto agar plates containing medium D, and the plates were incubated stationarily for 6 to 10 days. Then, each of the microbial colonies that appeared singly on the agar plates was picked by a platinum loop, inoculated into a test tube containing 5 ml of medium D, and cultivated for 2 to 3 days. The cultures thus obtained were kept at 5°C as the seed cultures of the microbial isolates.

In order to examine the fucoidan-degrading ability of the microbial isolates thus obtained, 30 μl of each of their seed cultures was transferred into a test tube containing 5 ml of medium D and cultivated for 3 to 5 days. Then neutral sugars in these cultures were assayed by phenol-H_2SO_4 reaction.

Of all the microbial isolates, the strain that most decreased the neutral sugars in its medium was selected as a microbial source of the fucoidan-digesting enzymes. The strain was a bacterial strain, SI-1234, and some of its bacteriological properties were examined by methods described previously (Collins and Jones, 1981; Krieg and Holt, 1984; Tamaoka and Komagata, 1984; Staley et al., 1989) at the Japan Food Research Laboratories (Tokyo).

Gene Sequencing and Phylogenetic Analyses

The 16S ribosomal DNA sequence of the bacterial strain SI-1234 was determined as follows. First, the 16S ribosomal DNA of the strain was amplified by polymerase chain reaction (PCR) directly from its bacterial cells by the method described previously (Saris et al., 1990), using bacterial 16S rDNA primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGCTACCTTGTTACGACTT-3′). The resulting PCR product was isolated by electrophoresis on a 1% low melting point agarose gel, and the amplified DNA thus isolated, about 1.5 kilobase pairs in length, was recovered using Easy Trap Version 2.0 (Takara Bio Inc.) according to the manufacturer's instructions. The amplified PCR product thus recovered was cloned into a plasmid vector, pT7Blue T-Vector (Novagen) according to the manufacturer's instructions. Then, using the plasmid vector thus obtained, competent *Escherichia coli* JM 109 cells (Takara Bio) were transformed and screened for the plasmid insertions by following the manufacturer's instructions. The transformed *E. coli* strain thus obtained was cultivated in 10 ml of L-broth (0.5% yeast extract, 1% peptone, and 0.5% NaCl) for 1 day, and its plasmid was purified using Miniprep DNA Purification Kit (Takara Bio) by following the manufacturer's instructions. Sequencing was performed on both strands of the cloned 16S rDNA using ABI 377 sequencer (Applied Biosystems Inc.), *Taq* Cycle Sequencing Core Kit (Takara Bio), and 6 kinds of DNA primers (*Bca*BEST Sequencing Primer M13-47, *Bca*BEST Sequencing Primer T7 (both from Takara Bio), and 4 other primers that were synthesized to sequence the 16S rDNA of the bacterial strain SI-1234). The resulting sequence, 1517 base pairs in length, was checked for similarities to DNA sequences in the database of DNA Data Bank of Japan (DDBJ) using a homology search program package, FASTA. Then a phylogenetic tree was constructed using 2 software packages, CLUSTAL W (Saitou and Nei, 1987; Thompson et al., 1994) and TREE VIEW (Page, 1996), to examine the phylogenetic positions of the bacterial strain SI-1234.

Results and Discussion

Preparation of Algal Fucoidan

The yield of the fucoidan from salted *C. okamuranus* was about 2.0% (wt/wt). The average relative molecular mass of

the fucoidan was estimated about 2×10^6 by size-fractionation HPLC. The analysis of neutral sugar composition of the fucoidan showed that fucose was its major neutral sugar, and it contained only trace amounts of xylose and mannose (data not shown). The absolute configuration of the fucose in the fucoidan was determined as levorotatory, based on the result that fucose content in the fucoidan assayed by cysteine-H_2SO_4 reaction and that assayed by using L-fucose dehydrogenase were nearly equal. Uronic acid was also detected in *C. okamuranus* fucoidan by carbazole reaction, and the uronic acid was identified as D-glucuronic acid by the results of sugar component analyses of the fucoidans before and after reduction of the carboxyl group of its uronic acid. The results of these analyses were as follows: D-glucose was not detected in nontreated *C. okamuranus* fucoidan but was detected in the fucoidan after reduction of the carboxyl group of its uronic acid, and the amount of D-glucose detected was nearly equal to the amount of uronic acid assayed by carbazole reaction in the nontreated fucoidan.

