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Isolation and Culture of a Marine Bacterium Degrading the Sulfated Fucans from Marine Brown Algae

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Abstract

Fucoidans are matrix polysaccharides from marine brown algae, consisting of an α-L-fucose backbone substituted by sulfate-ester groups and masked with ramifications containing other monosaccharide residues. In spite of their interest as biologically active compounds in a number of homologous and heterologous systems, no convenient sources with fucanase activity are available yet for the degradation of the fucalean algae. We here report on the isolation, characterization, and culture conditions of a bacterial strain capable of degrading various brown algal fucoidans. This bacterium, a member of the family *Flavobacteriaceae*, was shown to secrete fucoidan endo-hydrolase activity. An extracellular enzyme preparation was used to degrade the fucoidan from the brown alga *Pelvetia canaliculata*. End products included a tetrasaccharide and a hexasaccharide made of the repetition of disaccharidic units consisting of α-1→3-L-fucopyranose-2-sulfate-α-1→4-L-fucopyranose-2,3-disulfate, with the 3-linked residues at the nonreducing end.

Keywords: *Flavobacteriaceae* — fucalean brown algae — fucan oligosaccharides — fucoidan — fucoidanase — marine bacteria

Introduction

The main matrix polysaccharide of brown algae (Phaeophyta) is alginate, a linear polymer of β-D-mannuronic acid and its C_5 epimer, α-L-guluronic acid. Brown algae, especially Fucales, also comprise matrix polysaccharides known as fucoidans, made of sulfated-L-fucose residues (Mabeau and Kloareg, 1987). In the fucoid alga *Pelvetia canaliculata* fucoidans represent as much as approximately 40% of the cell wall dry weight (Kloareg, 1984). Fucoidans consist of a continuous spectrum of highly ramified polysaccharides with a complex and still somewhat elusive structure, ranging from high uronic acid, low-sulfate-containing polymers with significant proportions of D-xylose, D-galactose, and D-mannose to highly sulfated homofucan molecules (Kloareg and Quatrano, 1988; Mabeau et al., 1990).

In brown algal zygotes, sulfated fucans are thought to be involved with adhesion to the substratum as well as with cell polarization, through a transmembrane complex reminiscent of the focal adhesions of mammalian fibroblast and epithelial cells (Kropf et al., 1988; Goodner and Quatrano, 1993). Sulfated fucans have also been extensively investigated for their anticoagulant properties, which involve the inhibition of thrombin and/or the activation of antithrombin and heparin factor II (e.g., Millet et al., 1999; Pereira et al., 1999; Thorlacius et al., 2000). In addition, by interfering with cell–cell recognition in mammalian systems, they display a variety of biological activities. They inhibit the acrosomal reaction in human spermatozoa (Mahony et al., 1991, 1993) and block infections by such viruses as the human immunodeficiency virus (Béress et al., 1993; Hoshino et al., 1998), the vesicular stomatitis virus (Mayer et al., 1987), and the herpes simplex virus (Hoshino et al., 1998), as well as by other microbes, such as *Plasmodium knowlesi* (Dalton et al., 1991). Sulfated fucans are potent inhibitors of smooth muscle cell growth, both *in vitro* and *in vivo* (McCaffrey et al., 1992; Logeart et al., 1997), and they display antiproliferative activity in various cancer types, including a

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non–small–cell bronchopulmonary carcinoma line (Riou et al., 1996) and Erhlich carcinoma (Zhuang et al., 1995). Also, sulfated fucans dose-dependently but nonspecifically inhibit selectin-mediated leukocyte rolling and emigration, thus decreasing organ injuries in non infectious models of inflammation (Ley et al., 1993; Wikström et al., 1995; Ostergaard et al., 2000; Linneman et al., 2000). Finally, sulfated fucans may be used in crop disease control since oligomers from sulfated fucans induce systemic resistance in tobacco against tobacco mosaic virus (Klarzynski et al., 2003).

In this context, a reliable fucoidan-degrading enzyme preparation would be highly desirable to obtain oligofucoidans, both to further elucidate the fine chemical structure of fucoidans and to investigate their structure–activity relationships in homologous or heterologous systems. However, only a few attempts were made so far to isolate fucoidan-degrading enzymes. In the case of herbivorous molluscs, Thanassi and Nakada (1967) partially purified a fucoidanase with a molecular mass of between 100 kDa and 200 kDa from *Haliotus rufescens* and *H. corrugata*. These activities hydrolyzed the fucoidan from *Fucus gardneri* to oligosaccharides ranging from the decasaccharide to L-fucose. Fucoidanases were also partially purified from *Pecten maximus* (Daniel et al., 1999) but the activity contained in the digestive glands of this mollusc was essentially a highly active and unusually thermal stable exo-fucosidase of 200 kDa (Berteau et al., 2002), mixed with a putative fucoidanase activity (Daniel et al., 1999) as also reported for the bivalve *Patinopecten yessoensis* (Kitamura et al., 1992). As far as bacteria are concerned, a study of *Laminaria*-decomposing epiflora indicated that fucoidanase activities are inducible, weak, but not rare in marine bacteria (Uchida, 1995). A number of *Vibrio*-type, fucoidan-degrading bacteria were isolated from marine sediments (Morigana et al., 1981) and an exo-acting fucoidanase was purified from *Vibrio* sp. N–5 (Furukawa et al., 1992). Recently, Sakai et al. (2002) isolated from the Japan Sea a marine bacterium, *"Fucobacter marina" (Flavobacteriacea)*, which cleaved various fucoidans from the Laminariales *Kjellmaniella crassifolia*, *Undaria pinnatifida*, and *Lessonia nigrescens* but not from the Fucales *Fucus vesiculosus* and *Ascophyllum nodosum*. The enzyme was further characterized as a sulfated fucoglucuronomannan lyase (Sakai et al., 2003a). Finally, a bacterial strain belonging to the family *Verrucomicrobiaceae* was isolated from the gut of the sea cucumber *Stichopus japonicus* (Sakai et al., 2003b). This bacterium, *Fucophilus fucoidanolyticus*, was able to degrade a variety of fucoidans, suggesting it produces a number of fucoidan-digesting enzymes. Interestingly, *F. fucoidanolyticus* was not able to cleave the fucan of the host sea cucumber (Sakai et al., 2003b).

Altogether, no fucoidan-endohydrolase activity is conveniently available yet to degrade the fucoidans from fucalean brown algae. As Fucales are more likely than Laminariales to provide large amounts of sulfated fucoidans (Mabeau et al., 1990), we thus set out to search for convenient sources of such enzymes. Alginate-extraction plants are known to release liquid effluents that are enriched in brown algal sulfated fucoidans (Fleury and Lahaye, 1993). Muds in the water-treatment facilities used for the cleansing of these effluents could therefore be expected to be good candidates for the occurrence of fucoidan-degrading microorganisms. We here report on the isolation and the characterization, from this latter habitat, of a fucoidan-degrading bacterium. The fucoidanolytic activity from culture supernatant was capable of degrading sulfated fucoidans from various fucoid brown algae.

