SEROLOGICAL AND CHEMICAL STUDIES ON THE ANTIGENS OF BACTEROIDES FRAGILIS AND RELATED SPECIES

by

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DEDICATION

To my wife, , whose constant encouragement, support, and understanding, is deeply appreciated.

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INTRODUCTION

Although the presence of anaerobic bacteria had been described by Pasteur over a century ago, substantial gains in anaerobic bacteriology have lagged due to difficulties in technical procedures of culturing. However, with the anaerobic culture techniques now available (61, 85), many investigations have implicated the importance of anaerobes in numerous pathological and ecological processes (37, 85, 111). Consequently, a great deal of data is now emerging in the scientific literature dealing with the physiology, structure, and function of anaerobes.

One particular group of anaerobes, the '*Bacteroides fragilis* group', has been subject to intense research in recent years because its members are frequently encountered in clinical specimens and are part of the intestinal microflora of most persons (104, 136). Organisms belonging to this group have many phenotypic properties in common, an observation which led to their subdivision in terms of subspecies. This similarity in phenotypes, led many to investigate their serological properties. Depending on the serological tests employed, serogroups could be correlated with biochemical or biological properties. That some serogroups were associated with clinical specimens more frequently than others, implied the existence of particular virulent factors or properties that were associated with pathogenicity. Numerous properties have been studied (6, 12, 50, 98, 163). However, the means by which these anaerobes establish and augment pathological conditions is still unclear. Analyses on cell wall

components associated with antigenic (57, 75) and metabolic (91) functions have also been reported.

Recently, the taxonomy of this group has been clarified by DNA homology experiments and of over 300 "*B. fragilis*" strains examined, over 90% could be allocated to one of 10 major homology groups. Such a differentiation allows for an analysis of phenotypic traits that correlate with the genetic data.

Although the serology and to a lesser degree, the cell wall antigens of *Bacteroides* strains have been investigated before (5, 57, 74, 75, 76, 137), these studies examined strains that had been assigned to species on the basis of biochemical properties. A re-examination of their antigenic relationships seemed warranted because of the availability of strains which could be assigned to distinct DNA homology groups.

REVIEW OF THE LITERATURE

The literature review will be presented in two major parts under numerous subheadings. One part deals with lipopolysaccharide (LPS) structure in general, methods of antigen extraction, and DNA homology studies as they relate to LPS variation. The other summarizes the work that has been done on the serology and taxonomy of Bacteroides fragilis and related species.

LPS Structure

Polysaccharides, other than peptidoglycan, are important constituents of the cell wall of gram negative bacteria, where they occur principally as lipopolysaccharides. Lipopolysaccharides (LPS) are long chain phosphorus-containing heteropolymers, composed of a lipid moiety (Lipid A) covalently linked to a polysaccharide core region. To this are attached serologically active polysaccharide side chains (87, 90). Biosynthesis of this complex requires the participation of numerous genes (141). In addition, LPS can be chemically modified by a variety of enzymes coded for by genes of bacterial or viral origin (121, 141).

The elucidation of the structure of LPS was the result of work done by several independent laboratories. An example of the structure of a LPS is shown in figures 1 and 2. An idea of the overall organization of LPS was suggested by the early studies on antigen extraction. Boivin and Mesrobeanu (7), Morgan and Partridge (105), and Freeman (38) reported that a complex antigen could be split into polysaccharide

O-side Chain	Core Region		Lipid A
Repeating units of oligo-saccharides: neutral and amino sugars	neutral sugars amino sugars ethanolamine	KDO Heptose phosphate	hexosamine phosphorus fatty acids

Figure 1. Schematic structural diagram of lipopolysaccharides

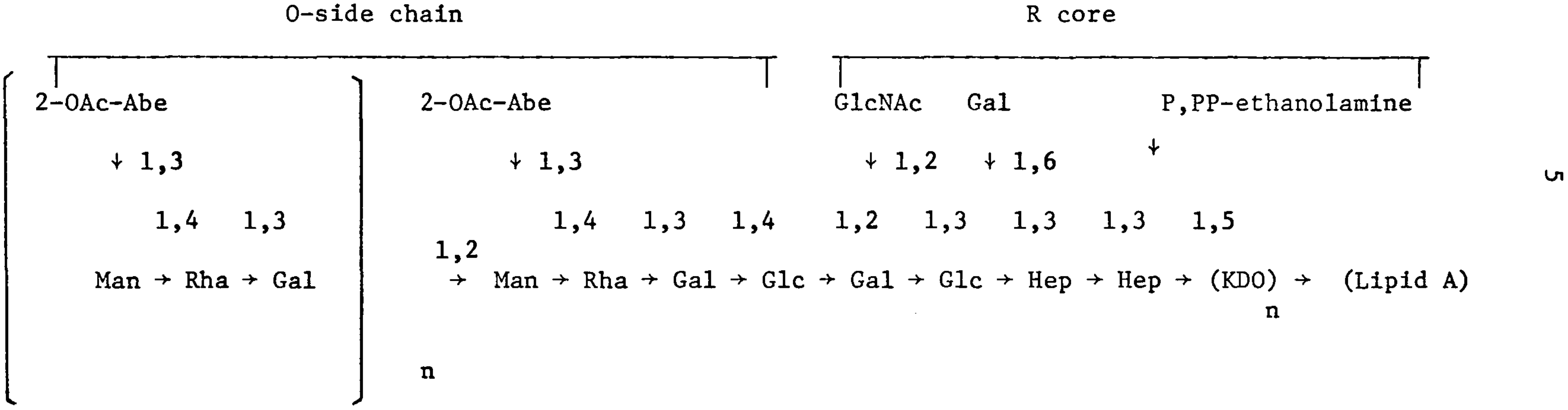

Figure 2. The structure of LPS from *S. typhimurium* taken from Nikaido (110). Abbreviations: Abe, abequose (3,6-dideoxy-D-galactose); Gal, D-galactose; Glc, D-glucose, GlcNAc, N-acetyl-D-glucosamine; Hep, L-glycero-D-mannoheptose; KDO, 2-keto-3-deoxyoctonic acid; Man, D-mannose; OAc, O-acetyl; P, phosphorus; PP, pyrophosphate; RHA, L-rhamnose.

and lipid moieties by mild acid hydrolysis. Westphal, Luderitz, and collaborators in 1960 proposed the first hypothesis dealing with the structure of LPS after a series of chemical and serological studies on mutant strains (88). Wild type Salmonella strains, possessing O-antigenic specificity and which formed smooth (S) colonies, could be transformed into mutants forming rough (R) colonies and which lacked O antigens. Comparison of the sugar composition of LPS isolated from both S and R forms disclosed that R forms possessed only five "basal" sugars, 2-keto-3-deoxyoctonic acid (KDO), heptose, D-glucose, D-galactose, and N-acetyl-D-glucosamine, regardless of the sugar composition of the parent S-form. Furthermore, a close relationship was noted between the serotype and chemotype. As a result, these authors hypothesized that wild type LPS was composed of a "variant" portion, containing a variety of sugars which determined the O-antigenic specificity, coupled to an "invariant" core, containing only the basal sugars. Thereby, R forms were those mutants that could synthesize the R core region but had lost the ability to synthesize side chains. Following detailed structural analyses on R-form mutants, the R core structure was depicted as that illustrated in figure 2. The structure of the O-specific side chains was originally proposed in 1962 by Robbins and Uchida (125), who concluded from immunochemical studies on Salmonella group E, that O-side chains were composed of repeating oligosaccharide units. Analyses were later extended to other groups, yielding similar findings (87, 90).

Although it was assumed that side chains were covalently linked to the R core, direct evidence on the nature of this linkage was not reported until 1969 by Nikaido (109). He reported that in Salmonella typhimurium, the reducing terminal sugar of the side chain was linked to an internal glucose residue in the core region rather than to the non-reducing terminus of the R core. At the reducing end of the core backbone, a lipid component, Lipid A, is covalently bound. Characterization of this region was the result of several studies (29, 30, 45, 113). These investigations provided evidence indicating that KDO occupied the reducing terminus of the core region and that KDO served to link the core to the lipid moiety probably through a ketosidic bond.

Chemical analyses have shown that the Lipid A component consists of hexosamine, phosphorus, and fatty acids, such as lauric, myristic, palmitic, and β-hydroxymyristic acid (87, 90). The chemical structure of the Salmonella Lipid A has been proposed by Luderitz et al (86) as having pyrophosphate bridges interlinking hexosamine units, which are esterified by the long chain fatty acids. This complex exhibits endotoxic activity and is immunogenic in rabbits. Recent investigations suggest that at least some LPS is covalently linked to protein through Lipid A although the mode of this linkage has yet to be clearly defined.

Methods of Antigen Extraction

Much of what is known about the chemical and biological properties of LPS has resulted from studies concerning extraction and

chemical modification of this complex. For this reason, the various methods of extraction will be briefly discussed.

Boivin and Mesrobeanu (7) were the first to describe a complex antigen extractable from gram negative bacteria using trichloroacetic acid (TCA). The antigen was immunogenic, possessed somatic O-specificity and endotoxic activity, and would sensitize red blood cells. Other extraction procedures were later reported by Morgan and Partridge (105) and Goebel et al (46) who used organic solvents, such as ethylene glycol and pyridine, under mild conditions, to obtain similar antigens to Boivin. These methods were empirically modified to form the basis for the methods of LPS extraction now in use.

The complex antigens obtained by the original methods possess proteins and lipids along with the LPS. These complexes, having molecular weights in the several millions, can further be dissociated into component parts using agents such as phenol, formamide, acetic acid, and alkali. Furthermore, these reagents can be used directly for the extraction of the specific components.

Direct extraction with phenol was introduced by Palmer and Gerlough in 1940 (115). This method was later improved by Westphal and Jann (165) and has since been used to extract LPS from smooth and rough variants of numerous genera (87, 90). When bacteria are extracted with a mixture of phenol and water at 68 C, the cells rapidly disintegrate releasing their LPS into the solvent. Upon cooling, three fractions are obtained: the aqueous layer, containing LPS, the phenol layer, and an insoluble layer, containing cell residue.

In addition to LPS, the aqueous phase may contain nucleic acids and capsular or glycan material which can be readily removed. The LPS isolated in this manner are highly aggregated, with a molecular weight in the several millions, and are devoid of protein, although nonessential peptides and amino acids may be present in some preparations. Serologically, phenol-extracted LPS possesses somatic O-specificity and will sensitize red blood cells. However, these properties, as well as the chemical composition and biological activity are dependent on the bacterial strain used. Phenol-water extraction has been shown to be more efficient than other LPS extracting agents, such as TCA or diethylene glycol, as cells exhaustively extracted with the latter two agents still release significant amounts of LPS into phenol-water (165). However, this efficiency has also been shown to be strain dependent.

With at least some bacteria, LPS can be recovered exclusively or largely from the phenol layer rather than from the aqueous layer (73, 90, 120). This unusual distribution has been explained in part by their lipophilic character, caused by their chemical composition. These unexpected findings led Galanos, et al (43) to develop a mild extraction method suitable for isolating phenol-soluble (lipophilic) LPS as well as R form (rough) LPS, in general. Using a monophasic mixture consisting of aqueous phenol, chloroform, and petroleum ether, (PCP), only R form LPS is extracted from whole cells. S-form LPS, proteins, nucleic acids, and polysaccharides are all insoluble in this mixture.

Formamide extraction of bacteria was introduced by Fuller in 1938 (42) as a means of extracting polysaccharides from streptococci. Formamide at 170 C dissolves most elements in the bacterial cells while disrupting the protein-lipid-LPS complex. The protein can subsequently be precipitated out, leaving degraded polysaccharide in solution. These polysaccharides possess O-specificity but are poorly immunogenic and will not sensitize red blood cells. Several investigations have dealt with the chemical action of formamide on cell wall material. Included in its effects, are the introduction of formyl groups on free amino groups and the partial destruction of amino sugars (117).

Somatic, degraded polysaccharides can be obtained by hot acetic acid hydrolysis of whole bacteria or isolated LPS (38). Hydrolysis in this manner, followed by a series of purification steps yields a mixture of haptenic (O-specific) polysaccharides, free of lipid and protein, having molecular weights ranging from 20,000 to 40,000. The polysaccharides are not immunogenic themselves, nor will they sensitize red blood cells. Polysaccharide preparations from certain bacterial strains, when fractionated on Sephadex gels, can be resolved into three peaks, representing serologically active side chain material, core region oligosaccharides, and a fraction containing free KDO (28, 106). The lipid liberated by acetic acid hydrolysis (Lipid A) can also be recovered by centrifugation or chloroform extraction. Hydrolysis can also be carried out using dilute mineral

acid, such as hydrochloric acid. Under such conditions, degraded polysaccharides, similar to those obtained using acetic acid, can be recovered.

Polysaccharides can also be obtained by alkali extraction of whole cells or isolated LPS. Extraction by this means leads to a deacylated polysaccharide-lipid complex of approximately 200,000 MW, which exhibits an enhanced affinity for red blood cells (107). This material is water soluble, non-toxic, and possesses O-specificity, although it lacks any alkali labile serological factors, such as O-acetyl groups.

Numerous other chemical agents have been used to extract antigens from gram negative bacteria. Among those are sodium chloride, urea, and the chelating agent ethylenediamine tetraacetic acid (EDTA). Some of the most simple and effective methods of antigen preparation are heating cells in fluid culture medium, water, saline, or phosphate buffer (126). All yield a highly antigenic water soluble complex containing proteins, lipids, and polysaccharides. LPS is also released when cells are treated with urea (153) or with EDTA (47, 83). The latter agent liberates approximately half of the total LPS. The success of these agents in extracting LPS indicates indirectly the nature of some of the forces involved in the association of the LPS with the cell wall, i.e., noncovalent physical forces, such as hydrophobic and ionic bonds.

Extraction of whole cells very often results in contamination with protoplasmic material, such as nucleic acids or intracellular

glycans. Pretreatment with TCA or formalin has been shown to reduce levels of contaminating nucleic acids in phenol-water extracts (112). Alternatively, extraction can be performed on isolated cell walls. In the course of cell wall preparation, many potential contaminants are destroyed or removed. Furthermore, extraction of LPS has been shown to be more complete from cell walls than from whole cells, yielding material, in many cases, of higher quality (41, 60).

Cell wall analyses were introduced by Salton and Horne in 1951 (128). Since then, numerous methods for cell wall prepration have been developed (170). Three most commonly used procedures are ultrasonic disruption, shaking with glass beads, and extrusion at high pressure. Cell breakage involves a large expenditure of energy resulting in heat production. Thus, adequate cooling of the cell suspension is necessary.

Ultrasonic vibrations (20KC/sec. or higher) are required to shear bacterial cells. However, prolonged exposure to these vibrations leads to excess heating, greater disintegration of cell fragments, and possibly, destruction of some structural features.

Shaking cells with glass beads (0.1 mm to 0.2 mm) at high speeds for various time intervals is sufficient to disrupt most bacteria. Disintegration is rapid and, in the case of the Braun disintegrator, can be performed under aerobic or anaerobic conditions. The glass beads are separated from the cell debris by filtration.

Disruption of bacterial cells can be achieved by forcing a bacterial suspension or frozen cell paste through a narrow orifice

under high pressure. Although it is very practical for breaking gram negative bacteria, it is less efficient for gram positive organisms, particularly gram positive cocci.

Following disruption, the crude cell walls are separated from intact cells by differential centrifugation, digested with nucleases and proteases, washed, and finally lyophilized. The pure cell wall preparations can then be used for chemical extraction or serological studies.

DNA Homology Studies and LPS Variation

Nucleic acid reassociation techniques have been used in taxonomy as a means of measuring overall similarities between genotypes of different organisms. Present techniques, which originated from the reassociation studies of Doty et al (26) and Marmur and Lane (93), can determine mole per cent Guanine plus Cytosine content (mole % G+C) (92), degree of complementary base pairing (70), and genome size (44). Numerous groups of aerobic and anaerobic bacteria have been examined by nucleic acid hybridization (103). Such studies have demonstrated the diversity of phenotypic characteristics that can exist in a genetically defined group of organisms. As a result, an assessment of phenotypic characters, as they relate to the differentiation of a given group of organisms can be made. Among the phenotypic properties that have been correlated with DNA homology values is cell wall composition.

Cell walls have long been used in taxonomy. Organisms have been classified on the basis of cell wall properties and components which include permeability, isomers of diaminopimelic acid (DAP), cell wall sugars, LPS, and antigenic specificity. Unfortunately, only a limited number of studies have directly related these properties with the genetic relationships. Correlation have been reported between serology, cell wall components, and DNA homology values among certain clostridia and propionibacteria (19, 69). Similar comparisons can be made among gram negative bacteria, by correlating various independent studies of LPS structure and homology relationships.

Within the genus *Neisseria*, three distinct DNA homology groups have been described (78, 79), representing the pathogenic *Neisseria*, non-pathogenic *Neisseria*, and the species *N. catarrhalis*. Very little intergroup homology was reported. Analyses on LPS from members belonging to these three groups disclosed qualitative and quantitative differences in their polysaccharide and lipid composition (1, 171). Differences in LPS composition between the genetically unrelated oxidase positive and negative moraxellas have also been reported (2, 67). Examination of LPS from one strain belonging to each group disclosed marked differences in hexosamine composition, thus suggesting possible structural differences paralleling the genetic division. DNA homologies among *Pseudomonas* species have shown that *P. aeruginosa* represents a cluster of highly related strains (90 to 100% interhomology) distinct from the other species (114). Serological and

chemical studies on the LPS complexes from P. aeruginosa strains suggest that side chains, determining the various serological specificities, are attached to a core region, whose structure is shared by all members of this species (15, 28, 96, 167).

In addition to the qualitative differences mentioned in the examples above, detailed structural variations also exist. Much of this information has resulted from immunochemical analyses on the enteric bacteria. Since their genetic relationships have also been investigated, this group can provide examples regarding DNA homology and antigenic structure.

The family, Enterobacteriaceae, comprises a group of organisms of similar morphology but of diverse distribution, virulence, antigenic make-up, and biochemical capacity. Twelve genera currently belong to this family and can be differentiated using mole % G+C, serological properties, and a few biochemical and morphological characteristics (17). Genetic studies indicate a greater uniformity than phenotypic properties suggest; often to such a degree as to suggest that separate species designations are not warranted. Among the Salmonella species (with the exception of atypical strains), homology values ranged from 85 to 100%. Similar levels of intragroup homologies were observed within Escherichia (E. coli being the only species) and Shigella (8, 9, 10, 11). When these three groups were tested for relatedness to each other, E. coli and Shigella strains showed considerable overlapping of homology values, whereas Salmonella strains were distinct by having only 50% homology to either Escherichia

or Shigella. A similar grouping was observed among Arizona and Salmonella (atypical and typical) strains which were 70 to 80% related to each other. Furthermore, strains of Citrobacter occupied a central position, being 50% related to both the E. coli-Shigella and the Salmonella-Arizona groups, while Proteus strains, having a lower mole % G+C, showed less than 20% homology to the other groups. If the segment of genetic material responsible for the biosynthesis of the LPS complex is measurable by DNA homology techniques, then the above genetic relationships may manifest themselves by variation in the LPS structure.

Variations in LPS Core Regions

Almost all enteric bacteria and members of several non-enteric gram negative genera, possess a number of sugar components in common, namely, glucosamine, KDO, L-glycero-D-mannoheptose, galactose, and glucose (87, 89, 90). The fact that these components are usually associated with the core region led to the hypothesis that LPS from most gram negative bacteria possess similar core regions. However, quantitative and later structural analyses have provided evidence that core regions may differ at the genus, species, and occasionally at the strain level. Although analyses on a large number of strains are needed before any general conclusions can be drawn, numerous chemical, serological, and genetic studies on various Salmonella species (serotypes) suggest that many, if not all, have identical or very similar core structures (90, 130, 131, 143). Similar observations were made

on Shigella LPS (64). There exist, however, core variations within E. coli and Proteus. At least two different core structures have been identified in Proteus and four within E. coli (87, 90). Some E. coli strains, having identical O-side chains, were shown to differ in their core regions (90). Of the known core types of E. coli, one was structurally identical to that of Salmonella, two (R1, R2) were chemically similar to Salmonella, and the last (R3), had much lower levels of galactose than Salmonella. However, all, except R1, cross reacted with Salmonella core LPS, suggesting similar structural features. Furthermore, core regions of Shigella, Arizona, and Citrobacter also cross reacted with Salmonella (130). However, these results must be interpreted with caution since many of these studies were conducted on only a single strain from each genus.

Chemical, serological, and genetic properties of an Arizona core LPS were found to correspond in all respects to those of the Salmonella type, thus correlating well with their close genetic relationships observed in the DNA homology studies. Also, a Shigella strain (probably about 50% homologous to Salmonella) possessed the same core sugar sequence as that found in the core backbone of Salmonella, but differed in regard to their linkages (90, 110). A similar grouping based on chemical and genetic properties was reported between the Citrobacter and E. coli R3 cores (130). In addition, a core region was described in Proteus mirabilis of similar composition to the E. coli R3 core (25). If these structures are very similar, then genetic unrelatedness does not necessarily imply marked differences in core

structure, as Proteus is only 20% homologous to E. coli. Thus, the data indicate that there is variation in the LPS core regions that is expressed in terms of chemical composition or structure. However, these differences occur between strains which are distantly related as well as strains which are very similar. Therefore, differences in core region cannot be accurately predicted on the basis of DNA homology values.

Variations in O-Specific Side Chains

O-specific side chains contain hexoses, deoxysugars, and sometimes rare components, such as dideoxysugars, dideoxyaminosugars, and uronic acids in combinations specific for one or more groups. The chemical, and consequently the serological diversity, is much more pronounced in this structure than in the R core region, both within and between genera. The hundreds of serological specificities of the enteric bacteria can be defined in terms of approximately fifty chemotypes (87, 90). Organisms belonging to one serogroup always belong to one chemotype, however, the reverse is not always true. For example, a single chemotype represents eighteen serogroups within E. coli and sixteen additional specificities of Salmonella, Arizona, and Shigella. Some, but not all, cross react with each other. Likewise, O-specific side chains of six Shigella flexneri serotypes contain the same sugar residues (90). Thus, the serological specificity is determined not only by sugar composition, but by structural features, such as branch points, linkages, and modifications, e.g., acetylation.

Immunochemical analyses on O-specific serotypes have supported this concept (90).

Similarities in chemotypes and serological properties are frequent among the E. coli, Salmonella, Arizona, and Shigella. However, for taxonomic relationships, arrangement, rather than chemical composition or immunological specificity provides the most valuable information. On serological grounds, Salmonella sendai and S. paratyphi are quite distinct. However, O-side chain structures of these two organisms were found to differ only in one sugar. Where tyvelose is found in S. sendai, paratose is located in S. paratyphi. The fact that tyvelose is synthesized by a one step conversion from the nucleoside-diphosphate derivative of paratose suggests that S. paratyphi is a single step mutant of S. sendai and therefore, a very close relative of it. Similarly, Salmonella serogroups G, U, N, and L show no intergroup cross reactions but, excluding serogroup L, all belong to the same chemotype. However, structural analyses of O-side chains revealed a greater similarity between groups G and L than the other groups, which were quite distinct and unrelated. Finally, genetic relatedness, as manifested in O-side chains, may be masked by the expression of, or lack of, modification enzymes coded for by genes of bacterial or viral origin. For example, converting phages can alter side chain structures to similar or totally unrelated sequences, thereby affecting both the serotype and chemotype (90, 121).

Strains of different genera occasionally possess identical side chains (90). If antigenic structure of O-side chains represents

a fraction of the genome that is measurable by DNA homology techniques, then greater relatedness should be observed in strains possessing similar antigens. Such relationships were observed in three groups of *E. coli* associated with certain types of infections and possessing similar O antigens (11). Furthermore, strains of a rarely encountered serotype of *Shigella*, also showed greater relatedness to each other in contrast to other *Shigella* and *E. coli* strains tested (9). However, this relationship is not always observed. Studies on *E. coli* and *Shigella* strains possessing identical or related antigens and causing diseases by similar mechanisms were no more related to each other than to any other *E. coli* or *Shigella* strain (8, 10). Therefore, among a group of organisms displaying various levels of genetic relatedness, a great number of antigenic specificities can exist. Antigenic differences may be expressed in terms of chemotypes or antigenic structure. Such differences do exist between closely related strains, as among the species of *Salmonella*. On the other hand, similarities in antigenic structure, and in some cases the possession of identical antigens, do not necessarily imply genetic relatedness. This is most vividly illustrated by the observation that *Salmonella* serogroup U and *E. coli* 086 possess sugar sequences in their side chains very similar to those found in human blood group substance B.

Taxonomy of *Bacteroides fragilis*

The taxonomic status and nomenclature of this group of organisms has been confusing and subject to change since the species was

originally described by Veillon and Zuber in 1898 (149). Recently, the taxonomy of *Bacteroides fragilis* and its subspecies, as described in the eighth edition of Bergey's Manual of Determinative Bacteriology (58), has been modified so that this author feels that a brief consideration of the subject is appropriate at this point.

Veillon and Zuber, using morphological, colonial, and pathogenic characteristics, described an anaerobic gram negative nonsporing rod that had been isolated from abdominal abscesses. The species, designated by those authors as *Bacillus fragilis*, was later transferred to the genus *Bacteroides* by Castellani and Chalmers in 1919 (13).

Since then, numerous synonyms have been used by different authors to describe organisms having phenotypic characteristics and morphology similar to those described by Castellani and Chalmers. In 1933, Eggerth and Gagnon (34) differentiated *Bacteroides* in terms of eighteen species, including *B. convexus*, *B. vulgatus*, *B. distasonis*, *B. thetaiotaomicron* and *B. ovatus*. Similarly, the genera *Ristella* and *Eggerthella* were proposed by Prevot (118) and Beerens (4), respectively, to represent the same group. In 1974, Holdeman and Moore, having studied over three hundred strains of bacteria fitting the early descriptions of *Bacteroides fragilis*, described a continuum of variants with apparent clustering relative to certain phenotypic characteristics (58). Such clusters were designated subspecies of *B. fragilis*, i.e. ss. *fragilis*, ss. *distasonis*, ss. *vulgatus*, ss. *thetaiotaomicron* and ss. *ovatus*. Recently, however, Johnson (65)

demonstrated that these five subspecies are sufficiently distinct genetically to warrant being given species rank. This has been done by Cato and Johnson (14), and, henceforth, the nomenclature used in this work will be that of Cato and Johnson.

Serology of *Bacteroides fragilis*

The early serology of *Bacteroides fragilis* is difficult to assess due to the vague and confusing descriptions of *Bacteroides* species reported in the literature. Such groups most likely included strains recognized by present criteria as *B. fragilis* in addition to several other species. Furthermore, culture techniques and methods of antisera and antigen preparation were quite inconsistent. Such variations have been shown to have significant effects on the serological properties. Dowell et al (27) noted that higher titer antisera were obtained if rabbits were immunized with *B. fragilis* grown in Schaedler broth as compared with cells grown in NIH Thioglycollate broth. Similarly, rabbits immunized with whole broth cultures of *B. thetaiotaomicron* produced higher titer antisera than rabbits immunized with saline cell suspensions. Dworcznski et al (33) also demonstrated that methods of antigen preparation influence the antibody response. Rabbits, immunized with formalinized rather than heated cell suspensions, produced higher titer antisera as measured by agglutination, hemagglutination, complement fixation, or immunodiffusion.

Finally, Dalland et al (20) investigated the production of cell bound antigens on _B. fragilis_ employing a chemostat. Conditions for optimal microbial growth were also found to be favorable for highly active antigen preparations. The pH was the most significant factor influencing antigen activity.

