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Immunochemical and Biological Characterization of Three Capsular Polysaccharides from a Single *Bacteroides fragilis* Strain

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Although *Bacteroides fragilis* accounts for only 0.5% of the normal human colonic flora, it is the anaerobic species most frequently isolated from intra-abdominal and other infections with an intestinal source. The capsular polysaccharides of *B. fragilis* are part of a complex of surface polysaccharides and are the organism's most important virulence factors in the formation of intra-abdominal abscesses. Two capsular polysaccharides from strain NCTC 9343, PS A1 and PS B1, have been characterized structurally. Their most striking feature is a zwitterionic charge motif consisting of both positively and negatively charged substituent groups on each repeating unit. This zwitterionic motif is essential for abscess formation. In this study, we sought to elucidate structural features of the capsular polysaccharide complex of a commonly studied *B. fragilis* strain, 638R, that is distinct from strain 9343. We sought a more general picture of the species to establish basic structure-activity and structure-biosynthesis relationships among abscess-inducing polysaccharides. Strain 638R was found to have a capsular polysaccharide complex from which three distinct carbohydrates could be isolated by a complex purification procedure. Compositional and immunochemical studies demonstrated a zwitterionic charge motif common to all of the capsular polysaccharides that correlated with their ability to induce experimental intra-abdominal abscesses. Of interest is the range of net charges of the isolated polysaccharides—from positive (PS C2) to balanced (PS A2) to negative (PS 3). Relationships among structural components of the zwitterionic polysaccharides and their molecular biosynthesis loci were identified.

Intra-abdominal infections present serious clinical conditions that are difficult to treat, frequently require surgical intervention, and often result in short-term and long-term complications. *Bacteroides fragilis* accounts for only 0.5% of the normal human colonic flora but is the anaerobic bacterium most frequently isolated from clinical infections in the abdominal cavity (9, 25). The capsular polysaccharides of *B. fragilis* are the most important virulence factors responsible for the formation of intra-abdominal abscesses by this organism (17, 19).

The capsular polysaccharides of *B. fragilis* exist on the organism's surface as part of a capsular polysaccharide complex (CPC). For the *B. fragilis* type strain, NCTC 9343, two polysaccharides of the CPC, PS A1 and PS B1, have been elucidated structurally (2, 31). The gene loci for biosynthesis of PS A1, PS B1, and a third, structurally unknown, capsular polysaccharide, PS C1, have recently been sequenced (3; M. J. Coyne, A. O. Tzianabos, B. E. Mallory, D. L. Kasper, and L. E. Comstock, submitted for publication). The most striking feature of all of the *B. fragilis* polysaccharides characterized to date is the presence of both positively and negatively charged substituent groups on each repeating unit. This specific charge motif (zwitterionic) is essential for the unique biological activity of this class of molecules (13, 30), including promotion of the formation of intra-abdominal abscesses (30).

Studies with monoclonal antibodies (MAb) and polyclonal antiserum have demonstrated, however, that the capsular polysaccharides of *B. fragilis* are quite heterogeneous (15, 26, 28). The *B. fragilis* strain that has been best studied, 638R, is known to produce a CPC that is immunologically distinct from the CPC of the type strain, NCTC 9343 (3, 21, 23, 27). However, the polysaccharides composing the 638R CPC have not been characterized. The biosynthesis loci of PS C from strain NCTC 9343 (PS C1) and strain 638R (PS C2) were the first loci to be cloned and sequenced. These loci are flanked by genes common to both strains; however, the genes involved in polysaccharide biosynthesis are distinct (3–5).

In this study, we sought to elucidate structural features of the *B. fragilis* strain 638R CPC to develop a clearer picture of the diversity of polysaccharides in the species and of the basic structure-activity and structure-biosynthesis relationships of abscess-inducing polysaccharides. To our knowledge, this is the first report of the isolation of three different surface-expressed capsular polysaccharides—PS A2, PS C2, and PS 3—from one bacterial strain. The immunochemical characteristics and abscess-inducing potentials of these capsular polysaccharides are described and brought together with information about their biosynthetic pathways.

MATERIALS AND METHODS

Bacterial strain and isolation of capsular polysaccharides of *B. fragilis* strain 638R. *B. fragilis* strain 638R (27) was maintained in peptone-yeast broth at −80°C. The production of CPC was enhanced and the glycogen content in extracts was minimized by the serial passage of bacteria five times through rat spleens (male Wistar rats; 175 to 200 g; Charles River Laboratories, Wilmington, Mass.) (14). Initially, 10^8 organisms in phosphate-buffered saline (PBS) were injected intraperitoneally into a rat and recovered 24 h later by dispersing the spleen. The organisms were cultured overnight anaerobically on brucella agar with 5% sheep blood (PML Microbiologicals, Mississauga, Ontario, Canada).