Materials and Methods

Preparation of Fucoidans. Thalli of *Pelvetia canaliculata* (Dcne et Thur.) (1 kg fresh weight) were collected during low tide at Roscoff (Brittany, France), freed from epiphytes, and washed with distilled water. They were crushed in a mortar in the presence of liquid nitrogen and macerated overnight in 1 L of ethanol/formaldehyde/H_2O (80:5:15 vol/vol). Algal fragments were then extracted with 2 L of ethanol/formaldehyde/H_2O followed by 2 L of acetone. The resulting pellet was dried at 60°C and extracted twice for 3 h at 70°C with a 0.01 N HCl solution supplemented with 4% (wt/vol) $CaCl_2$. The extract was filtered, concentrated with a rotary evaporator, and neutralized with ammonium carbonate, and fucoidans were precipitated with 2.5 volumes of ethanol. The precipitate was redissolved in water and freeze-dried (Lyolab ALSL, Secfroid). This crude fucoidan fraction (FS28) typically exhibited a total carbohydrate content of approximately 55%, as assayed colorimetrically according to Tillmans and Phillipi (1929), and the proportion of fucosyl residues was approximately 30% in dry weight, as measured with the cysteine method (Disches and Schettles, 1948). Sulfate accounted for 26% of dry weight (as determined from the sulfur content by elementary analysis, CNRS, Service Central d'Analyse, Vernaison, France), whereas the uronic acid content was 2%, using the modified *m*-hydroxydiphenylsulfuric acid method with mannuronic acid lactone as stan-

dard (Blumenkrantz and Asboe-Hansen, 1973). Unless stated otherwise, this cruder fucoidan fraction was used in all experiments. Alternatively, more purified fucans were obtained from *P. canaliculata*, *Ascophyllum nodosum*, and *Fucus spiralis*, using the cetylpyridinium chloride (CPC) purification method as described previously (Mabeau et al., 1990). They contained 32%, 28%, and 37% of fucose and 33%, 27%, and 35% of sulfate, respectively.

Isolation of Fucoidan–Degrading Bacteria. Muds were collected from the water treatment facility of an alginate extraction plant (Danisco-Ingredients, Landerneau, Brittany, France) and precultivation of fucan-degrading bacteria was carried out as follows. Erlenmeyer flasks (100 ml) containing 10 ml of seawater/fucan (SWF) medium, consisting of sterile seawater supplemented with 0.2% (wt/vol) fucoidan from *P. canaliculata* (FS28 fraction) were inoculated with 400 µl of the muds and incubated aerobically at 22°C with shaking at 200 rpm on a rotary shaker (New Brunswick Scientific). Aliquots were taken at daily intervals for 7 days and tested for the presence of fucoidanolytic activity as follows. The culture supernatant (200 µl) was added to 2 ml of acid albumin solution (3.26 g of sodium acetate, 4.56 ml of glacial acetic acid, and 1.0 g of bovine serum albumin (Sigma) dissolved in 1 L of water, pH adjusted 3.72 to 3.78). Failure to develop a white turbidity because of the polysaccharide–albumin interaction indicated the presence of fucoidan-degrading activity (Kitamikado et al., 1990).

Active cultures were then plated onto Petri dishes with ZoBell medium (ZoBell, 1941) containing 0.3% (wt/vol) fucoidan and solidified by 0.7% (wt/vol) agar, and incubated at 22°C for 1 week. The various colonies were then transferred aseptically into 10 ml of Zobell-Fucan (ZF) medium, consisting of one volume of filtered seawater supplemented with 0.4% (wt/vol) fucoidan and one volume of ZoBell medium, incubated at 22°C, and assayed daily for fucoidanase activity (see below). The fucoidan-degrading isolates were subjected to repeated cultivation on ZF medium, eventually leading to the isolation of two different fucoidan-degrading strains, referred to as SW1 and SW5. They were stored at –80°C in ZF medium.

Biochemical and Molecular Characterization of SW5 Bacterial Strain. The morphological features of strain SW5 were investigated by microscopy (A100PL, Olympus BH-2) with cells in the exponential phase in ZoBell medium. To determinate the respiratory type, bacteria were inoculated in Veillon tubes containing ZoBell medium solidified with 0.6% (wt/vol) agar. Oxygen was removed from the medium by boiling. To determine the oxidative or fermentative behaviour, bacteria were inoculated into a modified Hugh and Leifson O-F medium (Hugh and Leifson, 1953; Smibert and Krieg, 1981) containing 0.5% glucose. Oxidase activity was assayed with disks impregnated with dimethylparaphenylene diamine oxalate (Diagnostic Pasteur). Catalase activity was assayed by mixing one colony from a ZoBell agar plate with a drop of 10% (v/v) hydrogen peroxide. Production of flexirubin was assessed by flooding a 4-day plate culture with 5 N potassium hydroxide followed by the observation of changes in colony color from yellow to red or brown (Reichenbach et al., 1974). Other phenotyping tests were performed using API 20 NE strips (API System, Bio-Mérieux) and Biolog GN microplates (Micromer, France).

The 16S ribosomal DNA sequence of the strain SW5 was determined as previously described for another polysaccharide-degrading marine bacterium (Barbeyron et al., 2001). The 16S rDNA gene was amplified by polymerase chain reaction from the bacterial genomic DNA with the 8F primer (5′-AGAGTTTGATCCTGGCTCAG-3′) (Hicks et al., 1992) and the 1492R primer (5′-GGTTACCTTG TTACGACTT-3′) (Kane et al., 1993). PCR products were cloned in vector pCRII2.1 and both strands of 16S rDNA were sequenced using Texas-red labeled primers with a Vistra 725 sequencer. The sequence, 1,241 bp in length, was analysed for similarities to rDNA sequences in the DNA database. The phylogenetic tree was constructed as previously described (Barbeyron et al., 2001).

Culture of SW5 and Detection of Fucoidanolytic Activity. SW5 was inoculated from a –80°C stock into 10 ml of ZF medium in 250-ml Erlenmeyer flask. After incubation for 48 h at 20°C with vigorous rotary shaking, the culture was transferred to 250 ml of ZF medium in a 1-L Erlenmeyer flask and incubated under the same conditions. The culture was then centrifuged at 2,000 *g* for 15 min and the pellet was suspended in 10 ml of ZF medium and inoculated into 5 L of ZF medium, in a 6.6-L fermenter jar. The culture was maintained for 6 days at 20°C (Bioflo 3000, New Brunswick Scientific). The pH was regulated at 7.85 with HCl and NaOH solutions (1 N) and aeration was 1 vvm (5 L/min), regulated at 70% by rotary shaking ranging from 200 to 800 rpm.

Attempts to quantitatively assay for fucoidanolytic activity by monitoring the release of fucoidan

oligosaccharides by a conventional reducing sugar assay (Kidby and Davidson, 1973) were unsuccessful. Fucoidan-degrading activity was thus monitored by a carbohydrate-polyacrylamide gel electrophoresis (C-PAGE) assay of the release of anionic oligosaccharides, according to the procedure of Zablackis and Perez (1990). Briefly, 0.2% (wt/vol) fucoidan in 20 mM Tris-HCl buffer (pH 7.5) was incubated at room temperature with the enzyme and the products of hydrolysis (20 µl) were frozen at −20°C to stop the reaction. Samples were then mixed with the loading buffer (10% sucrose and 0.01% phenol red) and electrophoresed through a 6% (wt/vol) stacking and a 27% running, 1-mm thick, polyacrylamide gel in 50 mM Tris-HCl, 2 mM EDTA buffer (pH 8.7) and stained with alcian blue followed by silver nitrate (Min and Cowman, 1986). Fucoidanase activity was detected by the occurrence of anionic oligosaccharide bands in the running gel.