Despite these inconsistencies, the serological studies on _B. fragilis_ can arbitrarily be divided into two areas corresponding to their taxonomic and clinical relevance. As a taxonomic tool, serology has been used as an adjunct to phenotypic and biochemical tests. Eggerth and Gagnon (34) attempted to use serology (tube agglutination) in their classification scheme; however, only low titer, strain specific antisera could be generated. A few years later, more successful findings concerning antigenic relationships were reported by Henthrone, Thompson, and Beaver (52) in 1936 and by Weiss and Rettger (160) in 1938. Henthrone et al demonstrated serological cross reactivity between three of four _B. fragilis_ strains by agglutination and absorption studies. Weiss and Rettger, using a classification scheme that was based primarily on serology (tube agglutination), were able to classify seventy-four human fecal isolates, including several of the species described by Eggerth and Gagnon, into four groups showing little cross reaction. The antigenic relationships among _B. fragilis_ strains were further examined by Sonnenwirth (137). Employing the more sensitive serologic techniques of immunodiffusion and hemagglutination, he demonstrated a high level of cross reactivity between biochemically characterized strains of

B. fragilis and little or no cross reaction between B. fragilis and other species. By the nature of the tests and antigen extracts employed, his results indicated that species specificity resided in thermostable polysaccharide antigens. Additional support was lent to the above findings by the agglutination and immunodiffusion studies of Beerens (4), Reinhold (122, 123), Werner (161, 162), Werner and Sebald (164), and DeLa Cruz (23). All implicated the involvement of thermostable polysaccharide antigens in conferring species specificity on B. fragilis, B. distasonis, B. vulgatus, B. thetaiotaomicron, and B. ovatus. Few cross reactions between species were reported by these authors. However, within the species B. fragilis, B. vulgatus, and B. thetaiotaomicron, a complex antigenic interrelationship between strains was suggested. In addition to strain specific antigens, absorption and immunodiffusion studies disclosed a sharing of one or more antigens among the strains. A similar antigenic interrelationship was also described by Sharpe (135). In an investigation on rumen Bacteroides that included four B. fragilis strains, Sharpe, using precipitin reactions, reported that multiple antigens (thermostable) were shared among the four B. fragilis strains. Furthermore, no cross reactions were observed between the B. fragilis strains and the rumen organisms.

The serological interrelationships of Bacteroides were also illustrated by Beerens et al (5) in a study that resulted in the serological classification of one hundred and thirty-one Bacteroides strains. A serotyping scheme was devised by employing six antisera

directed against either B. fragilis, B. distasonis, B. vulgatus, B. thetaiotaomicron or B. ovatus. Five of the six strains used for immunization were biochemically defined neotypes (3), while the sixth was a clinical isolate, biochemically identified as B. fragilis. Both agglutination and immunodiffusion were used to characterize the six reference strains. High titers and multiple precipitin lines were observed in the homologous agglutination and immunodiffusion reactions, respectively. Although a low level of cross agglutination was reported between some of the reference strains, immunodiffusion studies failed to detect precipitin lines in any heterologous cross. Group antigens defined by the homologous reactions of the six reference strains were designated by capital letters (see table 32). The survey strains to be typed were analyzed biochemically and serologically (tube agglutination). Depending on the degree to which they were agglutinated by each of the six typing sera, the strains were assigned capital or lower case letters to designate the possession of major or minor antigens. Although 23% of the strains reacted with only a single antiserum, most strains possessed a mosaic antigen composition having one major and several minor antigens. Those showing two major antigen specificities were all confined within the two B. fragilis serogroups and shared both E_1 and E_2 antigens. Not all strains were typable. Eighteen strains (14%) failed to react with any antisera or were autoagglutinable. The most prevalent antigen was the B. fragilis E antigen, E_1 or E_2, being demonstrated on 84% of the typable strains. Biochemically, all of the serogroups, except one, were heterogeneous.

The success of Beerens' serotyping scheme provided an impetus to shift the emphasis of research from taxonomic to clinical studies. In an extension of Beerens' study, Sedallani et al (132) and Romond et al (127), surveyed clinical and normal stool isolates and reported a high occurrence of the E antigen, either as a major (60%) or minor (93%) component, in the clinical isolates. In normal stool specimens, the E antigen was rarely observed. These authors suggested that based on their findings, *Bacteroides* strains possessing the E antigen should be considered pathogenic. Similarly, the serological properties of *B. thetaiotaomicron* isolated from cattle infected with contagious foot rot were investigated by Hartwigk (51). Both agglutination and complement fixation tests (CFT) demonstrated a close antigenic relationship among these isolates.

With the accumulation of evidence concerning the clinical importance of *B. fragilis*, numerous studies were initiated to examine the immune response to *Bacteroides* infections and to identify such pathological conditions. Among the methods used were fluorescent antibody techniques (FA). Griffin (48) noted that the *B. fragilis* conjugates were species-specific and gave positive results with all of the specimens in which *B. fragilis* was identified culturally.

Further work on FA techniques, as applied to *Bacteroides*, was reported by Dowell, Lombard, and Jones (27). After preparing species-specific *B. fragilis* conjugates, they were able to detect 41 of the 43 *B. fragilis* strains tested (95%). Subsequently, Jones and Dowell (71) reported the preparation of a polyvalent *B. fragilis*

conjugate, allowing for the identification of 100% of the *B. fragilis* strains surveyed.

Indirect fluorescent antibody technique (IFA) has also been evaluated as a means of identification of *Bacteroides*. In a report by Stauffer, Hill, Holland, and Altemeier (140), clinical samples from suspected *Bacteroidaceae* infections were examined by IFA, employing both mono- and polyvalent antisera. Presumptive diagnoses correlated well with culture results.

The immune response to *Bacteroides* infections has been investigated by Danielson et al (21), Monson (102), and Lambe et al (82), using agglutination, IFA, immunodiffusion, and passive hemagglutination. Circulating agglutinins and precipitins against the infecting *Bacteroides* strains were demonstrated in all of the patients' sera but not in sera taken from healthy adults. Some degree of cross reactivity was observed between patients' sera and heterologous strains of *B. fragilis* and other *Bacteroides* species. As noted by these authors, such cross reactions could be attributed to a common or related antigen(s) or past infections with the strains in question. When monitored over a period of time, a decrease in the immune response was observed which paralleled the course of the infection. A serological means for detecting *B. fragilis* infections was also investigated by Rissing et al (124). Ultrasonicate extracts of two *B. fragilis* strains and one *B. thetaiotaomicron* strain were used in immunodiffusion studies to detect precipitins in the sera of patients suffering from *B. fragilis* infections. Sera from 50% of patients with bacteremia and 53% of those having abscesses, formed precipitin

lines against extracts of _B_. _fragilis_, _B_. _thetaiotaomicron_, or both, suggesting some degree of antigenic similarity. No precipitins reacting with _Bacteroides_ extracts could be demonstrated in sera from patients infected with aerobic gram-negative organisms nor in normal control sera.

At approximately the same time that antibodies to _Bacteroides_ were detected in patients with diagnosed infections, other laboratories were attempting to demonstrate antibody to members of the normal flora in healthy individuals. In 1972, Quick et al (119) reported detectable levels of antibody against _B_. _fragilis_ and two _Fusobacterium_ species in normal human sera. Antibodies could only be detected accurately by using indirect hemagglutination and were shown to be primarily of the IgM class. Further evidence for natural antibodies to _B_. _fragilis_ was reported by Hofstad (54), who surveyed sera from over one hundred healthy persons using indirect hemagglutination. Antibodies, specific for purified _B_. _fragilis_ lipopolysaccharide, were detected in most of the sera and were also of the IgM class of immunoglobulins.

In addition to the heat stable polysaccharides, other antigens have been reported that may contribute to the serological properties of _B_. _fragilis_. Heat labile antigens have been described by several investigators, however, few have performed systematic studies to determine the importance of these. Some evidence which suggests that common group or genus specificity resides in heat labile antigens was furnished by Reinhold (122, 123). Using unheated sodium carbonate and

ethyl ether extracts, a high level of cross reaction was observed between B. fragilis and B. thetaiotaomicron. These heterologous reactions were abolished on heating of the extracts.

Capsules have also been discussed as important factors influencing the serological properties of B. fragilis. Beerens et al (4) noted that all of the Eggerthella (Bacteroides) strains examined by him possessed some degree of capsulation. Preliminary studies demonstrated that the capsule was serologically active, as demonstrated by the Quellung reaction, and was shown to cross react with heterologous antisera. Recently, Kasper (75) has purified and characterized capsular material from Bacteroides fragilis. Using immunodiffusion analyses, he demonstrated reactions of identity between the carbohydrate moiety of the capsule and material similar in composition to LPS extracted by the aqueous phenol method. In subsequent studies, Kasper, using a radioactive antigen-binding assay, detected antibodies to this capsular material in antisera from rabbits immunized to eight heterologous strains of B. fragilis and one strain of B. thetaiotaomicron. Antibodies of similar specificity could not be demonstrated in rabbits immunized with B. vulgatus, B. distasonis or B. melaninogenicus.

Kasper (76) and Hofstad (56) have reported immunochemical analyses on the outer membrane complex and on the O-antigen specificity of LPS extracted from B. fragilis. The outer membrane complex was shown to be composed of two antigenic fractions, the capsule (described above) containing carbohydrate and protein, and a lipopolysaccharide-like

component. Furthermore, the carbohydrate and the protein components of these fractions were all associated with species-specificity. Hofstad examined LPS preparations from three B. fragilis strains by indirect hemagglutination and absorption studies. A minimum of six distinct antigenic determinants were identified, some being present on more than one strain, and all were destroyed by periodate oxidation.

Chemical Composition Studies on B. fragilis Antigens

Biological and chemical studies were reported as early as 1961 on antigenic components isolated from anaerobic oral bacteria (99). Such studies prompted similar investigations on other anaerobes, including B. fragilis. An indication of the nature of the antigens involved was suggested by the early serological tests and antigen extracts. From these studies, both heat labile and stable antigens were shown to participate in agglutination and precipitation reactions. Similarly, species-specificity was demonstrated using polysaccharide containing extracts which were sensitive to periodate oxidation.

A detailed study on the morphology and chemistry of the cell wall of B. convexus (B. fragilis) was reported in a series of publications by Ushijima et al (146, 147) beginning in 1970. Electron micrographs disclosed a five layer cell wall separated from the cytoplasmic membrane by an electron transparent layer. Surrounding the cell wall was a dense crust. Using numerous chemical agents and enzymes in combination with electron microscopy, the authors identified and subsequently isolated the various cell wall components. The chemical composition

of purified mucopeptide was found to be very similar to other gram negative bacteria. The molar ratio of amino sugar and amino acid components was 1:1:1:1:1:1 for glucosamine, muramic acid, L-alanine, D-glutamic acid, meso DAP, and D-alanine, respectively. Electron micrographs of LPS extracted from whole cells revealed a triple layered ribbon-like structure. In addition, the monosaccharide composition of outer membranes prepared by enzymatic digestion of cell walls, was chemically similar to LPS extracted by aqueous phenol. The predominant sugars were glucose, galactose, fucose, rhamnose, and xylose. Futhermore, heptose, but not KDO was detected. The authors also noted that most of the glucose associated with the cell wall was in the form of a glucan.

In the same year that Ushijima first published his work, Hofstad and Kristofferson (57) reported on the chemical composition of endotoxin extracted from B. fragilis. The yield of LPS extracted from whole cells was shown to be dependent on extraction conditions and ranged from 1 to 4%. Similarly, qualitative and quantitative composition of LPS was also shown to be dependent on extraction conditions. In particular, both glucose and protein content increased with prolonged extraction times. The sugars identified in the endotoxin were essentially the same as those reported by Ushijima, except that xylose was not detected. In addition, traces of mannose and two hexosamines, glucosamine and galactosamine, were identified as components. The authors also noted that neither KDO nor heptose were present in the extracted LPS and that the endotoxic potency in rabbits was low.

From these two investigations, came several significant observations. The cell wall of *B. fragilis* is similar to facultative gram negative bacteria, in that it is composed of a peptidoglycan and an outer membrane layer. Furthermore, a LPS complex can be extracted from the outer membrane having endotoxic properties and possessing monosaccharide constituents commonly found in LPS of other gram negative bacteria. However, LPS of *B. fragilis* apparently lacks KDO, and probably heptose. In addition, extraction conditions affect both the qualitative and quantitative composition of LPS, an observation which could explain the discrepancies between the two sets of results concerning the presence of particular constituents. Finally, both studies suggested the presence of a glycan which is either cell wall associated or intracellular. Additional support for the existence of this glycan was later reported by Seryczynska and Meisel-Mikolojczyk (133).

Subsequent investigations for the most part have been concerned with the presence of KDO and heptose, and with the sugar composition of LPS. To date, the question of whether or not KDO and heptose are present has yet to be resolved. In contrast to the previous studies, Sonnenwirth (138) in 1972 detected KDO in two *B. fragilis* strains using the thiobarbituric acid colorimetric method of Warren (154). Similarly, Kedzierska et al (77) reported the presence of KDO in three strains of *Eggerthella convexa* (*B. fragilis*) using both colorimetric and paper chromatographic techniques. The authors also demonstrated heptose colorimetrically and by paper chromatography in the same three strains. However, evidence against the presence of

these two components has since been reported by Bjornson and Hill (6), Hofstad (55), and Kasper (74). Bjornson and Hill, studying the biological properties of Bacteroidaceae cell wall components were unable to demonstrate KDO in isolated Bacteroides LPS. In a more systematic investigation, Hofstad surveyed twenty Bacteroides strains including representatives of B. fragilis, B. distasonis, B. thetaiotaomicron, and B. vulgatus and did not detect heptose or KDO in phenol-water extracts by colorimetric means or by paper or gas-liquid chromatography. Finally, Kasper (74) reported the absence of KDO and heptose in EDTA-extracted endotoxin from B. fragilis.

Several studies have dealt with the sugar constituents of LPS. Meisel-Mikolajczyk and Dworczynski (97) analyzed four Eggerthella convexa (B. fragilis) endotoxins having similar chemical composition i.e., protein, hexose, hexosamine, and lipid content, for sugar constituents by paper and TLC chromatography. Endotoxins were prepared by phenol-water extraction of whole cells and were not subjected to ultracentrifugation. Glucosamine, galactose, glucose, mannose, and ribose, were demonstrated in all four endotoxins. In addition, two preparations exhibited spots corresponding to uronic acid and rhamnose. The remaining two extracts lacked these components but contained an unidentified sugar component which was absent in the uronic acid-containing preparations. Neither galactosamine nor fucose were detected in any of the endotoxins.

Hofstad (56), investigating the serological specificity of three LPS preparations from B. fragilis, reported that the three preparations

contained the same neutral sugars, rhamnose, fucose, glucose, galactose, and mannose, but in different relative amounts. In addition, both glucosamine and galactosamine were present in all three preparations.

Lastly, Kasper (74, 75) presented evidence suggesting that capsular material isolated from B. fragilis possesses similar but not identical sugar constituents to those found in LPS. Glucose and galactose were conspicuously lacking in LPS and capsular material, whereas an additional component, sialic acid, was demonstrated in the capsular material.

Very little work has dealt with the lipids of B. fragilis beyond the stating of relative proportions of lipid found in isolated LPS. Hofstad and Kristofferson (57) and Meisel-Mikolajczyk and Dworczynski (97) reported that lipid represented approximately 20% of the endotoxin, expressed as fatty acid esters. Similar findings were reported by Kasper (74), however, the author demonstrated that most of this lipid was loosely bound. Very preliminary data on the lipid composition of LPS have been reported by Bjornson and Hill (6). These authors noted that the fatty acid content of LPS and Lipid A from a Bacteroides and an E. coli strain was found to be similar as were their β-hydroxy myristic acid content.

The complex lipids of Bacteroides and Sphaerophorus have been studied by Fritsche (39, 40), and Stoffel et al (142). Whole cell lipid extracts from strains of B. fragilis, B. thetaiotaomicron, B. vulgatus, and two additional Bacteroides species were characterized

by the presence of sphingolipids, a component lacking in Sphaerophorus strains. Similar differentiation between the two genera was observed in whole cell fatty acid profiles of their complex lipids.

DNA Homology of B. fragilis

All of the Bacteroides strains used in this investigation were chosen on the basis of the DNA homology studies of Johnson (66). They represent the five species, B. fragilis, B. distasonis, B. vulgatus, B. thetaiotaomicron, and B. ovatus. With the exception of B. thetaiotaomicron and B. ovatus, which were 40% homologous with each other, homology values indicated very little interspecies relatedness (< 20%). Within the species B. fragilis, strains belonged to one of two subgroups, designated I and II. These two groups were 65 to 70% homologous to each other and displayed a high intragroup homology (80 to 90%). Similarly, the B. distasonis and B. vulgatus groups showed the same high intragroup homology. However, within the B. thetaiotaomicron and B. ovatus groups, a wide range of homology values were observed (60 to 90%), suggesting multiple subgroups differing from each other in a manner similar to the two B. fragilis subgroups.

The above homology relationship were interesting in view of the phenotypic characteristics displayed by this group. On the whole, the species are quite similar phenotypically. Differentiation of the species can be achieved using the criteria of indole production and the fermentation of a few sugars. These phenotypic characters correlate well with the homology divisions. However, the genetic

distinction between some of the homology subgroups is not expressed as clearly using the phenotypic characters tested to date. For example, strains belonging to the two _B. fragilis_ subgroups, essentially displayed the same reactivity for a given trait (68) yet genetically, there was measurable heterogeneity. Similar observations were reported for strains belonging to the _B. thetaiotaomicron_ and _B. ovatus_ groups. On the other hand, a high level of intragroup homology did not necessarily indicate phenotypic homogeneity. Within the _B. distasonis_ homology group, which showed 80 to 90% intrahomology, only 71% of the strains utilized ribose. Thus, on the basis of the phenotypic tests used in this _Bacteroides_ study, no general correlations between levels of intergroup homologies and phenotype were observed.

Summary

Numerous studies have dealt with the serology and, to a lesser degree, the characterization of cell wall antigens of *Bacteroides fragilis*. These studies demonstrated that *B. fragilis* possesses a multiplicity of antigens, conferring strain, species, and occasionally group specificity. In addition to thermal stable cell wall polysaccharides, other antigens were shown to contribute to the serological properties, including cell wall associated proteins and capsules. Chemical studies have suggested that *B. fragilis* possesses a typical gram negative cell wall, although its LPS complex may differ in regard to certain structural features. Furthermore, a few studies have attempted to relate chemotype to serotype, although they have been limited to only a few strains belonging to *B. fragilis*. The strains used in all of these studies had been assigned to species on the basis of biochemical properties. Recently, over three hundred '*B. fragilis*' strains have been assigned to DNA homology groups, allowing phenotypes to be directly correlated to genotypes. Using a selection of these strains, the present investigation was undertaken for the purpose of establishing correlations between serological groupings and DNA homology groups. In addition, an effort was made to define a chemical basis for the serological reactions and to determine gross similarities and differences between cell wall components of facultative gram negative bacteria and the '*B. fragilis* group' of organisms.

MATERIALS AND METHODS

Bacterial cultures. Sources and designations of the bacterial organisms used in this study are given in table 1. Strains from the VPI Anaerobe Laboratory Culture Collection will be referred to by their VPI numbers and other identifying numbers will be provided where available. The Bacteroides strains represent six species, as defined by their DNA homology values (66) and phenotypic characteristics (58). Escherichia coli ATCC 11775, Proteus vulgaris, ATCC 13515, Proteus mirabilis, PM-1, and Pseudomonas aerugenosa, ATCC 15152, were chosen as representatives of the facultative gram negative group of organisms.

Materials. Reagents and recipes for materials used in this study are listed in Appendix 1. The source of all chemicals and solvents was Fisher Scientific Company unless specified.

Pre-reduced media. Pre-reduced media were prepared as outlined in the VPI Anaerobe Laboratory Manual (59). The compositions of all the media used are given in Appendix 1. For tubed media, the ingredients were placed in an Erlenmeyer flask of sufficient size to minimize air space above the fluid (e.g., 750 ml in a 1 liter flask). The flask was fitted with a removable chimney and heated with stirring until the resazurin turned colorless. The flask was removed from the heat, the chimney was replaced by a cannula delivering O_2-free CO_2 into the fluid and the medium was cooled to room temperature in an ice bath. With O_2-free CO_2 still bubbling through the medium, cysteine-HCl was added

Table I. Sources and Designations of Organisms Used in This Study

VPI No.	Source and other designations	Isolated from
B. fragilis type I		
2553*	ATCC 25285 VPI 2553 Sonnenwirth[a] EN2 NCTC 9343	appendix abscess
0439	Prevot[b] 2342A	liver abscess
1582	CDC[c] 1313E	wound
2554	Sonnenwirth EN3 NCTC 9344	septic wound
2556-1	Sonnenwirth FB-2	appendix abscess
2758-A	Balows (LC) 11-139-7	rectal abscess
3277-1	Medical College of Virginia (MCV)[f] Bacteriology Lab	blood culture
3389	Prevot 3053	pleurisy
3390	Prevot 640-A	?
3625	MCV Bacteriology Lab	uterus
4082	Prevot 2359	ovarian abscess
4128	Prevot 3020	ascitic fluid
4147	Prevot 3953	appendectomy
4361	Beerens[g] 376	?
4366	Prevot 2006	septicemia
4517	Balows[e] (SJH) 1-356-8	wound
4736	Lambe[h] IR426	surgical wound
4912	Lambe 164-058U	blood
5383	ATCC 23745 NCTC 10581	pleural fluid
5631	Gunderson Clinic, LaCrosse, WI	mitral valve
5711	French Hospital, N.Y.	?
5785-1	CDC, NCDC 3820	
5807	New Hanover Hosp., Wilmington, N.C. 244	blood
6057-B	Tuscon Medical Ctr.	buttock drainage
6059	St. Anthony Hospital, Terre Haute, IN 805	?
6123	Corning, N.Y. 3321	abdominal drainage
6195	VA Hospital Richmond, VA 227-10-3654	blood
6754	Baroness Erlanger Hosp., Chattanooga, TN 7475	blood
6805	Tuscon Medical Ctr.	appendix
6815	Dalton[f] MCV	lung tissue
6851	Mercy Hospital, San Diego, CA	?
6957	Forsyth Memorial Hosp., Winston-Salem, N.C.	blood
9032	Adult Leukemia Ctr.	blood
E323	Beerens	
B. fragilis type II		
2393	Beerens 12	?
2360	Reinhold[d] 234	lung
2552	Sonnenwirth EN1 NCTC 8560	appendectomy
2647-J2	VPI	hog cecum
3392	Prevot 4392	?
4076	Prevot 2228	blood
4112	Prevot 3748	buttock abscess
4117	Prevot 4026	?
4225	Prevot 3804	septicemia
B. thetaiotaomicron		
5482	ATCC 29148 VPI 5482 NCTC 10582 Werner E50	feces
0489	ATCC 12290	infection after appendectomy
0940-1	VPI 5MF95	feces
2808-B	Balows (LC) 1-33-7	rectal abscess
6212	Corning, N.Y. 3679	abdominal wound
7330-1	Lambe Emory University N38-72D	stab wound
0911A1	VPI 2MF95A	feces
3089	Memorial Hospital, Danville, VA	blood culture

Table I. Continued.

VPI No.	Source and other designations	Isolated from	VPI No.	Source and other designations	Isolated from
2297	13M	cecum	*B. vulgatus*		
6180A	Corning Hospital	peritoneal cavity	4245*	ATCC 8482	feces
8651	?		0959-1	VPI 7MF95	feces
B1-46		human fecal	2277	VPI 13M	large intestine
C9-11		human fecal	2365	Barnes AN21-27	
C11-15		human fecal		Norwich, England	chicken cecum
C11-16		human fecal	4506-1	Balows (SJH)	
C22-15		human fecal		I-497-8	abdominal cavity
			5710	French Hospital	
B. ovatus				New York, N.Y. 12	
0038-1*	ATCC 8483	feces	6370	Sprue S747	fecal
C12-31		human fecal	C6-7		human fecal
T4-7		human fecal	C7-2		human fecal
2822	Balows (IC) I-84-7	rectal abscess	C10-6		human fecal
			OC-13		human fecal
'3452-A' group			R4-24B		human fecal
3452-A	VPI	feces	6598-B	Corning, N.Y. 1499	wound
2308	VPI 13M	rectal contents			
8608	Isolated from		*B. distasonis*		
	VPI 6460C	ear infection	4243*	ATCC 8503	feces
3596	NASA, Douglas	fecal specimen	0052	AEC 9A	mouse feces
OC-9		human fecal	B1-20		human fecal
			C18-7		human fecal

* Type or neotype strains

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[h]D. W. Lambe, Jr., Emory University, Atlanta, GA.

and the pH adjusted to a final value of 6.9 with either 8N NaOH or 5N HCl. Once the final pH was obtained, O_2-free CO_2 was replaced by O_2-free N_2 and the medium was dispensed in appropriate amounts. No. 1 rubber stoppers (Fisher) were used to close the tubes. The stoppered tubes were placed in a press and autoclaved for the specified time at 121 C, 15 lbs/sq. in., using fast exhaust.

Media for batch cultures were prepared in 3 liter flasks in a similar manner with a few modifications. After all ingredients were mixed, including cysteine-HCl, and water added to obtain the final volume (2.5 L), the flasks were fitted with a black rubber stopper having right angled inlet and outlet tubes. To the outer ends of these tubes were attached, by rubber tubing, short cotton-filled glass tubing which could be clamped off to maintain an anaerobic environment. The stopper was held in place by means of a horizontal bar attached to a metal collar extending around the neck of the flask (19). The media was autoclaved 45 minutes at 121 C, 15 lbs/sq. in., using slow exhaust, and cooled under O_2-free N_2 gas.

Blood agar medium for plates was prepared by melting Brain Heart Infusion Agar (BHIA) tubes, adding 0.4 ml sterile sheep blood per 10 ml medium, mixing, and pouring into plastic petri dishes. Plates were used as soon as the agar solidified.

Anaerobic culture techniques. Anaerobic culture techniques as outlined in the VPI Anaerobe Laboratory Manual (59) were used. All anaerobic culture manipulations were performed under O_2-free CO_2 using the VPI Anaerobic Culture System (Bellco Glass, Inc., P. O. Box

"B", Vineland, New Jersey 08360), except where specified. Stock cultures were maintained in chopped meat medium and periodically subcultured into fresh medium. For serological surveys, cells were grown in trypticase-yeast extract-glucose (TYG) medium, or Brain Heart Infusion (BHI) broth, 25 ml per tube. Cultures for the isolation and analysis of cell wall components were grown batchwise as described by Cummins and Johnson (19), in high trypticase-yeast extract-glucose (hTYG) medium. Each batch was inoculated under O_2-free N_2 gas with 40 mls of an 18 to 24 hour culture grown in TYG medium and 40 mls of 10% bicarbonate. The cultures were inoculated at 37 C with stirring and harvested at the beginning of stationary phase (18 to 24 hours).

Aerobic media. The composition of media used for growth and maintenance of aerobic organisms is listed in Appendix 1. Where media of similar composition to anaerobic media were needed, they were made in the same way as for the anaerobic media except that resazurin and cysteine were not added and the media were not boiled. Media prepared in flasks were stoppered with gauze-covered cotton plugs, capped with aluminum foil, and autoclaved under the same conditions as the anaerobic media.

Aerobic culture techniques. Aerobes were routinely maintained on soft agar stabs or trypticase-soy agar slants. Aerobic batch cultures were inoculated aerobically with 40 mls of an 18 to 24 hour culture grown in aerobic TYG medium.

Harvesting of the cells. After reaching stationary phase, the cells were killed by the addition of 2% (v/v) formalin and a smear

was stained by Gram's method to determine purity. Cells were harvested by centrifugation, washed twice with distilled water, resuspended in a minimal volume of distilled water, and stored at 4 C with 0.1% sodium azide until used for immunization, agglutination tests, or the preparation of antigen extracts.

Preparation of cell walls. Cell walls were prepared from whole cells as described by Cummins and Johnson (19). Formalin-treated cells were washed twice with EDTA-saline and suspended in a final volume of 10 or 20 mls. An equal volume of glass beads (0.11 mm dia.) was added to the cell suspension and the mixture was shaken in a Braun disintegrator for 2 to 5 minutes. The beads were removed by filtration through a course sintered glass filter and the filtrate digested with pronase (c. 1 mg/ml) for 2 hours at 55 C. A gram stain was made on the digested mixture and any preparation showing intact cells was redisintegrated and redigested as described above. The final material was digested with RNase and DNase (c. 1 mg/ml) for 2 hours at 37 C, washed twice with distilled water, and centrifuged at 2000 X g to remove debris and dirt. The resulting cell walls were then lyophilized.

Immunological Techniques

Immunization and preparation of antisera. Two albino rabbits were used for the preparation of antiserum to each strain. The animals were immunized intravenously with a dense suspension of washed formalin-killed cells, in phosphate-buffered-saline, pH 7.5 (PBS). Injections were given twice a week in a stepwise manner according to the following schedule: 0.5, 1.0, 1.5, 2.0, and 2.5

mls. One week after the last injection, a test bleeding was made from the ear of each animal. Rabbits showing a significantly high titer ($\geq$ 640), as determined by whole cell agglutination tests, were exsanguinated by cardiac puncture. Rabbits showing little antibody response were rested one week and then reimmunized according to the same schedule as mentioned above.