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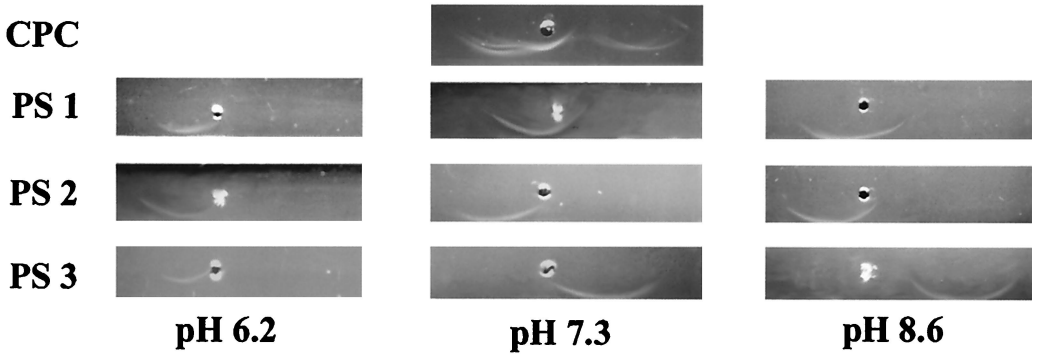

FIG. 1. IEP of the CPC and separated, purified polysaccharide components PS 1, PS 2, and PS 3 of the capsule of *B. fragilis* strain 638R at different pH values. Each antigen preparation was reacted with polyvalent rabbit anti-*B. fragilis* strain 638R antiserum.

The inoculation and recovery processes were repeated. Enhanced production of capsule was confirmed by electron microscopy studies with polyvalent antiserum to whole bacteria (data not shown). After the passages, the bacteria were grown anaerobically in a 16-liter batch culture as described previously (22, 31). The cells were harvested by centrifugation (average wet weight, 244 g) and suspended in water. CPC was extracted from the bacterial cells with hot phenol-water and digested extensively with RNase, DNase, and pronase. The dialyzed product (average dry weight, 2 g) was chromatographed on a column of Sephracyl S-400 HR (Amersham Pharmacia Biotech, Piscataway, N.J.) in a 3% deoxycholate acid-containing buffer at pH 9.8 to separate lipopolysaccharide (LPS) from CPC (22, 31). Chromatographic fractions were analyzed throughout the isolation procedure by measurements of UV absorbance at 280, 260, and 206 nm; refractive index; and protein by the bicinchoninic acid method (Pierce, Rockford, Ill.), silver staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (16), dot blot assay, immunoelectrophoresis (IEP), and immunodiffusion (20, 22). After gel filtration chromatography with S-400 HR, silver-stained SDS-PAGE showed fractions with a high-molecular-mass antigen (>160 kDa according to protein standards) representing CPC. Subsequently eluted fractions showed antigens smaller than 20 kDa at the gel front that represented LPS and digested protein and nucleic acid. Fractions eluted between CPC and LPS contained both materials. Fractions containing capsular polysaccharide exclusively and reacting with antiserum to the whole bacteria by double immunodiffusion were pooled, concentrated, alcohol precipitated, dialyzed, and lyophilized. The CPC (average dry weight, 0.2 g) was examined by IEP (in 50 mM Tris buffer at pH 7.3), which showed three dominant components, two positively and one negatively charged, and two weak, distinct components migrating towards the anode and cathode (Fig. 1).

Acid-treated (5% acetic acid at 100°C for 1 h), dialyzed, and lyophilized CPC was loaded onto a column containing Q-Sepharose FF (Amersham Pharmacia Biotech) in 50 mM Tris buffer at pH 7.3. Antigens were eluted with Tris buffer and an NaCl gradient (Fig. 2). Further purification steps required anion-exchange chromatography with S-Sepharose (Amersham Pharmacia Biotech) in water at pH 4, Q-Sepharose FF in 50 mM Tris buffer at pH 8.6, gel-filtration chromatography with Sephracyl S-300 HR (Amersham Pharmacia Biotech) in 50 mM PBS at pH 7.3, and isoelectric focusing. Separated polymers maintained their reactivity with the polyclonal antiserum. Fractions that appeared identical by the different measurements were pooled, dialyzed, lyophilized, and analyzed by high-resolution (500-MHz) proton nuclear magnetic resonance (NMR) spectroscopy. The final product was found to be essentially free of contaminating protein, nucleic acid, LPS, and lipids (29). The capsular polysaccharides were prepared in sterile, pyrogen-free saline for administration to animals.

Chemical characterization of capsular polysaccharides. Polysaccharides were analyzed for monosaccharide constituents and structure by both gas chromatography-mass spectroscopy (GC-MS) and NMR, as described elsewhere (32, 34). For GC-MS analysis, the polysaccharides were hydrolyzed completely and converted to alditol acetate derivatives. Analyses were performed on an HP 6890/5973 GC-MS spectrometer (Hewlett Packard, Wilmington, Del.) with a capillary DB17 column (J&W Scientific, Folsom, Calif.). GC was started at 150°C and held for 2 min; after an increase to 260°C at a rate of 5°C/min, GC was held at the final temperature for 20 min. Mass detection was obtained by electron ionization at 70 eV, and ions were scanned from 45 to 550 *m/z*. NMR experiments were performed on a Unity 500 spectrometer (Varian, Palo Alto, Calif.) with a proton resonance frequency of 500 MHz. All ^1H spectra were recorded at 70°C in ^2H_2O, and chemical shifts were referenced in relation to H^2HO resonance at 4.36 ppm.

The molecular-size distribution of the polysaccharides was determined by gel filtration chromatography on Superose 6 columns calibrated with dextran standards according to the fractionation ranges of the gel (Amersham Pharmacia Biotech) at a flow rate of 0.5 ml/min (13, 24, 33).

Preparation of polyclonal antiserum. Antiserum to *B. fragilis* strain 638R was prepared by intravenous immunization of New Zealand White rabbits three times a week for 3 weeks followed by a single booster injection during the fifth week and bleeding after 7 weeks. Each injection consisted of ~10^9 bacteria that were grown in basal medium, killed with formalin, and resuspended in 1 ml of sterile saline.