At the end of culture, the medium was centrifuged at 5,000 *g* for 30 min and the supernatant (5 L) was concentrated by ultrafiltration with an Aminco cassette (10-kDa cutoff). The cassette was washed with 1 L of fucoidanase buffer (pH 7.5) containing 20 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The retentate (500 ml) was brought to 40% (wt/vol) saturation with $(NH)_2SO_4$ and precipitated overnight at 4°C. The suspension was centrifuged at 12,000 *g* for 30 min and the resulting supernatant was brought to 60% (wt/vol) saturation with $(NH_4)_2SO_4$. After precipitation overnight at 4°C, the precipitate was collected by centrifugation at 20,000 *g* for 1 h, dissolved in approximately 20 ml of fucoidanase buffer, dialyzed (10-kDA cutoff) against the fucoidanase buffer for 2 or 3 days at 4°C, and stored at –20°C. The enzyme optimal pH and temperature were estimated by hydrolysis of the FS28 fucoidan fraction for 1 h, the ammonium sulfate fraction and the C-PAGE assay over the range 5.0 to 8.0 and 17 to 40°C, respectively.

Preparation of Fucoidan Oligosaccharides. An aliquot (8 ml) of the partially purified enzyme fraction [40% to 60% $(NH_4)_2SO_4$ fraction] was added to 1 L of 20 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 5 mM $CaCl_2$ and 50 mM NaCl containing 5 g of fucoidan from *P. canaliculata* (FS28 fraction) and the mixture was incubated at 25°C for 24 h. The hydrolysate was diluted in 20 L of distilled water and then ultrafiltered on a 10-kDa membrane (Millipore). The filtrate (17 L) was refiltered on a 500-Da membrane (Pall Filtron) with a 2 bars pressure. The filtrate (~15 L) was concentrated on a rotary evaporator and freeze-dried.

Aliquots (250 mg) of the resulting powder, referred to as OF fraction, were resuspended in distilled water (5 ml) and applied onto a DEAE Sepharose CL6B (Pharmacia, 1.6 × 10 cm, 1.1 ml/min) equilibrated with distilled water. Elution was first performed with water (330 ml), then with a linear gradient of 0 to 2 M NaCl (660 ml). Fractions (11 ml each) were assayed colorimetrically for total sugars (Thibault, 1979) and the carbohydrate-containing fractions were pooled, concentrated by evaporation, then applied on a BioGel P6 column (Bio-Rad, 4.4 × 100 cm, 1 ml/min) equilibrated with 50 mM sodium nitrate, NaN_3 0.01% (1,500 ml), and eluted with the same buffer. Detection of carbohydrates was performed by refractometry (ERC 7510). Carbohydrate-containing peaks, referred to as peaks 1 to 4, were collected, concentrated, desalted on a Sephadex G10 column (Pharmacia, 3.2 × 100 cm, 1.9 ml/min), and freeze-dried.

The neutral monosaccharide composition of the purified oligosaccharide fractions was analysed after hydrolysis and reduction by gas chromatography (Autosystem XL, Perkin Elmer) on an OV-225 column (30 m × 0.32 mm, 0.25 µm, DB 225J and W, USA) according to Blakeney et al. (1983). These fractions (250 µg/ml) were further analyzed by high-performance anion-exchange chromatography (HPAEC) and conductivity detection, using a Dionex chromatograph in the configuration DX500 (Dionex, Sunnyvale, CA) and equipped with an anion micromembrane supressor AMMS, a 50 µl injection loop and an AS11A column (4 × 250 mm). Elution was performed at 0.5 ml/min by a linear gradient from 125 mM to 350 mM NaOH for 25 min and 350 mM NaOH for 5 min. Desalted fucoidan oligosaccharides were freeze-dried twice with deuterium oxide (99.9% D, Aldrich) and dissolved in 1 ml of D_2O (100% D, Aldrich) to replace exchangeable protons by deuterium. C and H NMR spectra were recorded at 25°C with a Bruker ARX 400 spectrometer operating at 100.62 MHz and 400.13 MHz. Chemical shifts were assigned relative to internal acetone at 2.225 ppm and 31.45 ppm for H and C, respectively. Pulse sequences for 1D and 2D experiments were used as specified by the manufacturer.

Results

Isolation and Characterization of a Fucoidan-Degrading Bacterium. Muds collected from a water-treatment facility for the recycling of the effluents of an alginate extraction plant were searched for the occurrence of fucoidan-degrading bacteria. Of the 51 bacterial strains that were isolated for

their ability to grow on fucoidan-enriched media, only two, referred to as SW1 and SW5, were active in the crude fucoidan-BSA enzymatic test (Kitamikado et al., 1990). These two strains were maintained on fucoidan-enriched ZoBell medium solidified with agar and then tested in liquid medium for their ability to degrade fucoidans. The SW5 strain, which displayed the higher extracellular fucoidanolytic activity, was retained for further studies.

SW5 appeared as an orange-pigmented, gram-negative, rod-shaped, nonflagellated bacterium, unable to grow on the freshwater Luria Bertani medium (Maniatis et al., 1982). Strain SW5 was shown to be a strictly aerobic, seawater-requiring, chemoorganotrophic and heterotrophic organism, with an oxidative metabolism which used oxygen, but not nitrate, as the electron acceptor. The bacterium synthesized β-glucosidase and β-galactosidase but did not synthesize flexirubin, cytochrome oxydase or gelatinase and it was not able to degrade agarose and κ-carrageenan. In contrast, ι-carrageenan was hydrolyzed (data not shown).

The 16S rDNA sequence of strain SW5 was found to be related, with sequence identities of 96% and 93%, respectively, to those of the strains BSD RB 42 (GenBank Accession No. AY259505) and BSA CS 02 (GenBank Accession No. AY259501), which belong to the *Flavobacteriaceae* family. In the phylogenetic analysis of its 16S rDNA gene, SW5 16S rDNA clusters with heterologous genes from unidentified strains. This group forms a clade with the psychrophyllic bacteria *Psychroserpens burtonensis*, *Gelidibacter algens and G. mesophilus* (Figure 1).

Production of Fucoidanolytic Activity from SW5. To produce fucoidanolytic activity, the SW5 strain was grown in a fermenter in a fucoidan-enriched ZoBell medium. The generation time was of 12.5 h at 20°C and the culture medium reached an OD_{600nm} of approximately 1.0 after 3 days of cultivation (Figure 2A). Fucoidanase activity was detected by C-PAGE in the culture supernatant as early as at the end of the exponential phase and was maintained during the stationary phase (Figure 2B). In contrast, intracellular fucoidanolytic activity was very low throughout the culture (data not shown).