Blood was collected aseptically, allowed to clot at 37 C, and refrigerated overnight at 4 C. Serum was pipetted off into sterile centrifuge tubes, clarified by centrifugation, and preserved with 0.1% sodium azide. The antisera were dispensed in 10 ml portions and stored at either 4 C or -20 C until use.

Antisera against strains 6805 and 6851 were gifts of Dr. L. DS. Smith.

Absorption of antisera. Diluted antisera (1:10) were absorbed with whole cell suspensions or lyophilized Autoclaved extracts of whole cells.

i.) Absorption with whole cells: The amount of whole cells needed to absorb out the cross reactions was determined empirically from the results of the agglutination tests, e.g., see table 3. 4245 whole cells at a density equal to 80% T (560 nm) cross react with AS2553 to a dilution of 1/20; therefore, to absorb out cross reaction at 1/10, a two fold greater concentration of 4245 cells is needed.

A cell suspension of appropriate density was centrifuged and the pellet resuspended in the diluted antiserum. This mixture was incubated at 37 C for 1.5 hours and was agitated

at 10 minute intervals. After incubation, the mixture was centrifuged at 22,000 x g for 20 minutes and the same procedure was carried out using suspensions of the other cross reacting strains. Following all absorptions, Lyphogel (Gelman Instrument Company) was used to reconcentrate the diluted serum to its original volume. Agglutination tests were performed on the absorbed serum to determine whether any cross reactions remained.

ii.) Absorption with lyophilized Autoclaved extracts: Diluted antiserum (1:10) was mixed with a weighed amount of extract, incubated with agitation at 37 C for 1 to 2 hours, and then kept refrigerated (4 C) for 24 hours. Following centrifugation at 12,000 x g for 20 minutes, the clarified antiserum was reconcentrated to its original volume and examined for cross reactions by immunodiffusion. Before batch absorption was performed preliminary tests were done to determine the minimum level (mg/ml) of antigen which would eliminate the cross reacting antibodies.

Characterization of surface antigens. Since agglutination tests were detecting surface antigens, it was desirable to characterize what kinds of structures, e.g. proteins, polysaccharides, were involved in the agglutination reaction. Therefore, cell suspensions were treated with heat, proteolytic enzymes, or periodic acid to determine what affects these agents would have on the agglutination potential of the cells. In all treatments, control cell suspensions were run simultaneously. Cell suspensions made to 75-80% T at 560 nm in PBS (PBS represents 100% T) were treated with heat or enzymes. The cells were heated at neutral pH at 100 C for 0.5 to 1 hour. Similarly

the cell suspensions were incubated with pronase or trypsin (Sigma) at a final concentration of 1 mg per ml for 24 hours at 37 C. Following incubation, the cells were pelleted by centrifugation, washed three times with PBS, and resuspended to their original volume with PBS for use in the agglutination studies.

A cell suspension of 40% T at 560 nm in 0.1 M phosphate buffer, pH 7.0 was mixed with an equal volume of 0.01 M periodic acid. After mixing, aliquots were removed at regular intervals and transferred to tubes containing an equal volume of 20% glucose solution. Cells were pelleted by centrifugation, washed three times with PBS and resuspended to a density equal to 75-80% T at 560 nm for the agglutination studies.

Agglutination tests. Each antiserum was titered by tube agglutination. Serial two-fold dilutions of antiserum, 0.5 ml per tube, were mixed with an equal volume of whole cell or cell wall suspension adjusted to a fixed density of 75-80% T at 560 nm. The tubes were incubated at 56 C in a water bath for 2 hours, then allowed to stand overnight at room temperature before reading the results. Titers were expressed as the reciprocal of the final dilution which gave macroscopically visible agglutination. Each tube was scored ++++, +++, ++, +, ±, tr, or -, indicating the degree of agglutination (± indicating the smallest particles easily visible to the naked eye). Slide agglutination was performed on glass microscope slides. One drop of a cell suspension adjusted to a fixed density of 50% T at 560 nm was mixed with a drop of antiserum and rotated until agglutination

was observed. Buffer and positive controls were run simultaneously. Agglutination was scored in a similar manner to the tube agglutination.

Immunodiffusion tests. The two dimensional immunodiffusion method of Ouchterlony (72) was carried out on 2 x 3 inch glass slides in 1% ion agar buffered with PBS, pH 7.5. Reagent wells were 2 mm deep, 5 mm in diameter, with centers 10 mm apart. Plates were incubated at room temperature in a water saturated atmosphere. Antigen solutions were adjusted to equivalent carbohydrate concentrations (c. 1 mg/ml) as determined by the phenol-sulfuric acid assay of Dubois (31). Plates were read at 24, 48, 72 and 96 hours.

Hemagglutination tests. i) Sensitization of red blood cells (RBCs). Autoclaved extracts of whole cells or LPS were used to sensitize sheep red blood cells. Various antigen concentrations were used to study the sensitization process, however, for routine tests using the Autoclaved extracts, an antigen concentration of 1 mg carbohydrate per ml of extract, as determined by the phenol-sulfuric acid assay, was used. Washed packed RBCs were suspended in an antigen solution to give a final concentration of 2.5% RBC and incubated at 37 C for 1 hour. During this time the tubes were gently shaken at 15 minute intervals. The sensitized cells were then washed three times with PBS and further diluted with PBS to obtain a 0.5% suspension. The treated cells were used immediately for the hemagglutination assay.

ii) Hemagglutination assay. These were carried out in microtiter plates (Cooke Engineering Co.). Serial two-fold dilutions of antisera, 50 μl per well, were mixed with an equal volume of 0.5%

sensitized cells. The plates were incubated at 37 C for one hour with agitation every 15 minutes. Plates were read at one hour and allowed to stand overnight at 4 C before the final reading. Titers were expressed as the reciprocal of the final dilution which gave macroscopically visible agglutination. Agglutination was scored ++, +, ±, or -, where ± indicated approximately 50% of the sensitized RBCs were agglutinated. Each plate included the following controls: 1) PBS with treated cells, 2) antiserum with untreated cells, and 3) normal rabbit serum with treated cells. Before being used in hemagglutination tests, all antisera were routinely absorbed at a dilution of 1:10 with washed untreated red blood cells at 37 C for 1 hour.

Antigen Extracts

Several different antigen extracts were made from either whole cells or cell walls. Their method of preparation are given below.

Autoclaved extract. Formalin-treated whole cells or cell walls (50 - 100 mg, dry wt) were suspended in 4.0 ml PBS and autoclaved at 15 lbs/sq. in. 121 C for 15 minutes. The mixture was stored overnight at 4 C, centrifuged, and the resulting supernatant was preserved with 0.1% sodium azide at 4 C until use.

TCA extract. Lyophilized whole cells (100 mg) or cell walls (10 to 20 mg) were suspended in 1 ml of 10% TCA and placed in a 55 C water bath for 30 minutes. After cooling, 2 mls of distilled water were added and the mixture was centrifuged at 2000 x g for 10 minutes. The supernatant was filtered through a 0.8 μ millipore filter before 5

volumes of acetone and a few drops of 5.0% potassium acetate were added. After 24 hours at 4 C, the precipitated polysaccharides were recovered by centrifugation (12,000 x g for 10 minutes), redissolved in 1 ml of distilled water, and neutralized to phenol red with 0.1 N NaOH. The neutralized extracts were kept at 4 C and preserved with 0.1% sodium azide.

Formamide extract. Formalin-treated whole cells were suspended in 1 ml of formamide and heated in an oil bath at 165 - 170 C for 15 minutes. After cooling, 2.5 mls of acid-alcohol (0.5 ml conc. HCl + 100 ml 95% ethanol) were added and the mixture was centrifuged at 1200 x g for 10 minutes. To the supernate, 5 ml of acetone were added along with a few drops of 5.0% potassium acetate until precipitation began. After 30 minutes at room temperature, the precipitates were recovered by centrifugation and redissolved in 1 ml of distilled water. The extracts were neutralized to phenol red with 0.1 N NaOH and preserved with 0.1% sodium azide.

Sonication. An ultrasonicate extract was made from whole cells. A 20% (wet wt/vol) cell suspension in PBS was sonicated with a Biosonic III (Bronwill Scientific) at 45% maximum (680 watts/in^2 = 100%) for five minute intervals while cooled on ice. Cell breakage was monitored by phase contrast microscopy. The mixture was centrifuged at 20,000 x g for 30 minutes and the pellet discarded. The supernate was preserved with sodium azide (0.1%) and kept refrigerated until use.

Phenol-water extract. Lipopolysaccharide was extracted from cell walls with 45% phenol according to the method of Westphal and Jann

(165). Approximately 4 grams dry weight of cell walls were suspended in 50 mls of distilled water and heated to 65 C. To this mixture, an equal volume of phenol, preheated to 65 C, was added with vigorous stirring and the extraction was allowed to proceed for 20 to 30 minutes at 65 C. After 30 minutes, the mixture was cooled to 10 C in an ice bath, centrifuged at 8000 x g for 45 minutes, and the aqueous layer aspirated off. The phenol and insoluble layers were reextracted as described above with another 50 mls of distilled water. Both aqueous layers containing the LPS were combined, dialyzed against tap and distilled water until all traces of phenol were removed, and lyophilized.

HCl extracts. Washed formalin-treated cells were extracted with 0.05 N HCl at 100 C for 10 minutes. After cooling, a cell-free extract was obtained by centrifugation and neutralized to phenol red with 1 N NaOH. The extracted polysaccharides were precipitated with 5 volumes of acetone, harvested by centrifugation, and redissolved in a final volume of 1 ml of distilled water.

Acetic acid extract. Approximately 10 g wet cells were suspended in 100 mls of 1% (v/v) acetic acid and heated under reflux at 100 C for 1 to 1.5 hours. After cooling, the mixture was centrifuged at 27,000 x g for 20 minutes and the supernate was lyophilized. The dried material was redissolved in 10 mls of distilled water, recentrifuged, and the supernate was again lyophilized. This was denoted as the primary extract and used for the gel-filtration studies. As a modification of the above protocol, high molecular weight material (Peak I) was isolated by adding one volume of

acetone to the supernatant resulting from the first centrifugation. The precipitates were collected by centrifugation, redissolved in distilled water, and used in this form for the column separations and chemical studies.

Isolation of peptidoglycan. Peptidoglycan was prepared by the method of Weidel (159). The insoluble residue of the phenol-water extract was dispersed by a tissue grinder and dialyzed against tap and distilled water until all traces of phenol were removed. After dialysis, the material was washed twice with distilled water, digested with pronase, 2 mg per ml, at 37 C for 24 hours, and finally re-washed twice with distilled water. Following the final washing, the pellet was resuspended in 0.4% sodium dodecyl-sulfate (SDS) and disintegrated for 1 to 2 minutes using the Biosonic III (Bronwell Scientific) at 80% maximum. The sediment was collected by centrifugation, washed twice with distilled water, and lyophilized for use in the chemical studies.

Colorimetric Chemical Assays

The chemical composition of the different LPS preparations were determined, in part, by colorimetric chemical assays. A complete listing of reagents and protocols is given in Appendix 2.

Carbohydrate estimation. Total carbohydrate was determined by the phenol-sulfuric acid and α-napthol assays of Dubois (31) and Molisch (168), respectively, using glucose as a standard.

Protein estimation. Protein concentrations were estimated by the procedure of Lowry (84) using Bovine Serum Albumin (BSA) as a standard.

Protein content was also estimated spectrophotometrically by measuring the absorbance at 260 nm and 280 nm.

Lipid content. Lipid, as expressed in terms of fatty acid esters, was estimated by the hydroxylamine reaction described by Shapiro (134). Methyl palmitate was used as a standard.

Nucleic acid estimation. Nucleic acid content was determined spectrophotometrically by measuring the absorbance at 260 nm and 280 nm. Isolated DNA was used as a standard.

Phosphorus estimation. Total phosphorus was estimated according to the method of Fiske and Subbarow (36) using KH_2PO_4 in 0.1 N H_2SO_4 as a standard.

Hexosamine estimation. Hexosamine content was estimated by the modified Rondle and Morgan procedure (168). Samples were hydrolyzed in 3 N HCl for 2 hours in a sealed glass test tube. After cooling, the hydrolysates were dried under a heat lamp, taken up in 3 ml distilled water, and used directly for the colorimetric test.

Amino sugar analyses were also performed on LPS samples using an automated amino acid analyzer equipped with Autolab System AA Integrator. Dried samples were hydrolyzed in 8 N HCl for 2 hours at 95 C and evaporated to dryness in vacuo. The residue was redissolved in 0.5 N, pH 2.2 citrate buffer and used directly for analysis according to the method of Walborg et al (152) as modified by Gum (49).

Heptose estimation. Heptose content was estimated by the cysteine-sulfuric acid assay of Osborn (113) and by the method of Vaskovsky and Isaz (148) for the determination of formaldehyde release

after periodate oxidation. D-Mannoheptulose (Nutritional Biochemicals Corporation) was used as a standard.

KDO estimation. Quantitative determination of KDO in the LPS preparations was performed using the thiobarbituric acid assays of Warren (154) and Waradarsky and Saslaw (156). In addition, the method of Vaskovsky and Isaz (148) for the determination of formaldehyde release was also used for KDO estimation in the gel filtration studies. Authenic KDO (Sigma) was used as a standard.

6-Deoxyhexose (methyl pentose) estimation. 6-deoxysugar content was estimated by the cysteine-sulfuric acid assay of Dische and Shettles (24) using rhamnose as a standard.

Chromatography

Paper chromatography. Approximately 10 to 20 mg of lyophilized extract (LPS, Autoclaved extract, TCA extract, or peak I material) were hydrolyzed for sugar and amino acid determinations.

For sugar analysis, extracts were hydrolyzed in 5 ml of 2 N H_2SO_4 for 2 hours at 100 C in a sealed glass test tube. After cooling, the mixture was neutralized with solid barium carbonate, centrifuged, and the supernatant poured off and retained. The $BaSO_4$ deposit was washed twice with distilled water and these washings were combined with the original supernatant. After a final centrifugation, the washings were evaporated to dryness in vacuo over phosphorus pentoxide (P_2O_5). The residue was taken up in 0.3 ml of distilled water, centrifuged, and the supernatant saved for spotting chromatograms. 25 µl aliquots were spotted on Whatman No. 1 paper and developed

with solvent systems A and B (see Appendix 3 for a complete listing of solvent systems). For the chromatography of KDO, solvent systems A, B, and C were used. Solvent systems A, B, C, and D were used in the heptose studies. The sugars were detected by the aniline hydrogen phthalate method, alkaline silver, or ninhydrin (for amino sugars). A complete listing of detection reagents is given in Appendix 4.

Amino acids and DAP were detected by hydrolyzing 10-20 mg of sample in 6 ml of 6 N HCl for 18 hours at 100 C in a sealed glass test tube. The hydrolysates were cooled, filtered by gravity through a Whatman No. 1 filter, and evaporated to dryness under a heat lamp. The residue was taken up in 0.3 ml of distilled water. 25 μl aliquots were spotted on Schleicher and Schuell paper, No. 2043A, or Whatman No. 1 paper and developed with solvent E. Amino acids and DAP were visualized with the ninhydrin reagent.

Two dimensional ascending chromatography was done on 8 inch squares of Whatman No. 1 paper. 10 to 25 μl of hydrolysate was spotted at the origin and developed in one dimension with solvent F and in the second dimension, with solvent G. Amino acids were visualized with the ninhydrin reagent.

Molecular-sieve chromatography. Gel filtration studies were carried out on Sephadex gels (Pharmacia Fine Chemicals) of different exclusion limits with either Ammonium acetate buffer, 0.02 M, pH 5.9, or pyridine acetate solution (4 ml pyridine + 10 ml acetic acid in 1 liter of distilled water), pH 5.9, as eluant. Approximately 20 mg of sample (primary extract) were fractionated on a column (1.5

x 4.5 cm) with a flow rate of 2 ml per hour. 1.5 ml fractions were collected and assayed for total carbohydrate by phenol-sulfuric acid and for heptose and KDO by the formaldehyde assay of Vaskovsky and Isaz (148). Fractions containing high molecular weight (peak I) material were pooled, dialyzed against distilled water for 24 hours at 4 C and lyophilized. Fractions containing peak II and III material were combined and lyophilized.

High voltage electrophoresis. 5 to 25 μl of sample to be analyzed were applied to a 1.5 cm starting line on Whatman 3MM paper. Buffer systems H or I (see Appendix 3) were used. The voltage applied was 3000 volts for 1 hour or until the desired separation was achieved. Spots were visualized with ninhydrin, alkaline silver, aniline hdyrogen phthalate, or the thiobarbiturate reagents.

Gas chromatography. Neutral sugars of LPS were determined as their alditol acetate derivatives by gas liquid chromatography (GLC). Derivatives were made according to the methods of Sawardekar et al (129) and Metz et al (100) with xylose as internal standard. Analyses were performed on a Hewlett-Packard 5830 A gas chromatograph equipped with a 18850A GC terminal. The gas chromatograph was fitted with a glass column (2 m x 2 mm) packed with 3% SP 2340 100/120 mesh Supelcoport (Supelco, Inc.). The injection temperature was 260 C, the flame ionization detector (FID) temperature 260 C, and the carrier gas flow (nitrogen) 30 ml per minute. The column was temperature programmed with an initial temperature of 200 C rising to 245 C at a rate of 3 degrees per minute. The retention times of the acetates derived

from the LPS preparations were identical to those of acetates prepared from standard sugar solutions. The response of the FID detector to all the glycitol acetates was found to be linear. The relative peak areas were determined for each sample and used to calculate the molecular ratios for the constituent sugars.

Electron microscopy. For sections, LPS specimens (approximately 20 mg) were prefixed in 5% glutaldehyde at 4 C overnight. After being rinsed with double distilled water, specimens were postfixed with 1% osmium tetroxide, centrifuged in agar into a pellet (53), and stained with 0.5% uranyl acetate. Samples were dehydrated in a graded alcohol series, washed with acetone, infiltrated with acetone-Spurr solution and embedded in Spurr (139). Ultrathin sections were cut on a Sorvall MT2-B Ultra-microtome and stained with uranyl acetate and lead citrate (157, 150). Specimens were viewed on a Jeol 100B electron microscope.

RESULTS

Initial serological studies dealt with the devising of workable serological schemes for the analysis of the six Bacteroides reference strains. Their interhomology relationships are given in table 2. After the antigenic relationships between the reference strains were determined, a preliminary survey was conducted on randomly selected strains belonging to the different homology groups with emphasis on the two B. fragilis groups.

Serological Studies on the Reference Strains

Reference antisera. The four week immunization scheme was sufficient to elicit a good antibody response, as monitored by tube agglutination, in all rabbits except those immunized with B. distasonis, 4243, and B. ovatus, 0038-1, see table 3. At the time of the initial test bleeding, antisera titers from the rabbits inoculated with 4243 and 0038-1 were 20 and 40, respectively. These rabbits were rested one week and then reimmunized for a second course of inoculations. At the completion of this period, antisera titers had increased significantly to a value of 1280 for the 4243 antiserum, yet the highest titer obtainable against 0038-1 was only 160. A shorthand designation that will be used in this text to represent a particular antiserum will be the two capital letters 'AS' followed by the VPI number of the bacterial strain used to generate the antiserum, e.g., AS2393.

Agglutination studies. Tube agglutination was used to determine the interrelationship between the reference strains with respect to

Table 2. DNA Homology Values (%) for _Bacteroides fragilis_ and Related Species[a]

VPI No.	Reference DNA					
	B. fragilis Group II 2393	_B. fragilis_ Group I 2553	_B. distasonis_ 4243	_B. vulgatus_ 4245	_B. thetaiota-omicron_ 5482	_B. ovatus_ 0038-1
Competitor DNA: _B. fragilis_ Group II 2393	100	67	17	8	19	4
Competitor DNA: _B. fragilis_ Group I 2553	66	100	5	10	21	16
Competitor DNA: _B. distasonis_ 4243	8	18	100	8	5	3
Competitor DNA: _B. vulgatus_ 4245	14	24	9	100	9	9
Competitor DNA: _B. thetaiota-omicron_ 5482	19	21	7	13	100	40
Competitor DNA: _B. ovatus_ 0038-1	15	37	4	9	40	100

[a]Results of Dr. J. L. Johnson, Anaerobe Laboratory, VPI & SU (65)

their surface antigens. Table 3 illustrates the agglutination pattern obtained in homologous-heterologous crosses using unabsorbed antisera and whole cells as antigens. The reference strains were clearly differentiated with antiserum titers of 1280 or 2560, except for _B. ovatus_, whose titer was only 160. A low level of cross reactivity was observed between some of the strains and in particular, whole cells of _B. vulgatus_, 4245, were agglutinated to various degrees by all of the antisera. However, all cross reactions were insignificant in comparison to the homologous reactions and could be removed by absorbing the antiserum with whole cells without significantly lowering the homologous titers, see table 4. A similar agglutination pattern, shown in table 5, was observed when cell walls were used as antigens, although cell wall agglutination titers were lower than in the whole cell system, and the agglutination was characterized by a much heavier flocculation. With cell wall agglutination, the only cross reactions observed were between the two _B. fragilis_ strains, 2393 and 2553, and those were insignificant with respect to the homologous reactions. Likewise, a similar differentiation of strains was achieved using slide agglutination. Table 6 shows the slide agglutination pattern using both unabsorbed and absorbed antisera. The results are very similar to those obtained in the whole cell tube agglutination tests. All cross reactions were removed by absorption.

Characterization of surface antigens. Cell suspensions were subjected to heat, pronase, trypsin, and 0.01 M periodic acid to

Table 3. Whole Cell Tube Agglutination Results of *Bacteroides* Species

		Unabsorbed Antisera					
	VPI No.	*B. fragilis* Group II 2393	*B. fragilis* Group I 2553	*B. distasonis* 4243	*B. vulgatus* 4245	*B. thetaiota-omicron* 5482	*B. ovatus* 0038-1
Formalin-treated whole cells	*B. fragilis* Group II 2393	1280[a]	<20	0	0	0	0
	B. fragilis Group I 2553	<20	2560	0	<20	<20	0
	B. distasonis 4243	0	0	1280	40	0	0
	B. vulgatus 4245	80	20	40	2560	<20	<20
	B. thetaiota-omicron 5482	0	<20	<20	20	1280	0
	B. ovatus 0038-1	0	0	0	0	<20	160

[a]Titers expressed as reciprocal of dilution

Table 4. Whole Cell Tube Agglutination Results Using Absorbed Antisera

VPI No.	Absorbed Antisera: *B. fragilis* Group II 2393	*B. fragilis* Group I 2553	*B. distasonis* 4243	*B. vulgatus* 4245	*B. thetaiota-omicron* 5482	*B. ovatus* 0038-1
Formalin-treated whole cells: *B. fragilis* Group II 2393	640[a]	-	-	-	-	-
B. fragilis Group I 2553	-	640	-	-	-	-
B. distasonis 4243	-	-	640	<20	-	-
B. vulgatus 4245	-	-	-	640	-	-
B. thetaiota-omicron 5482	-	-	-	-	640	-
B. ovatus 0038-1	-	-	-	-	-	80

[a]Titers expressed as reciprocal of dilution

Table 5. Cell Wall Tube Agglutination Results

	VPI No.	Unabsorbed Antisera					
		B. fragilis Group II 2393	*B. fragilis* Group I 2553	*B. distasonis* 4243	*B. vulgatus* 4245	*B. thetaiota-omicron* 5482	*B. ovatus* 0038-1
Cell Walls	*B. fragilis* Group II 2393	160[a]	<20	0	0	0	0
	B. fragilis Group I 2553	20	320	0	0	0	0
	B. distasonis 4243	0	0	320	0	0	0
	B. vulgatus 4245	0	0	0	160	0	0
	B. thetaiota-omicron 5482	0	0	0	0	40	0
	B. ovatus 0038-1	0	0	0	0	0	40

[a]Titers expressed as reciprocal of dilution.

Table 6. Slide Agglutination Using Unabsorbed and Absorbed Antisera

VPI No. (Formalin-treated whole cells)	Antisera: *B. fragilis* Group II 2393	*B. fragilis* Group I 2553	*B. distasonis* 4243	*B. vulgatus* 4245	*B. thetaiota-omicron* 5482	*B. ovatus* 0038-1
B. fragilis Group II 2393	+/+[a]	+/-	-/-	-/-	-/-	-/-
B. fragilis Group I 2553	+/-	+/+	-/-	-/-	-/-	-/-
B. distasonis 4243	-/-	-/-	+/+	-/-	-/-	-/-
B. vulgatus 4245	+/-	+/-	+/-	+/+	+/-	-/-
B. thetaiota-omicron 5482	-/-	-/-	+/-	+/-	+/+	-/-
B. ovatus 0038-1	-/-	-/-	-/-	-/-	-/-	+/+

[a]Unabsorbed antisera/Absorbed antisera; + = agglutination

determine what effect these agents would have on agglutination. The results are summarized in table 7. Heating decreased the size of flocculation and lowered the titers of all strains. B. vulgatus, 4245, showed the greatest drop in titer. Proteolytic enzymes had a less dramatic effect on agglutination causing no change or only a slight drop in titer, while enhancing the size of flocculation. Destruction of the carbohydrate moieties by periodate oxidation decreased both the titers and size of flocculation. In addition, it caused the cell suspensions of B. distasonis 4243 to autoagglutinate. Therefore, both protein and polysaccharide antigens were available to the cell surface and may participate in the agglutination reactions. The spectrum of sensitivities that the organisms displayed toward these agents implied that the relative proportion of proteins and polysaccharides exposed to the cell surface varied from strain to strain.