Immunological assays. Double diffusion in agarose was performed by the method of Ouchterlony (20) with 1% agarose in 50 mM Tris (pH 7.3). IEP was performed in 1% agarose in 50 mM Tris (pH 7.3), 50 mM phosphate buffer (pH 6.2), and 0.2 M barbital buffer (pH 8.6). The polysaccharide antigens were used as 1-mg/ml solutions. Each antigen preparation was reacted with polyvalent rabbit anti-*B. fragilis* strain 638R antiserum (10). SDS-PAGE was performed

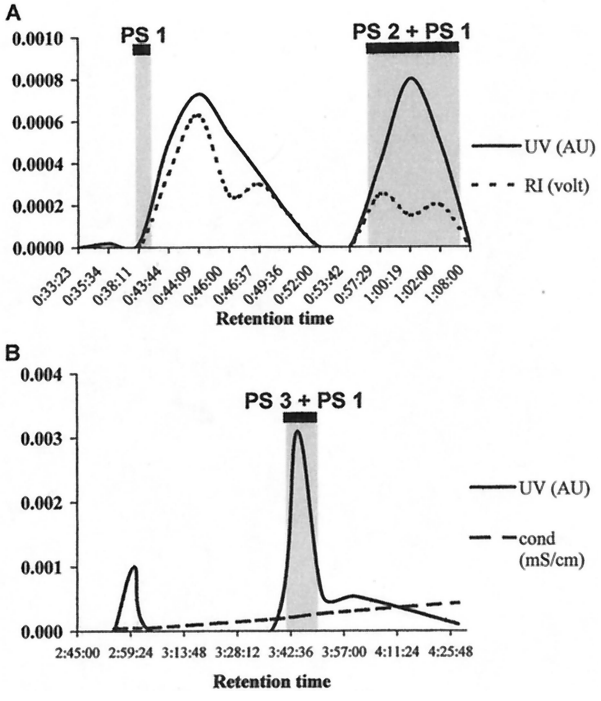

FIG. 2. Recorded profile of UV absorbance at 280 nm, refractive index (RI), and conductivity (cond) of ion-exchange chromatography (Q-Sepharose FF) of the capsular polysaccharide complex of *B. fragilis* strain 638R with 50 mM Tris (pH 7.3) (A) and during elution with an NaCl gradient (B). The polysaccharide-containing fractions were identified by dot blot assay, IEP, immunodiffusion, and NMR.

according to the method of Laemmli (16) with 4 to 20% gradient gels. The gels were stained with silver stain (Bio-Rad, Hercules, Calif.). Dot blotting was performed by applying 2.5 μl of capsular polysaccharide from a 1-mg/ml solution to a nitrocellulose paper as described previously (22).

For pooled chromatography fractions and purified polysaccharides, an enzyme-linked immunosorbent assay (ELISA) with and without inhibition was performed as described previously (11, 21). In brief, polystyrene microtiter plates (Immulon A; Dynatech Laboratories, Chantilly, Va.) were coated with poly-L-lysine-coupled polysaccharides. After nonspecific binding was blocked, polyvalent rabbit anti-*B. fragilis* strain 638R antiserum, MAb 4D5 (21), or control antibody with or without *B. fragilis* strain 638R polysaccharides was added. The reactions were visualized with an alkaline phosphatase-conjugated anti-rabbit or anti-mouse polyclonal antibody (Sigma, St. Louis, Mo.) and by subsequent addition of the phosphatase substrate *p*-nitrophenyl phosphate (Sigma).

For the electron microscopic visualization of the capsules of bacterial cells, bacteria were grown as described above, washed extensively, and suspended in 15 mM PBS. Drops of bacterial solutions (5 μl) were placed on Parafilm, and 200-mesh Formvar-carbon-coated copper grids (Electron Microscopy Sciences, Fort Washington, Mass.) were placed on top of the drops for 1 min. The grids were then blocked by placement on a 15-μl drop of 0.5% fish skin gelatin in 15 mM PBS with 0.1% Tween 20. Application of the primary antibody, polyvalent rabbit antiserum to *B. fragilis* strain 638R diluted 1:1,000 in blocking buffer, with or without inhibiting capsular polysaccharide, to the grids in a fashion similar to that described above was followed by incubation for 20 min at room temperature and four washes with 15 mM PBS. Normal rabbit serum, mouse serum, or isotype-matched antibodies were used as controls. Gold-conjugated protein A (with gold particles 20 nm in diameter) was applied at a dilution of 1:20 in blocking buffer. After the grids were incubated for 15 min at room temperature, they were washed twice with 15 mM PBS and deionized water. Staining was performed in quadruplicate. The grids were examined by an observer unaware of the experimental design. Two hundred bacteria on each grid were counted, and the number of gold-labeled bacteria was recorded. The inhibition of labeling was expressed as a percentage.

Isolation of capsular polysaccharide PS A1 from *B. fragilis* NCTC 9343. PS A1 from *B. fragilis* NCTC 9343 was prepared as described previously (2, 22, 30, 31). The polysaccharide was isolated by hot phenol-water extraction, enzyme digestion, gel filtration, and anion-exchange chromatography, and isoelectric focusing. PS A1 was prepared in sterile, pyrogen-free saline for administration to animals.