The protein ammonium sulfate precipitate from the culture supernatant of SW5 extensively degraded fucoidans purified with cetylpyridinium chloride from the fucoid alga *Pelvetia canaliculata* as well as those from two other fucoid algae, *Fucus spiralis* and *Ascophyllum nodosum* (Figure 3A). Based on the C-PAGE assay of the release of fucoidan oligomers from the FS28 fucoidan fraction, the optimal pH and temperature were estimated at 7.5 and 20° to 25°C, respectively. Although the digestion profiles depended on the fucoidan under investigation, they were all found to share two low molecular weight components with similar electrophoretic motilities (referred to as bands 3 and 4 on Figure 3A). The kinetics of the degradation of *P. canaliculata* FS28 fucoidan fraction by the enzyme ammonium sulfate fraction is shown in Figure 3B. Oligofucoidans were detected as early as after 2.5 min of enzymatic digestion, in the form of several discrete bands, referred to as 3, 2, 8, 9, and 10. With the exception of band 2, the amount of the high molecular weight fucoidan oligosaccharides rapidly decreased, while new oligofucoidan products appeared (bands 1, 5, 6, and 7). However, the terminal product (band 4) was always present in the reaction medium. No significant increase was observed in the OD_{235nm} of the reaction mixture (data not shown).

Fractionation of Fucoidan Oligosaccharides. The crude fucoidan from the fucoid alga *P. canaliculata* (FS28) was hydrolyzed to completion by the ammonium sulfate enzyme fraction and the products were ultrafiltered through a 500-Da membrane (OF fraction in Figure 3C), with a recovery of 66% in mass relative to the mass of the initial substrate. The OF fraction was fractionated by anion-exchange chromatography on a DEAE Sepharose CL6B column, resolving three major peaks (Figure 4A), which, on the basis of their levels in total sugars, represented approximately 87% of the initial oligosaccharide mixture. The main fucoidan oligosaccharide fraction, which was eluted at 0.6 to 1.6 M NaCl (fraction numbers 85 to 100) and represented 55% of OF in total carbohydrates, was further fractionated by gel filtration onto a Biogel P6 column (Figure 4B). It consisted of a mixture of unresolved fucan oligosaccharides eluting close to the column void volume as well as of four distinct low molecular mass fractions. Based on their sugar content, these latter oligosaccharides represented approximately 10% of OF fraction in total carbohydrates, that is, about 7% of the mass of initial polysaccharide. Whereas the OF fraction contained, besides fucose (83.8 mol%), some xylose (5.9 mol%) and galactose (10.4 mol%) as well as traces of mannose and glucose, fucose was the only monosaccharide detected by gas chromatography in the purified oligosaccharide fractions 2, 3, and 4.

After desalting on a Sephadex G10 column, fractions 3 and 4 appeared homogeneous upon C-PAGE analysis (Figure 3C). This was confirmed by high-performance anion-exchange chromatography (HPAEC) analysis. They were eluted essentially

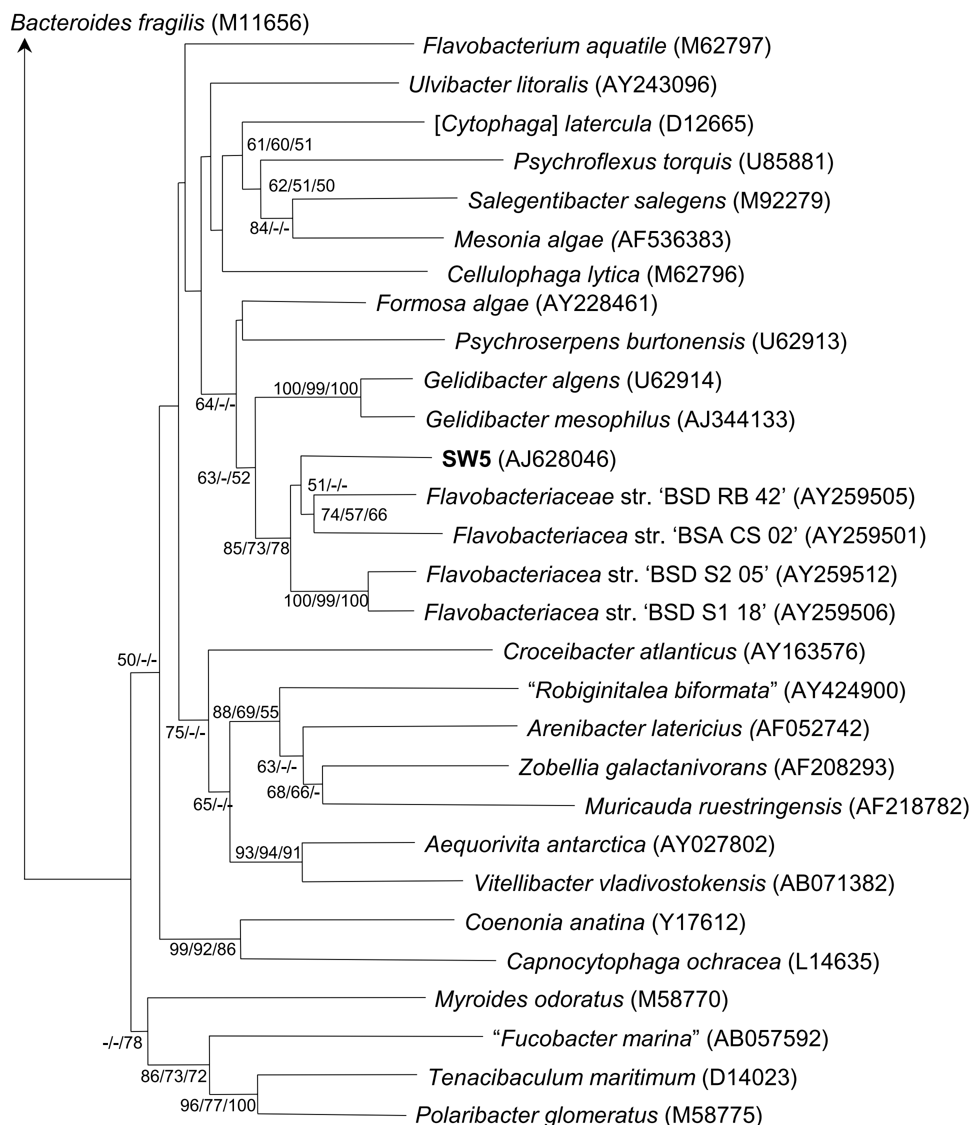

Fig. 1. Phylogenetic relationships of strain SW5 to some marine representatives of the family *Flavobacteriaceae*. Square brackets indicate a generically misnamed taxon and quotation marks indicate a name not yet validated. Accession numbers of 16S rDNA sequences are given in brackets. The topology shown is the tree obtained using the neighbor-joining method (Jukes and Cantor distance correction). Numbers at the nodes refer to the bootstrap values (100 replicates) as obtained in distance, maximum-likelihood, and maximum parsimony analyses, respectively, while dashes instead of numbers indicate that the node was not observed in the corresponding analysis. The scale bar represents the expected number of changes per sequence position.

as single peaks, with retention times of 18.5 and 13.2 min, respectively (Figure 4C). In contrast, although fractions 1 and 2 appeared as homogeneous by C-PAGE analysis (Figure 3C), they featured several peaks on HPAEC chromatography, with their main components eluting at 19.9 and 20.9 min, respectively (Figure 4C). Discrete bands on C-PAGE gels may thus contain more than one fucoidan oligosaccharide, with various structures, but having the same electrophoretic motility.