Antigen extracts. Several kinds of antigen extracts were made from whole cells and cell walls of the six Bacteroides reference strains. These were compared by two dimensional immunodiffusion to determine the best extract(s) for further studies and to characterize the antigens involved in this serological test. Figure 3 illustrates the precipitin patterns observed when the various antigen extracts made from each of the reference strains were compared. In all cases, the complexity and sharpness of the precipitin line patterns were dependent on the methods of extraction, incubation period, and particularly, on the concentration of the reactants. All extracts gave at least one major precipitin line and in most cases several

Table 7. Gross Chemical Characterization of Surface Antigens

	UNTREATED Control		HEAT 1 hr, 100 C		PRONASE 1 mg/ml, 24 hr, 37 C		TRYPSIN 1 mg/ml, 24 hr, 37 C		PERIODIC ACID 0.01 M, 60 min	
Strain	Titer	Max. Degree Floc.	Titer	Max. Degree Floc.	Titer	Degree Floc.	Titer	Degree Floc	Titer	Degree Floc.
2393	1280[a]	+++[b]	640	+	320	+++	1280	+++	40	+
2553	2560	+++	1280	++	1280	+++	1280	+++	40	+
4243	1280	++	160	++	320	++	640	++	R[d]	R
4245	2560	+++	160	+	640	+++	1280	++++	80	+
5482	1280	+++	320	+	640	+++	640	++++	40	+
0038-1	160	++	80	+	160	++	N.D.[c]	N.D.	20	+

[a]Agglutination titer when cells were tested against homologous antiserum; titers expressed as reciprocal of dilution

[b]Maximum degree of flocculation observed

[c]Not determined

[d]Autoagglutinated

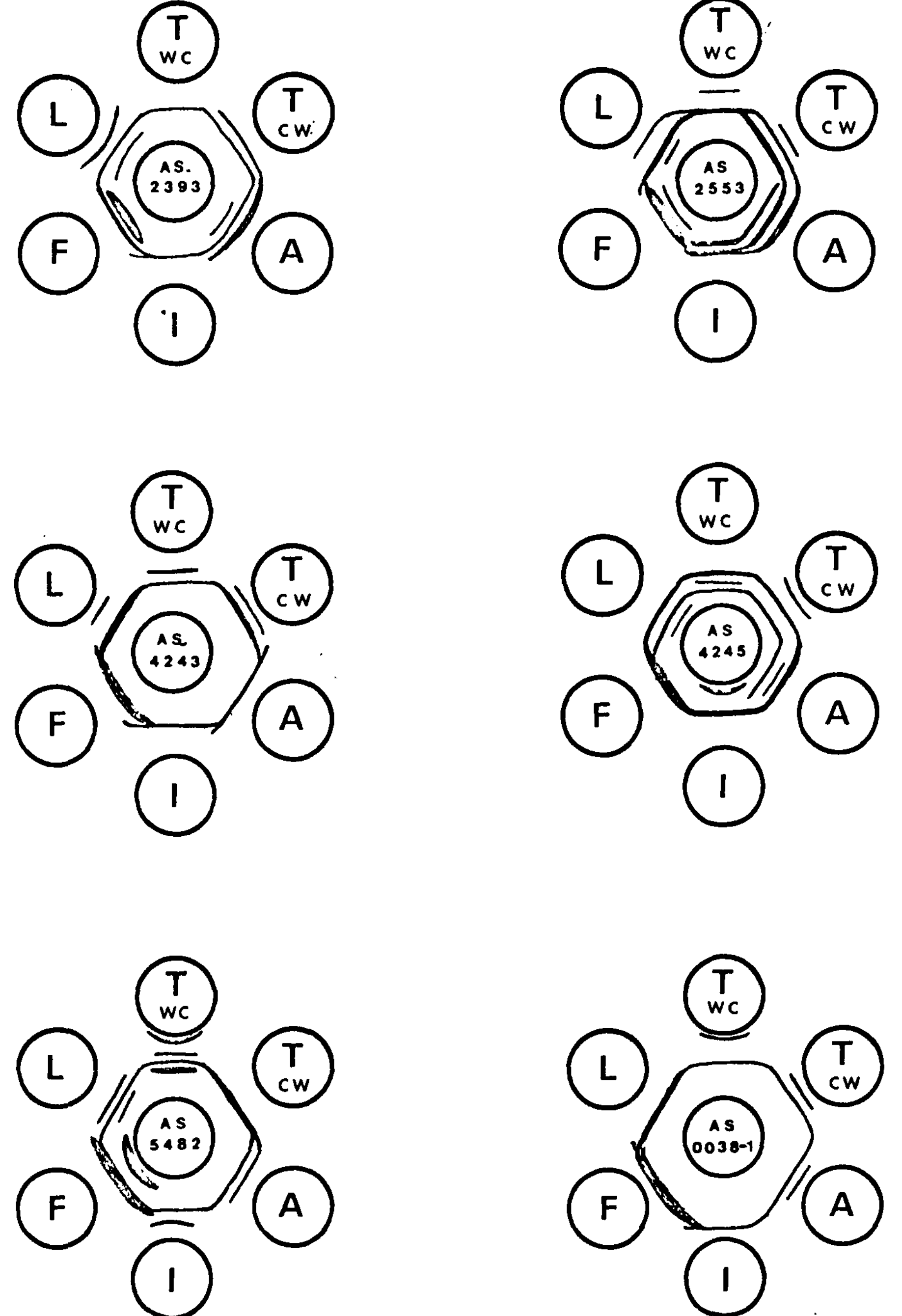

Figure 3. Immunodiffusion in Homologous Systems: Relationships of Antigen Extracts. Abbreviations: Twc, TCA extraction of whole cells; Tcw, TCA extraction of cell walls; A, autoclaved whole cells; I, peak I material from Sephadex G-50; F, formamide extraction of whole cells; L, material isolated from the aqueous phase of 45% phenol extraction of cell walls.

minor lines. The antigens extracted by all methods were apparently very similar but were not always identical as indicated by reactions of partial identity (spurring). The reactions of identity between the TCA extracts of whole cells and of cell walls make it very likely that the extracted antigens represent major cell wall polysaccharides. Similar reactions of identity were observed between Autoclaved extracts of whole cell and cell walls (not shown). Furthermore, LPS material, which was extracted from purified cell walls, gave lines of partial or full identity with the other whole cell and cell wall extracts. Two antigen preparations not shown in the diagrams were prepared by extracting whole cells with dilute HCL and with ultrasonication. The major antigens extracted by these methods also gave reactions of partial or full identity to the precipitin lines of the other extracts. However, a more diffuse and complex precipitin line pattern was observed with the ultrasonicate extract and this therefore seemed a less satisfactory method of preparing extracts.

The extracts obtained by autoclaving whole cells, TCA extraction of either whole cells or cell walls, and 45% phenol extraction of cell walls (LPS) consistently gave strong reactions and were used for the further characterization of the reference strains.

Immunodiffusion studies. Tables 8-11 compare the reference strains in terms of the different extracts. Since the number of lines is dependent on several factors, the tabulated numbers represent the maximum number of lines observed when the extracts were tested under

various conditions, e.g., different concentrations and incubation times. Generally, all of the extracts gave similar precipitin patterns. Homologous reactions were characterized by multiple lines. Not all of the cross reactions observed between reference strains in the agglutination studies were represented with precipitin lines. For example, the wide cross reactivity of B. vulgatus, 4245, observed with tube agglutination, was not present in the immunodiffusion studies. However, the presence of certain lines was often dependent on the extraction method. For example, precipitin lines were observed between AS2393 and 2553 when Autoclaved and 45% phenol (LPS) extracts of 2553 were used but not when TCA extracts and Peak I material (high molecular weight fraction resulting from acetic acid hydrolysis of whole cells) were used. Similar situations existed in the other antigen systems, such as 4245 and 2393. Only one cross reaction was observed in the immunodiffusion studies that was not detected by tube agglutination. That was the reaction between Peak I material of 2553 and AS4243. Except for the cross reactions between the two B. fragilis groups, which sometimes displayed two precipitin lines, heterologous cross reactions were characterized by only single lines.

Hemagglutination studies. Both phenol-extracted LPS and autoclaved extracted material would sensitize sheep red blood cells. The sensitization potential of both types of extracts was determined. As indicated in figures 4 and 5, the maximum titers obtained in the homologous reactions of B. fragilis 2393 and 2553, using red blood cells sensitized with Autoclaved extracts, were 20,480 and 10,240, respectively. The plateauing of the titers suggests that the maximum

Table 8. Immunodiffusion Results Using Autoclaved Whole Cell Extracts

	VPI No.	Unabsorbed Antisera					
		B. fragilis Group II 2393	*B. fragilis* Group I 2553	*B. distasonis* 4243	*B. vulgatus* 4245	*B. thetaiotaomicron* 5482	*B. ovatus* 0038-1
Antigen Extract	*B. fragilis* Group II 2393	5[a]	1	0	0	0	0
	B. fragilis Group I 2553	1	3	0	0	0	0
	B. distasonis 4243	0	0	3	0	0	0
	B. vulgatus 4245	0	0	1	2	0	0
	B. thetaiotaomicron 5482	0	0	0	0	2	0
	B. ovatus 0038-1	0	0	0	0	0	2

[a]Maximum number of precipitin lines observed

Table 9. Immunodiffusion Results Using TCA Whole Cell and Cell Wall Extracts

	VPI No.	Unabsorbed Antisera					
		B. fragilis Group II 2393	*B. fragilis* Group I 2553	*B. distasonis* 4243	*B. vulgatus* 4245	*B. thetaiotaomicron* 5482	*B. ovatus* 0038-1
Antigen Extracts	*B. fragilis* Group II 2393	3[a] (2)[b]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	B. fragilis Group I 2553	0 (0)	3 (3)	0 (0)	0 (0)	0 (0)	0 (0)
	B. distasonis 4243	0 (0)	0 (0)	3 (2)	0 (0)	0 (0)	0 (0)
	B. vulgatus 4245	0 (0)	0 (1)	1 (0)	3 (3)	0 (0)	0 (0)
	B. thetaiotaomicron 5482	0 (0)	0 (0)	0 (0)	0 (0)	4 (2)	0 (0)
	B. ovatus 0038-1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (2)

[a] Maximum number of precipitin lines observed when whole cell TCA extracts were antigens

[b] Cell wall results

Table 10. Immunodiffusion Results Using LPS[a]

VPI No.	Unabsorbed Antisera: *B. fragilis* Group II 2393	*B. fragilis* Group I 2553	*B. distasonis* 4243	*B. vulgatus* 4245	*B. thetaiotaomicron* 5482	*B. ovatus* 0038-1
Antigen Extract						
B. fragilis Group II 2393	4[b]	1	0	0	0	0
B. fragilis Group I 2553	2	3	0	0	0	0
B. distasonis 4243	0	0	3	0	0	0
B. vulgatus 4245	1	0	0	3	0	0
B. thetaiotaomicron 5482	0	0	0	0	3	0
B. ovatus 0038-1	0	0	0	0	0	2

[a]Material isolated from aqueous phase of 45% phenol extraction.

[b]Maximum number of lines observed

Table 11. Immunodiffusion Results Using Peak I Material[a]

	Unabsorbed Antisera					
VPI No.	*B. fragilis* Group II 2393	*B. fragilis* Group I 2553	*B. distasonis* 4243	*B. vulgatus* 4245	*B. thetaiota-omicron* 5482	*B. ovatus* 0038-1
B. fragilis Group II 2393	2[b]	1	0	0	0	0
B. fragilis Group I 2553	0	4	1	0	0	0
B. distasonis 4243	0	0	4	0	0	0
B. vulgatus 4245	0	0	1	2	0	0
B. thetaiota-omicron 5482	0	0	0	0	2	0
B. ovatus 0038-1	0	0	0	0	0	1

[a]High molecular weight material from fractionation of 1% acetic acid extracted material on Sephadex G-50.

[b]Maximum number of precipitin lines observed.

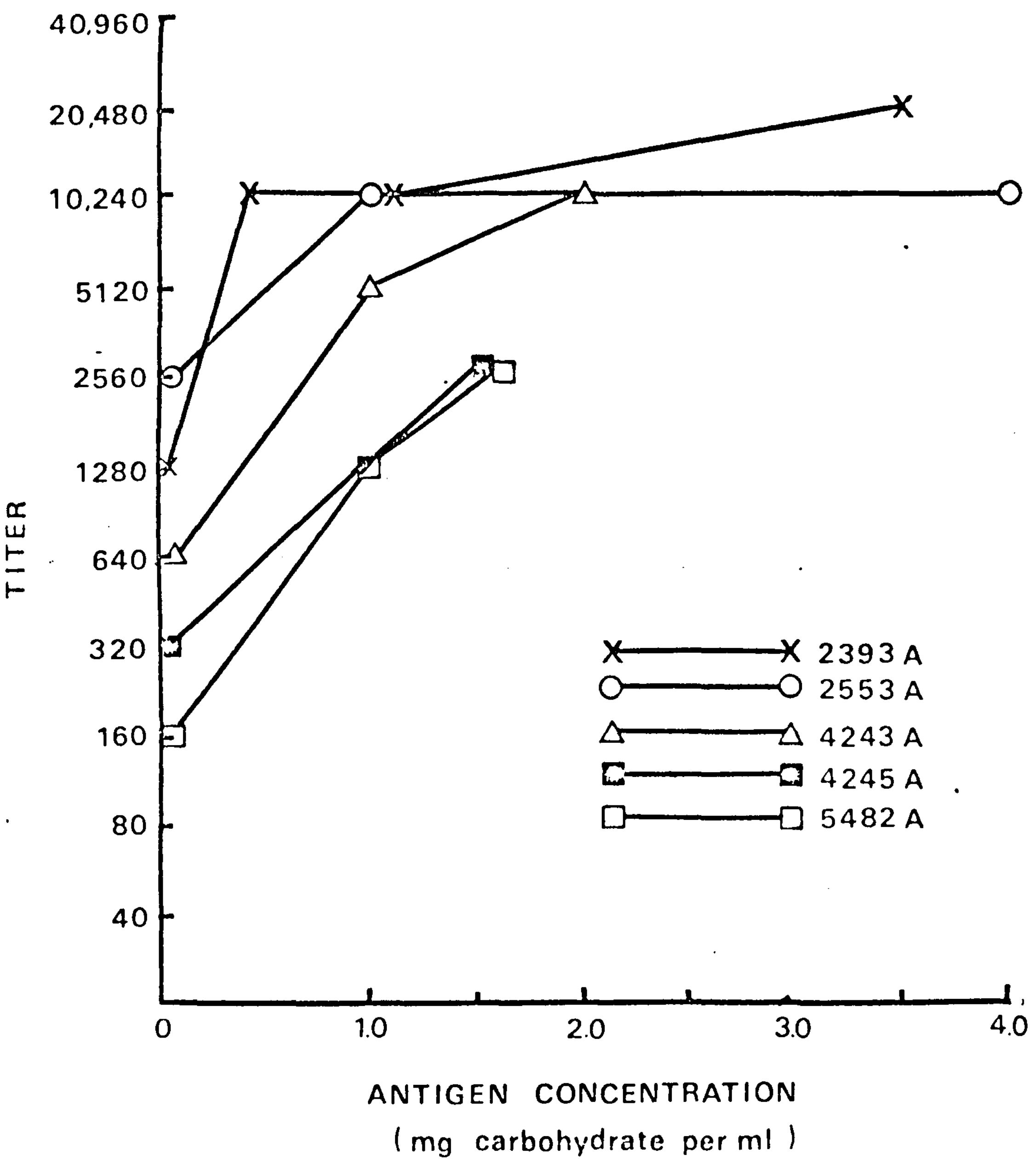

Figure 4. Hemagglutination Sensitization Potential of Crude Autoclave Extracts.

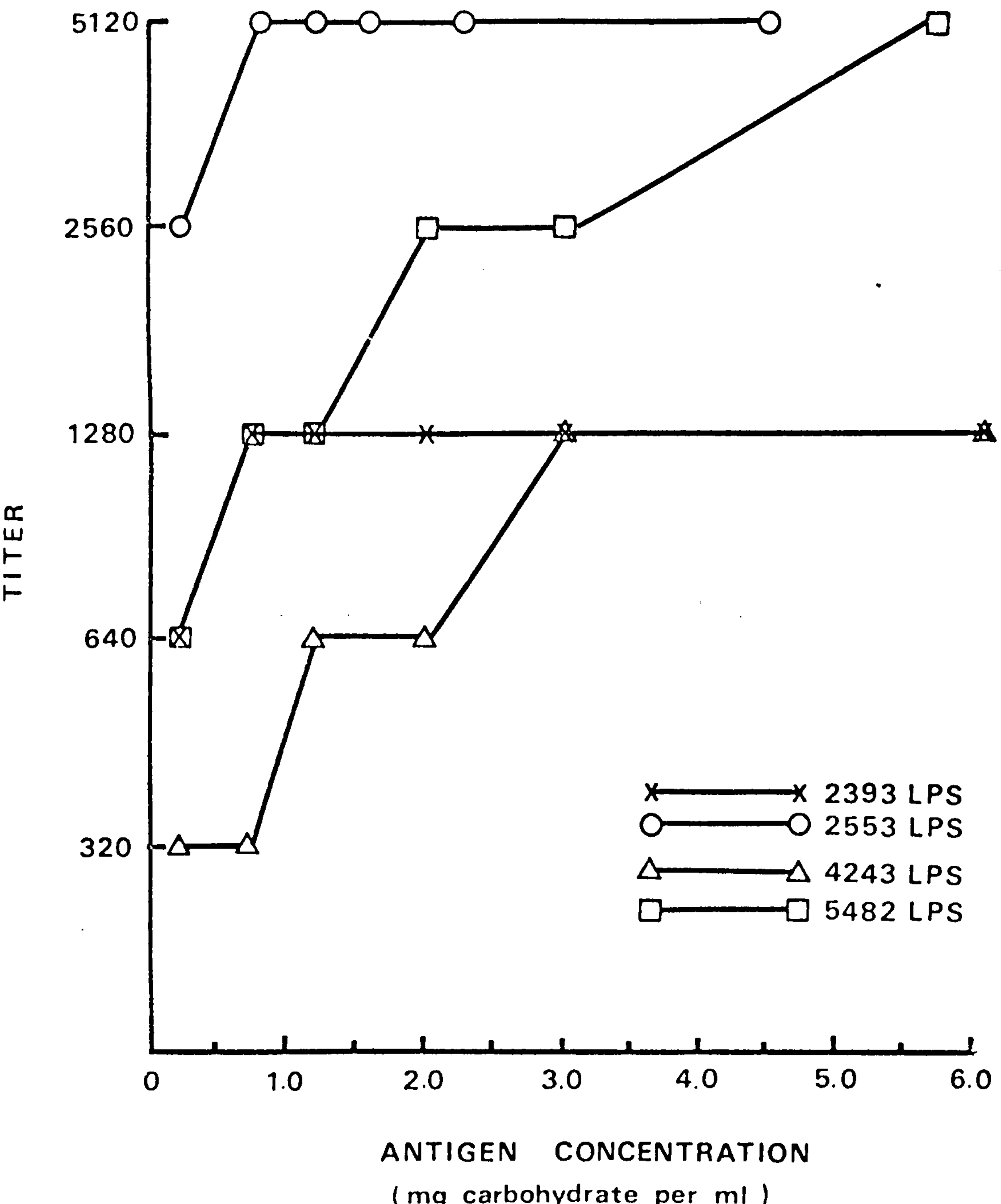

Figure 5. Hemagglutination Sensitization Potential of Aqueous Phenol-Extracted LPS.

sensitization in these systems was obtained at antigen concentrations greater than 1 mg of carbohydrate per ml. The maximum sensitization potential of the extracts from the other Bacteroides species could not be determined since no plateau was observed with the extract concentrations used in this experiment. However, at antigen concentrations comparable to those of the B. fragilis extracts, titers were one to two fold lower. Only one antigen concentration of the 0038-1 extract was tested, 1 mg carbohydrate per ml, and the titer obtained was only 40. The lower sensitization potential of the extracts from strains other than the two B. fragilis organisms may be the result of several factors. These include the inability of the antigens to absorb onto the RBC due to their physical or chemical properties, or differences in the activities of the antisera.

Hemagglutination titers were generally lower when phenol-extracted LPS was used to sensitize the red blood cells. Maximum sensitization was achieved at an antigen concentration of approximately 1 mg carbohydrate per ml for 2393 and 2553, and of 3.5 mg per ml for 4243. No plateauing was observed in the 5482 system and LPS of 4245 and 0038-1 were not tested.

When the hemagglutination assay was used to compare reference strains, good differentiation with high titers was achieved with five of the six strains, see table 12. The cross reactions between the two B. fragilis groups, observed with the other serological tests, were also present in this assay at levels insignificant with respect to the homologous reactions. There was also a low level of cross reactivity observed between the B. fragilis antisera, AS2393 and

Table 12. Hemagglutination Results of *Bacteroides* Species

VPI No.	Antisera					
	B. fragilis Group II 2393	*B. fragilis* Group I 2553	*B. distasonis* 4243	*B. vulgatus* 4245	*B. thetaiota-omicron* 5482	*B. ovatus* 0038-1
Sensitized RBC's: *B. fragilis* Group II 2393	10,240[a]	40	-	-	-	-
B. fragilis Group I 2553	20	10,240	-	-	-	-
B. distasonis 4243	160	320	10,240	160	20	-
B. vulgatus 4245	-	-	-	1280	-	-
B. thetaiota-omicron 5482	-	-	-	-	1280	-
B. ovatus 0038-1	<20	20	-	-	-	40

[a]Titers expressed as reciprocal of dilution.

AS2553, and red blood cells sensitized with 0038-1 extract. The most striking observation was the cross reactions displayed by cells sensitized with B. distasonis. These reactions were absent in the other serological tests. This agglutination may be of a nonspecific nature, since cells sensitized with 4243 extract were agglutinated by two normal sera and six non-Bacteroides antisera, see table 13.

Survey of Bacteroides Strains

Fifteen different unabsorbed Bacteroides antisera were used to survey randomly selected strains belonging to the different homology groups. The tests employed were the agglutination, immunodiffusion, and hemagglutination techniques used to characterize the six reference strains.

Agglutination survey. Both slide and tube methods were initially conducted. However, difficulty was encountered in reading the slide agglutination reactions and consequently, the technique was discontinued. Surveys were generally performed using a two tube dilution scheme. The results are summarized in table 14.

Except with B. fragilis, cross reactions were for the most part limited to within homology groups. In groups other than B. fragilis, intergroup cross reactions were characterized by low titers and were not restricted to any particular group.

Within each homology group, members were tested against at least two antisera (only one antiserum each was used for the 3452A and 0038-1 groups). Except for the B. ovatus group, an entire spectrum of intragroup reactivity, ranging from no reaction to titers comparable to the

Table 13. Hemagglutination Activity of *B. distasonis* Survey Strains

	Antisera										
VPI Strain No.	*B. distasonis* 4243	normal 1	normal 2	Vibrio sp.	*P. avidum* 0589	*P. granulosum* 0507	*P. acnes* 3706	Calf serum	Human serum	*Clostridium beijerinckii* 2983	Treponema sp.
4243	10,240[a]	20	40	80	20	80	–	–	80	20	20
0052	320	20	40	ND[b]	40	20	20	–	20	ND	ND
B1-20	80	20	40	ND	20	20	40	–	40	ND	ND
C18-7	640	40	80	ND	20	40	80	–	160	ND	ND
T3-25	320	20	40	ND	20	20	20	ND	ND	ND	ND

[a]Hemagglutination titers expressed as the reciprocal of dilution

[b]Not determined

Table 14. Tube Agglutination Survey of Bacteroides Homology Group Members[a]

	Antisera														
	B. fragilis Group II			*B. fragilis* Group I			*B. distasonis*			*B. vulgatus*		*B. thetaiota-omicron*		*B. ovatus*	3452A
Strain	2393	2360	4117	2553	6195	6805	6851	4243	B120	4245	C7-2	5482	2808B	0038-1	3452A
B. fragilis Group II															
2393	**1280**[b]	20	-[c]	20	-	-	-	-	-	-	-	-	20	-	20
4076	200	20	20	200	20	20	20	20		20		20		-	
3392	200	20	-	200	-	20	200	-	-	-	-	-	-	-	
4225	200	20	20	200	-	20	-								
2360	—	**2560**	20	-	200	-	-	-	20	-	20	-	-	-	
2647J2	200	200	-	20	20	200	20	20	-	20	-	-	-	-	
4117	20	-	**320**	-	-	-	-	-	-	-	-	-	-	-	
4112	200	-	-	20	-	-	-	-	-	-	-	-	-	-	
2552	20	20	-	20	20	20	-	20	-	20	-	20	-	-	
B. fragilis Group I															
2553	20	20	-	**2560**	-	20	-	-		20		20	20	-	20
E323	20	20	20	200	-	200	20					20	-		
6195	200	20	20	20	**2560**	-	-		-		-		-		
5807	200	20	200	200	20	200	200	-		-		-		-	
3277-1	20	200	20	20	-	200	20	-		-		-	-		
4912	R[d]			R		R		R		R		R		R	
4366	20	20	20	20	200	200	200	-		-		-		-	
6123	200	200	20	200	20	200	200	-		20		20	20	-	
4147	200	20	20	20	-	200	200	-		20		20	-	-	
2556-1	20	20	20	20	20	20	20	-		-		-		-	
6805	20	20	20	-	-	**640**	20	-		20		-		-	
1582	20	-	20	20	20	200	-	-		-		-	-	-	
3390	20	200	-	20	-	200	-	-		-		-	-	-	
6957	-	20	20	-	-	20	20	-		-		-	-	-	
4517	20	-	20	20	-	20	20								
5785-1	200	20	20	20	200	20	20	-		-		-		-	
4082	20	-	20	-	-	-	20	-		-		-		-	
6851	200	20	-	200	20	200	**320**	-		20		20	-	-	
5631	200	-	-	200	-	200	-								
6059	20	20	20	20	20	-	20	-		-		-			
4128	20	20	20	-	-	20	-	-		-		-		-	
6754	200	200	20	20	20	20	20								

Table 14. Continued

	Antisera														
												B. thetaiota-omicron		*B. ovatus*	3452A
	B. fragilis Group II			*B. fragilis* Group I			*B. distasonis*			*B. vulgatus*					
Strain	2393	2360	4117	2553	6195	6805	6851	4243	B120	4245	C7-2	5482	2808B	0038-1	3452A
6057B	20	20	20	20	20	20	20	20	-		-		-		-
4736	200	20	-	200	200	200	200			20		20			
6815	20	-	20	200	20	-	-	-		-		20	-		
B2-22	200	20	-	20	20	200	200	20		20		20		-	
2758A	200	20	-	200	200	200	20	-		-		-		-	
B. distasonis															
4243	-	-	-	-	-	20	-	1280	-	20	20	-	-	-	20
0052	-	-	-	20	-	-	-	200	-	-	20	-	-	-	
B1-20	-	-	-	-	-	-	-	-	1280	20	-	-	-	-	
T3-25	-	-	-	-	-	-	-	20	-	-	20	-	-	-	
C18-7	-	-	-	-	-	-	-	20	-	-	-	-	-	-	
B. vulgatus															
4245	20			20		20		20		2560	200	20		20	20
C7-2	-	-	-	-	-	-	-	-	-	20	640	-	-	-	
6370	20	-	-	-	-	-	-	-	-	-	-	-	-	-	
R4-24B	20	-	-	-	-	-	-	-	-	20	200	20	20	-	
0959-1	-			-						200	20				
5710	-	-	-	-	-	-	-	-	-	20	200	20	20	-	
4506-1	20	-	-	-	-	20	-	200	-	20	-	200	20	-	
2365	-			-				-		-	-	-			
C10-6	-			-				-		200					
2277	R			R				R		R		R		R	
0C-13	20	-	-	-	-	20	-	-		-	-	-			
C6-7	-	-	20	20	-	-	-	-	200	20	-	20	20	-	
6598B	20	-	-	-	-	20	-	20	20	-	-	20	-	-	
B. thetaiotaomicron															
5482	-	-	-	20	-	20	-	20	-	20	-	1280	20	-	20
C9-11	-	-	-	-	20	-	-	-	-	-	-	200	200	-	
8651	20	-	-	20	-	-	20	-	-	-	-	20	20	-	
C11-15	20	-	-	-	-	-	-	-	-	-	-	20	20	-	
C22-15	-	20	-	-	20	-	-	20	-	-	-	20	20	-	
0489	-	-	-	-	-	20	-	-	-	-	-	20	-	-	
2808B	20	-	-	20	-	-	-	-	-	-	-	-	1280	200	-

Table 14. Continued.

Strain	*B. fragilis* Group II			*B. fragilis* Group I			*B. distasonis*			*B. vulgatus*		*B. thetaiotaomicron*		*B. ovatus*	3452A
	2393	2360	4117	2553	6195	6805	6851	4243	B120	4245	C7-2	5482	2808B	0038-1	3452A
6212	-	-	-	-	-	-	20	-	-	20	-	-	-	-	-
0911A1	-	-	-	-	-	-	-	-	20	-	-	-	20	-	
0940-1	-	-	-	20	-	-	20	-	-	-	-	200	200	-	-
6180A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
B1-46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
7330-1	20	-	-	-	-	20	20	-	-	-	20	-	-	-	-
2297	-	-	-												
C11-16	20	-	-	-	-	20	-	-	-	-	-	200	200	200	-
B. ovatus															
0038-1	-	-	-	-	-	-	-	-	-	-	-	-	-	**160**	-
T4-7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C12-31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2822	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3524	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Survey was conducted using unabsorbed antisera and a two dilution (1/20 and 1/200) antisera scheme. Homologous reactions (bold numerals) were titered out completely.

[b]Titers expressed as reciprocal of dilution.

[c]Indicates no agglutination

[d]Indicates autoagglutinating cell suspensions.

homologous reaction, was observed, see tables 19 and 20. This reactivity did not correlate with DNA homology values, as strains showing low homology to a given reference strain were agglutinated often with titers higher than strains displaying high homology values, e.g., B. distasonis 0052 and T3-25, B. thetaiotaomicron 2808B and C9-11, B. fragilis 3390 and 2554.

Within and between the two B. fragilis groups, various degrees of reactivity were observed. In the two-tube survey most strains were agglutinated by AS2393, AS2553, and AS6805. Only one B. fragilis strain, 4912, was autoagglutinable.

Members of these two homology groups were tested in a little more detail against AS2393 and AS2553, see table 19. All but one 2393 group (B. fragilis group II) member was agglutinated by AS2393. Members belonging to group II and which were agglutinated by AS2553 always displayed titers with AS2393 equal to or greater than those with the former antisera. Seven 2553 group (B. fragilis group I) strains were not agglutinated by AS2553. Group I strains reacting with both antisera generally displayed comparable titers with both antisera or slightly higher titers with AS2553. However, two strains, 5785-1 and 6123, gave significantly higher titers with AS2393 as compared to AS2553.

With respect to surface antigens, the other homology groups were relatively homogeneous, as most strains belonging to a particular homology group were agglutinated by at least one of their respective antisera. However, the B. ovatus group appeared to be more

heterogeneous than the others, since five members of this group that were tested failed to be agglutinated by AS0038-1. Furthermore, these strains were not agglutinated by any of the 14 other heterologous antisera.

Comparison of the antisera used in each homology group study suggested that certain antisera, such as AS2393, AS6805, AS4243, and AS2808B, were more reactive, i.e., agglutinating more strains with higher titers, than the other antisera.

