Chemical and Immunochemical Analyses of Bacteroides fragilis Lipopolysaccharides

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Lipopolysaccharides (LPSs) from 17 different Bacteroides fragilis strains were extracted in a two-step procedure. The first step was a hot phenol-water extraction of whole bacteria, resulting in a crude aqueous phase, which after lyophilization in a second step was extracted with a phenol-chloroform-light petroleum mixture. The resulting LPSs, which were essentially free from contaminating nucleic acid, proteins, and capsular polysaccharide, were investigated for their (i) qualitative and quantitative sugar and fatty acid composition, (ii) immunochemical specificity by enzyme-linked immunosorbent inhibition assays, and (iii) particle weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Of the 17 strains, 13 had LPSs which all contained l-rhamnose, d-glucose, d-galactose, and d-glucosamine in approximately the same ratio. Three of these LPSs also contained d-galactosamine. The fatty acid composition was also similar in that the same fatty acids, although in slightly varying proportions, were found in all LPSs. The 13 strains also showed the same specificity in inhibition studies by enzyme immunoassay with rabbit anti-LPS antisera and LPS antigen. The LPS particle weights were also very similar, in the range of what is found for LPSs from rough mutant strains of enterobacteria. Our results suggest that most strains of B. fragilis have similar, if not identical, LPSs with relatively short polysaccharide chains.

The cell envelope of Bacteroides fragilis bacteria has been shown to contain two major saccharide containing components, a lipopolysaccharide (LPS) and a capsular polysaccharide (13). The chemical composition and immunochemical properties of B. fragilis LPS have been subject to investigations, but the results have varied (7, 10, 11). A main reason for the observed variations has probably been the difficulty in obtaining pure LPS preparations. Most extraction procedures, such as phenol-water extractions or outer membrane extraction, result in preparations which contain both LPS and capsular polysaccharide (13). A further possibility for variation is the observation that upon several in vitro passages of the B. fragilis strain ATCC 23745, the capsule was replaced by a glycan which was coextracted with the LPS (12).

We recently showed that B. fragilis LPS can be isolated in pure form by first making a phenol-water extraction of the bacteria, followed by a phenol-chloroform-light petroleum (PCP) extraction of the lyophilized aqueous phase (13). Although the results from chemical analyses of the B. fragilis LPS have varied, it is clear that this antigen differs in its composition and biological activity from most aerobic gram-negative bacteria (7). The polysaccharide part of the LPS appears to lack both a conventional 3-deoxy-d-manno-octulosonic acid and 1-glycerol-d-mannoheptose (5). The fatty acid composition of the lipid part of B. fragilis is also different from that of lipid A of enterobacterial LPS since it does not contain 3-hydroxytetradecanoic acid, a major fatty acid in aerobic gram-negative bacteria (25).

The finding that an antiserum elicited by a purified B. fragilis LPS could identify approximately 90% of clinical isolates of B. fragilis, raised the question of common antigenic determinants (one or more) in the LPS (23). In the present study, we describe the carbohydrate and fatty acid composition as well as particle weight of LPS purified by PCP extraction from 17 different B. fragilis strains, most of which have previously been analyzed in DNA homology studies (9). Of these 17 strains, 13 (76%) had a similar monosaccharide composition both qualitatively and quantitatively and were shown to be similar, if not identical, in immunochemical studies with a rabbit antiserum-B. fragilis LPS inhibition system.

MATERIALS AND METHODS

Bacterial strains. The B. fragilis strains used are listed in Table 1.

Growth conditions. All strains were grown in a 4-liter fermentor (Ultraferm 160; LKB Produkter, Bromma, Sweden). The composition, preparation of the medium, and the growth conditions were essentially as described earlier (13). The medium in this study did not contain fetal calf serum.

Extraction of LPS. Pelleted organisms were suspended in water and extracted with phenol-water (24). After dialysis against tap water for 6 days and against distilled water overnight, the aqueous phase was lyophilized. The LPS was obtained from the lyophilized water phase with PCP extraction (3). The LPS was subsequently precipitated from the phenol phase with distilled water, and the precipitate was washed once with 80% aqueous phenol (wt/vol) and four times with acetone. After drying, the LPS was dissolved in distilled water, dialyzed for 3 days against distilled water at 4°C, and lyophilized.

Chemical methods. The protein content was determined by the method of Lowry et al. (17) with bovine serum albumin as the standard. The presence of nucleic acids was determined by UV spectroscopy (16).