NMR Analysis of Fucoidan Oligosaccharides. From its COSY and HMQC NMR spectra, oligosaccharide 4 featured chemical shifts assignable to five different α-L-fucopyranosyl residues (Bock et al., 1984), referred from ***a*** to ***e*** (Table 1). The ROESY spectrum (Figure 5) showed cross-peaks from H1 of residue ***b*** with H3 of residue ***d*** (***b1/d3***), from H1 of residue ***d*** to H4 of residue ***c*** (***d1/c4***), and from H1 of residue ***c*** to H3 of residues ***a*** and ***e***. As previously reported for 3-linked sugars in the

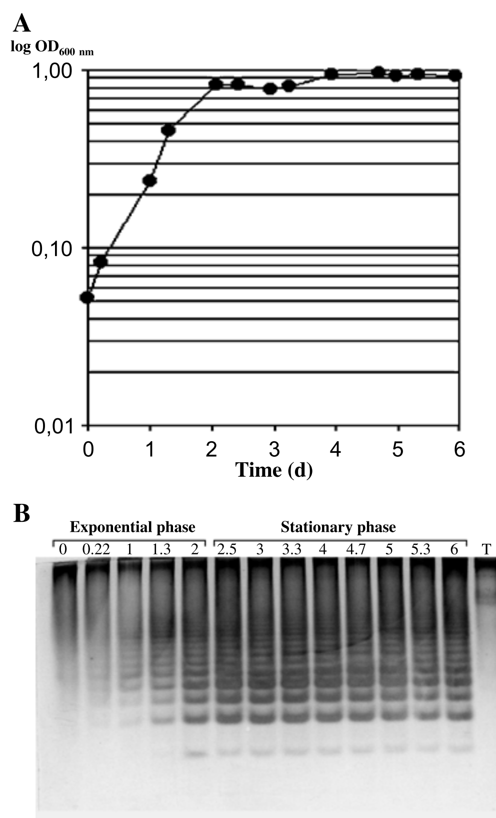

Fig. 2. Growth of the SW5 strain (**A**), as seen from the culture optical density measured at 600 nm. SW5 was grown in a 5-L fermenter in the presence of ZoBell medium supplemented with fucoidan from *Pelvetia canaliculata*. (**B**) Fucoidanolytic activity in the culture supernatant was monitored using C-PAGE analysis. Culture aliquots (2 ml) were centrifuged, supernatant aliquots (20 µl) were incubated for 3 h with 100 µl of 0.2% (wt/vol) fucoidan from *P. canaliculata* , and the hydrolysates (5 µl aliquots) were analyzed by C-PAGE. Numbers above the lanes refer to the culture duration (in days), with T corresponding to unhydrolyzed fucoidan.

galacto-configuration (Lipkind et al., 1988; Bock and Thøgerson, 1982), cross-peaks were also observed between H1 of residue ***b*** and H4 of residue ***d*** and from H1 of ***c*** and H4 of residue ***a***. The HMBC spectrum (data not shown) corroborated the presence of α-1→3-and α-1→4-linked fucose. The anomeric protons of ***b***, ***c***, ***d***, at 5.40, 5.39, and 5.30 ppm, correlated with C3 of ***d*** and ***a***, at 73.6 and 74.8 ppm, and with C4 of ***c***, at 80.4 ppm (Table 1). Conversely, the anomeric carbons ***b1***, ***c1,*** and ***d1*** correlated with protons ***d3***, ***a3,*** and ***c4***, respectively. From these results, oligosaccharide 4 consists of the following carbon backbone: α-L-Fuc*p*-1→3-α-L-Fuc*p*-1→4-α-L-Fuc*p*-1→3-α/β-L-Fuc*p*.

Protons of α-L-fucopyranosyl units were identified from the COSY spectrum of oligosaccharide 3 and C chemical shifts were deduced from the HMQC spectrum (Table 2). Chemical shifts were similar to those of oligosaccharide 4, with the exception of two groups of signals (***c′*** and ***d′***), indicating the presence of an additional disaccharide unit in this oligosaccharide. The actual sequence of oligosaccharide 3 was deduced from its HMBC spectrum (Figure 6) and by comparison with C and H of oligosaccharide 4. HMBC showed cross-peaks from H3 of residues ***d***, ***d′***, and ***a*** to C1 of residues ***b***,

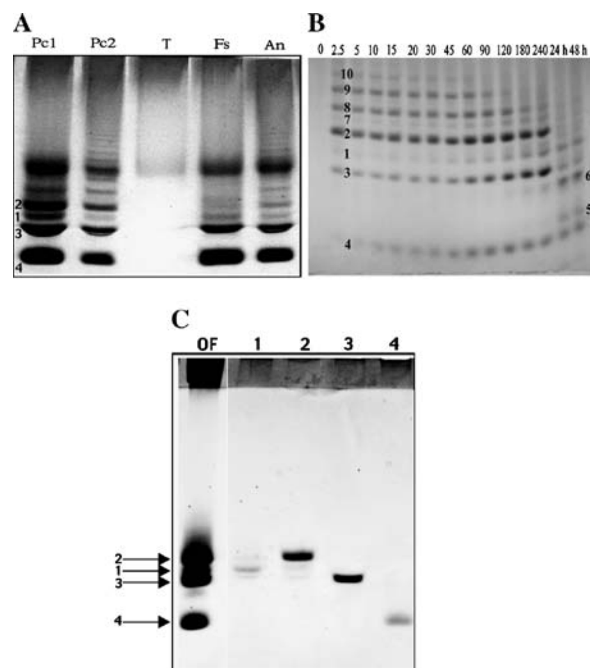

Fig. 3. (**A**) C-PAGE electrophoresis of the hydrolysates by SW5 ammonium sulfate fucoidanase fraction of various brown algal sulfated fucans (0.5%): the FS28 fraction of *Pelvetia canaliculata* fucoidan (Pc1), a CPC-purified fraction from the same species (Pc2), and fucoidans purified using CPC from *Fucus spiralis* (Fs) and *Ascophyllum nodosum* (An). The control (T) consisted of the FS28 fraction incubated with boiled enzyme. Bands numbers were assigned relative to their elution order on gel filtration (see Figure 4B) and by comparison with ***C***. (**B**) C-PAGE analysis of the hydrolysis kinetics of *P. canaliculata* fucoidan (FS28) by SW5 ammonium sulfate fucoidanase fraction. The fucoidan (400 µl, 0.2%) was incubated with the ammonium sulfate enzyme fraction (40 µl) and aliquots (20 µl) of this mix were boiled and electrophoresed as described previously. Band numbers were assigned by comparison to ***A*** and ***C***. Numbers at the top of the gel refer to the duration of hydrolysis (in minutes and hours). (**C**) C-PAGE analysis of purified low molecular weight fucoidans. *P. canaliculata* fucoidan (FS28) was hydrolyzed with SW5 fucanase and the products were recovered by ultrafiltration through a 500-Da membrane (OF fraction). This fraction was further purified by chromatography on DEAE Sepharose CL6B and then on Biogel P6, and carbohydrate-containing peaks 1, 2, 3, and 4 (see Figure 4B) were submitted to C-PAGE (lanes 1 to 4). Arrows indicate the corresponding oligosaccharide bands in the OF fraction.