Immunodiffusion survey. The survey was continued with immunodiffusion techniques using Autoclaved whole cell extracts and the fifteen antisera. The tests were conducted using an arbitrary antigen extract concentration of 1 mg carbohydrate per ml. The results are shown in tables 15, 19, and 20. In general, the antigenic relationships between and within homology groups, as visualized by immunodiffusion, were in agreement with the agglutination results. Most reactions were confined to within homology groups although the high level of antigenic similarities between the two *B. fragilis* groups, observed by agglutination, was also manifested in the immunodiffusion tests. Within the homology groups, strains agglutinating with a particular antiserum usually gave precipitin lines when tested against that same antiserum, see tables 19 and 20. High titers were generally correlated with multiple precipitin lines, although this was not always the case. For example, strains 5710 and R4-24B of the *B. vulgatus* group, gave multiple precipitin lines when their respective agglutination titers were only 80 and 20, see table 20. Similar

Table 15. Immunodiffusion Survey of Bacteroides Homology Group Members[a]

	Antisera													
	B. fragilis Group II			*B. fragilis* Group I			*B. distasonis*			*B. vulgatus*		*B. thetaiotaomicron*		*B. ovatus*
Strain	2393	2360	4117	2553	6195	6805	6851	4243	B120	4245	C7-2	5482	2808B	0038-1
B. fragilis Group II														
2393	3[b]	1^{w}	0	1	0	0	1^{w}	0		0		0	0	0
4076	1	0	0	0	0	0	0	0		0		0	0	0
3392	3	1^{w}	0	1^{w}	0	0	1	0		0		0	0	0
4225	3	1^{w}	0	1	0	0	0	0		0		0	0	0
2360	1	2	0	0	2	0	0	0		0		0	0	0
2647J2	0	1	0	1^{w}	1	0	1	0		0		0	1^{w}	0
4117	0	0	2	0	0	0	0	0		0		0	0	0
4112	2	1	0	0	0	1	0	0		0		0	1^{w}	0
2552	3	1	0	1^{w}	0	0	0	0		0		0	0	0
B. fragilis Group I														
2553	1	0	0	3	0	1	1^{w}	0		0		0		0
6195	0	2	0	0	3	1	0	0		0		0		0
5807	3	0	0	1	1^{w}	1	1	0		0		0		0
3277-1	1	0	0	2	0	1^{w}	1^{w}	0		0		0		0
4912	2	1	0	1	1	1	0	0		0		0		0
4366	1	1	1^{w}	1	2	3	1	0		0		0		0
6123	1	0	0	0	1	1	1	0		0		0	1^{w}	0
4147	1^{w}	0	1^{w}	1^{w}	0	2	1	0		0		0		0
2556-1	1	2	0	0	1	1^{w}	0	0		0		0		0
6805	2	0	0	1^{w}	0	3	0	0		0		0	0	0
1582	2	0	0	1^{w}	0	2	0	0		0		0		0
3390	0	0	0	0	0	1	0	0		0		0		0
6957	1	0	0	0	0	1	1	0		0		0		0
4517	3	0	0	0	0	1	0					0		0
5785-1	0	0	0	0	1^{w}	2	0	0		0		0	0	0
4082	0	0	0	0	0	0	1^{w}							0
6851	1	0	0	1	0	0	2	0		0		0	0	0
5631	2	0	0	0	0	0	0			0		0		0
0439	1	0	0	1^{w}	0	1	0	0		0		0		0
6754	0	0	0	0	1^{w}	2	1^{w}							0
6057B	0	0	0	0	0	2	1^{w}	0		0		0		0

Table 15. Continued.

Strain	*B. fragilis* Group II 2393	2360	4117	*B. fragilis* Group I 2553	6195	6805	6851	*B. distasonis* 4243	B120	*B. vulgatus* 4245	C7-2	*B. thetaiota-omicron* 5482	2808B	*B. ovatus* 0038-1
4736	0	0	0	0	0	0	0							0
6815	1	1	0	0	0	0	0	0		0		0		0
B2-22	1	0	0	1^{w}	0	3	1	0	0	0		0		0
2758A	1	0	0	2	0	0	1^{w}							
3625	1	0	0	2	0	0	1^{w}							
B. distasonis														
4243	-			-			2	1	0		0		0	
0052	1^{w}			1^{w}				1	0	0		0		0
B1-20	0			0				1	2	0		0		0
T3-25	0			0				0	0	0		0		0
C18-7	0			0				1	1	0		0		0
B. vulgatus														
4245	0			0				1^{w}		2	1	0	0	0
C7-2	0			0				0		1	2	0	0	0
6370	1^{w}			0				0		1^{w}	–	0	0	0
R4-24B	0			0				0		2	2	0	0	0
0959-1	0			0				0		2	1	0	0	0
5710	0			0				0		2	1^{w}	0	0	0
4506-1	0			0				0		1^{w}	1	0	1^{w}	0
2365	0			0				0		1^{w}	1^{w}	0	0	0
C10-6	0			0				0		1^{w}	1^{w}	0	0	0
2277	0			0				0		0	1	0	0	0
OC-13	1^{w}			0				0		0	0	0	0	0
C6-7	0			0				0		0	0	0	0	0
6598B	0			0				0		0	1^{w}	0	0	0
B. thetaiotaomicron														
5482	0			0				0		0	0	3	2	0
C9-11	0			0				0		0	0	1	2	0
8651	0			0				0		0	0	1	2	0
C11-15	0			0				0		0	0	2	3	0
C22-15	0			0				0		0	0	1^{w}	1	0
0489	0			0				0		0	0	1^{w}	1	0

Table 15. Continued.

Strain	*B. fragilis* Group II			*B. fragilis* Group I			*B. distasonis*			*B. vulgatus*		*B. thetaiota-omicron*		*B. ovatus*
	2393	2360	4117	2553	6195	6805	6851	4243	B120	4245	C7-2	5482	2808B	0038-1
2808B	0			0				0		0	1[w]	1	2	0
6212	0			0				0		0	0	0	1	0
0911A1	0			0				0		0	0	0	1	0
0940-1	0			0				0		0	0	1	2	0
6180A	0			0				0		0	0	0	1	0
B1-46	0			0				0		0	0	0	1	0
7330-1	0			0				0		0	0	0	1[w]	0
2297	0			0				0		0	0	0	1[w]	0
C11-16	0			0				0		0	0	1	2	0
B. ovatus														
0038-1	0			0				0		0		0		2
T4-7	0			0				0		0		1[w]		0
C12-31	0			0				0		0		0		0
2822	0			0				0		0		0		0

[a]Survey was conducted using unabsorbed antisera and autoclaved whole cell extract, 1 mg carbohydrate/ml.

[b]Number of precipitin lines observed after 72 hours.

[c]Indicates weak reaction.

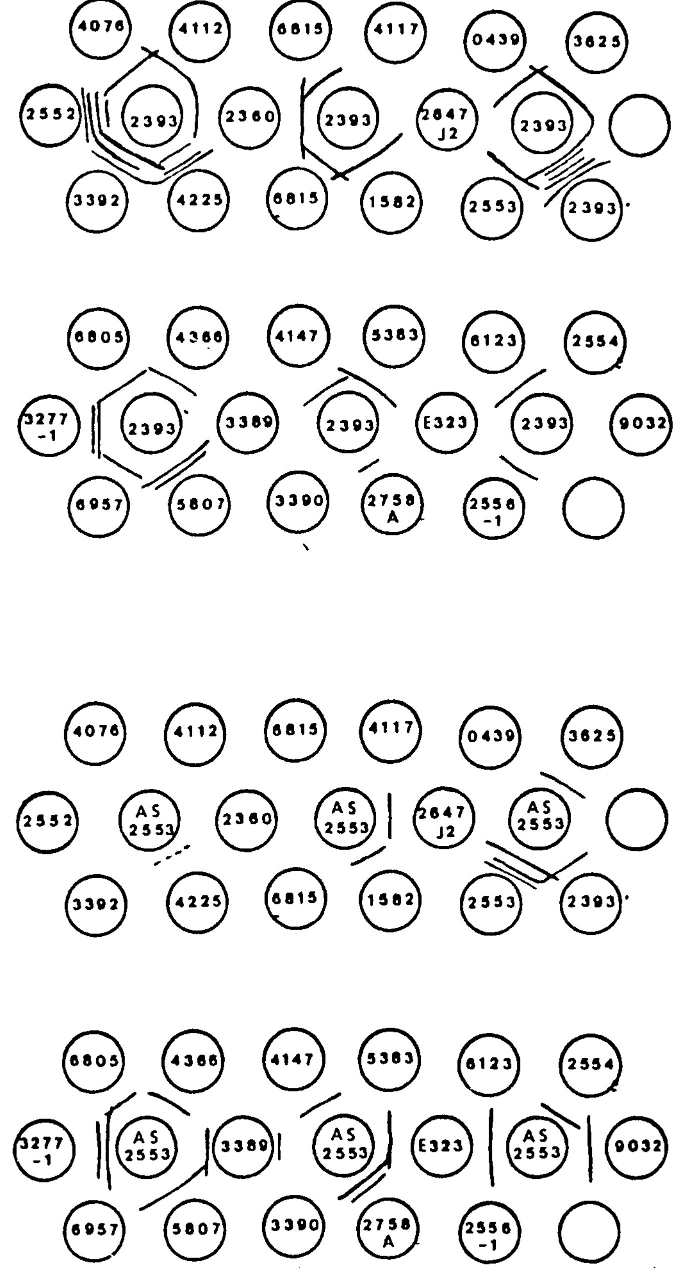

Figure 6. Immunodiffusion Survey of *B. fragilis*

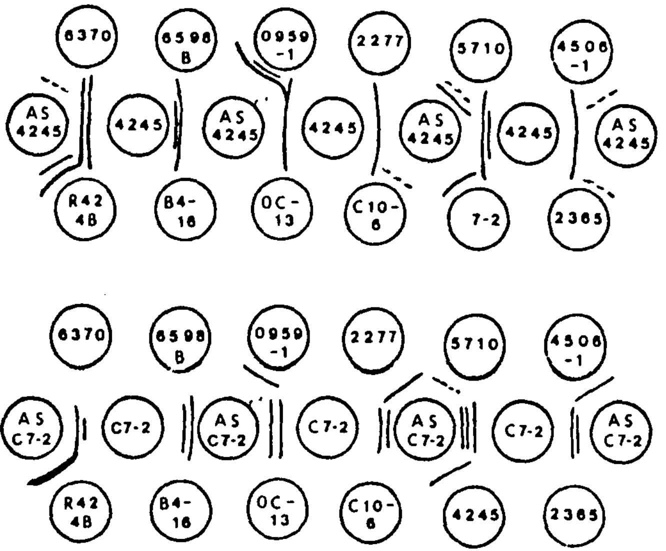

Fig. 7a. B. vulgatus Survey Strains

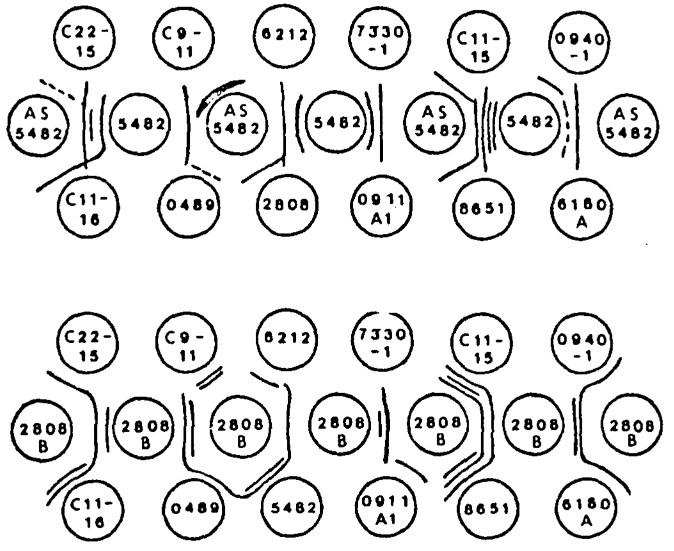

Fig. 7b. B. thetaiotaomicron Survey Strains

Figure 7. Immunodiffusion Survey of B. vulgatus and B. thetaiotaomicron

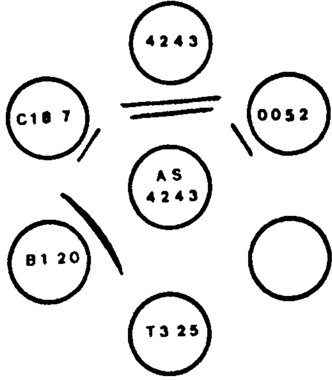

Fig. 8a. Survey of B. distasonis

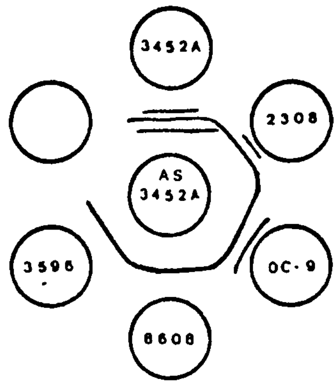

Fig. 8b. Survey of '3452A' Group

Figure 8. Immunodiffusion Survey of B. distasonis and the '3452-A' Group.

reactions were observed with B. thetaiotaomicron strains 5482, 8651, and C11-15, and with B. fragilis strains 5361, 2552, 6059, 3277-1, 6805, 1582, and 4517, see tables 19 and 20. Conversely, B. fragilis strains 4076, 2556-1, 4736, 5631, 6754, and 5785-1, were agglutinated with a given antiserum to high titers, yet failed to give any precipitin lines against the same antiserum. As noted in the agglutination studies, serological reactivity within species did not correlate with intragroup homologies, i.e. there were no apparent groupings correlating with homology values.

Some but not all of the intergroup reactions observed in the agglutination studies were detected by immunodiffusion. These reactions, other than those of the B. fragilis groups, were characterized by single weak precipitin lines, often appearing only after 48 hours. It is important to note that when immunodiffusion tests were conducted with antigen extract concentration less than 1 mg carbohydratee, per ml, many of the precipitin lines could not be detected.

The reactions of members belonging to the two B. fragilis groups, given in tables 15 and 19, suggest a complex interrelationship of antigenic components. Seven B. fragilis antisera were used to survey the strains. Each antisera showed different degrees of reactivity. Of the seven, AS2393 and AS6805 seemed to be the most reactive. However, AS6805 reacted with only one group II strain, whereas AS2393 reacted with numerous extracts of members from both groups. Of all the group I and II strains tested, all reacted with at least one of the antisera and many, such as 4912, reacted with three or more. These reactions

were characterized by both single and multiple precipitin lines. Comparison of the precipitin line patterns obtained from the reactions between a single extract and several antisera, indicated that in some cases different antigens were being detected. Figure 9 provides several examples. However, in other cases, the antigens were identical. Similar observations were made from preliminary absorption studies. If AS2553 was absorbed with 2393 extract and then used in conjunction with the unabsorbed antiserum, certain precipitin lines would disappear while others would not be affected, see figure 10.

In a similar manner, comparison of extracts from several strains which reacted with one given antiserum indicated that there was a multiplicity of antigens present on some strains but absent on others. Figure 11 illustrates this type of antigenic relationship observed in several antiserum systems. Three precipitin lines are present in the homologous reaction of 2553. All of the other extracts gave one or more precipitin lines against AS2553. However, the combinations are different for each strain. A similar relationship can be observed in the other systems.

Multiple precipitin lines in heterologous crosses were more often observed with AS2393 than with AS2553. In addition, precipitin lines not detected in the homologous reaction were occasionally observed in heterologous reactions. A minimum of ten different precipitin lines, denoted antigens "a" through "k", have been identified using AS2393 and six lines, denoted 1 through 6, using AS2553. As indicated in

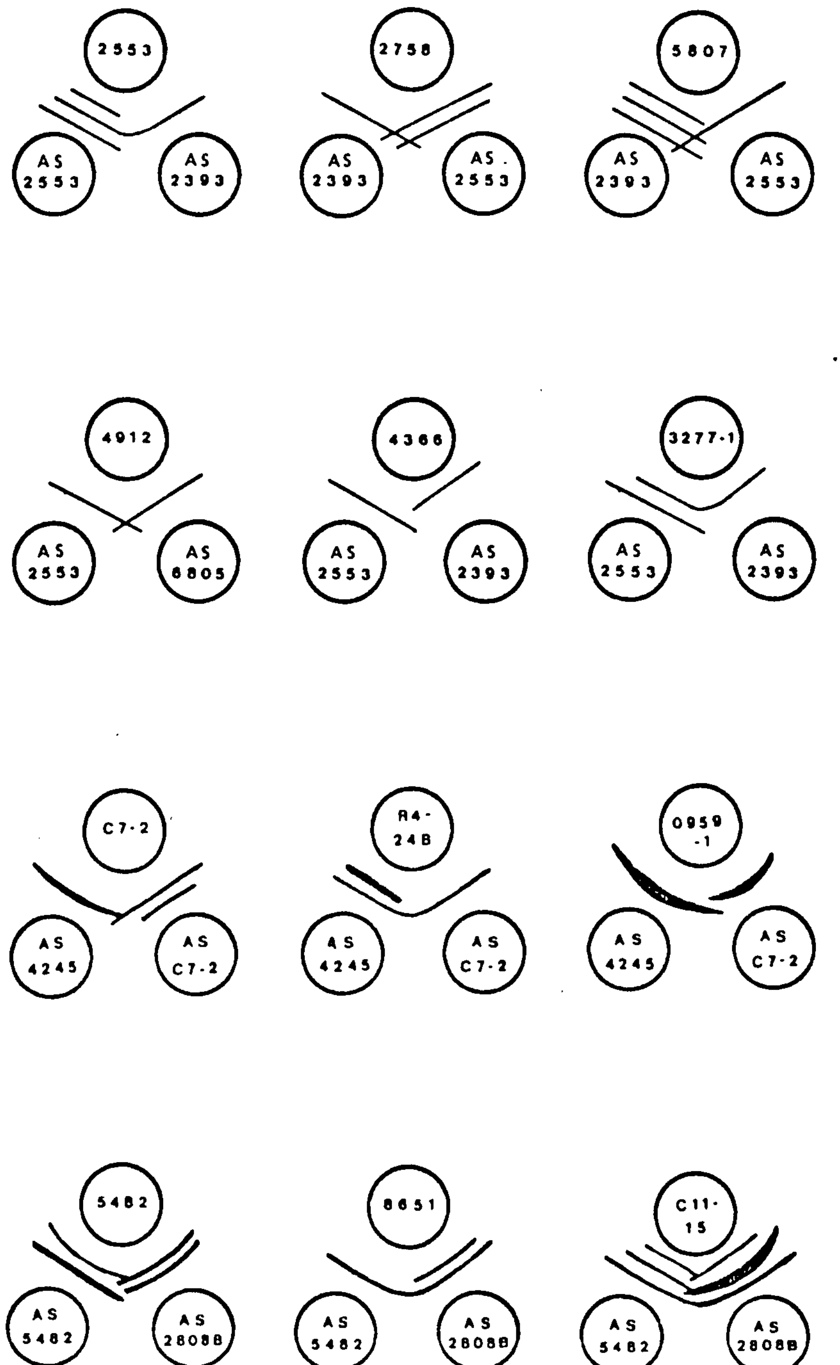

Figure 9. Immunodiffusion in Heterologous Systems: Comparison of Precipitin Line Patterns of Various Antisera Reacting With One Survey Strain.

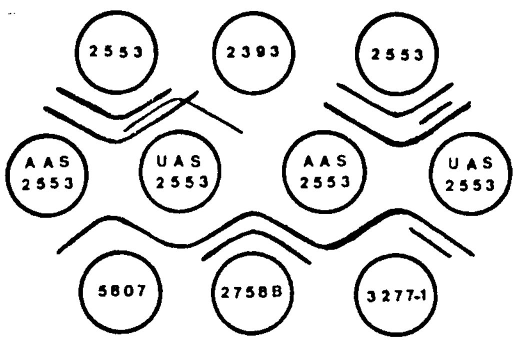

Figure 10. Immunodiffusion in Heterologous Systems: Comparison of Precipitin Line Patterns of Absorbed and Unabsorbed Antisera. Abbreviations: UAS, unabsorbed AS2553; AAS2553, 2553 antiserum absorbed with 2393 Autoclaved extract.

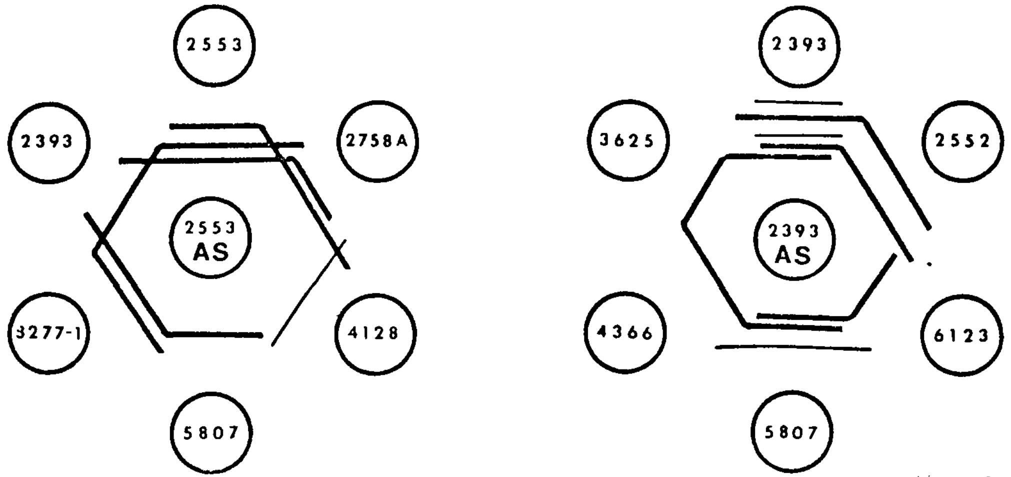

Fig. 11a. B. *fragilis* Survey Strains

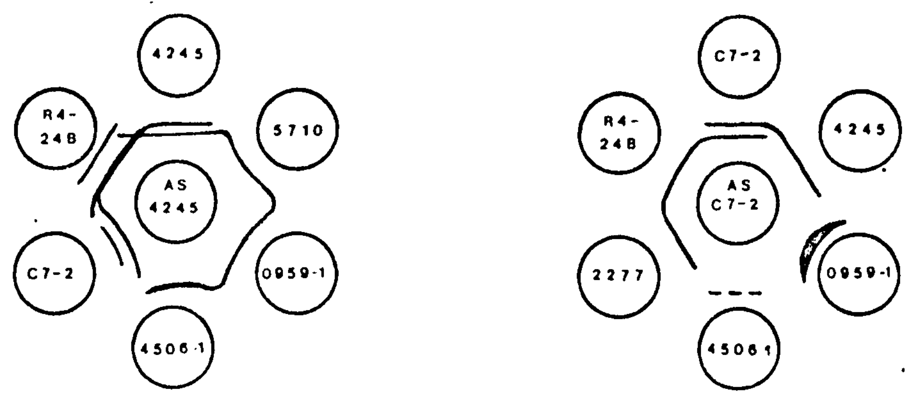

Fig. 11b. B. *vulgatus* Survey Strains

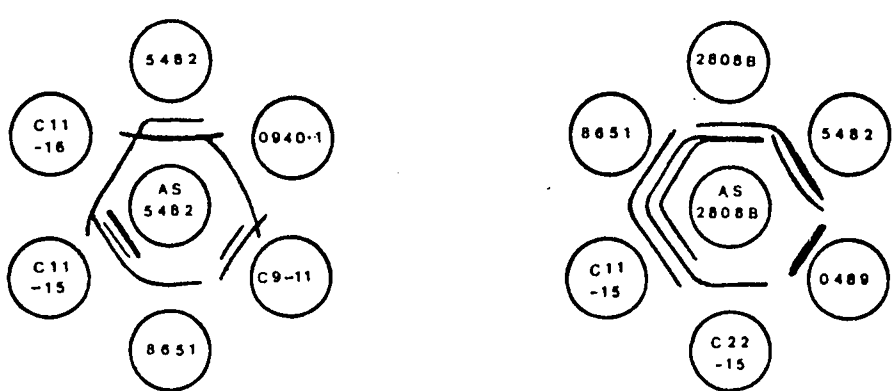

Fig. 11c. B. *thetaiotaomicron* Survey Strains

Figure 11. Immunodiffusion in Heterologous Systems: Comparison of Precipitin Line Patterns of Various Survey Strains Reacting with One Antiserum.

tables 16 and 17, some degree of segregation was observed among the strains into groups defined by the precipitin lines.

A quantitative relationship of antigenic make-up between the *B. fragilis* strains was also suggested by these results. Since antigen extracts were standardized at a fixed carbohydrate concentration, differences in number of lines and strength of the precipitin line patterns reflected, in part, the concentration (or number) of antigens capable of reacting with the specific antibodies. For example, the antigen extract of 3277-1, when tested against AS2393 and AS2553, produced a strong precipitin reaction, whereas, against AS6805 and AS6851, only weak reactions were visualized, and against AS2360, AS4117, and AS6195, no reactions were observed.

A mosaic type of antigenic makeup was also observed in the other homology groups. Figures 11b, 11c, and 8b illustrate the same kind of antigen distribution as observed in the *B. fragilis* groups. Certain antisera, such as AS2808B, AS3452A, and AS4245 reacted more widely than others. As observed in the *B. fragilis* groups, comparison of precipitin line patterns obtained from reactions between a single extract and two antisera indicated that in some cases different antigens were being detected, see figure 9.

Hemagglutination survey. Surveying several strains from each of the homology groups disclosed a similar antigenic relationship to that observed in the other tests, see table 18. Most cross reactions, excluding those between the two *B. fragilis* groups were at low levels, i.e., ≤ 20. Of the five groups examined, members of the *B. distasonis*

Table 16. Antigenic Factors of B. fragilis 2393* and Their Distribution Among B. fragilis Survey Strains

Antigenic Factors										
a	b	c	d	e	f	g	h	i	j	k
2393	2393	2393	2393	2393	5383	6815	2360	4225	4076	4147
	2553	2758A				0439	4112	3392		
		3625				6805		2552		
		3392				4366		6123		
								5807		
								1582		

* Antigens a-k are necessarily possessed by 2393 since immunization with 2393 whole cells has elicited antibodies to these antigens; however, under conditions employed, only antigens a-e were detected in extracts of 2393.

Table 17. Antigenic Factors of *B. fragilis* 2553* and Their Distribution among *B. fragilis* Survey Strains

Antigenic Factors					
1	2	3	4	5	6
2553	2553	2553	1582	2554	4128
2758A	2393	2758A	4225		0439
	3625	E323			3625
	3277-1	3277-1			4147
		4912			
		5807			

*Antigens 1-6 are necessarily possessed by 2553 since immunization with 2553 whole cells has elicited antibodies to these antigens; however, under conditions employed, only antigens 1-3 were detected in extracts of 2553.

Table 18. Hemagglutination Survey Results[a]

VPI Strain No.	Antisera *B. fragilis* Group II 2393	*B. fragilis* Group I 2553	*B. distasonis* 4243	*B. vulgatus* 4245	*B. thetaiota-omicron* 5482	*B. ovatus* 0038-1
B. fragilis Group II						
2393	**10,240**[b]	40	–	–	–	–
4076	**5120**	20	–	–	–	–
3392	**5120**	320	–	–	–	–
2552	**1280**	20	20	–	–	–
B. fragilis Group I						
2553	20	**10,240**	–	–	–	–
9032	20	**5120**	–	–	20	–
3390	320	**20**	–	–	–	–
5383	2560	**160**	–	–	–	–
5807	640	**10,240**	–	–	–	20
B. distasonis						
4243	200	200	**10,240**	200	20	20
B1-20	20	320	**80**	20	–	–
0052	20	20	**320**	20	20	–
C18-7	20	20	**640**	20	20	20
T3-25	20	20	**320**	160	–	20
B. vulgatus						
4245	–	–	–	**2560**	–	–
0959-1	20	20	–	**2560**	–	20
OC-13	20	20	–	–	–	–
5710	20	20	–	**640**	–	–
2365	20	–	–	**40**	–	–
C10-6	20	20	–	**40**	–	–
R4-24B	20	20	–	**2560**	–	–
B. thetaiotaomicron						
5482	–	–	–	–	**2560**	–
0940-1	–	–	–	–	**20**	–
2808B	–	–	–	–	–	–
C9-11	20	–	–	–	**160**	–
0911A1	20	–	–	–	**40**	–
8651	20	–	–	–	**640**	–
7330-1	20	–	–	–	**40**	–

[a]Survey was conducted using a two dilution (1/20 and 1/200) scheme for interspecies reactions. Within species reactions were carried out with two-fold dilutions, 1/20 to 1/20, 480.