Animal model for abscess induction. In two independent experiments, 0.5 ml of injection volume containing 100, 10, 1, or 0.1 μg of capsular polysaccharide and sterile cecal contents (dilution, 1:4 in PBS) was administered intraperitoneally by injection through an 18-gauge needle to groups of six outbred male Wistar rats (150 to 175 g; Charles River Laboratories). As the control, PBS was substituted for the capsular polysaccharide. Six days after challenge, the rats were sacrificed and then examined for macroscopically visible intraperitoneal abscesses by observers unaware of the treatment status. Grossly visible abscesses were confirmed microscopically. The development of one or more abscesses was considered a positive result.

Statistical analysis. In vivo experiments were analyzed in a structured logistic regression model that permitted evaluation of separate dose-response relationships (dose in micrograms) and direct interference on the median abscess inducing dose (AD^{50}), which is the theoretical dose of polysaccharide required to induce abscesses in 50% of animals (6, 13, 30). Likelihood ratio tests were performed for hypotheses concerning commonality of dose-response slopes and AD^{50}s. Rats that died within 2 days after challenge were not included in the analysis because their deaths were due to anesthesia.

RESULTS

Isolation of polysaccharides. Attempts to separate the differently charged polymers by isoelectric focusing or application of ion-exchange or gel filtration chromatography were not successful, whereas mild acid hydrolysis and further processing by ion-exchange and gel filtration chromatography and isoelectric focusing led to the isolation of distinct capsular polysaccharides.

The flow of the Q-column run yielded a purified polysaccharide that was temporarily designated PS 1 (average molecular mass, 110 kDa). A distinct polysaccharide, termed PS 2, also eluted from this column, but the preparation was contaminated with PS 1 (Fig. 2A). In addition, fractions that eluted with a low NaCl concentration (conductivity, 22 μS/cm) yielded a third polysaccharide, designated PS 3, which was also contaminated with PS 1 (Fig. 2B).

TABLE 1. GC-MS analysis of PS 1, PS 2, and PS 3 of *B. fragilis* strain 638R

Polysaccharide	Saccharide
PS 1	3-Acetamido-3,6-dideoxyglucose
	2-Amino-4-acetamido-2,4,6-trideoxygalactose
	N-Acetylmannosamine
	Fucose
	Mannoheptose
	3-Hydroxybutanoic acid
PS 2	Fucosamine[a]
	Pentose
	Mannose
	Uronic acid of 2-amino[a]-2,6-dideoxyhexose (e.g., fucosaminuronic acid)
	Uronic acid of hexosamine[a] (e.g., mannosaminuronic acid)
	Unidentified amino sugar
PS 3	Galactose
	Mannosamine[a]
	Rhamnose
	Uronic acid of 6-deoxyhexosamine[a]
	Uronic acid of hexosamine[a] (two)

[a] N-acetylated form indistinguishable by GC-MS analysis.

Further processing of the fractions containing PS 2 by chromatography using S-300 HR and 2 M NaCl elution of S-Sepharose FF (distilled H_2O [pH 4]) permitted purification of PS 2. PS 2 was found to have an average molecular mass of 250 kDa.

Separation of PS 3 from PS 1 was achieved by chromatography with Q-Sepharose FF (50 mM Tris at pH 8.6 and 5% octyl-β-glucoside [Pierce]). PS 3 (average molecular mass, 185 kDa) was eluted with a low concentration of salt (conductivity, 29.5 μS/cm). After dialysis, PS 3 was purified by isoelectric focusing with a pH gradient of 3 to 10 in 1% ampholyte (Bio-Lyte; Bio-Rad), where it migrated at pH 3 toward the anode.

Immunochemical characterization of polysaccharides. IEP of the isolated polysaccharides at acid, neutral, and basic pHs showed distinct patterns reflecting different structural compositions and net charge motifs. The distinct patterns represented the main components of the CPC described above (Fig. 1).

PS 1 showed a predominant neutral-to-positive charge motif at neutral pH that was unchanged at pH 6.2 and slightly shifted toward the anode at pH 8.6. Efforts to determine the exact isoelectric point of PS 1 by isoelectric focusing showed a wide distribution over a pH range between 4.5 and 10 (data not shown). However, the structure and conformation of PS 1, as determined by a combination of various analyses, showed a polymer with one free amino group and one carboxyl group per repeating unit, properties that give the polymer an average net neutral charge motif and classify it as a balanced zwitterionic polysaccharide (34). PS 1 is composed of five residues, namely, 2-amino-4-acetamido-2,4,6-trideoxygalactose (AAT), fucose (Fuc), mannoheptose (Hep) replaced with 3-hydroxybutanoic acid, *N*-acetylmannosamine (ManNAc), and 3-acetamido-3,6-dideoxyglucose (ADG) (GC-MS analysis [Table 1]). NMR spectroscopy confirmed five anomeric proton signals at 5.26, 4.97, 4.84, 4.79, and 4.70 ppm (Fig. 3). Further studies by two-dimen-