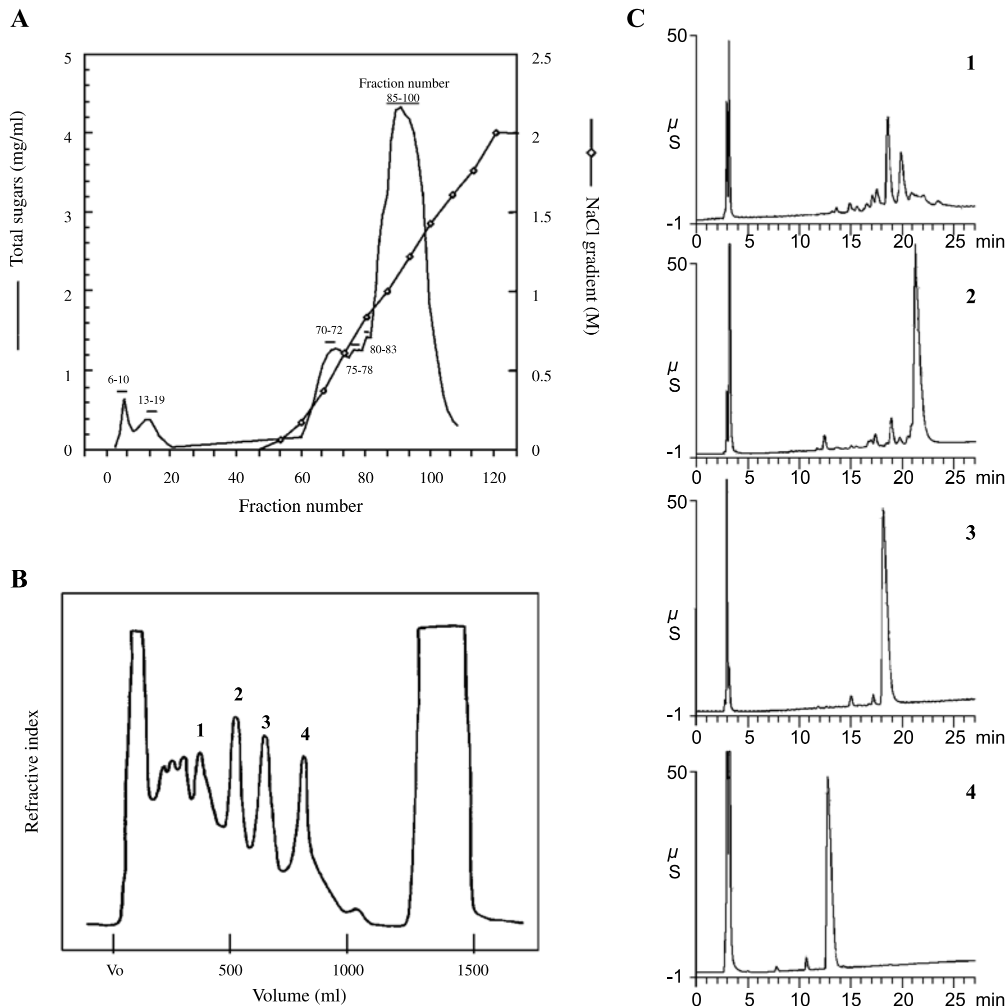

Fig. 4. Purification of the end-products of the hydrolysis of *Pelvetia canaliculata* fucoidan (FS28) by the SW5 ammonium sulfate fucanase fraction. (**A**) Fractionation of the hydrolysate (OF fraction) from *P. canaliculata* fucan on DEAE Sepharose. The OF fraction (250 mg) was chromatographed on DEAE Sepharose CL6B with a 0 to 2 M NaCl gradient, eluted fractions were assayed for total sugars, and carbohydrate-containing fractions (fractions 85 to 100) were collected. (**B**) Fractions 85 to 100 were pooled and then chromatographed on Biogel P6 using 50 mM NaNO3 as eluent. The four peaks resolved as fractions 1 to 4 were collected. (**C**) HPAEC elution profiles of peaks 1 to 4 from fractions 85 to 100 on Biogel P6 chromatography. Fractions were desalted on Sephadex G10 and injected at 250 μg/ml on an AS11A anion-exchange column, using a NaOH linear gradient and conductivity detection.

c, and ***c***′, and from H4 of residues ***c*** and ***c***′ to C1 of residues ***d*** and ***d***′. Cross-peaks between ***c***, ***c***′ and ***d***, d′ corroborated the presence of α-1→4-linkages. However, it was not possible to specify whether the ***c***′-***d***′ units were close to the reducing end or the nonreducing end in the oligosaccharide. The proposed sequence for oligosaccharide 3 is α-L-Fuc*p*-1→3-α-L-Fuc*p*-1→4-α-L-Fuc*p*-1→3-α-L-Fuc*p*-1→4- α-L-Fuc*p*-1→3-α/β-L-Fuc*p*.

The position of sulfate ester substituents in oligosaccharides 4 and 3 was deduced from the downfield shifts of H2 (0.7 to 0.9 ppm), which were consistent with those reported for the 2-*O*-sulfated fucans from the egg jelly layer echinoderms (Vilela-Silva et al., 2002), and of C2 (4 to 5 ppm), relative to unsubstituted α-L-Fuc*p* residues (Table 3; Bock and Thøgerson, 1982). Sulfation on H3 of the 4-linked α-L-Fuc*p* 2-sulfate was deduced from the downfield shift observed for H3 (~0.5 ppm) and C3 (~9 ppm) of this residue compared with the same signals in 4-linked α-L-Fuc*p*-2-sulfate in *Strongylocentrotus droebachiensis* sea urchin fucan (Vilela-Silva et al., 2002). Furthermore, the proton and carbon chemical shifts for oligosaccharides 3 and 4 were in good agreement with those reported for alternating 3-linked α-L-Fuc*p*-2-sulfate and 4-linked α-L-Fuc*p*-2,3-disulfate in fucoidan oligomers from *Ascophyllum nodosum* (Chevolot et al., 2001).

Based on the attribution of the signals for proton of oligosaccharides 4 and 3, the mean degree of poly-

Table 1. Chemical Shifts (ppm) for the Fucan Oligosaccharide 4

Residue	*H1*[a]	*H2*	*H3*	*H4*	*H5*	*H6*
a (α)	5.51	4.55	4.08	4.11	4.25	1.25
e (β)	4.73	4.35	3.91	4.06	3.83	1.30
c	5.39	4.66	4.78	4.30	4.57	1.42
d	5.30	4.60	4.22	4.14	4.46	1.31
b	5.40	4.58	4.77	4.24	4.61	1.26
	C1[b]	*C2*	*C3*	*C4*	*C5*	*C6*
a (α)	91.68	74.53	74.84	70.28	67.08	16.60
e (β)	96.32	78.34	77.55	69.28	71.76	~16.6
c	96.18	73.61	75.05	80.38	69.03	16.78
d	99.86	76.53	73.61	70.56	68.20	16.46
b	95.81	73.52	75.05	71.76	67.693	16.38

[a]The ^{1}H spectrum was recorded at 400 MHz, 298 K, chemical shifts are relative to internal $\delta^{1}H$ acetone 2.225.
[b]The ^{13}C spectrum was recorded at 400 MHz, 298 K, chemical shifts are relative to internal $\delta^{13}C$ acetone 31.45.