[b]Hemagglutination titers expressed as reciprocal of dilution.

group appeared to be the most cross reactive, however, only 4243, B1-20, and T3-25 were agglutinated to high titers with the non-*B. distasonis* antisera and as indicated in table 13, these strains were also agglutinated with normal sera and several non-*Bacteroides* antisera. The two *B. fragilis* antisera, AS2393 and AS2553, were reactive against most of the strains tested and as observed in the other serological tests, AS2393 was the more reactive of the two.

The intragroup hemagglutination results are shown in tables 19 and 20. In general, the hemagglutination results were in good agreement with both the agglutination and immunodiffusion results of the groups. Within the *B. fragilis* groups, the titers ranged up to 20,480, with AS2393 appearing to be the more reactive of the two antisera. Generally, strains such as 2758A, 5807, and 4366, whose extracts gave multiple precipitin lines or a strong single line in immunodiffusion, usually showed moderate to high hemagglutination titers. However, with a few strains, such as 5631, 1582, and 6805, this was not the case. Alternatively, several extracts from strains, such as 3390 and E323, gave no lines in immunodiffusion but displayed significant hemagglutination activity, however, this activity never exceeded a hemagglutination titer greater than 320. On the basis of their hemagglutination titers, the *B. fragilis* survey strains could arbitrarily be assigned to one of five groups, as shown in table 19. Three strains out of forty-three, or 7%, gave high titers against both AS2393 (Group II) and AS2553 (Group I). All were group I strains. Thirteen strains, or 30%, gave high titers only with

Table 19. Serological Reactions of *B. fragilis* Groups 1 (2553) and 2 (2393)[a]

Strain	% Homology Value to		T. A. Titer[b]		I.D. No. Lines[d]		H.A. Titer[d]	
	2393	2553	AS2393	AS2553	AS2393	AS2553	AS2393	AS2553
4361	73	91	20	1280	2	2	5,120	5,120
4366	68	94	40	20	1	1	640	640
4912	71	94	R[e]	R	2	1	10,240	5,120
2393	100	68	1280	40	5	1	10,240	40
4076	90	72	640	320	1	0	5,120	20
2360	90	68	–	–	1	0	2,560	–
2552	89	68	20	40	3	1[w]	1,280	20
4225	94	65	320	160	3	1	10,240	320
3392	93	72	2560	320	3	1[w]	5,120	320
6059	71	88	80	80	3	0	10,240	20
3625	70	80	320	320	2	1	2,560	20
4128	73	87	80	–	1	1	2,560	20
6815	76	90	20	160	1	0	5,120	40
6957	71	91	–	–	1	0	1,280	–
3390	76	90	80	20	0	0	320	20
4517	72	86	20	40	3	0	160	–
2553	69	100	320	1280	1	3	20	10,240
2758A	68	87	1280	1280	1	2	640	10,240
5807	74	92	1280	1280	3	1	640	10,240
3277-1	74	89	20	20	1	2	20	5,120
E323	77	92	40	640	0	2	160	20,480
2554	68	85	160	320	0	1	20	1,280
9032			ND	ND	0	1	20	5,120
4112	92	68	160	40	2	0	320	80
5383	67	91	40	–	1	0	2,560	160
6123	76	87	1280	160	1	0	640	160
2556-1	69	89	20	320	1	0	80	160
6805	74	89	20	–	2	1[w]	40	80
4736	72	89	640	320	0	0	20	80
2647J2	85	68	160	20	0	1[w]	40	160
6851	71	90	320	640	1	1	20	20
B222	70	87	320	80	1	1[w]	20	20
0439	69	88	20	–	2	2[w]	20	20
1582	69	89	20	20	2	1[w]	20	20
4147	76	90	320	80	1[w]	1[w]	20	–
5631	69	89	640	320	2	0	20	20
3389	70	88	–	–	0	1[w]	20	–
6195	72	88	–	40	0	0	40	–
5785-1	73	87	320	40	0	0	20	–
4082	74	91	20	–	0	0	20	–
6057B	72	87	20	20	0	0	20	20
6754	74	92	320	80	0	0	20	20
4117	80	64	20	–	0	0	–	20

[a]Strains are arbitrarily grouped on the basis of their hemagglutination titers

[b]Tube agglutination titer against designated antisera

[c]Number of precipitin lines observed after 48 hours in immunodiffusion studies with designated antisera.

[d]Hemagglutination titer against designated antisera

[e]Autoagglutinating cell suspension.

Table 20. Serological Reactions of *B. distasonis*, *B. vulgatus*, *B. thetaiotaomicron*, and '3452A' Homology Groups.

Strain	% Homology Value To 4243	T.A. Titer[a] AS4243	T.A. Titer[a] ASB1-20	I.D. No. Lines[a] AS4243	I.D. No. Lines[a] ASB1-20	H.A. Titer[c] AS4243	H.A. Titer[c] ASB1-20
4243	100	1280	–	2	1	10,240	640
0052	78	160	–	1	0	320	640
B1-20	81	–	1280	1	2	80	5120
C18-7	80	80	20	1	1	640	1280
T3-25	88	40	–	0	0	320	160
Strain	4245	4245	C7-2	4245	C7-2	4245	C7-2
4245	100	2560	320	2	1	2560	320
0959-1	84	320	80	2	1	2560	640
OC-13	85	–	20	0	0	–	–
5710	87	80	160	2	1^w	640	80
C7-2	86	20	640	1	2	320	1280
4506-1	86	–	–	1^w	1^w	40	–
2365	86	20	–	1^w	1^w	40	–
C10-6	84	–	–	1^w	1^w	40	20
R4-24B		20	320	2	2	2560	2560
Strain	5482	5482	2808	5482	2808	5482	2808
5482	100	1280	80	3	2	2560	320
0940-1	91	160	640	1	2	20	40
2808B	93	20	1280	1	2	–	320
0489	82	20	–	1^w	1	–	20
7330-1	86	–	20	0	1	40	320
C9-11	86	320	640	1	2	160	320
C11-16	88	320	320	1	2	320	320
8651	91	80	80	1	2	640	2560
C11-15	92	40	20	2	2	20	160
C22-15	86	20	20	1^w	1	40	160
0911A1	74	–	20	0	1	40	20
Strain	3452A	AS3452A		AS3452A		AS3452A	
3452A	100	1280		3		2560	
2308	90	–		2		20	
OC-9	91	20		2		320	
8608	89	80		1		20	
3596	99	640		2		2560	

[a]Tube agglutination titer against designated antisera

[b]Number of precipitin lines observed after 48 hours in immunodiffusion studies with designated antisera.

[c]Hemagglutination titer against designated antisera.

AS2393 and of these thirteen, seven belonged to group I. Seven strains reacted in the reciprocal manner, i.e. high titers with only AS2553, and all belonged to group I. Another group of seven strains, of which six belonged to group I, had intermediate hemagglutination reactivity and the last group, comprising of thirteen strains, displayed low hemagglutination activity to both antisera. However, of these thirteen strains, seven gave precipitin lines to one or both of the *B. fragilis* antisera.

Within the other homology groups, multiple precipitin lines generally correlated with high hemagglutination titers, see table 20. Exceptions to this were *B. vulgatus* R4-24B, *B. thetaiotaomicron* 0940-1, and a 3452A group member, 2308. However, hemagglutination titers were generally lower than those obtained in the studies on the *B. fragilis* groups. All of the *B. distasonis* group members displayed hemagglutination titers to both of their group antisera, however, extracts of two of its members, 0052 and T3-25, failed to give precipitin lines in the immunodiffusion studies when tested against the same antisera. A similar situation was observed with *B. thetaiotaomicron* 7330-1 and 0911A1. However, in these two cases, titers were only 40. On the other hand, extracts of *B. vulgatus* 4506-1, 2365, and *B. thetaiotaomicron* 2808B and 0489 gave precipitin lines against their respective antisera but did not display hemagglutination activity.

Gel-filtration Studies of Products of Mild Acetic Acid Hydrolysis

Profiles. Studies with *E. coli* (106), *Proteus* (25), and *Pseudomonas* (15) have shown that polysaccharides obtained by mild acetic

acid hydrolysis of either whole cells or isolated LPS can be fractionated into core and O-specific side chain regions by molecular sieve chromatography. Similar fractionation studies were performed on the six reference Bacteroides strains.

A comparison between elution profiles obtained from hydrolysates of LPS from E. coli and B. fragilis 2553 on Sephadex G-50 is shown in figure 12. Equal amounts of material were applied to the column in each case and the profiles represent total carbohydrate, as determined by the phenol-sulfuric acid method. Peak I of the E. coli profile was largely eluted in the void volume and has been demonstrated by other investigators to be O-specific side chain material (106). Peaks II and III, which represented about 40% of the material, contain core region material. Elution profiles similar to that of E. coli were obtained with Proteus vulgaris and P. mirabilis. Peak I of the B. fragilis profile accounted for roughly 75% of the material fractionated and was sharper than the comparable peak for E. coli. There was very little material corresponding to E. coli peak II although B. fragilis had larger amounts of lower molecular weight, peak III, material.

The profiles were also monitored for KDO and heptose using the formaldehyde assay of Vaskovsky and Isay (148). For both the E. coli and the two Proteus samples, formaldehyde was released by periodate from material in peaks II and III after periodate oxidation, whereas in the B. fragilis profile, liberation of formaldehyde could be detected only with material eluting around fraction number 60, which

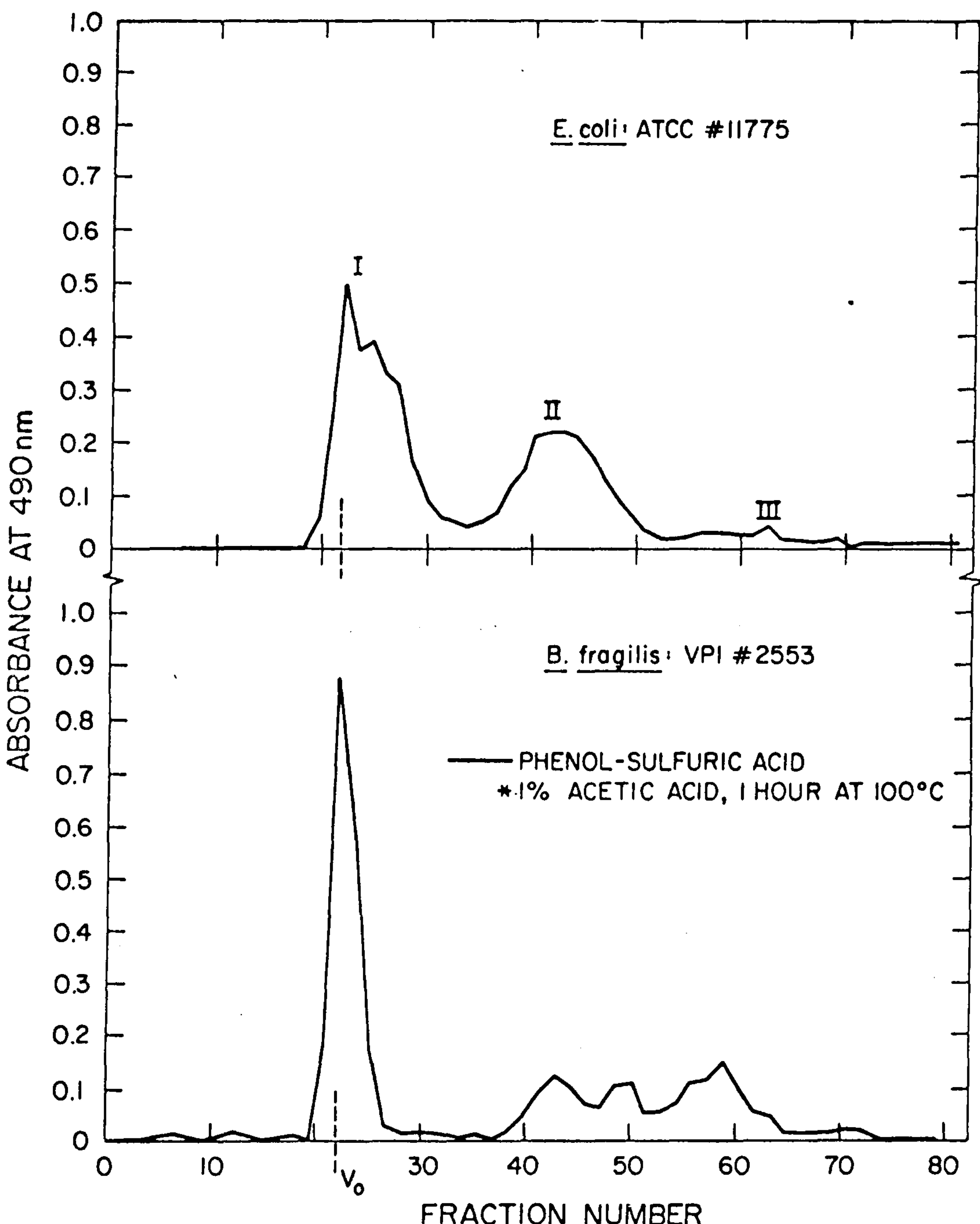

Figure 12. Fractionation of Acetic Acid Extracts from Escherichia coli and Bacteroides fragilis in Sephadex G-50.

was slightly behind peak III. This elution position corresponded to the point at which ribose eluted.

Whole cells of *E. coli* and *B. fragilis* 2553 strains were subjected to hydrolysis by 1% acetic acid for various times ranging from 30 to 150 minutes and the resultant polysaccharides were chromatographed on the G-50 column. Very little difference in profiles of both organisms was observed over the experimental conditions tested. The only change was a slight decrease in peak I with a corresponding increase in lower molecular weight material as hydrolysis time lengthened. However, the serological activities of the *B. fragilis* peaks I, II, and III were not altered over the range of hydrolysis times when monitored by immunodiffusion.

Figure 13 compares the profiles of the six *Bacteroides* strains when equal amounts of material were fractionated on the column. All had a major peak appearing in the void volume and up to three smaller peaks showing varying degrees of retardation. Strains 4245, 5482 and 0038-1 had a peak eluting between fractions 30 and 40 which was absent in the other three strains. Marked differences were observed in the peak I content. Profiles of 4243, and 5482 indicated smaller amounts of high molecular weight peak I material, while 0038-1 appeared to have slightly more. When the various fractions were analyzed for formaldehyde release, the results were consistent with those observed with *B. fragilis* 2553, that is, formaldehyde was demonstrated only with material in fractions around 60, slightly behind peak III.

Peak I material from all of the strains was collected and rechromatographed on Sephadex G-75. Material from *E. coli* and the two

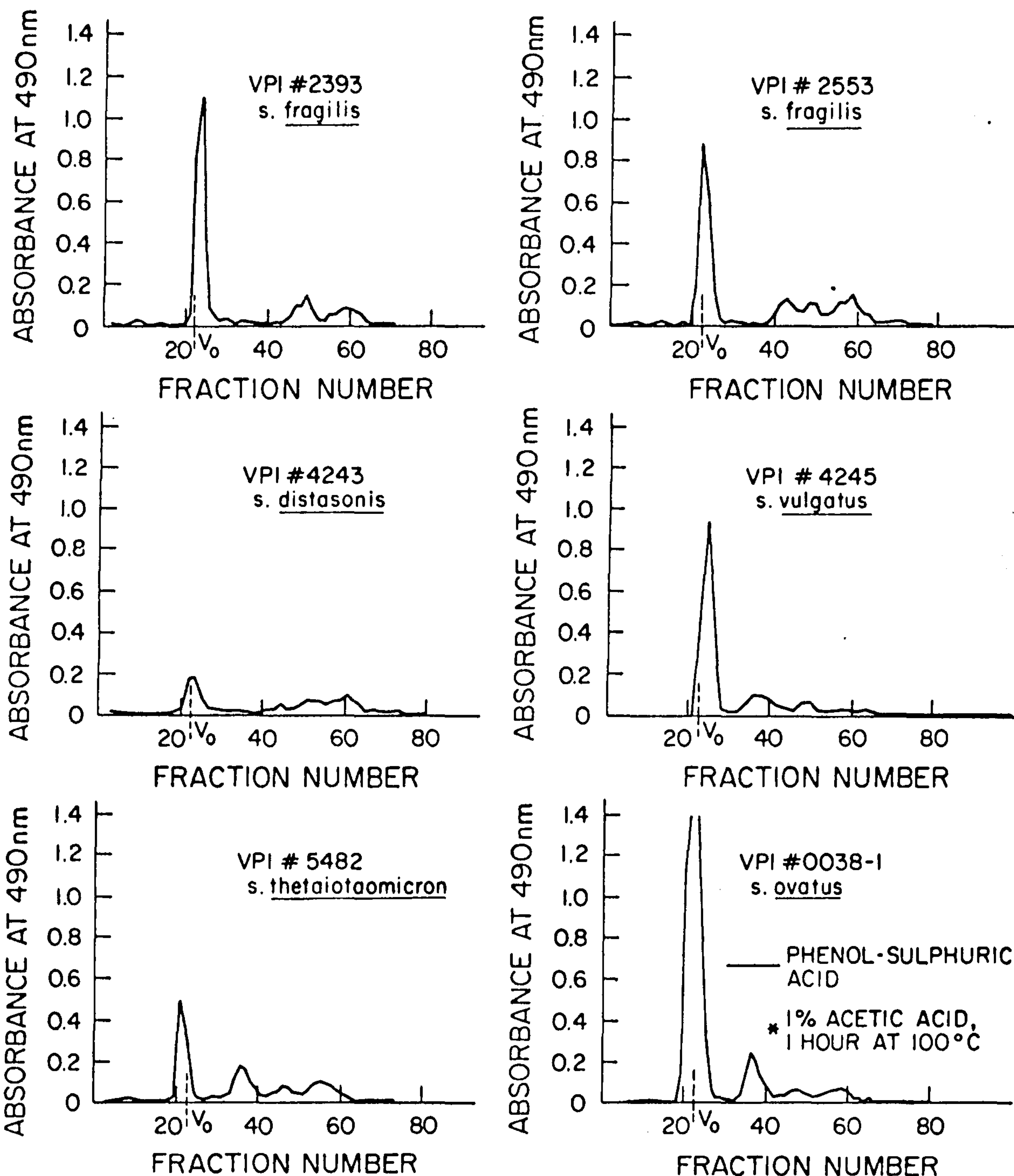

Figure 13. Fractionation Profiles of Acetic Acid Extracts from Bacteroides fragilis and Related Species.

Proteus strains split into two peaks; material from all the Bacte roides strains still eluted in the void volume as sharp peaks.

Serological activity. The Bacteroides profiles were monitored serologically by immunodiffusion. Serological activity was largely associated with peak I. Although precipitin lines were formed using fractions taken from peaks II and III, these lines were always the same as those present in peak I fractions although generally weaker. Immunodiffusion comparisons of peak I with the other antigen extracts from the same organism indicated a high level of similarity, see figure 3. Moreover, the precipitin line pattern obtained when comparing the antigenic interrelationship of peak I material between reference strains was very similar to those obtained using the other antigen extracts, see tables 8-11. Multiple precipitin lines were observed in all of the homologous reactions, except for B. ovatus. Of the three cross reactions observed, only the reaction between AS4243 and 2553 peak I had not been observed before using any of the other extracts.

Chemical Composition Studies

Sugar composition of phenol-extracted LPS. Bacteroides and E. coli LPS material was analyzed by descending paper chromatography and gas-liquid chromatography. Both methods yielded similar findings. Their results are summarized in tables 21-27. The major neutral and amino sugars in the LPS preparations were the two hexosamines, galactosamine and glucosamine, the hexoses, galactose, glucose, and mannose, and the two 6-deoxyhexoses, fucose and rhamnose. Although

Table 21. Comparison of Sugar Constituents in Various Antigen Extracts of _Bacteroides fragilis_ 2393

	Amino Sugars		Neutral Sugars				
ANTIGEN EXTRACT	Galactos-amine	Glucos-amine	Galactose	Glucose	Mannose	Fucose	Rhamnose
LPS[a]	tr(12)[b]	+(88)	+(6.15)[c]	+++(79.3)	+(3.13)	+(4.72)	++(6.7)
Autoclave[d]	-	±	±	+	±	+	±
TCA[e]	tr	±	+	++	±	±	±
Peak I[f]	tr	+	+	+	++	++	+++

[a]Material isolated from aqueous phase of 45% phenol extraction

[b]% composition of amino sugars in LPS as determined by amino acid analyzer programmed for hexosamine determination

[c]% neutral sugar composition in LPS as determined by GLC

[d]Autoclaved whole cell extract

[e]TCA extract of whole cells

[f]Peak I material from fractionation of 1% acetic acid extracted material on Sephadex G-50

Table 22. Comparison of Sugar Constituents in Various Antigen Extracts of *Bacteroides fragilis* 2553

ANTIGEN EXTRACT	Amino Sugars		Neutral Sugars				
	Galactosamine	Glucosamine	Galactose	Glucose	Mannose	Fucose	Rhamnose
LPS[a]	-(0)[b]	++(100)	++(24.0)[c]	+++(66.6)	-(2.76)	+(3.65)	tr(2.98)
Autoclave[d]	-	±	±	+++	-	tr	-
TCA[e]	-	+	++	+	-	±	-
Peak I[f]	+	+++	+++	+	-	+++	+

[a]Material isolated from aqueous phase of 45% phenol extraction

[b]% composition of amino sugars in LPS as determined by amino acid analyzer programmed for hexosamine determination

[c]% neutral sugar composition in LPS as determined by GLC

[d]Autoclaved whole cell extract

[e]TCA extract of whole cells

[f]Peak I material from fractionation of 1% acetic acid extracted material on Sephadex G-50

Table 23. Comparison of Sugar Constituents in Various Antigen Extracts of <u>Bacteroides distasonis</u> 4243

ANTIGEN EXTRACT	Amino Sugars		Neutral Sugars				
	Galactosamine	Glucosamine	Galactose	Glucose	Mannose	Fucose	Rhamnose
LPS[a]	+(62)[b]	tr(38)	tr(1.69)[c]	++++(95.1)	tr(0.75)	tr(0.73)	tr(1.74)
Autoclave[d]	tr	tr	-	+++	tr	-	-
TCA[e]	tr	tr	-	++	-	-	-
Peak I[f]	++	+	+	++	tr	tr	+

[a]Material isolated from aqueous phase of 45% phenol extraction

[b]% composition of amino sugars in LPS as determined by amino acid analyzer programmed for hexosamine determination

[c]% neutral sugar composition in LPS as determined by GLC

[d]Autoclaved whole cell extract

[e]TCA extract of whole cells

[f]Peak I material from fractionation of 1% acetic acid extracted material on Sephadex G-50

Table 24. Comparison of Sugar Constituents in Various Antigen Extracts of Bacteroides vulgatus 4245

ANTIGEN EXTRACT	Amino Sugars		Neutral Sugars				
	Galactosamine	Glucosamine	Galactose	Glucose	Mannose	Fucose	Rhamnose
LPS[a]	-(0)[b]	+(100)	+(10.6)[c]	+++(53.5)	++(10.4)	+(6.56)	+++(18.96)
Autoclave[d]	±	±	+	+	tr	+	-
TCA[e]	++	++	+++	+++	+	+++	-
Peak I[f]	++	++	+++	+++	tr	+++	+

[a]Material isolated from aqueous phase of 45% phenol extraction

[b]% composition of amino sugars in LPS as determined by amino acid analyzer programmed for hexosamine determination

[c]% neutral sugar composition in LPS as determined by GLC

[d]Autoclaved whole cell extract

[e]TCA extract of whole cells

[f]Peak I material from fractionation of 1% acetic acid extracted material on Saphadex G-50

Table 25. Comparison of Sugar Constituents in Various Antigen Extracts of *Bacteroides thetaiotaomicron* 5482

ANTIGEN EXTRACT	Amino Sugars: Galactosamine	Amino Sugars: Glucosamine	Neutral Sugars: Galactose	Neutral Sugars: Glucose	Neutral Sugars: Mannose	Neutral Sugars: Fucose	Neutral Sugars: Rhamnose
LPS[a]	tr(14)[b]	++(86)	tr(3.13)[c]	+++(93.5)	tr(2.73)	-(0.32)	-(0.33)
Autoclave[d]	-	±	-	+	-	-	-
TCA[e]	-	+	tr	++	±	-	-
Peak I[f]	+	+++	+	+	tr	-	+

[a]Material isolated from aqueous phase of 45% phenol extraction

[b]% composition of amino sugars in LPS as determined by amino acid analyzer programmed for hexosamine determination

[c]% neutral sugar composition in LPS as determined by GLC

[d]Autoclaved whole cell extract

[e]TCA extract of whole cells

[f]Peak I material from fractionation of 1% acetic acid extracted material on Saphadex G-50

Table 26. Comparison of Sugar Constituents in Various Antigen Extracts of *Bacteroides ovatus* 0038-1

	Amino Sugars		Neutral Sugars				
ANTIGEN EXTRACT	Galactos-amine	Glucos-amine	Galactose	Glucose	Mannose	Fucose	Rhamnose
LPS[a]	tr(54)[b]	tr(46)	+(12.31)[c]	+++(39)	++(29.8)	++(13.69)	tr(5.16)
Autoclave[d]	-	-	+	+	+	±	-
TCA[e]	-	tr	±	±	±	±	-
Peak I[f]	-	+++	-	+++	+++	++++	tr

[a]Material isolated from aqueous phase of 45% phenol extraction

[b]% composition of amino sugars in LPS as determined by amino acid analyzer programmed for hexosamine determination

[c]% neutral sugar composition in LPS as determined by GLC

[d]Autoclaved whole cell extract

[e]TCA extract of whole cells

[f]Peak I material from fractionation of 1% acetic acid extracted material on Saphadex G-50

Table 27. Comparison of Neutral and Amino Sugar Components in LPS[a] from E. coli and Bacteroides Species

VPI No.	Hexosamines		Hexoses			6-Deoxyhexose	
	$GalNH_2$	$GlcNH_2$	Gal	Glc	Man	Fuc	Rha
B. fragilis, Gp. II 2393	12	88[b]	6.15[c]	69.3	3.13	4.72	6.7
B. fragilis, Gp. I 2553	0	100	24.01	66.6	2.76	3.64	2.98
B. distasonis 4243	62	38	1.69	95.1	0.75	0.73	1.74
B. vulgatus 4245	0	100	10.61	53.5	10.39	6.56	18.96
B. thetaiotaomicron 5482	14	86	3.13	93.5	2.73	0.33	0.32
B. ovatus 0038-1	54	46	12.31	39.0	29.8	13.69	5.16
E. coli, ATCC 11775	0	100	4.17	92.95	2.69	0.19[d]	

[a]Material isolated from the aqueous phase of 45% phenol extraction of cell walls

[b]% Composition of Neutral Sugars

[c]% Composition of Amino Sugars

[d]% Composition of Colitose

lacking the 6-deoxyhexoses, the E. coli LPS had the 3,6-dideoxyhexose, colitose. In addition to these components, ribose was detected in varying amounts in all of the preparations and was attributed to RNA contamination. An unidentified component that ran similarly to colitose was also detected in the B. thetaiotaomicron preparation.

Glucose was the predominant sugar in all of the preparations representing at least 50% of the hexose and deoxysugar content. In 4243, 5482, and E. coli, it accounted for greater than 90%. Consequently, the remaining components, particularly the deoxysugars, fucose and rhamnose, and the dideoxysugar, colitose, accounted for less than 3% of the sugars. Of the hexosamines, glucosamine was the predominant amino sugar, although four of the six Bacteroides strains had galactosamine as an additional component in their LPS.

Distinct differences in chemotype were evident between strains. Among the Bacteroides strains, 4243 and 5482 had much lower levels of fucose and rhamnose, the latter sugar being not quite as low in 4243. As a further distinction between the two strains, galactosamine was the predominant amino sugar in 4243 whereas in 5482, glucosamine predominated. The two B. fragilis strains, 2393 and 2553, could be differentiated by galactose, mannose, and galactosamine content, while 4245 and 0038-1 had much higher levels of rhamnose and fucose, respectively, than any of the other strains. Furthermore, these strains were also distinct from the others by their high mannose content and could be distinguished from each other by their hexosamine content, i.e., 0038-1 possessed galactosamine which was lacking in 4245.

Sugar composition of antigen extracts. Four serologically active antigen extracts were analyzed by paper chromatography to determine the variation in sugar patterns resulting from differences in extraction method. Three of the four extracts, the Autoclaved, TCA, and Peak I, were whole cell extracts although all, including the phenol-extracted LPS had similar serological activity and chemical components, see tables 21-26. Variation was for the most part quantitative rather than qualitative. Components identified in LPS as trace amounts were generally not detected in the Autoclaved or TCA extracts, although in the case of 4245, rhamnose was present in large amounts in the LPS material but was not detected in either of the Autoclaved and TCA whole cell extracts. In addition, these latter two extracts generally contained less glucose as compared to the LPS material.

