Immunochemical Evidence for Multiple Serotypes of *Bacteroides fragilis*

ROBERT CHERNIAK,^{1*} GEORGE L. LOMBARD,² AND V. R. DOWELL, JR.²

¹Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, and ²Enterobacteriology Branch, Bacteriology Division, Bureau of Laboratories, Center for Disease Control, Atlanta, Georgia 30333²

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An immunochemical comparison of outer membrane antigens obtained from five select and biochemically defined strains indicated that there are several serotypes of *Bacteroides fragilis*. Each strain was serologically defined by individual or by combinations of determinant groups composed of carbohydrates in the form of polysaccharides or glycoproteins. The carbohydrate constituents were tentatively identified as glucose, galactose, fucose, rhamnose, glucosamine, galactosamine, and traces of mannose. Strains were observed to have minor qualitative and major quantitative variations in carbohydrate composition.

The development of improved cultural techniques for characterizing anaerobic bacteria led to the reclassification of the *Bacteroides fragilis* group. In the new system each subspecies is elevated to species level (4). *B. fragilis*, formerly *B. fragilis* subsp. *fragilis*, is common in the human lower intestinal tract as part of the normal microflora and is the most frequently encountered nonsporeforming anaerobe in clinical specimens associated with disease in humans.

Romond et al. (16) and Beerens et al. (3) suggested a serological classification scheme for the *B. fragilis* group based on the serological activities of specific antisera raised against six prototypic strains. Most (84%) of the 49 strains studied, identified biochemically as *B. fragilis*, were found to possess E₁, E₂, or E₁E₂ major antigens when specific antisera were used (16) (E₁ = NCTC 9343 and E₂ = IPL E323). The specific antisera raised against NCTC 9343 and IPL E323 cross-agglutinated with IPL E323 and NCTC 9343, respectively, at a fourfold lower dilution than the homologous titer. Other *Bacteroides* species were frequently observed to agglutinate when their specific antisera were used at low dilution (3). In addition, lines of identity were observed when the two *B. fragilis* strains were compared by double diffusion in gel (3).

The distribution of major antigens among species was indicated by the fact that strains classified biochemically as *B. fragilis* did not always have homologous serotypes. For example, most of the *B. fragilis* isolates which did not contain E antigens did contain the major B antigen of *B. ovatus* ATCC 8483 (16). An obvious complex antigenic relationship within and among species was indicated.

An approach similar to that of Beerens et al.

was used by Lambe and Moroz (13) in establishing a serological classification scheme for *B. fragilis* based on the presence of one or more of seven heat-stable constituent antigens. They were able to classify 100% of the *B. fragilis* strains investigated and observed no cross-reaction among *B. distasonis*, *B. thetaiotaomicron*, and *B. vulgatus*. Most strains of the 98 investigated contained more than one antigenic determinant, and 5 strains contained all seven antigenic components. Finding strains which contained the entire complement of antigenic determinant groups specific for all *B. fragilis* strains was of particular interest in subsequent efforts to develop a diagnostic reagent. However, a fluorescent antibody reagent Jones prepared against one of these strains (192-76 = CDC 9053) stained only 66% of the *B. fragilis* strains investigated (G. L. Jones, Ph.D. thesis, University of North Carolina, Chapel Hill, 1974; and see below).

A comprehensive study by Jones of 103 biochemically characterized *B. fragilis* strains was performed with direct immunofluorescence (Jones, Ph.D. thesis). Three fluorescent conjugates prepared to CDC 14462, CDC 9053, and CDC 5462 and used at a twofold dilution lower than the strain-specific titer stained 85% of the *Bacteroides* strains tested. The remaining 15% of the strains were detected with the same set of reagents used at 1:3 dilution. Cross-reactions were minimal when the *B. fragilis* conjugates were used at their working dilutions. However, cross-reactions with other species, notably, *B. distasonis* and *B. vulgatus*, were occasionally observed when the *B. fragilis* reagents were diluted 1:3. Contrary to the results of Romond et al., discussed above, a fluorescent conjugate

to *B. ovatus* CDC 11296 did not cross-react with any of the *B. fragilis* strains.

Abshire et al. (1) prepared trichloroacetic acid extracts of nine biochemically characterized *B. fragilis* strains and compared them by double diffusion in gel. The complex antigenic relationship among strains of *B. fragilis* was demonstrated by the formation of precipitin lines of identity and nonidentity. Of particular interest was the observation that the antigen preparations individually failed to form precipitin reactions with two or more of the heterologous antisera.