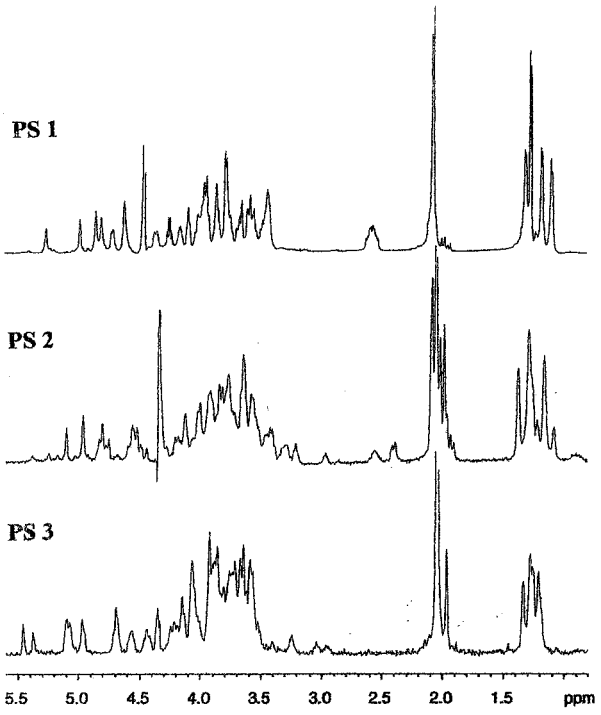

FIG. 3. ^{1}H NMR spectra of PS 1, PS 2, and PS 3.

sional NMR spectroscopies, chemical methods, gas chromatography, and molecular modeling elucidated both the chemical and three-dimensional structures described elsewhere (34). The repeating unit of PS 1 is a branched pentasaccharide with the sequence of →2-Hep-(1→3)-ManNAc-(1→4)[Fuc-(1→2)]-ADG-(1→3)-AAT-(1→.

On the basis of the structure of PS 1 and a partial analysis of the biosynthesis region in the same area of the 638R chromosome that encodes PS A1 of NCTC 9343, PS 1 was redesignated PS A2 (Coyne et al., submitted). PS A2 was reactive with polyclonal antiserum to whole bacteria. In an inhibition ELISA, polyclonal antiserum adsorbed with PS A2 (25 μg/ml) showed a dose-dependent reduction of up to 100% of reactivity with PS A2. A dose of 0.06 μg/ml inhibited the reaction by 50% (Fig. 4). The reactivity of polyclonal antiserum with whole organisms in electron microscopy was reduced by an average of 52% by adsorption with the purified PS A2.

Electrophoretic mobility suggested that a PS 2 molecule has a net positive charge (Fig. 1). Both GC-MS analysis and NMR indicated that PS 2 is slightly contaminated—predominantly with PS 1. Unfortunately, we could not completely separate PS 2 from this polysaccharide because of the limited quantity of PS 2 synthesized by the bacterial strain (purified amount, 5 mg). Adsorption of polyclonal antiserum with PS 2 reduced immunolabeling in electron microscopy by an average of 15%. However, by subtractive analysis, we were able to determine the composition of PS 2, which is definitely distinct from the other two sugars: GC-MS analysis identified pentose, mannose, fucosamine, an unidentified amino sugar, and uronic acids of 2-amino-6-dideoxyhexose and of hexosamine (Table 1). NMR analysis showed structural differences between PS 2 and both PS 1 and PS 3. These differences were most evident from the proton chemical shifts between 4.5 and 5.5 ppm. PS 2 showed several acetyl signals around 2.05 ppm, indicating that PS C2 has several N- or O-acetylated hexosamines. Resonances at ~1.3 ppm arise from methyl groups of 6-deoxy sugars (Fig. 3).

A polysaccharide biosynthesis locus involved in the synthesis of an unidentified polysaccharide of NCTC 9343 was sequenced. Because this locus was shown not to be involved in the synthesis of PS A1 or PS B1, it was designated the PS C1 biosynthesis locus. The same area of the 638R chromosome was sequenced and was similarly designated the PS C2 biosynthesis locus because the genes involved in polysaccharide biosynthesis were distinct from those of the PS C1 locus (5). A mutant with a transposon in the PS C2 locus was unable to produce the capsular polysaccharide that reacts with MAb 4D5. We have demonstrated that among our collection of 50 *B. fragilis* strains, only those with a PS C locus genetically similar to 638R react with 4D5, indicating that this MAb is specific for PS C2. PS 2 was demonstrated by dot blot assay, double immunodiffusion, IEP, and ELISA to be reactive with MAb 4D5 and was therefore designated PS C2. Based on the genetic complement of the PS C2 locus, the composing sugars were predictive. Inhibition ELISA with polyclonal antiserum and PS A2 and PS C2 as inhibitors showed a 10-fold difference in inhibition between the two polysaccharides. PS C2 inhibited the reactivity of the PAS with PS A2 by 50% at a theoretical dose of 0.6 μg/ml (Fig. 4).

Because neither biosynthesis nor a flanking region has been identified for the third isolated polysaccharide, for the time being this polymer is called PS 3. The net charge of PS 3 is negative, as shown by IEP (Fig. 1), and its behavior during the purification process by ion-exchange chromatography predicted a negatively charged polymer (Fig. 2). GC-MS analysis identified galactose, mannosamine, rhamnose, and three uronic acids, one of a 6-deoxyhexosamine and two of hexosamines (Table 1). NMR spectroscopy showed resonances between 4.5 and 5.5 ppm from six anomeric carbons and chemical-shift signals around 1.3 ppm originating from sugars, such as rhamnose and 3-acetamido-3,6-dideoxyglucose (Fig. 3). Ad-

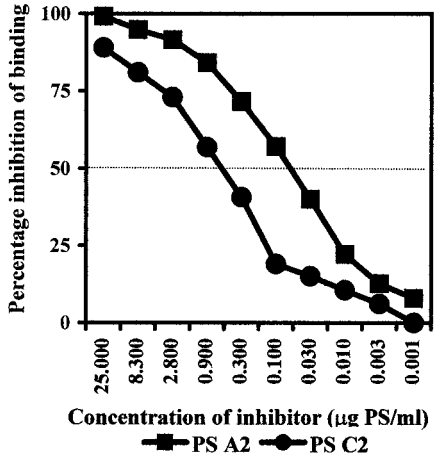

FIG. 4. Inhibition by PS A2 and PS C2 of binding of rabbit polyclonal antiserum raised to whole bacteria of *B. fragilis* strain 638R on microtiter wells coated with PS A2. The values are the means of duplicate determinations.