The neutral and amino sugar pattern of peak I material was also very similar to that observed in the LPS preparations, including the unidentified fast running component of 5482. Partial hydrolysis, 15 minutes at 100 C, on both LPS and peak I material indicated that this unidentified component and the 6-deoxysugars of the other strains were liberated before the other sugars. Peak I generally contained more hexosamine and deoxysugars than the LPS material. In particular, there was a definite enrichment of rhamnose in both the 4243 and 5482 material. Galactosamine, a component not observed in the LPS preparations of 2553 and 4245 was detected in significant amounts in peak I material. Comparison of the *E. coli* LPS with its peak I material disclosed a lack of galactose in the peak I material, while

the colitose content was enriched. This was taken as further evidence that the peak I material represents side chain material since galactose is known to be found only in the core region while colitose is an important side chain component.

Analysis for KDO and heptose. As mentioned earlier, the profiles obtained in the gel filtration studies were monitored for heptose and KDO. The presence of these compounds was suggested by the formaldehyde release in peak II or III regions of both the facultative gram negative bacteria and all of the *Bacteroides*. More substantial evidence for their presence was sought using paper chromatography, electrophoresis, and colorimetric tests.

Pooled material from peaks II and III was chromatographed in solvent systems A, B, and C and the chromatograms were stained with alkaline silver, and the thiobarbituric acid reagents. KDO could be detected in the *E. coli* (Peak II and III) material only as a very weak spot migrating with a similar mobility to authentic KDO. Heptose could not be identified in Peaks II and III of any strain. Electrophoretic separation of KDO from the other components in peaks II and III of *E. coli* could be achieved in a buffer system H, using a potential of 3000 volts for 1 to 2 hours, see figure 14. As was observed using paper chromatography, the presence of KDO was not detected in the *Proteus* or *Bacteroides* strains.

As a final means of detecting the two sugars, the thiobarbituric acid assays of Warren (154), and Waravdekar and Saslaw (156) were used to test for KDO; the cysteine-sulfuric acid assay of Osborn (113)

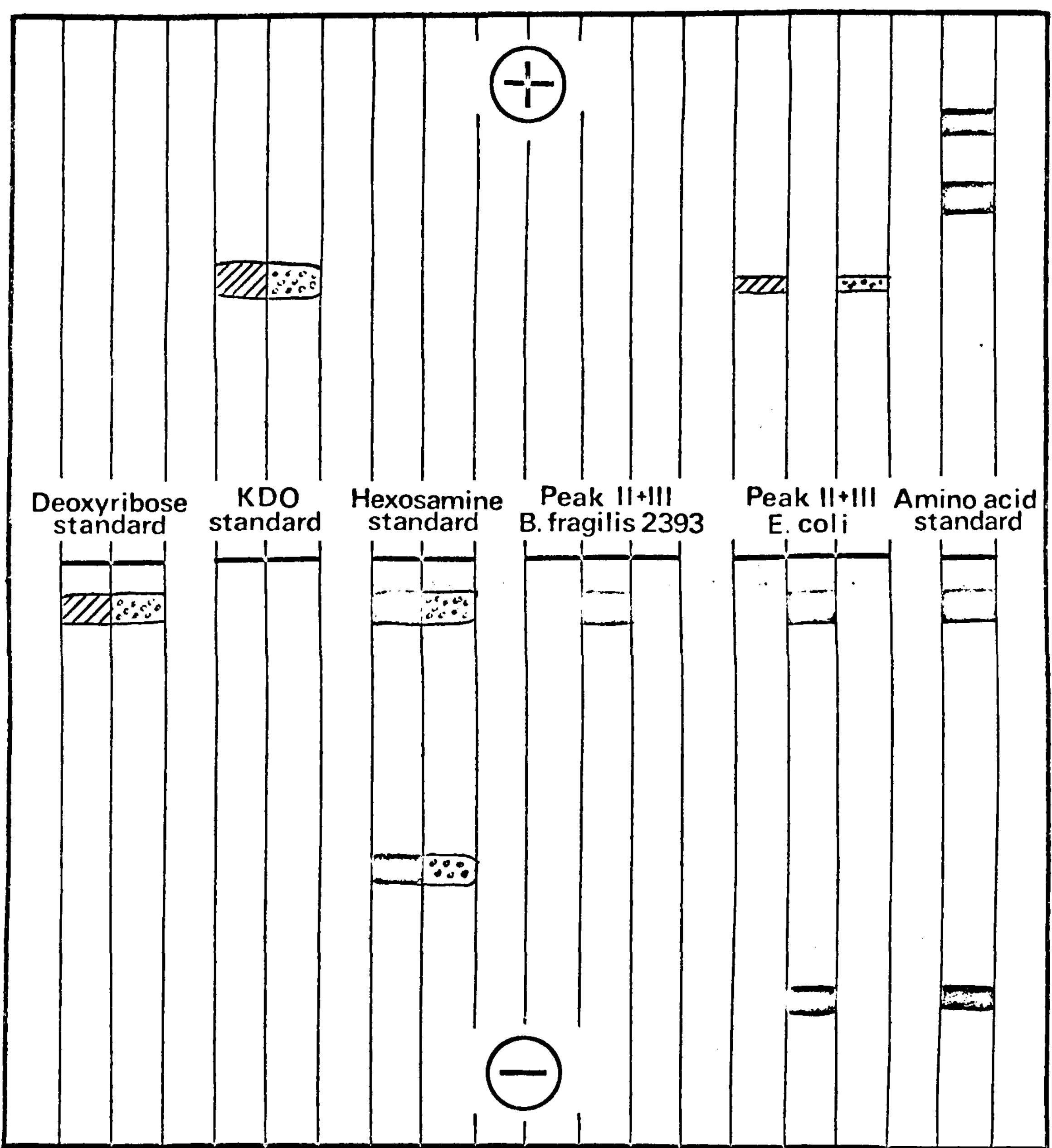

Figure 14. Electrophoretic Separation of KDO. A potential of 3000 volts applied for 1-2 hours, using solvent system H, see APPENDIX 3. ▭ = developed with ninhydrin; ▦ = developed with alkaline silver; ▨ = developed with thiobarbituric acid spray reagent. Hexosamine standard: Glucosamine, galactosamin, glucose; Amino acid standard: aspartic acid, glutamic acid, serine, lysine.

was used for heptose. These colorimetric tests were performed on pooled peak II and III material and phenol-extracted LPS. KDO was detected in the *E. coli* peak II and III material but not in any of the *Bacteroides* or *Proteus* samples. However, in LPS, it represented approximately 8 and 2% of the total dry weight of *E. coli* and *Proteus*, respectively. Similarly, heptose was demonstrated only in the *E. coli* and *Proteus* LPS samples and accounted for approximately 1% of the total dry weight.

Gross chemical composition of LPS. Phenol-extracted LPS from five of the six *Bacteroides* strains and three facultative organisms were analyzed to determine similarities in chemical composition. The gross chemical composition of all of the preparations is given in table 28.

The yield of LPS from the whole cells ranged from 2 to 5% for the *Bacteroides* and 3 to 8% for the facultative bacteria. However, nucleic acids were present in all of the preparations and varied in content from 3 to 24% dry weight depending on the preparation. To this extent the yield values are misleading. Carbohydrate was the major component of the *Bacteroides* LPS comprising 30 to 50% while for the facultative organisms it represented less than 20% of the material. Protein content also varied, representing less than 10% of the material in all but the *B. distasonis*, *E. coli*, and *Proteus mirabilis* preparations. Although less phosphorus was detected in the *Bacteroides* LPS, the hexosamine and lipid (fatty acid ester) content of both the *Bacteroides* and facultative organisms were generally very similar.

Table 28. Chemical Composition (%) of LPS[a] from *Bacteroides* and Enteric Organisms

Strain	Cell Wall Yield	LPS Yield	Total Carbohydrate	6-Deoxy-hexoses	Hexo-samine	Protein	Phos-phorus	Lipid (Fatty acid Ester)	KDO	Heptose	Nucleic Acid
B. fragilis 2393	13.7[b]	4.0[b]	47.6 (39.9)[c]	4.9	7.3 (5.3)[d]	3.1[e]	1.8	18.2	0[f]	0	3.0
B. fragilis 2553	18.0	5.0	26.4 (20.8)	3.0	6.0 (5.0)	4.2	2.6	23.0	0	0	23.7
B. distasonis 4243	12.1	2.6	45.2 (37.5)	1.1	5.3 (4.9)	12.1	1.8	25.6	0	0	6.3
B. thetaiota-omicron 5482	12.3	5.0	37.8 (31.0)	0.9	7.1 (6.3)	8.0	1.7	21.8	0	0	5.0
B. ovatus 0038-1	15.4	6.3	28.5 (26.7)	3.6	6.2 (5.3)	4.8	2.6	17.7	0	0	18.6
E. coli ATCC 11775	19.0	4.0	17.3 (15.0)	0.1	7.6 (6.8)	12.2	3.2	17.3	7.5	1.4	8.8
Proteus mirabilis PM-1	35.0	4.6	14.7 (12.3)	0.2	11.6	17.8	2.8	24.8	2.1	1.0	24.5
Pseudomonas aeruginosa ATCC 15142	29.0	7.5	17.0 (14.7)	2.1	8.4	8.2	3.8	33.2	3.0	0.4	20.0

[a]Material isolated from the aqueous phase of 45% phenol extraction of cell walls

[b]Based on whole cells, dry weight

[c]Results from the α-napthol colorimetric test

[d]Results from the amino acid analyzer

[e]Results of Lowry colorimetric test and spectrophotometric values were comparable

[f]Results of both thiobarbituric acid colorimetric tests were comparable

The hexosamine content as determined by the modified Rondle and Morgan method was slightly higher but in fairly good agreement with the values obtained from the amino acid analyzer. Hexosamines accounted for approximately 5% to 12% of the extracted material. Fatty acid ester content ranged from 17 to 26% for all but the Pseudomonas preparation, in which it was 33%. The 6-deoxyhexose content correlated well with the paper chromatography results. In particular, lower levels were detected in both the B. distasonis and B. thetaiotaomicron preparations.

As mentioned earlier, KDO and heptose were found in detectable amounts only in the LPS from the facultative organisms. In both the thiobarbituric acid assays, considerable color, characteristic of the chromophore derived from 2-deoxysugars, was observed in all of the Bacteroides preparations.

The presence of DAP. The insoluble fractions obtained from the aqueous phenol extraction of cell walls were processed further (see Materials and Methods) to determine the isomer of DAP present in the Bacteroides strains. All contained the meso isomer of DAP as did E. coli, Proteus, and the Pseudomonas strains. However, for none of the strains could a peptidoglycan preparation containing only the basic components, glucosamine, muramic acid, L-alanine, D-glutamic acid, meso-DAP, and D-alanine, be achieved. In all cases, the material obtained still appeared to contain extraneous protein.

Electron Microscopy of LPS

LPS extracted from the Bacteroides by aqueous phenol appeared very similar to that extracted from *E. coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. A trilaminar, dark-light-dark, pattern was observed in all of the sectioned preparations, see figures 15-23. However, a less electron dense crust was frequently associated with the outer edges of the *Bacteroides* ribbon-like structures.

Observations on Capsulation

Many representative strains from several of the homology groups were observed to be encapsulated, see figure 24. Capsulation of cells grown in broth was easily demonstrated by the India ink wet-mount technique of Duguid (32) or by negative staining dry mounts. The size of the capsule varied within a single preparation and between the different strains, but it was approximately one-half to one times the cell diameter. Occasionally, capsules up to four times the cell diameter were observed. Most were well defined and appeared rigid. Cell suspensions of entirely encapsulated cells stored for over two years at 4 C still retained a well defined, apparently intact capsule, as visualized by the India ink wet mount. However, certain strains possessed copious slime-like layers and could easily be identified by their viscous broth cultures. With most strains capsulated cells accounted for approximately 10% or less of the total cell number; however, a few strains, such as *B. ovatus*, 0038-1, were entirely encapsulated. On solid media, no difference in colonial morphology was observed which could be related to capsulation. Each colony, on

Figure 15. Electron Micrograph of B. *fragilis* 2393 LPS. 113,000 X.

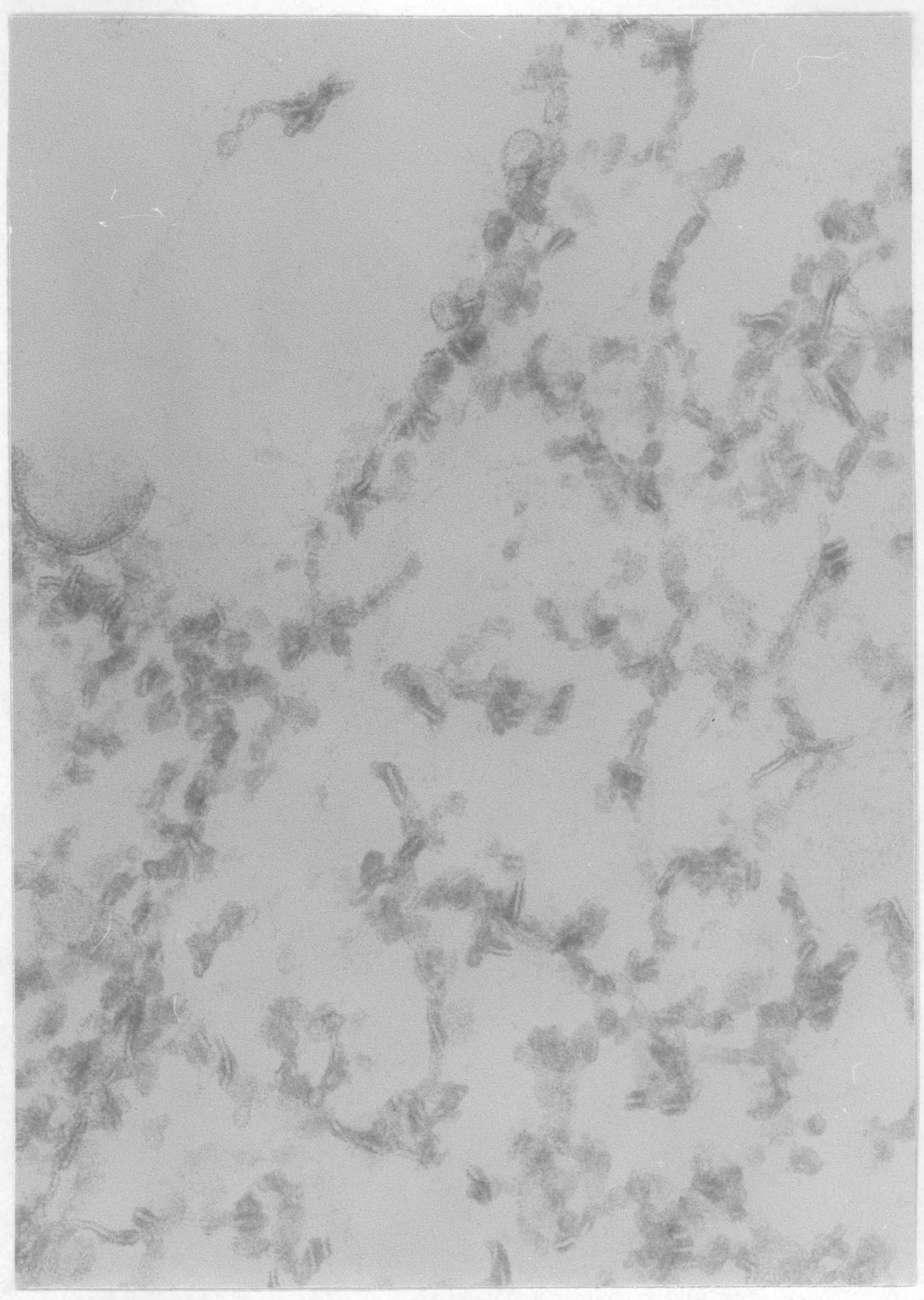

Figure 16. Electron Micrograph of B. *fragilis* 2553 LPS. 130,000 X.

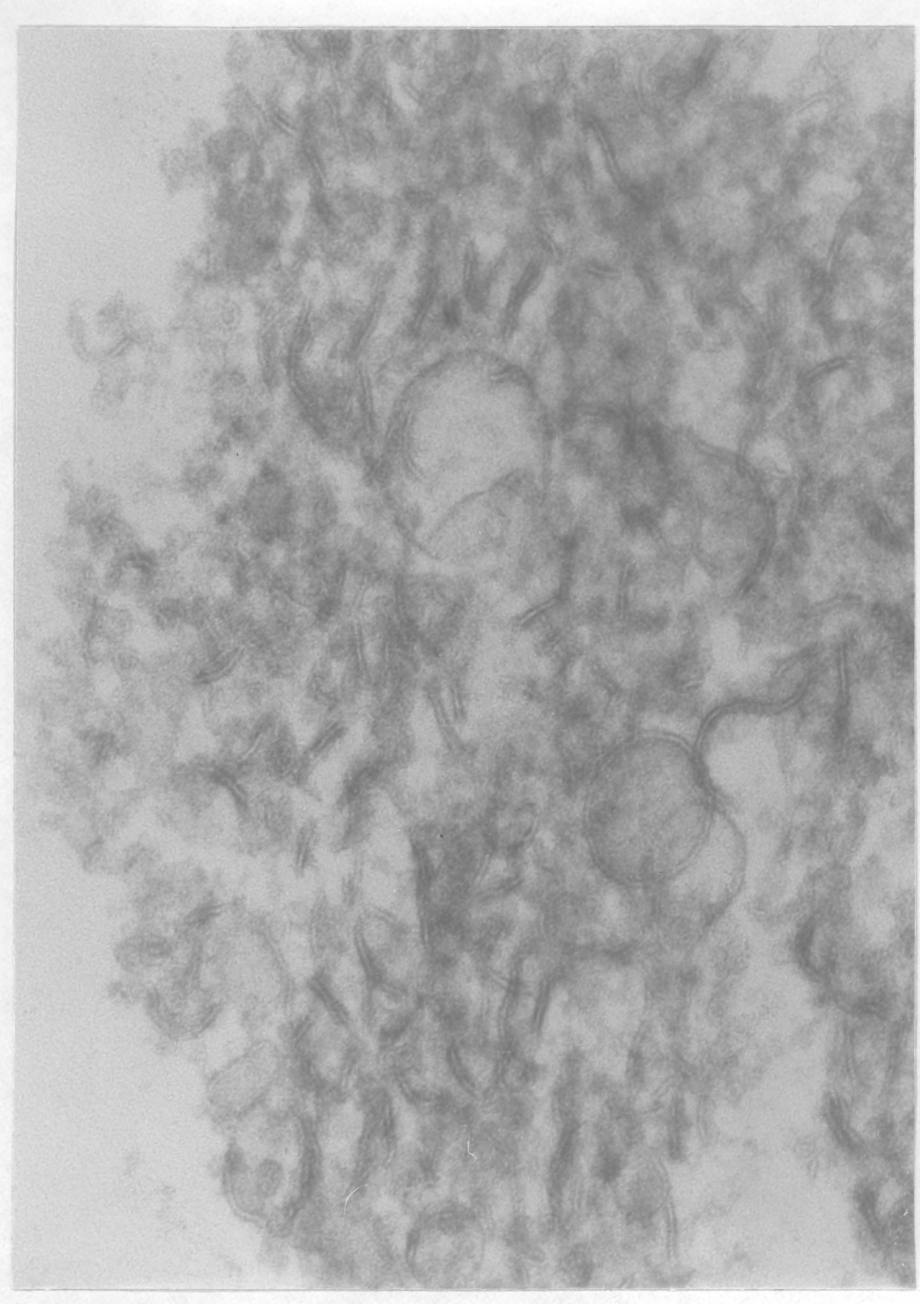

Figure 17. Electron Micrograph of B. distasonis 4243 LPS. 130,000 X.

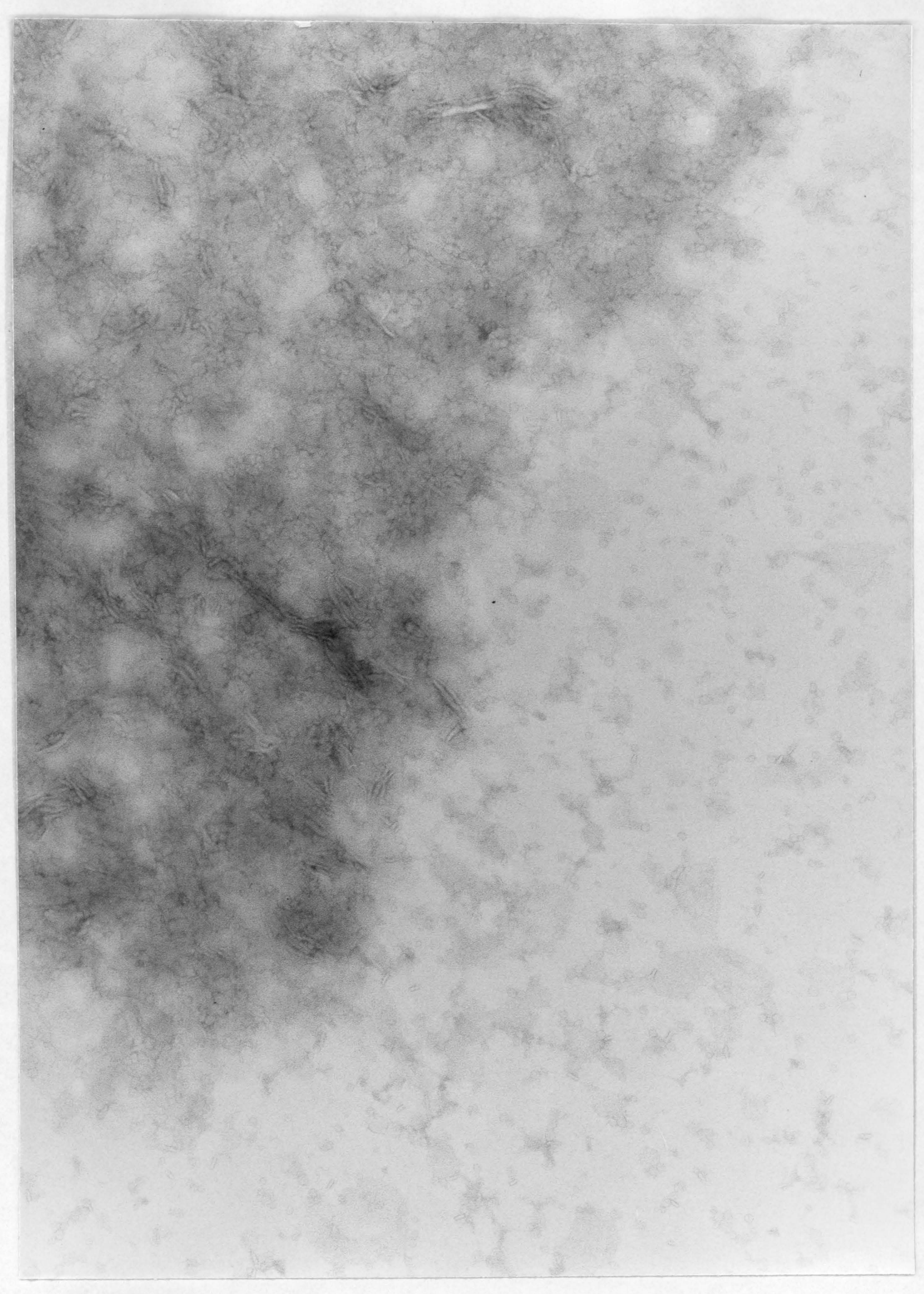

Figure 18. Electron Micrograph of B. vulgatus 4245 LPS. 63,000 X.

Figure 19. Electron Micrograph of B. thetaiotaomicron 5482 LPS. 128,000 X.

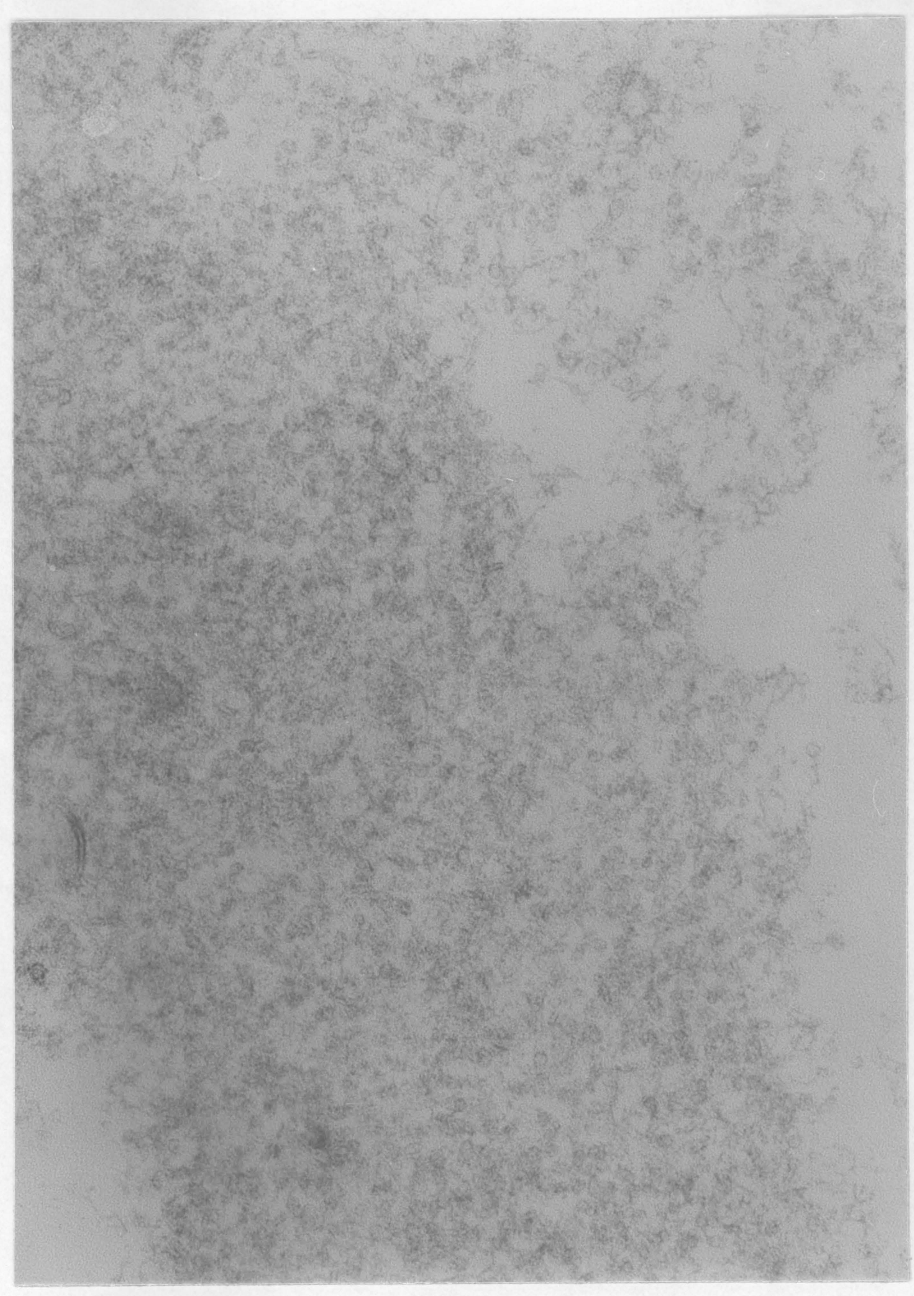

Figure 20. Electron Micrograph of *B. ovatus* 0038-1 LPS. 130,000 X.

Figure 21. Electron Micrograph of *Escherichia coli* ATCC 11775 LPS. 105,000 X.