In contrast to the results cited above, Kasper and Seiler (12) and Kasper et al. (11) presented evidence for a common capsular antigen of *B. fragilis* which allows essentially all strains to be identified with a single antiserum. Outer membrane antigens isolated from several strains of *B. fragilis* consistently gave two precipitin lines of identity by double diffusion in gel (12). Clinical isolates (125) consisting of *B. fragilis* (83 strains), *B. distasonis* (8 strains), *B. vulgatus* (11 strains), *B. thetaiotaomicron* (13 strains), *B. ovatus* (5 strains), and unclassified (5 strains) were examined with an indirect fluorescent antibody procedure, using antisera specific for ATCC 23745 (CDC 14462). The selectivity for identifying *B. fragilis* was essentially 100%; 1 strain of *B. thetaiotaomicron* also reacted positively (11).

The emphasis being placed on the development of fluorescent antibody reagents for identifying the commonly encountered species of *Bacteroides* dictates that the ambiguity concerning the degree of heterogeneity of *B. fragilis* be resolved.

The objective of this study was to prepare outer membrane antigens from a select group of *B. fragilis* strains, using the methods described by Kasper and Seiler (12), and to partially define their immunochemical relatedness.

MATERIALS AND METHODS

Bacterial strains. *B. fragilis* strains CDC 14462 (ATCC 23745), CDC 5462, CDC 9053 (VPI 2553, NCTC 9343), CDC 12330, and CDC 13940 were obtained from the lyophilized culture collection of the Anaerobe Section, Center for Disease Control, Atlanta, Ga. Each strain was confirmed to be *B. fragilis* by selecting an isolated colony from blood agar and characterizing it by colony characteristics, Gram reaction, microscopic morphology, a complete set of biochemical tests, and gas-liquid chromatographic analysis of the fatty acid products in peptone-yeast extract-glucose broth (6). Working cultures, maintained in chopped meat-glucose medium (7), were incubated anaerobically in a glove box at 35°C and transferred routinely every other day.

Cultivation. The selected strains were cultivated

anaerobically in Schaedler broth (Baltimore Biological Laboratory). One milliliter of a 1-day-old culture was transferred to each of six tubes of broth (10 ml per tube), equilibrated to 35°C in a glove box, in the evening. The next morning this material was used to inoculate six flasks containing 125 ml of broth (one tube of culture per flask), which served to seed six flasks containing 1,600 ml of medium each. The latter flasks were incubated in an anaerobic glove box overnight at 35°C, and organisms were harvested the next morning by continuous-flow centrifugation. After centrifugation the pellet was washed with 0.15 M sodium chloride.

Isolation of the outer membrane. Outer membranes of the selected strains were isolated by the method of Kasper and Seiler (12) immediately after the cells were harvested. We chose to repeat the low-speed-centrifugation step several times before pelleting the membrane fraction at 80,000 × *g*. The clear, gel-like pellet was removed carefully from any opaque residue, suspended in water, dialyzed against distilled water, and lyophilized.

Antisera. Antisera used in this study were prepared by Abshire et al. (CDC 12330, CDC 12103, CDC 12336, and CDC 12959) (1) and Jones (CDC 14462, CDC 5462, and CDC 9053) (Ph.D. thesis), using formalized cells. In addition, antisera were prepared against live cells and *B. fragilis* outer membrane (CDC 14462) by intravenous injection according to Kasper and Seiler (12).

ID. Immunological specificity was determined with specific rabbit antisera by double diffusion in gel. Plates were prepared by pipetting 4.5 ml of 0.75% agarose-2% polyethylene glycol containing 1.0 M NaCl-0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.2, on microscope slides (3.5 by 7.6 cm) which had been coated previously with a thin film of 0.2% Noble agar. Auto-Gel (Grafar Corp.) was used to cut a pattern arranged with six peripheral wells (5-mm outer diameter) which were placed 10 mm apart on centers around a central well (6-mm outer diameter). In those cases where a peripheral well contained antiserum, a 6-mm punch was used. The antisera and antigens were compared with a Piazzi plate (15) to determine the optimum conditions for immunodiffusion (ID) analysis. All antisera were used undiluted.

Antigens were suspended in distilled water or 0.5% sodium deoxycholate (20 mg/ml). Reactants were added with 22.9-cm disposable transfer pipettes. Plates were held in a humid chamber at 4°C and interpreted after 16, 24, and 48 h. A permanent record of the results was obtained by photographing the unstained slides.

The outer membrane preparations were tested for their stability to chemical oxidation by NaIO₄ or proteolytic digestion by Protease (Sigma P5380) by previously reported procedures (5).

Analytical procedures. We used the phenol-sulfuric acid methods of Dubois et al. (8) for the detection of neutral carbohydrate. The carbohydrate constituents were tentatively identified on the basis of results of descending paper chromatography on Whatman no. 1 paper with butanol-pyridine-water (6:4:3) as the solvent (2). Gas-liquid chromatography of the per(trimethylsilyl) ethers of the monosaccharide com-

ponents was performed as previously described (14).