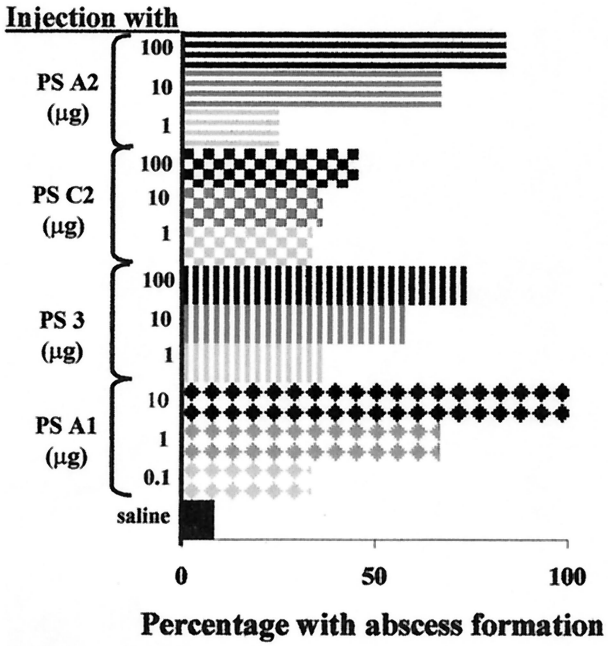

FIG. 5. Induction of intra-abdominal abscesses in rats with different doses of capsular polysaccharides PS A2, PS C2, and PS 3 of *B. fragilis* strain 638R, PS A1 of *B. fragilis* NCTC 9343, and saline. The rat model of intra-abdominal-abscess formation was used in two independent experiments with six rats per group in each experiment.

sorption of the polyclonal antiserum with PS 3 reduced immunolabeling of whole bacteria by an average of 41% in electron microscopy.

Biological activity of polysaccharides in experimental abscess formation. All the polysaccharides in the tested dose ranges induced intra-abdominal abscesses in a dose-dependent fashion (testing for no dose effect; PS A1, PS A2, and PS 3, $P < 0.001$; PS C2, $P = 0.19$) (Fig. 5). PS A1 of *B. fragilis* NCTC 9343 was the most potent abscess-inducing zwitterionic polysaccharide, with an AD^{50} of 0.28 μg. The AD^{50} of the zwitterionic polysaccharide PS A2 of strain 638R was 4.79 μg—17 times higher. At a theoretical dose of 3.58 μg, the predominantly negatively charged polysaccharide PS 3 would have induced abscesses in 50% of the animals. The AD^{50} of the predominantly positively charged polysaccharide PS C2 was 749.89 μg.

DISCUSSION

Studies with monoclonal and polyclonal antibodies have demonstrated that capsular polysaccharides of abscess-inducing *B. fragilis* strains, including the type strain, NCTC 9343, and the clinical strain 638R, are heterogeneous (3, 15, 21, 23, 26–28). For strain NCTC 9343, two polysaccharides of a CPC have been fully characterized (2, 31). Only a few bacterial organisms are known to produce such a complicated array of surface polysaccharides (1, 8, 12, 18). Recently, three polysaccharide biosynthesis loci have been identified for the strain NCTC 9343 (7; Coyne et al., submitted). In this study, we were able to isolate three different immunogenic capsular polysaccharides of the strain 638R.

Although the biological activity of PS A2 in vivo is very high, it is nevertheless 17 times lower than that of PS A1. Both polysaccharides have a balanced positive and negative charge motif on each repeating unit, and they have comparable molecular masses. However, several aspects of their three-dimensional structures might be different. Conformational modeling of PS A2 suggested a right-handed helix with two repeating units per turn and a pitch of 20 Å (34). Exposure of positive and negative charges on the outer surface of the polymer in a regularly spaced pattern renders them accessible to other molecules. In contrast to PS A2, PS A1 consists of only a tetrasaccharide instead of a pentasaccharide repeating unit, a difference that might contribute to a smaller pitch (31). The genes *wcfR* and *wcfS* are involved in the synthesis of the unusual sugar AAT and are found in the PS A1 locus of strain 9343 and in the PS A2 locus of strain 638R (Coyne et al., submitted). Although PS A1 and PS A2 have the α-D-AAT sugar in common, the amino group is at position 2 in PS A2 and at position 4 in PS A1. This and other compositional differences might lead to different charge accessibilities, to different configurations of large grooves, to a slightly more preferred configuration of PS A1 that enhances the interaction between polysaccharides and protein molecules required for biological activity (13), and to an altogether different host response. Another explanation for the lower abscess-inducing activity of PS A2 might be that, although PS A2 demonstrated an average balanced zwitterionic charge motif, isoelectric focusing showed that PS A2, in contrast to PS A1, had a heterogeneous charge distribution ranging from a negative to a positive net charged motif. This heterogeneity may indicate that PS A2 is being isolated in a form with molecules having variable degrees of acetylation.