For sugar analyses the LPS was hydrolyzed with 0.5 M trifluoroacetic acid at 100°C for 16 h, and the monosaccharides were analyzed with gas-liquid chromatography after conversion to alditol acetates essentially as described by Sawardeker et al. (21). Alditol acetates were separated isothermically with the following columns: 3% SP 2340 on 100/120 Supelcoport at 220°C and 3% OV 17 on 100/120 Gas-Chrom Q at 190°C (Supelco, Bellefonte, Pa.). The
TABLE 1. B. fragilis strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>9343 (VPI 2553)</td>
<td>National Collection of Type Cultures, London, England</td>
</tr>
<tr>
<td>23745 (VPI 5383)</td>
<td>American Type Culture Collection, Rockville, Md.</td>
</tr>
<tr>
<td>10584</td>
<td>National Collection of Type Cultures</td>
</tr>
<tr>
<td>E323</td>
<td>Institute Pasteur de Lille, Lille, France</td>
</tr>
<tr>
<td>TM 4000</td>
<td>Francis Tally, Tufts Medical School, Boston, Mass.</td>
</tr>
<tr>
<td>Ic650</td>
<td>Francis Tally</td>
</tr>
<tr>
<td>2556-1</td>
<td>Virginia Polytechnic Institute and State University (VPI), Blacksburg</td>
</tr>
<tr>
<td>6059</td>
<td>VPI</td>
</tr>
<tr>
<td>6851</td>
<td>VPI</td>
</tr>
<tr>
<td>6057-B</td>
<td>VPI</td>
</tr>
<tr>
<td>4361</td>
<td>VPI</td>
</tr>
<tr>
<td>2554</td>
<td>VPI</td>
</tr>
<tr>
<td>6815</td>
<td>VPI</td>
</tr>
<tr>
<td>4117</td>
<td>VPI</td>
</tr>
<tr>
<td>2552</td>
<td>VPI</td>
</tr>
<tr>
<td>4225</td>
<td>VPI</td>
</tr>
<tr>
<td>5631</td>
<td>VPI</td>
</tr>
</tbody>
</table>

columns were fitted to a Varian gas chromatograph (model 1400; Palo Alto, Calif.) equipped with a flame ionization detector and connected to a Hewlett-Packard integrator (model 3380A; Palo Alto, Calif.). The sugars were identified by cochromatography with authentic standards. For quantitative analyses t-xylene was used as an internal standard.

The fatty acids were liberated and converted to methylesters by methanolysis with 2 M HCl in water-free methanol at 85°C for 18 h (20). The fatty acid methylesters were then extracted twice with n-hexane, and the volume was reduced under a stream of nitrogen. The fatty acid methylesters were separated isothermically (170°C) on a WCOT OV 101 glass capillary column (25 m; Chrompack, Middelburg, Netherlands). The column was fitted to a Varian gas chromatograph (model 3700) which was equipped with a flame ionization detector and connected to a Hewlett-Packard integrator (model 3380A). For quantitative analyses heptadecanoic acid (methylester) and 3-hydroxydecanoic acid (methylester) were used as internal standards.

**SDS-polyacrylamide gel electrophoresis.** The discontinuous gel system used was essentially as described previously (14). The separation gel (142 by 120 by 1.5 mm) contained 20% acrylamide and 0.45% bisacrylamide. The LPS and capsular polysaccharide samples in water were mixed with an equal volume of 0.1 M Tris-hydrochloride buffer (pH 6.8) containing 2% (wt/vol) sodium dodecyl sulfate, 10% (vol/vol) glycerol, 2% (vol/vol) 2-mercaptoethanol, 0.1% (wt/vol) EDTA, and 0.001% (wt/vol) phenol red. The mixtures were heated at 100°C for 5 min, and 10- to 20-μl samples containing 5 μg of LPS or 20 μg of capsular polysaccharide were applied to the sample wells. The gels were stained by the silver method (22).

**Immunochromatography methods.** Rabbit antisera against B. fragilis NCTC 9343 and B. fragilis ATCC 23745 LPSs were prepared as described earlier (13). The enzyme-linked immunoabsorbant assay (ELISA) was performed as described previously (16). In ELISA inhibition experiments, serum was preincubated for 1 h with different concentrations of the LPS assayed. The 50% inhibitory value was recorded as the concentration of inhibitor needed to obtain a 50% lowering of the optical density at 40 nm as compared to control tubes with no inhibitor added.