These results clearly showed that the uronic acid residue in *C. okamuranus* fucoidan was only D-glucuronic acid residue. These results and the results of the assays of sulfate and acetyl residues in the fucoidan showed that the molar ratio of the 4 residues L-fucose, D-glucuronic acid, sulfate, and acetyl residue was about 4:1:2:1. Prior to our study, Nagaoka et al. (1999) also extracted *C. okamuranus* fucoidan by diluted hydrochloric acid at 100°C for 15 minutes, and the molar ratio of the above 4 residues in their fucoidan was about 6:1:3:1. This ratio is slightly different from that in our fucoidan. However, a small difference between them would not directly reflect a difference in absolute structures of these 2 fucoidans because *C. okamuranus* fucoidan is a mixture of sulfated fucose-containing polysaccharides. One possible reason for the above difference between the 2 fucoidans is that a small amount of fucose-rich sulfated polysaccharides in the cell wall of the alga that was not extracted by our method (at pH 6.0) was partially degraded and extracted at low pH. The average relative molecular mass of our fucoidan (2×10^6) was much higher than that of their fucoidan (5.6×10^4). This large difference was caused by the difference of the conditions for its extraction because the fucoidan is not so stable against heat and acid. We extracted the fucoidan at pH 6.0 at 90°C in order not to degrade its native structure, so that the bacterial enzymes could recognize it, and our fucoidan was suitable for this purpose.

Isolation and Preliminary Characterization of Fucoidan-Degrading Bacterial Strain from Sea Cucumbers

Isolation

First, we tried A and B to cultivate the microorganisms living in the gut contents of the sea cucumbers. As they grew and decreased the fucoidan in their media, a portion of each of these cultures was transferred into medium C that contained 10 times as much fucoidan as that in media A and B so that the fucoidan-degrading microorganisms preferentially would grow. In medium C, although the microorganisms also decreased the fucoidan, they did not grow very well. To isolate a microorganism by using an agar plate, it should grow well and form a visible colony on the plate. Therefore, a portion of each of these cultures was transferred into medium D that contained 10 times as much peptone as that in medium C. Then, the cultivated medium D was spread onto agar plates, and the plates were incubated until many individual microbial colonies appeared. Then, 45 strains of microorganisms were isolated from the plates. Each of the microbial isolates was cultivated in medium D, and the amount of the fucoidan in the medium decreased by the isolate was estimated. Of the 45 isolates, the strain that decreased the fucoidan by the greatest amount (44%) was bacterial strain SI-1234, and it was selected as a bacterial source of the fucoidan-digesting enzymes.

The microorganisms in the gut contents of the sea cucumbers were first cultivated in media A and B, and their cultures were subcultured twice using other kinds of medium as described above. As a result, the bacterial strains that decreased the fucoidan in their media were isolated only from the subculture of the cultivated medium A. Probably such bacteria would grow preferentially in medium A rather in medium B because of the lower concentration of peptone. Conversely, higher concentrations of peptone might be necessary for the strain to form a visible colony on an agar plate. Actually, the cultivated media A and B described above were each spread onto agar plates containing media A and B, respectively, and 66 strains of microorganisms were isolated, but none of them could decrease the fucoidan in their medium, which indicated that the fucoidan-degrading microorganisms could not form a visible colony on these agar plates.

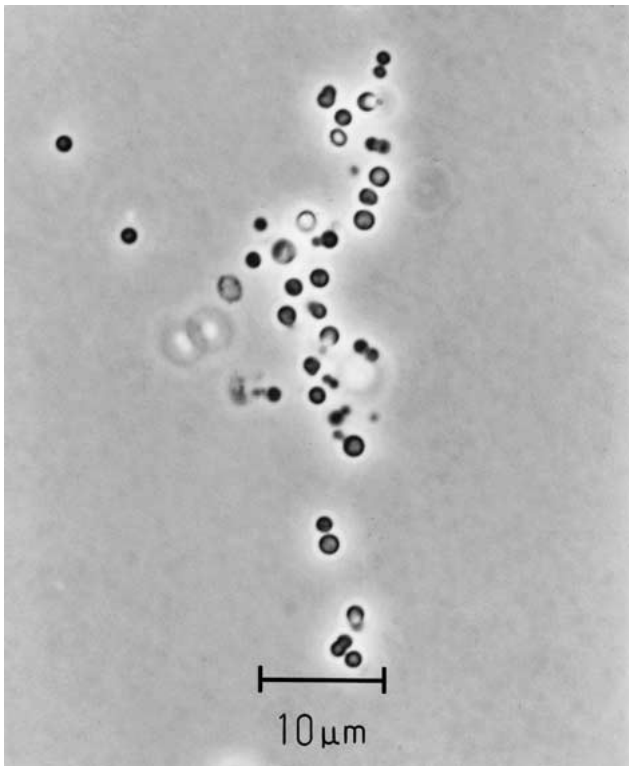

Figure 1. Microphotograph of the fucoidan-degrading bacterial strain SI-1234.