RESULTS

ID. When the outer membrane fractions were compared by double diffusion in gel against antiserum CDC 14462 (live cells), two strong precipitin lines formed with its homologous antigen and one line of partial identity formed with antigen CDC 5462 (Fig. 1a). The outer membrane preparations of CDC 9053, CDC 12330, and CDC 13940 were unreactive within 24 h (Fig. 1a), and a faint precipitin line appeared with CDC 13940 after 48 h. Similar experiments were carried out with the other available antisera. Antiserum CDC 12330 was monospecific for its homologous antigen (Fig. 1b), whereas antiserum CDC 5462 reacted weakly with its homologous antigen and with antigen CDC 14462 (Fig. 1c). A sharp line of precipitation was observed with antiserum CDC 12959 and antigen CDC 12330; antigen CDC 5462 reacted weakly, and antigens CDC 9053, CDC 13940, and CDC 14462 did not react at all (Fig. 1d). In an identical experiment, antiserum CDC 12336 reacted weakly with antigens CDC 5462 and CDC 13940 and was negative with the remaining outer membrane preparations (Fig. 1e). Antiserum CDC 12103 surveyed in the same manner had weak precipitin reactions with antigens CDC 13940, CDC 14462, and CDC 12330 (Fig. 1f and Table 1).

Antiserum available against CDC 9053 consistently produced weak precipitin lines with its homologous antigen. However, precipitin lines were discernible with antigens CDC 9053, CDC 5462, and CDC 12330 after 40 h (Fig. 1g). Table 1 summarizes the observations made at 24 and 48 h for the ID experiments described above.

The homologous and heterologous precipitin reactions were eliminated by pretreating the outer membrane antigens with NaIO_4 , except for antigen CDC 12330, the activity of which was altered but not destroyed (Fig. 2). Proteolytic digestion of antigens CDC 14462, CDC 12330, and CDC 5462 did not change the precipitin patterns normally observed by ID.

Carbohydrate composition. The yields of outer membrane fractions isolated from the selected strains of *B. fragilis* are shown in Table 2. The neutral sugar content represented between 7 and 12% of the dry weight of the isolated antigens. Chromatographic examination of the outer membranes demonstrated that glucose and galactose were the major neutral sugars present (Table 3). Galactose and glucose were always present in relatively equal quantities except in the case of CDC 9053, in which the glucose content was low and the galactose con-

FIG. 1. ID in gel of the outer membrane antigens. Wells 1 through 5 contained antigens from CDC 5462, CDC 9053, CDC 13940, CDC 14462, and CDC 12330, respectively. Well 6 contained a duplicate of the homologous antigen when available. The center wells of a through g contained specific antisera to CDC 14462, CDC 12330, CDC 5462, CDC 12959, CDC 12336, CDC 12103, and CDC 9053, respectively. The precipitin reactions were photographed after incubation for 24 h (a through f) or 40 h (g). Precipitin lines in c through f were artistically enhanced.

TABLE 1. ID in gel of outer membrane antigens of *B. fragilis*

Antigen source	Specific antiserum ^a						
	CDC 5462	CDC 9053	CDC 12103	CDC 12330	CDC 12336	CDC 12959	CDC 14462 ^b
CDC 5462	+1(+2)	(+1W)	-(+1W)	-(+1W)	+1	+1(+2)	+2
CDC 9053	-(+1W)	+2(+3)	-	-	-	-	-
CDC 12330	-(+1W)	-(+3W)	+1W	+1	-	+1	-
CDC 13940	-	-	+1	-	+1	-	-(+1W)
CDC 14462	+1	-	+1W	-	-	-	+2

^a +, Precipitin line present; -, precipitin line absent; W, weak precipitin line. Arabic numerals indicate the number of precipitin lines observed. Observations made after 48 h of incubation are in parentheses.

^b The observed precipitin lines were independent of the immunogen used for antisera preparation: live cells, formalized cells and outer membrane.

FIG. 2. ID in gel of outer membrane antigens before and after treatment with NaIO_4 . Wells 1 through 3 contain antigens from CDC 12330, CDC 14462, and CDC 5462 respectively. Wells containing antigens treated with NaIO_4 are indicated by the letter p. Wells 4 through 11 contain specific antisera to CDC 14462, CDC 12103, CDC 5462, CDC 12336, CDC 12959, CDC 14462, CDC 12330, and CDC 12959, respectively. The precipitin reactions were photographed after incubations of 24 h.

tent was seven times as high (Table 3). Fucose was present in all the antigen fractions in approximately the same relative quantity as glucose. Rhamnose concentrations in all the antigen preparations were about one-half those of glucose (Table 3). Galactosamine was present in all the antigens, and glucosamine was detected in CDC 5462 and CDC 13940 (Table 3). Ribose was found only in the outer membrane of CDC 13940.