**RESULTS**

**Isolation of LPSs.** All B. fragilis strains were grown in a 4-liter fermentor to late logarithmic phase. The yield of the bacteria was about 2 g liter (dry weight). The yield of crude aqueous phase after phenol-water extraction corresponded to about 5% of the bacterial mass dry weight (wt/wt). The LPS was subsequently isolated from the lyophilized crude aqueous phase after phenol-water extraction with PCP extraction. The yield of PCP-extractable LPS was about 0.5% of the bacterial mass (wt/wt). All LPS preparations were found to be virtually free of nucleic acids (<5 μg/mg of LPS) as assayed by UV spectroscopy. Furthermore, no proteins were present (<5 μg/mg of LPS) as determined by the method of Lowry et al. (17), with bovine serum albumin as standard.

**Carbohydrate analyses of LPSs.** After hydrolysis of the LPS preparations (0.5 M TFA, 16 h, 100°C), the resulting monosaccharides were reduced with NaBH₄, and after acetylation they were analyzed as alditol acetates by gas-liquid chromatography (Table 2).

The total amounts of carbohydrates in the different B. fragilis LPSs varied between 26 and 66%. In the quantitative and qualitative sugar analysis, five groups could be seen, all containing D-glucosamine, as follows: (i) group 1 of 10 strains with an L-rhamnose/D-galactose/D-glucose ratio of 1:3-5:1; (ii) group 2 with three strains having the same ratios as in group 1, but which in addition contained approximately one unit of D-galactosamine; (iii) group 3 with two strains with an L-rhamnose/D-galactose/D-glucose/D-galactosamine/D-glucosamine ratio of 1:1:1:1:2; (iv) one strain, VPI 4225, with a higher D-glucose content; and (v) one strain,

**TABLE 2. Sugar analysis of LPSs from 17 B. fragilis strains**

<table>
<thead>
<tr>
<th>Group</th>
<th>Strains</th>
<th>Sugar (mol %)*</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NCTC 9343, NCTC 10584, ATCC 23745, E323, TM 4000, Ic650, VPI 2556-1, VPI 6059, VPI 6851, VPI 6057-B</td>
<td>L-Rhamnose 12-18, D-Galactose 41-65, D-Glucose 10-14, ND Δ</td>
<td>8-32</td>
</tr>
<tr>
<td>2</td>
<td>VPI 6815, VPI 2554, VPI 4361</td>
<td>13-14, D-Galactose 43-54, D-Glucose 10-13, D-Galactosamine 7-11</td>
<td>10-20</td>
</tr>
<tr>
<td>3</td>
<td>VPI 2552, VPI 4117</td>
<td>12, D-Galactose 15-16, D-Glucose 17-18, D-Galactosamine 18</td>
<td>36-38</td>
</tr>
<tr>
<td>4</td>
<td>VPI 4225</td>
<td>17, D-Galactose 13, D-Glucose 33, D-Galactosamine 16</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>VPI 5631</td>
<td>32, D-Galactose ND, D-Glucose ND, D-Galactosamine ND</td>
<td>30</td>
</tr>
</tbody>
</table>

* Determined by gas-liquid chromatography as their alditol acetates.
Δ ND, Not detectable.
VPI 5631, lacking both d-galactose and d-galactosamine (Table 2).

Fatty acids in LPSs. The total amounts of fatty acids in the various LPS preparations varied from 24% (VPI 2552) to 52% (VPI 5631). The 9343 LPS preparation contained the following fatty acids: 13-methyltetradecanoic (15%), 3-hydroxypentadecanoic (16%), 3-hydroxyhexadecanoic (35%), 3-hydroxy-15-methylhexadecanoic (21%), and 3-hydroxyheptadecanoic (6%). The quantitative analysis of the LPSs revealed that the ratios between individual fatty acids were comparable (data not shown). The predominating fatty acids were identified as 3-hydroxyhexadecanoic and 3-hydroxy-15-methylhexadecanoic acids.