Table 2. Chemical Shifts (ppm) for the Fucan Oligosaccharide 3

Residue	*H1*[a]	*H2*	*H3*	*H4*	*H5*	*H6*
a (α)	5.49	4.53	4.07	4.10	4.23	1.24
e (β)	4.72	4.33	3.90	4.05	3.82	1.28
c′	5.39	4.64	4.76	4.28	4.56	1.40
d′	5.30	4.58	4.22	4.13	4.39	1.30
c	5.40	4.65	4.79	4.29	4.62	1.40
d	5.29	4.58	4.22	4.13	4.43	1.30
b	5.39	4.57	4.73	4.22	4.59	1.25
	C1[b]	*C2*	*C3*	*C4*	*C5*	*C6*
a (α)	91.64	74.57	74.57	70.16	67.06	16.56
e (β)*						
c′	95.73	73.57	75.06	80.87	68.98	16.76
d′	100.04	74.57	74.57	70.53	68.15	16.43
c	95.96	73.57	75.06	80.58	68.98	16.76
d	99.87	74.57	74.57	70.53	68.15	16.43
b	95.96	73.57	76.18	71.73	67.68	16.43

[a] The ^{1}H spectrum was recorded at 400 MHz, 298 K, chemical shifts are relative to internal $\delta^{1}H$ acetone 2.225.
[b] The 13C spectrum was recorded at 400 MHz, 298 K, chemical shifts are relative to internal $\delta^{13}C$ acetone 31.45.
*Not determined.

merization (DP) was estimated from the sum of the integral of H1 of the reducing-end sugar ***a*** (α-configuration) and H5 of the reducing-end sugar ***e*** (β-configuration) or that of H1 of ***a*** and (H5 + H3)/2 of ***e,*** in order to establish the integral value of one proton (***H***). The number of internal fucose residues (i.e., excluding nonreducing residue) (***B***) was estimated from the integral of signals between 5.25 and 5.40 ppm divided by the integral value for one proton (***B/*** **H**). The DP value was then calculated as (***B/H***) + 1. Using this calculation, oligosaccharides 4, 3, and 2, have a mean DP of 3.9, 5.9, and 6.8, respectively. The proton nuclear magnetic resonance (NMR) spectrum of oligosaccharide 2 indicated that the mixture did not contain oligosaccharides in the series homologous to oligosaccharides 4 and 3.

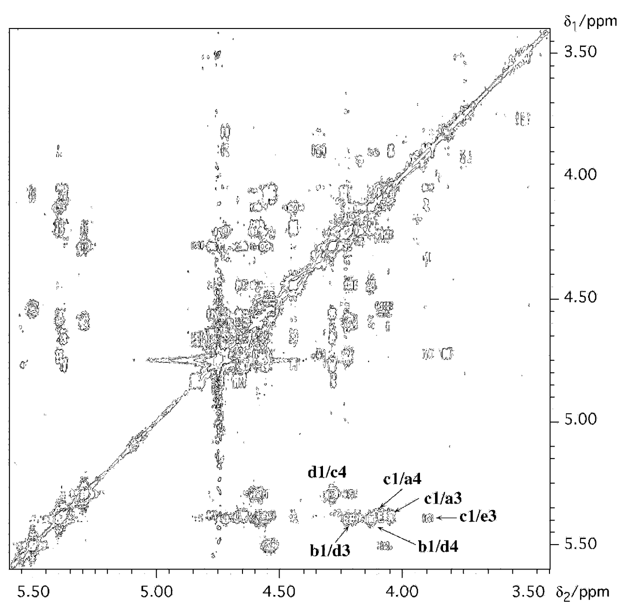

Fig. 5. Expansion of the double ^{1}H-^{1}H ROESY spectrum of oligosaccharide 4, at 400 MHz, 25°C, in D_2O. Cross-peaks between the various sugar residues are indicated as, for example: ***d1/c4*** for H1 of unit ***d*** with H4 of unit ***c***. 1024 experiments of 2000 data points and eight transients each with a recycling time of 1.16 s were recorded and transformed on 2 × 2 K data points with unshifted sine bell multiplication in both dimensions. The ROESY spinlock pulse duration was 700 ms.

Discussion

The SW5 Strain Is a Fucoidanolytic Flavobacteriaceae. We here report the isolation from the effluent treatment facility of an alginate plant of a novel bacterium referred to as SW5, which exhibits fucoidanase activity when grown in the presence of sulfated fucans from the fucoid *Pelvetia canaliculata* (Figure 2). The occurrence of such a fucoidanolytic bacterium in this habitat is likely to be accounted for by the presence of significant amounts of high molecular weight sulfated fucoidans in these effluents, as byproducts of the alginate-manufacturing process from fucoidan-containing brown algae such as *Laminaria digitata* and *Ascophyllum nodosum* (Fleury and Lahaye, 1993).

Based on its physiological and biochemical characteristics as well as on the phylogenetic analysis of its 16S rDNA gene (Figure 1), SW5 belongs in the order of *Flavobacteriales* and in the family *Flavobacteriaceae*. However, it is only remotely related to the only other fucoidan-degrading *Flavobacteriaceae* characterized so far, "*Fucobacter marina*" (Sakai et al., 2002). It is closely related to,