TABLE 2. Isolation of outer membrane of *B. fragilis*

Strain (CDC no.)	Cells (g, wet wt)	Outer membrane (mg, dry wt)	Carbohydrate ^a (% of outer membrane wt)
5462	41	59	8
9053	40	90	12
12330	25	60	8
13940	30	59	7
14462	20	76	9

^a Determined as glucose by the phenol- H_2SO_4 method.

All outer membrane antigens contained a major constituent that had a retention time just exceeding the value observed for α -D-glucose, for which no assignment could be made (Table 3, unknown). Chromatography indicated the presence of a uronic acid, but its identity has not been determined. The assignments shown in Table 3 should be considered tentative until confirmed by additional chemical characterization.

DISCUSSION

We prepared outer membrane antigens for CDC 14462 using Schaedler broth in this study after testing other media, such as NIH thioglycolate medium (Difco) and peptone-yeast extract-glucose broth (7). Schaedler broth produced a dense growth within 16 h of incubation. *B. fragilis* incubated aerobically at 35°C in a standard incubator grew to the same density as that obtained in the anaerobic glove box if a 15% inoculum was added carefully to 10 liters of freshly prepared medium. Outer membrane preparations from cells grown in the various media produced identical precipitin patterns by ID.

The high-speed pellet was a clear gel with a yellow tint. The gel was carefully removed with a spatula from the opaque residue which underlay it in order not to contaminate the outer membranes. The pellet was difficult to resus-

TABLE 3. Relative molar ratios of the carbohydrate constituents of *B. fragilis* outer membrane antigens^a

Antigen	Relative molar ratio								
	Glucose	Galactose	Rhamnose	Fucose	Glucosamine ^b	Galactosamine ^b	Mannose	Unknown	Ribose
14462	1	1.6	0.3	0.9	— ^c	1.0	0.1	0.5	—
5462	1	1	0.6	0.8	0.8	0.5	<0.1	0.4	—
9053	1	7	0.4	0.7	—	0.4	<0.1	1.8	—
12330	1	1	0.6	1.0	Trace	0.7	<0.1	0.4	—
13940	1	1.3	0.3	0.7	0.5	0.5	<0.1	0.4	0.3

^a Gas-liquid chromatography data were analyzed on a relative basis by comparing the area of each major constituent with that of D-glucose. No corrections were made for variations in detector responses to the various component sugars. Outer membrane antigens were hydrolyzed with 2 N trifluoroacetic acid for 3 h at 100°C.

^b Hexosamines were determined after hydrolysis with 2 N trifluoroacetic acid for 12 h at 100°C.

^c —, Not detectable.

pend and never regained its original opalescent appearance. Centrifugation at $10,000 \times g$ resulted in the loss of aggregated outer membrane. Recovery of outer membrane antigens was enhanced by repeating the $10,000 \times g$ centrifugation several times before sedimenting the membranes at $80,000 \times g$. Examination of the outer membrane fraction by electron microscopy showed many bilayer membranes. The general appearance of thin sections was similar to those described by Kasper and Seiler (12).

The precipitating antibodies to *B. fragilis* in immune rabbit sera were directed toward the outer membrane antigens. The reactivity of the antigen preparations in ID was enhanced by replacing distilled water with 0.5% sodium deoxycholate. However, the antigens still did not form a completely homogeneous solution. The fact that the observed precipitin lines were eliminated after oxidation with NaIO_4 indicated the importance of the carbohydrate components for the serological activity of the outer membrane of *B. fragilis*. The carbohydrate composition of CDC 9053 shown in Table 3 parallels that which Hofstad and Kristoffersen reported for the isolated lipopolysaccharide obtained from the same strain (NCTC 9343) (9). We did not detect glucosamine in CDC 9053 in our study.

The variation in carbohydrate was consistent with the heterogeneity of *B. fragilis* observed with ID. The relative serological significance of the carbohydrate moieties present in the outer membrane antigens cannot be determined until the pure antigenic fractions corresponding to specific precipitin lines observed with ID are isolated. Although the carbohydrate identifications have not been confirmed by isolation and characterization of the individual moieties, we present the data here to emphasize the related elements and individuality of the *B. fragilis* strains.

No definitive lines of identity were observed in the ID experiment. However, many of the

precipitin reactions were weak, and it is possible that under favorable experimental conditions a relationship between some of the antigens could be documented.

The major conclusion drawn from the data presented is that *B. fragilis* consists of at least several serotypes defined by individual or combinations of determinant groups composed of carbohydrate and present in the form of polysaccharides or glycoproteins.

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