SDS-polyacrylamide gel electrophoresis of different B. fragilis LPSs. The LPS preparations from all 17 B. fragilis strains were examined together with capsular polysaccharides from B. fragilis NCTC 9343 and ATCC 23745 by using SDS-polyacrylamide gel electrophoresis. LPSs from Shigella flexneri strains 3a and 4bR were used as internal standards. The LPS isolated from strain 3a is of the smooth type, with an O polysaccharide chain that contains several repeating units. The 4bR LPS is of the rough type; it lacks the O polysaccharide chain but contains the entire core structure (8). The S. flexneri 3a LPS was resolved into many bands, each representing a different number of repeating units in the LPS molecule, whereas the 4bR LPS gave one broad band only (Fig. 1).

All of the 17 B. fragilis LPS preparations were of about the same size as the S. flexneri 4bR LPS. Some of the LPS preparations were not homogeneous. The LPS from B. fragilis strains NCTC 9343, ATCC 23745, E323, Icc50, VPI 2554, VPI 4361, VPI 6815, and TM 4000 showed one major and one minor band. The pure capsular polysaccharides from B. fragilis NCTC 9343 and ATCC 23745 were included as controls (Fig. 1C). None of the two preparations contained any detectable LPS, and only the NCTC-9343 capsule was visible. As reported earlier (13), these two capsular polysaccharides differ both in chemistry and immunochemical specificity. The difference in the migration in the SDS-polyacrylamide gel electrophoresis can be caused by difference in molecular size or difference in charge or both.

Immunological analyses. The immunological specificity of the 17 LPS preparations was studied in ELISA inhibition experiments with rabbit antisera elicited against B. fragilis NCTC 9343 and ATCC 23745 LPSs. All investigations were done with the homologous LPS as coating antigen, and the concentration of inhibitors required for 50% inhibition was determined (Fig. 2). Only results with the B. fragilis NCTC 9343 LPS-antisum system are shown since studies with the ATCC 23745 LPS and serum system gave essentially identical results. LPS from 12 of the strains inhibited in the same range as the NCTC 9343 LPS; 0.05 to 0.3 μg of inhibitor was required for 50% inhibition. All strains belong to groups 1 and 2 in terms of sugar composition (Table 2). The LPSs from VPI 4117 and VPI 2552 were less potent; the concentrations required for 50% inhibition were 5 and 75 μg, respectively. When the LPS from VPI 2552 and VPI 5631 were used as inhibitors, none reached a 50% inhibition even with 100 μg (the highest concentration used).

DISCUSSION

The present investigation of LPSs extracted from 17 different characterized B. fragilis strains revealed more similarities than differences. Below we discuss the LPSs with respect to (i) their molecular size, (ii) their fatty acid composition, (iii) their monosaccharide composition, and (iv) their immunological specificity.

All B. fragilis LPSs moved extensively in the SDS-polyacrylamide gel electrophoresis (Fig. 1). They resolved into one, or at most two, band(s) which were seen on the gel in the same region as the LPS from S. flexneri 4bR. This is a rough mutant strain which contains the enterobacterial lipid A and a polysaccharide composed of approximately 10 sugar residues, including 3-deoxy-D-manno-octulosonic acid (8). Since the B. fragilis LPS sugar/lipid ratio is approximately 1:1 and thus similar to that found in LPSs from rough mutants of enterohepatic strains like S. flexneri 4bR, we expect the B. fragilis saccharide to be composed of approximately 10 sugar residues. It is therefore unlikely that we would find a polysaccharide chain composed of a varying number of repeating units in B. fragilis as found in the S. flexneri 3a LPS (Fig. 1) and in LPSs from smooth Escherichia coli (4) and Salmonella typhimurium (4) strains. The "rough" character of the B. fragilis LPS is further demon-
stratified by its ability to be extracted by the PCP mixture which preferentially extracts the more hydrophobic LPS found in rough enterobacterial bacteria (3). We assume that the inability of the PCP mixture to extract the *B. fragilis* LPSs from intact bacteria probably reflects physicochemical properties of the *B. fragilis* cell envelope; the LPS may be masked by the capsular polysaccharide.

Fatty acid analyses of the 17 different *B. fragilis* LPS preparations showed that all have a similar lipid composition (data not shown). The main fatty acids are isobranched pentadecanoic acid, 3-hydroxy-pentadecanoic acid, 3-hydroxyhexadecanoic acid, isobranched 3-hydroxyheptadecanoic acid, and 3-hydroxyheptadecanoic acid. The range of 3-hydroxy fatty acids found in the *B. fragilis* LPS is also quite unique, as found in an earlier study (25). The 3-hydroxytetradecanoic acid, which is the major fatty acid in enterobacterial LPS, is present in *B. fragilis* LPS only in trace amounts. Thus, the fatty acid composition as found in this study on 17 different LPSs is in agreement with what our earlier collaborative studies on a few strains have shown (13, 25). Although different from what has been found for enterobacterial LPSs both in fatty acid composition and in lack of common endotoxic activities (7), the large similarities in fatty acids, both qualitatively and quantitatively suggests that *B. fragilis* in this respect is a fairly homogenous bacterial species.