The PS C2 biosynthesis locus has been sequenced and found to be composed of 22 open reading frames (3). Two genes encoding products similar to aminotransferases were identified, suggesting that PS C2 contains at least two monosaccharides with free amino groups. This finding was confirmed by the results of the GC-MS analysis, NMR, and IEP. Complementation analysis has determined that the products of two genes, *mnaA* and *mnaB* (formerly *wcgC* and *wcgD*), are involved in the synthesis of *N*-acetyl-mannosaminuronic acid, which could be the uronic acid of hexosamine identified by GC-MS analysis. Three clustered genes, *wcgJ* to *wcgL*, encode products likely to be involved in the formation of *N*-acetylfucosamine. A possibly N-acetylated form of a fucosamine was identified by GC-MS analysis. Homology-based analysis identified five putative glycosyltransferases, suggesting that PS C2 contains at least five monosaccharides (3). GC-MS analysis identified six composing sugars. The AD^{50} of PS C2, at 749.89 μg, was unexpectedly high. C-substance, the group polysaccharide from *Streptococcus pneumoniae*, is another naturally occuring zwitterionic polysaccharide with a positive net charge. It has a tetrasaccharide repeating unit with a total of three positive and two negative charges, which give a highly charged density to the polysaccharide. C-substance was shown to be a potent abscess-inducing polymer with an AD^{50} of 5 μg (30). We hypothesize that contaminating polysaccharides isolated with PS C2 may be masking critical charges and configurations, resulting in a loss of abscess-inducing potency.

PS 3 was shown by GC-MS analysis to contain three sugars carrying uronic acids, to possess a net negative zwitterionic

charge motif demonstrated by IEP, and to be a potent abscess-inducing capsular polysaccharide. We were not able to determine by GC-MS analysis which of the hexosamines is N acetylated. PS 3 certainly has a high density of charged molecules, which renders the polysaccharide very capable of inducing intra-abdominal abscesses. Previously, the capsular polysaccharide of *S. pneumoniae* type 1 was shown to induce intra-abdominal abscesses in 50% of animals at a dose of 31 μg, and PS B1 of *B. fragilis* NCTC 9343 had an AD^{50} of 25 μg. Both of these capsular polysaccharides carry two negatively changed groups and one positively charged group per repeating unit (30).

We were able to isolate three distinct surface-expressed capsular polysaccharides from a single abscess-modulating bacterial strain, *B. fragilis* 638R. Compositional and immunochemical studies demonstrated a common zwitterionic charge motif in all of the capsular polysaccharides, with net charges ranging from predominantly positive to balanced to predominantly negative. This study reflects the importance of the determination of the structural composition of the CPC in a species in yielding information about biological activity, biosynthesis, and their relationships.