Characterization

In order to identify the bacterial strain SI-1234, some of its bacteriological properties were characterized. The cells of the strain were cocci, 1.2 to 1.6 μm in diameter (Figure 1). The strain was gram-negative and possessed menaquinone 7 as its predominant respiratory quinone, and the GC content of its genomic DNA was 52%. In addition to these properties, some other properties of the strain are shown in Table 1. However, considering those properties, the strain was not identified as a known species of bacteria (Holt et al., 1994). For example, the bacterial strains among Flavobacteriaceae are gram-negative, and some of them have menaquinones as their predominant respiratory quinones as in our strain; however, the GC contents of their genomic DNAs are more than 10% below that of our strain (Holt et al., 1994).

Gene Sequencing and Phylogenetic Analysis

The 16S ribosomal DNA sequence of the bacterial strain SI-1234, 1517 base pairs in length, was determined for phylogenetic analysis and deposited at the National Institute of Genetics (Shizuoka, Japan) under accession number AB073978. The search for the similarities between its 16S rDNA sequence and DNA sequences in the DDBJ database showed that there were not many phylogenetically related bacteria even including unculturable bacteria. Therefore, a phylogenetic tree was constructed including these unculturable bacteria, which indicated that our strain was a new member of the subdivision 4 of the division "Verrucomicrobia" (Figure 2). The division "Verrucomicrobia" is represented by only a few cultivated species, and the majority of its members are known only as 16S rDNA sequences recovered from a variety of habitats (Hedlund et al., 1997; Hugenholtz et al., 1998; Zwart et al., 1998; O'Farrell and Janssen, 1999). The closest cultivated relatives of the bacterial strain SI-1234 was *Opitutus terrae* (Chin et al., 2001). However, the percentage of similarity between the 16S rDNA sequence of our strain and those of the species *O. terrae* that have certain variations in 16S rDNA sequences (AJ229235, X99390, and X99392) ranged from 81.6% to 82.7%. These large phylogenetic distances suggested that our strain and *O. terrae* should not be included in the same genus. In addition to these phylogenetic distance, there were also 3 notable differences between our strain and *O. terrae*: the former is an aerobic bacterium, without a flagellum, and the GC content of its genomic DNA was 52%; the latter is an obligately anaerobic bacterium, with a flagellum, and the GC content of its genomic DNA was 74% (more than 20% above that of our strain). Thus, the phenotypic characteristics of our strain were quite different from those of *O. terrae*. Therefore, our strain was assumed to be a new member of the division "Verrucomicrobia." The novel bacterial strain was deposited at the National Institute of Biosciences and Human-Technology in August 1999 (Tsukuba, Japan; its current name is National Institute of Advanced Industrial Science and Technology) as *Fucophilus fucoidanolyticus* SI-1234 under accession number FERM BP-7495.

Degradation of Algal Fucoidans by Fucoidan-Degrading Bacterial Strain SI-1234

Fucoidan is a general name of various sulfated fucose-containing polysaccharides (SFCPs). Actually, the structures of fucoidans from different algae differ from each other (Fattah et al., 1974; Mori et al., 1982; Percival et al., 1984; Nishino and Nagumo, 1991; Fleury and Lahaye, 1993; Nagaoka et al., 1999; Pereira et al., 1999; Chevolot et al.,

Table 1. Characteristics of Fucoidan-Degrading Bacterial Strain SI-1234[a]

Test	Result	Test	Result
Cell shape	Cocci (1.2–1.6 μm)	Growth in 1% NaCl	−
Gram staining	—	Artificial seawater	+
Sporulation	—	Mol% of G+C	52
Motility		Oxidase	−
Flagellar	—	Catalase	+
Gliding	—	Color of colony	Transparent pale yellow to pale beige
Relation to oxygen	Aerobic		
Major quinone	Menaquinone-7	OF-test	O

[a] The properties of the bacterial strain SI-1234 were characterized at the Japan Food Research Laboratories (Tokyo). The bacterial strain was assumed to be a new member of subdivision 4 of the division "Verrucomicrobia" by the results of the phylogenetic analysis of its 16S ribosomal DNA sequence (Figure 2).