The qualitative and quantitative sugar analyses revealed that 16 of 17 strains analyzed contained L-rhamnose, D-galactose, D-glucose, and D-glucosamine (Table 2). For 10 of these strains, the ratios of these sugars were very similar (Table 2, group 1). Also, the LPSs from the three strains of group 2 had the same ratio but contained, in addition, D-galactosamine. All these 13 LPSs were in addition to similarities in saccharide composition shown to have the same immunochemical specificity (Fig. 2; see below). Four strains (VPI 4117, 2552, 4225, and 5631; Table 2) differed in their sugar composition by having either significantly reduced or no D-galactose in their LPSs. This coincided with a reduced or no inhibitory activity in the immunochemical assays (Table 2; Fig. 2).

Studies of the polysaccharide chain from the LPS of strain 9343 using methylation analysis have shown that it has the following structure (Weintraub et al., submitted for publication):

\[
\begin{align*}
\beta-D-Galp1 & \rightarrow 6-\beta-D-Galp1 \rightarrow 6-\beta-D-Galp1 \\
6-\beta-D-Galp1 & \rightarrow 4/6-\alpha-D-GlcP1 \rightarrow 2-\alpha-L-Rhap1 \\
4/6 & \rightarrow 1 \\
\end{align*}
\]

This structure has an L-rhamnose/D-galactose/D-glucose ratio of 1:5:1. The data in Table 2 show L-rhamnose/D-galactose/D-glucose ratios of 1:3 to 5:1. The difference in these results may, however, be explained by the fact that the PCP-extracted LPS from the 9343 and other strains was a mixture of two LPS populations (Fig. 1). We showed that the minor band which can be seen on the SDS-polyacrylamide gel electrophoresis corresponded to an LPS fraction in
which the β,1,6-linked galactose chain was lacking (submitted). This explains the observed variation in D-galactose content.

The chemical and immunochemical data do suggest that the common immunospecificity demonstrated for 13 of 17 B. fragilis LPS is a consequence of the galactose chain and that the β-galactosamine in group 2 strain LPSs does not interfere with this specificity.

All LPSs studied contained D-glucosamine (Table 2). The demonstration of amide-linked fatty acids in the B. fragilis LPS (25) indicates that this linkage occurs between the fatty acids and the amino sugar. This would be in accordance with the findings in enterobacterial lipid A in which the backbone is a β,1,6-linked D-glucosamine disaccharide (19).

The results of the ELISA inhibition experiments are in agreement with our own data showing that approximately 90% of more than 100 clinical isolates of B. fragilis could be identified by using the antisera elicited by the PCE-extracted LPS from the strain NCTC 9343 LPS (23). Earlier studies had suggested that the capsular polysaccharide was a common antigen for the B. fragilis species (18). This investigation unequivocally shows that instead the LPS is the common antigen, a hypothesis suggested in an earlier study (13). Our results also suggest that the various serotyping systems proposed for B. fragilis (2, 6, 15) have been largely based on different antigenic specificities in capsular polysaccharides rather than somatic O antigens (i.e., LPSs).

Most of the B. fragilis strains used in this study (except NCTC 10584, TM 4000, and IC650) have earlier been divided into two DNA homology groups (9), and the relationship between the DNA group and antigenic specificity of the cell envelope was studied (1). It is interesting to note that with one exception (the LPS from strain VPI 5631) we found all strains of DNA homology group I to have LPSs with the same immunochromogenic specificity. The exceptional strain, VPI 5631, which lacked D-galactose in its LPS, may well be a mutant which has lost the immunochromogenic active saccharide. Our immunochromogenic data are not entirely in agreement with those of Babb and Cummins (1). This may be explained by the fact that these investigators used sera elicited by whole bacteria in their serological tests and the antigens were either whole cells or crude cell wall fractions. Consequently antigens and antibodies representative of both the LPS and the capsular polysaccharide of the cell envelope were studied simultaneously.

ACKNOWLEDGMENTS

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LITERATURE CITED