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REFERENCES

1. **Abe, M., J. E. Sherwood, R. I. Hollingsworth, and F. B. Dazzo.** 1984. Stimulation of clover root hair infection by lectin-binding oligosaccharides from the capsular and extracellular polysaccharides of *Rhizobium trifolii*. J. Bacteriol. **160:**517–520.
2. **Baumann, H., A. O. Tzianabos, J. R. Brisson, D. L. Kasper, and H. J. Jennings.** 1992. Structural elucidation of two capsular polysaccharides from one strain of *Bacteroides fragilis* using high-resolution NMR spectroscopy. Biochemistry **31:**4081–4089.
3. **Comstock, L. E., M. J. Coyne, A. O. Tzianabos, and D. L. Kasper.** 1999. Interstrain variation of the polysaccharide B biosynthesis locus of *Bacteroides fragilis*: characterization of the region from strain 638R. J. Bacteriol. **181:** 6192–6196.
4. **Comstock, L. E., M. J. Coyne, A. O. Tzianabos, A. Pantosti, A. B. Onderdonk, and D. L. Kasper.** 1999. Analysis of a capsular polysaccharide biosynthesis locus of *Bacteroides fragilis*. Infect. Immun. **67:**3525–3532.
5. **Comstock, L. E., A. Pantosti, and D. L. Kasper.** 2000. Genetic diversity of the capsular polysaccharide C biosynthesis region of *Bacteroides fragilis*. Infect. Immun. **68:**6182–6188.
6. **Cox, C.** 1990. Fieller's theorem, the likelihood and the delta method. Biometrics **46:**709–718.
7. **Coyne, M. J., W. Kalka-Moll, A. O. Tzianabos, D. L. Kasper, and L. E. Comstock.** 2000. *Bacteroides fragilis* NCTC9343 produces at least three distinct capsular polysaccharides: cloning, characterization, and reassignment of polysaccharide B and C biosynthesis loci. Infect. Immun. **68:**6176–6181.
8. **Glazebrook, J., and G. C. Walker.** 1989. A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell **56:**661–672.
9. **Gorbach, S. L., and J. G. Bartlett.** 1974. Anaerobic infections (second of three parts). N. Engl. J. Med. **290:**1237–1245.
10. **Graber, P., and C. A. Williams.** 1953. Methode permettant l'etude conjuguee des proprietes electrophoretiques et immunochemiques d'un melange de proteins. Application au serum sanguin. Biochim. Biophys. Acta **10:**193.
11. **Gray, B. M.** 1979. ELISA methodology for polysaccharide antigens: protein coupling of polysaccharides for adsorption to plastic tubes. J. Immunol. Methods **28:**187–192.
12. **Gray, J. X., and B. G. Rolfe.** 1990. Exopolysaccharide production in *Rhizobium* and its role in invasion. Mol. Microbiol. **4:**1425–1431.
13. **Kalka-Moll, W. M., A. O. Tzianabos, Y. Wang, V. J. Carey, R. W. Finberg, A. B. Onderdonk, and D. L. Kasper.** 2000. Effect of molecular size on the ability of zwitterionic polysaccharides to stimulate cellular immunity. J. Immunol. **164:**719–724.
14. **Kasper, D. L., A. B. Onderdonk, B. G. Reinap, and A. A. Linberg.** 1980. Variations of *Bacteroides fragilis* with in vitro passage: presence of an outer membrane-associated glycan and loss of capsular antigen. J. Infect. Dis. **142:**750–756.
15. **Kasper, D. L., A. Weintraub, A. A. Lindberg, and J. Lonngren.** 1983. Capsular polysaccharides and lipopolysaccharides from two *Bacteroides fragilis* reference strains: chemical and immunochemical characterization. J. Bacteriol. **153:**991–997.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
17. **Lindberg, A. A., A. Weintraub, D. L. Kasper, and J. Lonngren.** 1982. Virulence factors in infections with *Bacteroides fragilis*: isolation and characterization of capsular polysaccharide and lipopolysaccharide. Scand. J. Infect. Dis. Suppl. **35:**45–52.
18. **Omar, A. S., J. Weckesser, and H. Mayer.** 1983. Different polysaccharides in the external layers (capsule and slime) of the cell envelope of *Rhodopseudomonas capsulata* Sp11. Arch. Microbiol. **136:**291–296.
19. **Onderdonk, A. B., D. L. Kasper, R. L. Cisneros, and J. G. Bartlett.** 1977. The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: comparison of the pathogenic potential of encapsulated and unencapsulated strains. J. Infect. Dis. **136:**82–89.
20. **Ouchterlony, O.** 1958. Diffusion-in-gel methods for immunological analysis. Prog. Allergy. **5:**1–78.
21. **Pantosti, A., R. Colangeli, A. O. Tzianabos, and D. L. Kasper.** 1995. Monoclonal antibodies to detect capsular diversity among *Bacteroides fragilis* isolates. J. Clin. Microbiol. **33:**2647–2652.
22. **Pantosti, A., A. O. Tzianabos, A. B. Onderdonk, and D. L. Kasper.** 1991. Immunochemical characterization of two surface polysaccharides of *Bacteroides fragilis*. Infect. Immun. **59:**2075–2082.
23. **Pantosti, A., A. O. Tzianabos, B. G. Reinap, A. B. Onderdonk, and D. L. Kasper.** 1993. *Bacteroides fragilis* strains express multiple capsular polysaccharides. J. Clin. Microbiol. **31:**1850–1855.
24. **Paoletti, L. C., and K. D. Johnson.** 1995. Purification of preparative quantities of group B streptococcus type III oligosaccharides. J. Chromatogr. A **705:**363–368.
25. **Polk, B. F., and D. L. Kasper.** 1977. *Bacteroides fragilis* subspecies in clinical isolates. Ann. Intern. Med. **86:**569–571.
26. **Poxton, I. R., and R. Brown.** 1986. Immunochemistry of the surface carbohydrate antigens of *Bacteroides fragilis* and definition of a common antigen. J. Gen. Microbiol. **132:**2475–2481.
27. **Privitera, G., A. Dublanchet, and M. Sebald.** 1979. Transfer of multiple antibiotic resistance between subspecies of *Bacteroides fragilis*. J. Infect. Dis. **139:**97–101.
28. **Reid, J. H., S. Patrick, and S. Tabaqchali.** 1987. Immunochemical characterization of a polysaccharide antigen of *Bacteroides fragilis* with an IgM monoclonal antibody. J. Gen. Microbiol. **133:**171–179.
29. **Tzianabos, A. O., R. W. Finberg, Y. Wang, M. Chan, A. B. Onderdonk, H. J. Jennings, and D. L. Kasper.** 2000. T cells activated by zwitterionic molecules prevent abscesses induced by pathogenic bacteria. J. Biol. Chem. **275:**6733–6740.
30. **Tzianabos, A. O., A. B. Onderdonk, B. Rosner, R. L. Cisneros, and D. L. Kasper.** 1993. Structural features of polysaccharides that induce intra-abdominal abscesses. Science **262:**416–419.
31. **Tzianabos, A. O., A. Pantosti, H. Baumann, J. R. Brisson, H. J. Jennings, and D. L. Kasper.** 1992. The capsular polysaccharide of *Bacteroides fragilis* comprises two ionically linked polysaccharides. J. Biol. Chem. **267:**18230–18235.
32. **Wang, Y., and R. I. Hollingsworth.** 1994. The structure of the O-antigenic chain of the lipopolysaccharide of *Rhizobium trifolii* 4s. Carbohydr. Res. **260:**305–317.
33. **Wang, Y., R. I. Hollingsworth, and D. L. Kasper.** 1998. Ozonolysis for selectively depolymerizing polysaccharides containing beta-D-aldosidic linkages. Proc. Natl. Acad. Sci. USA **95:**6584–6589.
34. **Wang, Y., W. M. Kalka-Moll, M. H. Roehrl, and D. L. Kasper.** 2000. Structural basis of the abscess-modulating polysaccharide A2 from *Bacteroides fragilis*. Proc. Natl. Acad. Sci. USA **97:**13478–13483.

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