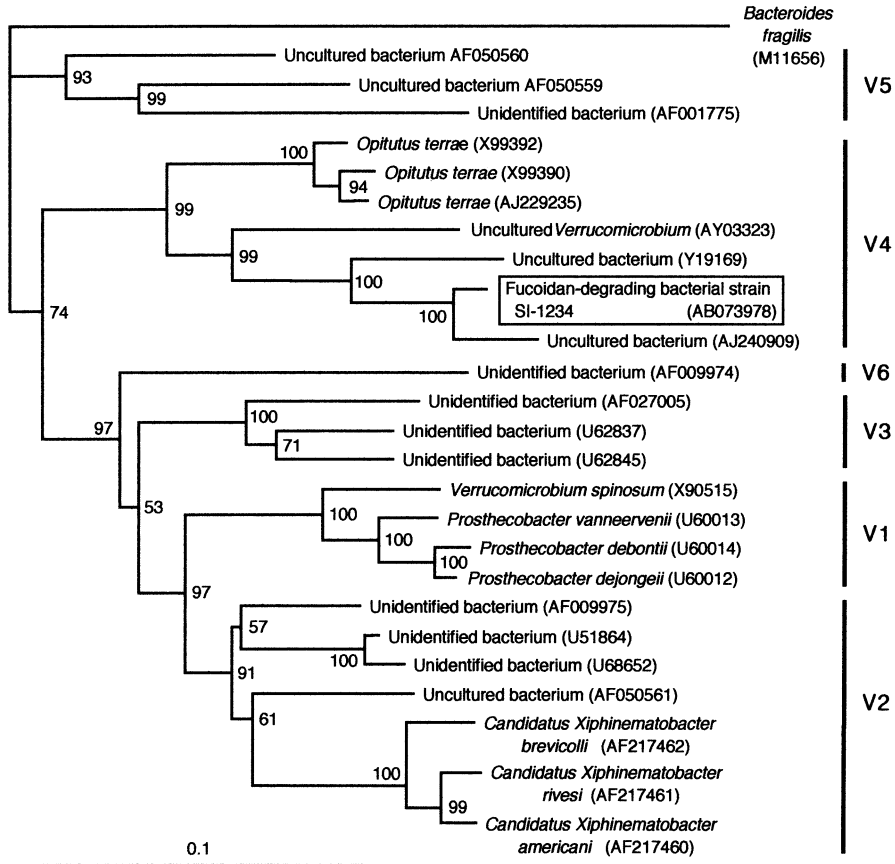

Figure 2. Phylogenetic relationships of 16S ribosomal DNA sequences of the fucoidan-degrading bacterial strain SI-1234 and other phylogenetically related bacteria. As there were not many bacteria phylogenetically related to strain SI- 1234, some unculturable bacteria were also included in the phylogenetic tree. *Bacteroides fragilis* was used as the outgroup. The scale bar represents 0.1 substitutions per base position. Numbers given at the nodes represent bootstrap values (%). V1 to V6 are verrucomicrobial subdivisions according to Hugenholtz et al. (1998) and Vandekerckhove et al. (2000).

2001; Sakai et al., 2003a). Although the bacterial strain SI-1234 was isolated as "*C. okamuranus* fucoidan-degrading bacterium," it may be able to degrade various kinds of fucoidans. If so, then it might be able to produce various kinds of fucoidan-digesting enzymes. Therefore, the fucoidan-degrading ability of bacterial strain SI-1234 was

examined using fucoidan preparations obtained from various algae, and the fucoidans in those media were decreased: *Nemacystus decipiens* (19%), *Laminaria japonica* (31%), *Kjellmaniella crassifolia* (23%), sporophyl of *Undaria pinnatifida* (22%), *Fucus vesiculosus* (42%), and *Ascophyllum nodosum* (61%). The results indicate that our strain is a possible bacterial source for many kinds of fucoidan-digesting enzymes. However, our strain did not decrease the SFCPs of the host sea cucumber in its medium, though the major SFCP of the sea cucumber is composed of α-L-fucosyl and sulfate residues like algal fucoidan (Pereira et al., 1999). These results indicate that the fucoidan-digesting enzymes of our strain strictly recognized the structural difference among various SFCPs.

In this report we have not presented any data about the fucoidan-digesting enzymes of bacterial strain SI-1234. However, the strain produces very useful enzymes for structural analyses of *C. okamuranus* fucoidan. In the next report, the chemical structures of the oligosaccharides obtained by digestion of *C. okamuranus* fucoidan with the intracellular enzymes of bacterial strain SI-1234 will be shown clearly (Sakai et al., 2003c).

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