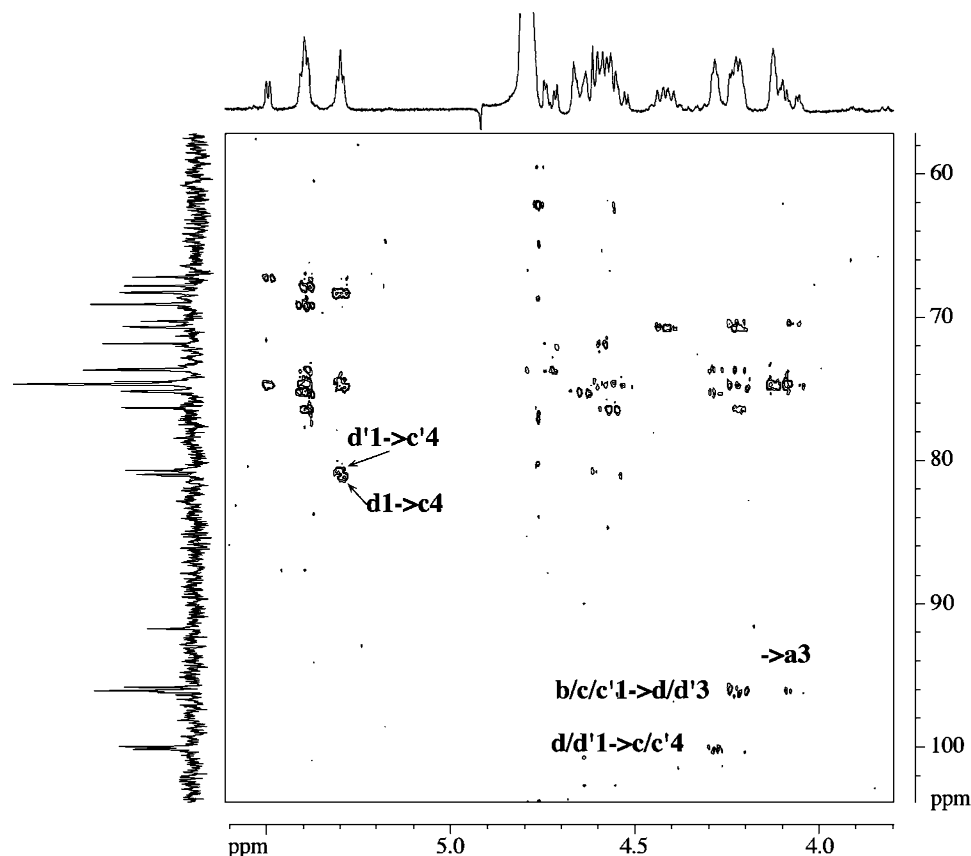

Fig. 6. Expansion of the double ^{1}H-^{13}C HMBC spectrum of oligosaccharide 3, at 400 MHz, 25°C, in D_2O. Cross-peaks between the various sugar residues are indicated using the same nomenclature as in Figure 5. 512 experiments of 2000 data points and 64 transients, each with a recycling time of 1.2 s, were recorded and transformed on 1 × 2 K data points on the F1 and F2 dimensions, respectively after exponential multiplication in F1 (line broadening of 0.1 Hz) and unshifted sine bell multiplication in F2. The evolution time for long range interactions was set at 75 ms.

but distinct from, the genera *Gelidibacter* and *Psychroserpens*, which were isolated from an Antartic sea-ice habitat (Bowman et al., 1997). All of these bacterial strains require the presence of salt for optimal growth, suggesting that they are marine in origin. Therefore, SW5 may have been a member of the natural epiflora of brown algae carried throughout the alginate-extraction process and seeded into the water-treatment muds, where it contributes to the depolymerisation of the sulfated fucoidans in the effluents. Alternatively, since the water treatment facility is located close to an estuary, SW5 may have been naturally introduced from the nearby estuarine bacterial communities.

SW5 Secretes Fucoidan Hydrolase Activity. The bacterium extracellular enzymes were subjected to fractionation by ammonium sulfate precipitation. The sulfated fucoidan from *P. canaliculata* was extensively degraded by the fraction of SW5 extracellular enzymes which precipitates between 40% and 60% ammonium sulfate saturation (Figure 3A). Fucoidan was first cleaved into a number of high molecular weight fucoidan oligosacchar-

Table 3. Comparison of the Chemical Shifts of Various Fucans

	H1	*H2*	*H3*	*H4*	*H5*	*H6*
Desulfated sea cucumber fucan[a]	5.08	3.92	4.00	nd	4.27	1.21
native sea cucumber fucan, residue A[a]	5.40	4.58	4.39	4.91	4.37	1.25
α-L-Fucose	5.2	3.8	3.8	3.8	4.2	1.2
α-L-Fucose residues of fucan oligosaccharides 3 and 4	4.7–5.5	4.4–4.7	3.9–4.0	4.0–4.1	3.8–4.2	1.2–1.3

[a]Data from *Ludwigothurea grisea*.

ides (Figure 3B), suggesting that this enzyme fraction contains hydrolases which randomly attack fucoidan chains.

As shown by DEAE chromatography and gel filtration followed by C-PAGE, HPAEC, and NMR analysis, the hydrolysis products contained a tetrasaccharide, α-L-Fuc*p*-2,3-(diOSO_3)-1→3-α-L-Fuc*p*-2-(OSO_3)-1→4-α-L-Fuc*p*-2,3-(diOSO_3)-1→3-α-L-Fuc*p*-2-(OSO_3), and a hexasaccharide, α-L-Fuc*p*-2,3-(diOSO_3)-1→3-α-L-Fuc*p*-2-(OSO_3)-1→4-α-L-Fuc*p*-2,3-(diOSO_3)-1→3-α-L-Fuc*p*-2-(OSO_3)-1→4-α-L-Fuc*p*-2,3-(diOSO_3)-1→3-α-L-Fuc*p*-2-(OSO_3), as well as higher molecular-weight fucoidan oligosaccharides. These findings are similar to those reported from a low molecular weight fucan fraction of *A. nodosum* (Chevolot et al., 2001), which harbors repetitions of the disaccharide 1→3-α-L-Fuc*p*-2(OSO_3)-1→4-α-L-Fuc*p*-2,3(diOSO_3), referred to as B-A and where the B fucosyl residue can more or less regularly bear a substituent at C-4 (sulfate, xylose, fucose or sulfated fucose). Similar fucan oligosaccharides with repeating units were also recognized in the fucoidans of *F. vesiculosus* (Chevolot et al., 2001), *F. evanescens* (Bilan et al., 2002) and *F. distichus* (Bilan et al., 2004). Since SW5 extracellular enzymes can degrade a variety of sulfated fucoidans from fucoid algae (Figure 3A), it is thus likely that the bacterium attacks fucoidan by cleaving linkages within blocks of alternating α-1→3 and α-1→4 L-sulfated fucose residues. This conclusion is only preliminary, however, since SW5 is likely to possess multiple fucoidan degradative activities, as suggested by the presence of different low molecular weight fucoidans at completion of the hydrolysis of *P. canaliculata* fucoidan (Figure 3B). Altogether it appears that SW5 features a fucoidan-degrading enzyme machinery distinct from that of "*Fucobacter marina*", which exhibits a fucoglucuronomannan lyase (Sakai et al., 2002, 2003a). Other fucoidan-degrading activities were reported from intracellular extracts of *Fucophilus fucoidanolyticus*, namely a fucoidan hydrolase, an α-D-glucuronidase and a fucose-deacetylase (Sakai et al., 2003b,c). The isolation of SW5 provides an alternative source for the production of extracellular enzymes to degrade fucoidan.

In conclusion, on the basis of its capacity to degrade brown algal sulfated fucoidans, we have isolated a flavobacteriacean strain, referred to as SW5, which secretes fucoidan-hydrolase activity in its culture medium. The end products of the degradation of *P. canaliculata* fucoidan comprised a tetrasaccharide, made of the repetition of two units of α-1→3-L-fucopyranose-2-sulfate, and α-1→4-L-fucopyranose-2,3-disulfate, with the 3-linked residues at the nonreducing end, and a hexasaccharide in the same homologous series. SW5 has now been successfully maintained for more than 5 years in our laboratory, where it is routinely used for the preparation of fucoidan-degrading enzymes. The strain was patented (Descamps et al., 1998) and deposited in the DSMZ bacterial collection, under the accession number 12171. SW5 fucoidan endohydrolase(s) should prove a useful tool for the preparation of biologically active low molecular weight fucans as well an interesting novel material for the isolation of enzymes for the structural analysis of fucoidans.

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