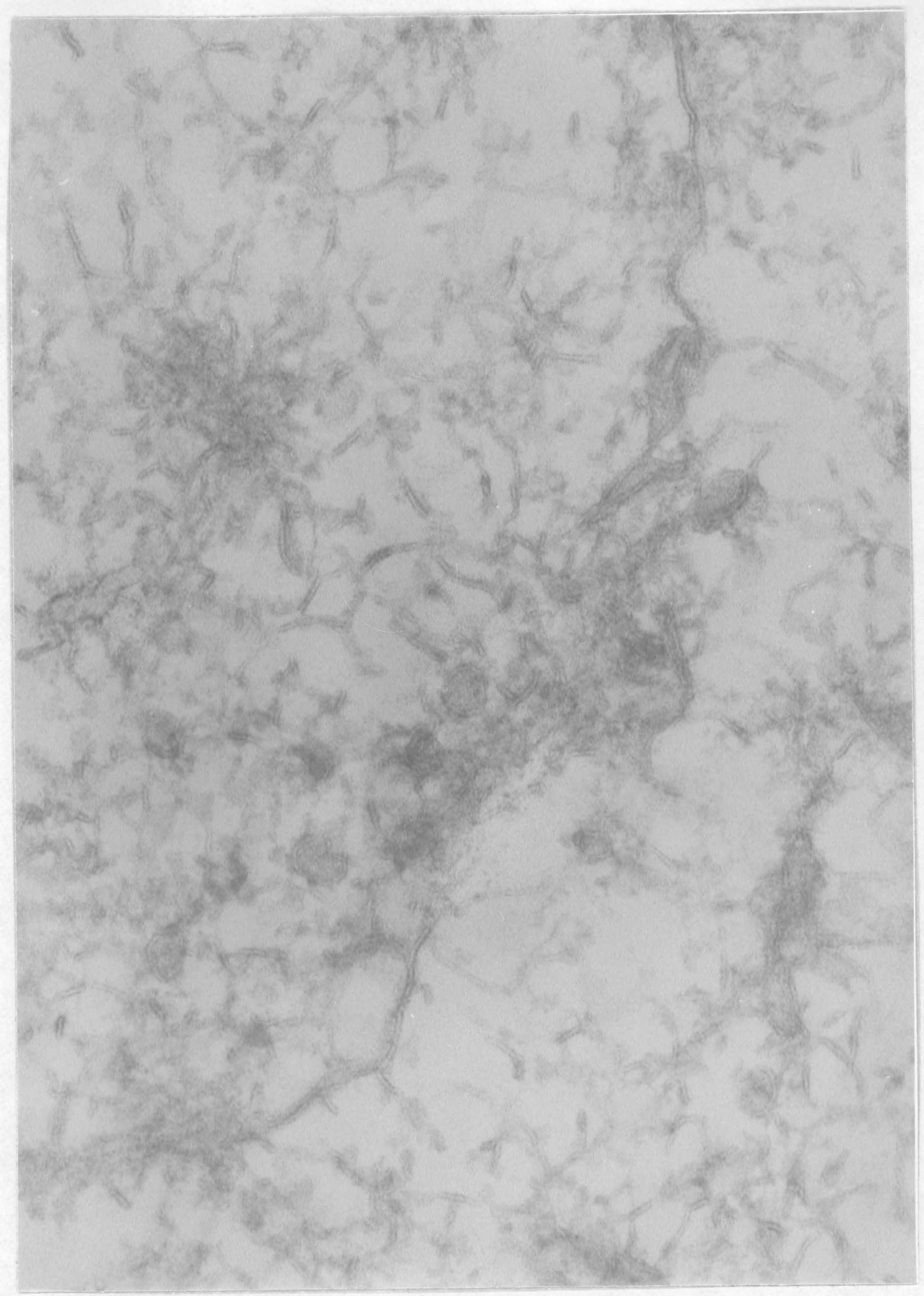

Figure 22. Electron Micrograph of Proteus mirabilis PM-1 LPS. 110,000 X.

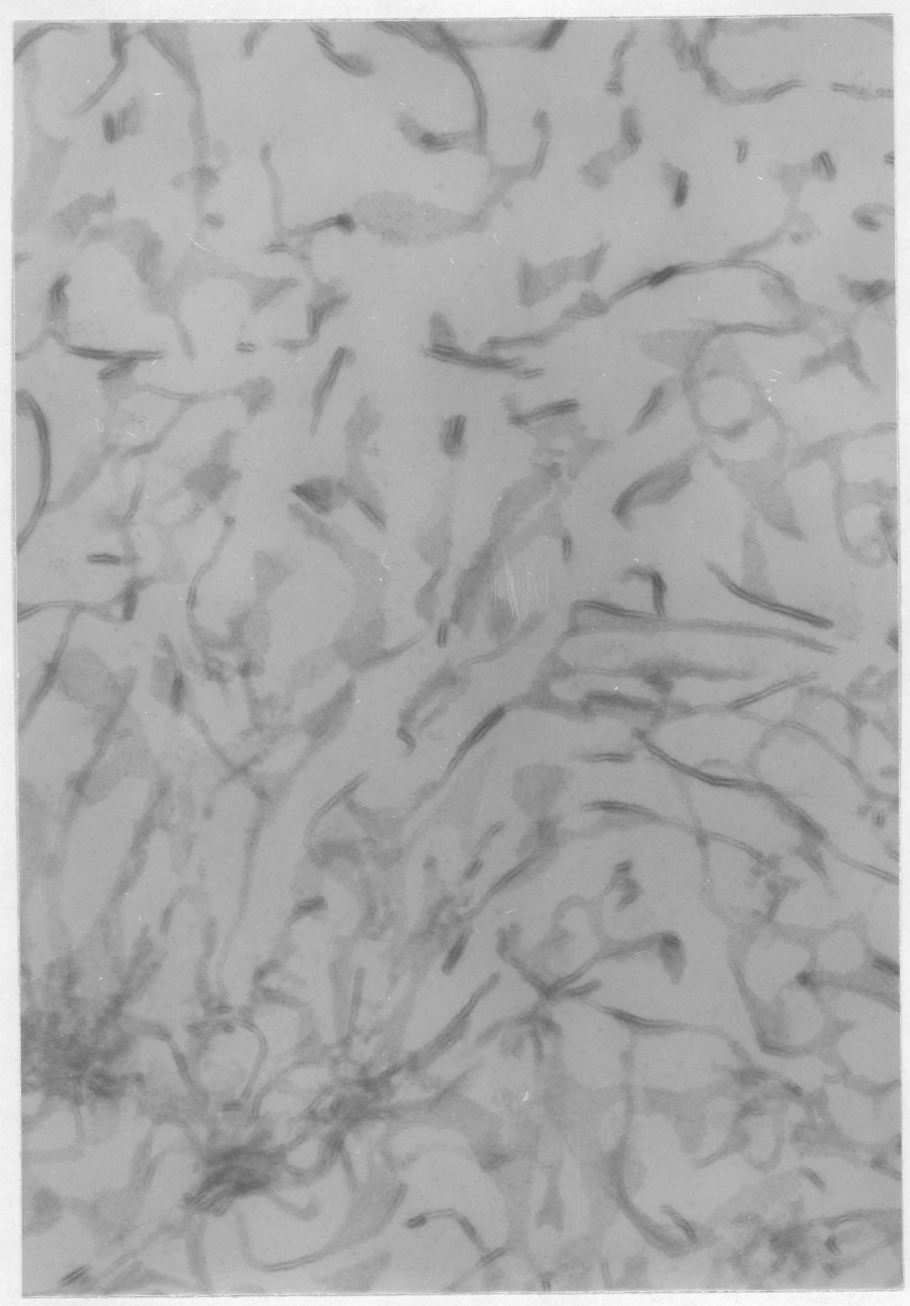

Figure 23. Electron Micrograph of Pseudomonas aeruginosa ATCC 15152 LPS. 110,000 X.

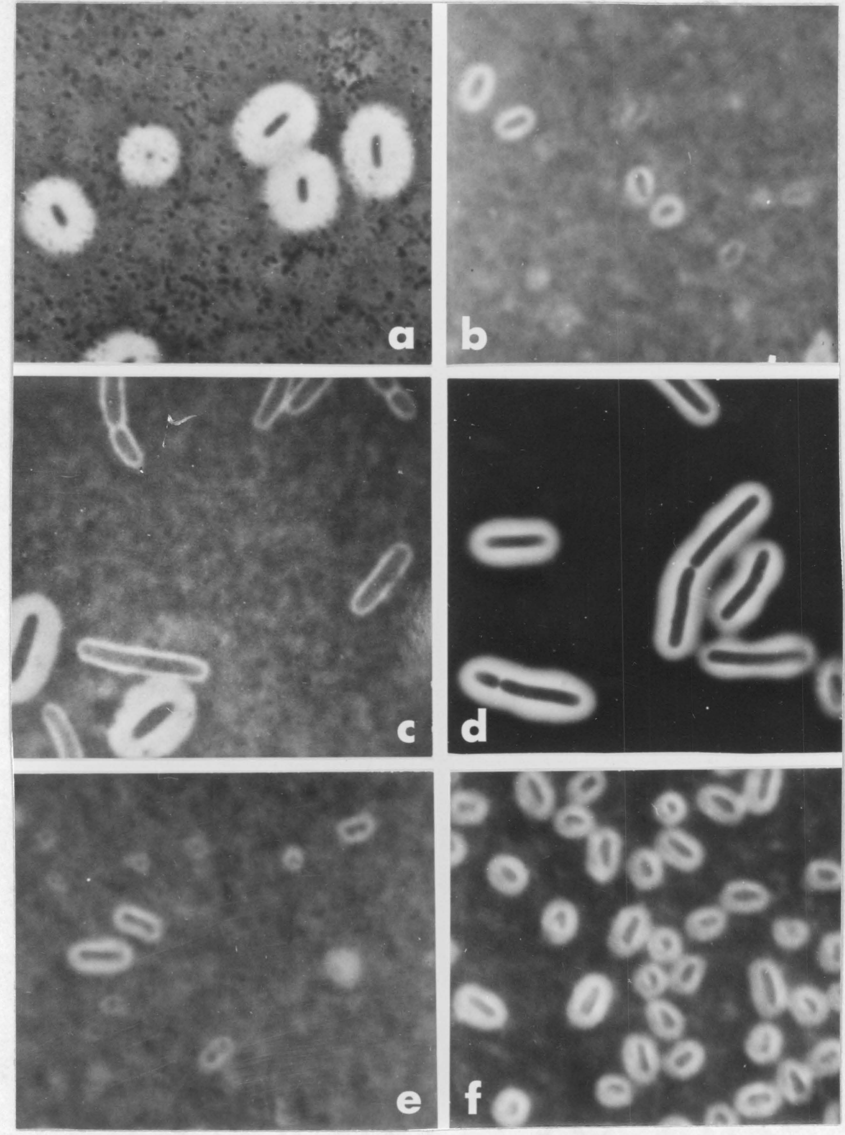

Figure 24. Capsulated cells of some Bacteroides species. a. B. fragilis 2553, 1000 X; b. B. fragilis 2392, 1000 X; c. B. vulgatus C7-2, 1200 X; d. B. vulgatus 4245, 1200 X; e. B. thetaiotaomicron 5482, 1000 X; f. B. ovatus 0038-1, 1000 X. All organisms grown in TYG medium.

microscopic examination, appeared to be comprised of both capsulated and non-capsulated cells in the ratio stated above.

Cell suspensions of entirely encapsulated cells were obtained by differential centrifugation. However, when these suspensions were used to inoculate broth cultures or restreak solid media, no enrichment of capsulated cells over non-capsulated cells was obtained.

Factors affecting capsulation. A host of organic and inorganic nutrients were tested along with various growth parameters, such as incubation temperature, time, and pH, to determine their influences on capsulation. Table 29 summarizes the results. The effect on capsulation by the various parameters was based on a comparison of cells grown under the experimental conditions to those grown in the normal TYG medium. Of all the factors tested, only a decrease in yeast extract concentration in the liquid media or growth in a simple glucose-salts medium increased capsulation up to approximately 30%.

Chemical studies on the capsule. Capsulated cells were treated with several agents under a range of experimental conditions, to determine what effect(s) these conditions would have on the physical appearance of the capsule. The results are presented in table 30. Fairly drastic conditions, such as heating in 0.1 N HCl at 100 C for 15 minutes, were needed to remove the capsule and in many cases, the cells were damaged, as determined by phase contrast microscopy, before the capsules were removed. Enzymatic treatment, either with the α-amylases, β-amylase, or pronase, had no visible effect on the capsule. However, mechanical shearing either by brief sonication

Table 29. Factors Affecting Capsule Production of *Bacteroides* Species[a]

Parameter	Effect on Capsulation	Parameter	Effect on Capsulation
Carbohydrate Substrate:[b]		Temperature:	
Glucose, 2%	none	19 C, 30 C, 45 C	none
Ribose	none	pH:[d]	
Xylose	none	7, 7.5, 8	none
Cellobiose	none	Time, Growth Phase:	
Mannose	none	early expodential thru late stationary	none
Fructose	none	Media:	
Inorganic Nutrients:[c]		PYG	none
Ca^{++}, 1M	none	GMB	slight stim. (up to 30%)
$MgSO_4$, 10^{-3}M	none	TYG, 0.25% yeast extract	"
$MnCl_2$, 10^{-3}M	none	PYG, 0.25% yeast extract	"
$MgSO_4$ + $MnCl_2$, 10^{-3}M	none		
Bicarbonate, 10%	none		
Rabbit Serum Supplement[c]			
1%, 5%, 10% (v/v)			

[a]Bacteroides strains examined were *B. fragilis* 2393, 2553, 3390, 3277-1, 4076, 4255, 6805, *B. distasonis*, 4243, *B. vulgatus* 4245, 2277, C7-2, OC-13, and *B. thetaiotaomicron* 5482, 2808B, 8651.

[b]TYG medium with 1% specified carbohydrate substrate replacing 1% Glucose

[c]TYG medium plus stated nutrient

[d]TYG medium buffered to specified pH with phosphate buffer.

Table 30. The Effects of Various Agents on Capsule Extraction

TREATMENT	EFFECT ON CAPSULE	COMMENTS
1% Trichloroacetic acid, 56 C 30 minutes	none	Cells slightly less refractile in phase microscopy
0.5N NaOH, 100 C, 10 minutes	capsule removed	complete loss of cell refractility
0.1N HCl, 100 C, 15 minutes	capsule removed	cells less refractile
0.01 M Periodic acid, 19 C, 60 minutes	none	no apparent loss of capsule
Phosphate Buffered Saline, pH 7.5 0.01 M, 100 C, 30, 60, 90 minutes	variable	Gradual thinning of capsule with longer heating times
Glycine-HCl-NaOH Buffer, pH 10, 0.1 M, 100 C, 30 minutes	none	Capsules still present; cells less refractile
α-amylase *Aspergillus oryzae* (Sigma) α-amylase, bacterial (Calbiochem) β-amylase, bacterial (Calbiochem) — 0.1M Phosphate Buffer, pH 7, 37 C, 2 hours	none	no apparent loss of capsule
Pronase (Sigma) in Phosphate buffered Saline, pH 7.3, 24 hours, 37 C	none	no apparent effect
Sorvall Omnimix, 3 x 5 minutes, Phosphate buffered saline, pH 7.3	variable	longer exposure, better removal
Sonication, (Biosonic III, 20% maximum, 10 sec intervals)	capsules removed	some cell damage with long exposure

or longer treatment with the Sorvall Omnimix extracted the capsule from the cells with minimal cell damage, as monitored by phase contrast microscopy and by absorbance at 260 nm. Consequently, sonication was adapted for the isolation of capsular material. Washed suspensions of capsulated cells in PBS, pH 7.3, obtained by differential centrifugation, were briefly sonicated, while being monitored by phase contrast microscopy. When the majority of cells had lost their capsules, the cell suspension was centrifuged and the supernatant containing the capsular material was retained. Five volumes of cold acetone were added to the supernatant and the precipitate was collected by centrifugation. This material was redissolved in distilled water, dialyzed against distilled water for 24 hours at 4 C and lyophilized for further characterization.

Capsular material from Bacteroides fragilis 2393, 2553, and from B. vulgatus, 4245, was isolated according to the method described above and analyzed for its chemical constituents by paper chromatography. Table 31 summarizes the results. Many of the same components observed in the LPS preparations were present in the capsules. All contained glucose, galactose, and fucose, although the presence of mannose, glucosamine, and galactosamine was variable. Rhamnose and uronic acids (see Appendix 4) were not detected in any of the capsules.

Serological studies. A suspension of entirely encapsulated cells of 2393, 2553, and 4245 was obtained by differential centrifugation and used in agglutination and immunodiffusion studies. In tube agglutination tests, capsulated cells were agglutinated with homologous

Table 31. Sugar Constituents of Capsular Material[a,b]

VPI No.	Galactos-samine	Glucos-samine	Galac-tose	Glu-cose	Man-nose	Fucose	Rham-nose
2393	++	+	++	++	-	+++	-
2553	+	+	++	++	++	++	-
4245	-	-	++	++	+++	++	-

[a]Capsular material isolated by sonication

[b]Constituents identified by paper chromatography

antisera to titers of only 20 or 40, as compared to titers of 1280 and 2560 when non-capsulated cells were used. Similarly, Autoclaved or TCA extracts, of capsulated cells generally gave weaker but similar precipitin lines against homologous antisera as compared to the extracts of non-encapsulated cells. Isolated capsular material failed to give precipitin lines when tested against homologous antisera. A suspension of capsulated 2553 cells was used to immunize a single rabbit. After a single course of immunizations, a test bleeding was done and the antiserum tested for activity. In agglutination studies with this serum capsulated cells were only agglutinated to titers of 20 and 40. This antiserum, when tested against whole cell extracts of both capsulated and non-encapsulated cells, produced common precipitin lines against both, although the lines were much stronger in the extracts of capsulated cells. Isolated capsular material was also tested against this antiserum but failed to give any precipitin lines.

As an effort to produce a stronger antiserum, the rabbit was rested one week and then begun on a second course of immunizations. Unfortunately, the rabbit died and consequently, further antisera was not obtained.

As a final test of serological activity, the antiserum against capsulated cells was tested against red blood cells sensitized with 2553 Autoclaved extract. This antiserum agglutinated the cells to a titer of only 160 whereas the same cell suspension was agglutinated to a titer of 10,240 with AS2553.

DISCUSSION

The purpose of this investigation was threefold: first to establish correlations between serological groupings and DNA homology groups; secondly to define a chemical basis for the serological reactions; and thirdly, to determine gross similarities and differences between cell wall components of facultative gram negative bacteria and the '*B. fragilis* group' of organisms.

Serological Groups and DNA Homologies

Numerous investigations have dealt with the serology of *Bacteroides fragilis* and related species, demonstrating that they possess a multiplicity of ill-defined antigens conferring strain, species, and occasionally group specificity. As a result, serogroupings could often be correlated with biochemical or biological properties depending on the particular serological test employed. The most noted example is Beerens' correlation of the 'E' antigen with pathogenicity (127). In hopes of clarifying such discrepancies, the serological techniques and many of the antigen extracts used in the previous studies were employed in this investigation. The strains examined were assigned to species on the basis of genetic properties, thus eliminating any potential bias resulting from interpretation of phenotypic characteristics. It should be noted however, that many of the strains used in this study were clinical isolates and therefore may represent a biased population.

In general, the five Bacteroides species were found to be antigenically distinct. There were low levels of reaction across species boundaries, however, most cross reactions were detected only by agglutination which suggested that surface antigens were primarily responsible. Species specific antigens were also available at the cell surface and the antibody response was largely directed toward them since absorption of the antisera with whole cells removed all cross reactions without significantly lowering the homologous titers. An attempt was made to characterize the types of antigens exposed at the cell surface by treating cell suspensions with heat, proteolytic enzymes, and periodic acid. It was evident that polysaccharides were particularly important surface antigens from the effect that periodic acid had on the antigenicity and stability of some of the cell suspensions. However, the effects of proteolytic enzymes and heat indicated that proteins were also exposed at the surface and that their presence often masked underlying thermostable antigens. The range of sensitivities exhibited by the different strains to these three agents implied that the relative proportions which polysaccharides and proteins occupy on the cell surface varied with the strain. That these protein antigens may be responsible for the interspecies cross reactions was suggested by the absence of such reactions in the cell wall agglutination studies (cell walls were digested with proteolytic enzymes) and by the observations on 4245, whose high level of cross reactivity in whole cell agglutination studies was correlated with the greatest sensitivity to heat. In addition, immunodiffusion tests

employing extracts selective for thermostable antigens failed to detect most of these interspecies reactions. These conclusions are in agreement with the serological studies of Beerens (5). On the basis of an agglutination survey, he reported that the antigenic make-up of *Bacteroides* organisms was a mosaic containing one major and several minor antigens of varying specificity. The interspecies cross reactions observed among Beerens' reference strains by agglutination are similar to those obtained in the present study; see tables 3 and 32. However, as in this study, only species specific antigens were demonstrated in immunodiffusion tests using extracts containing thermostable polysaccharide antigens. Therefore, it is likely that the interspecies reactions were the result of surface thermolabile antigens. Reinhold also correlated intragroup reactions with thermolabile protein antigens (122, 123). It is important to note that only one of Beeren's serogroups, that characterized by reference strain 5482, was biochemically homogenous. The other groups frequently contained strains biochemically classified as *B. thetaiotaomicron* (or *B. ovatus*) and *B. fragilis*.

The distinct antigenic divisions displayed by the five species were in accordance with their genetic relationships, i.e., low genetic relatedness, little antigenic similarity, see table 2. However, the serological properties did not appear to distinguish genetic heterogeneity at levels down to approximately 65% homology. Homology groups including the two *B. fragilis* subgroups were relatively homogeneous. This was an interesting observation since strains of

Table 32. Tube Agglutination Results Taken from Beerens, H., et al. (1971)

VPI No.	Antisera: B. fragilis Group II E323	B. fragilis Group I 2553	B. distasonis 4243	B. vulgatus 4245	B. thetaiota-omicron 5482	B. ovatus 0038-1
B. fragilis E323	1280	320	0	0	0	R
B. fragilis 2553	320	1280	40	0	0	0
Whole Cells: B. distasonis 4243	0	40	2560	40	0	20
B. vulgatus 4245	0	40	0	5120	0	20
B. thetaiota-omicron 5482	0	0	40	0	80	0
B. ovatus 0038-1	0	0	0	0	0	160
Serogroup	E_2	E_1	D	C	A	B

Enterobacteriaceae of similar levels of homology display extensive serological and chemical heterogeneity (e.g., see references 8 and 90). In contrast to Beerens' result many homology group members were not agglutinated to high titers with any particular antiserum. Yet extracts of most strains produced at least one precipitin line with antiserum against a member of the same homology group. Thus, the low agglutinating potential of these strains was believed to be related to the differences in accessibility of species specific antigens at the surface due to interferences with other surface structures such as proteins. Although most strains reacted with an antiserum directed against a member of the same homology group, immunodiffusion studies demonstrated that this homogeneity was not always represented by a single "common" antigen. In B. fragilis, B. vulgatus, and B. thetaiotaomicron there was a multiplicity of shared antigens, with each strain displaying a particular combination of antigens. This complex antigenic relationship had been implied by the studies of Sharp (135), and Werner (162). With B. vulgatus and B. thetaiotaomicron, at least three different antigenic factors were evident in each system, see figures 11b and c. Furthermore, the reactivity of certain antisera implied that certain strains possessed more antigens, both in kind and number, than others, e.g., ASC7-2 and AS4245, AS5482 and AS2808B, see tables 15 and 20. The mosaic type of antigen composition was also evident among the B. fragilis strains. The present work suggests that the minimum number of antigenic factors on B. fragilis 2393 and 2553 was ten and six, respectively (labelled a - k

and 1 - 6 in tables 16 and 17). There was apparent clustering regarding the possession of particular antigens but it was irrespective of intrahomology values. Until absorption studies are performed on these strains, the nature and distribution of these factors cannot be predicted accurately. Very recently, Hofstad reported on preliminary studies of this kind on three B. fragilis strains, indicating that a minimum of six factors were shared among the three strains (56).

Although most strains, excluding those belonging to the B. ovatus group, displayed serological activity in either the immunodiffusion or hemagglutination studies, some did not react with any antiserum directed against a member of the same homology group, e.g. 4082 in table 19, 0959-1 in table 20. These could represent other sero-groups.

The qualitative and quantitative variation which accompanies the mosaic antigen make-up of strains in the genus Bacteroides provides information relating to the interpretation and organization of serological studies. Beerens' species antigens A through E are most likely conglomerates of several antigenic factors. Therefore, it would be possible for two strains possessing, for example, the "E" antigen to have two completely different factors. Furthermore, certain antisera were shown to be more reactive toward survey strains than others. This implied that strains used for generation of these anti-sera perhaps were better immunogens or more importantly, that they possessed a more encompassing antigenic composition than other organisms. In either case, they would be the most favorable reference

strains for serological identification schemes. For example, in this study, AS2393 was superior to AS2553. In view of this, it is interesting to note that in Beerens' survey, strain E323 was the second *B. fragilis* reference strain. This strain was determined to be a member of the 2553 homology group (66). Therefore, the 2393 homology group was not represented in the survey by Beerens. In the present study, little antigenic similarity was detected between E323 and 2393, so it is very likely that certain *B. fragilis* strains were not detected in his survey.

In the immunodiffusion survey, non-reciprocal reactions were occasionally observed, see table 8, 4245 Autoclave extract versus AS4243. Furthermore, precipitin lines were often observed in heterologous crosses which were not detected in the homologous reactions, see figure 11b, R4-24B Autoclave extract versus AS4245; tables 16 and 17, factors f - k and factors 4 - 6, respectively. Several factors may contribute to these phenomena. A poor response of a particular rabbit to immunization and the degree to which an antigen is available to elicit an antibody response can result in reactions characteristic of the first example (table 8). Likewise, the absence of lines in homologous reactions observed in heterologous crosses can result from the inaccessibility of a particular antigen to react with the antibodies. In addition, extreme differences in amounts of antigen present in the antigen extracts can also lead to this problem. Antigens in excess can inhibit and/or mask precipitin line formation of antigens in lower concentrations (18). In addition incubation

time and temperature are among other factors that can affect immunodiffusion tests. Of crucial importance in this study was the concentration of reactants. Many of the precipitin lines formed in homologous and heterologous crosses were not detected at extract concentrations less than the arbitrary value chosen. This value was chosen on the basis of hemaagglutination studies which indicated maximum sensitization for the B. fragilis strains at this concentration. Consequently, this may not represent the optimum concentration for maximum sensitivity in immunodiffusion tests. This is particularly important with a mixture of reactants at different concentrations, since it is difficult to choose a concentration optimal for all of them at one time. Error in the determination of antigen concentration cannot be ruled out since the phenol-sulfuric acid assay is very sensitive to contaminating particles, such as dust, which may give falsely high levels of carbohydrate.

It was not possible to investigate the B. ovatus homology group in detail because of the failure to generate a high titer 0038-1 antiserum. Although both agglutination and precipitin reactions were observed, these were weak and could not be strengthened significantly by concentration of the reagents. Negative results in heterologous tests are therefore of doubtful significance in view of the weak homologous reaction (positive control). It is of interest to note that the AS0038-1 used in Beerens' investigation displayed a titer comparable to that in this study. The problem is believed to concern the presence of a capsule on this organism and will be discussed later.

Hemagglutination Tests

In 1960, Sonnenwirth, and later, Hofstad reported on the feasibility of hemagglutination as a serological test to investigate the *Bacteroides* species. Due to the success of their investigations, this test was employed in the present study to investigate some homology group members in an attempt to devise an identification scheme. Although sensitization was performed using extracts similar to those used in the earlier studies, much higher hemagglutination titers were obtained for some of the *B. fragilis* reference strains than those previously reported. This may reflect the use of stronger antisera due to immunization with formalin-treated cells, since Dworzynski reported that higher titered antisera could be obtained using formalin-killed bacteria (33). Immunization with *B. vulgatus*, *B. thetaiotaomicron* and *B. ovatus* systems did not produce such high titers. Three factors may have contributed to this. First, the antisera produced by the immunization scheme may not contain high levels of antibodies efficient in hemagglutination reactions, secondly, the maximum sensitization of RBC's was not achieved in the sensitization process, and thirdly, the antisera may not have been concentrated to levels comparable to those obtained with the *B. fragilis* antisera.

Hemagglutination results were generally in agreement with the other serological tests. However, hemagglutination activity was occasionally observed with strains whose extracts failed to yield precipitin lines against the same antisera. In such cases, hemagglutination titers were never greater than 320, and the results

probably reflect differences in the sensitivities of these two assays. Similar observations were made by Quick et al (119). There were also strains whose extracts yielded precipitin lines but displayed no hemagglutination activity. Numerous factors may contribute to this phenomenon. There may not have been enough material to adsorb onto the RBC's. This seems unlikely since the same extracts were active in the immunodiffusion tests. Assuming the antigen concentrations were accurate and the sensitization conditions were standardized, there may be differences in sensitization potential, resulting, in part, from chemical differences of the antigens. Although the adsorption process is not fully understood, molecular size, degree of aggregation, configuration, and O-acetyl content are among the factors believed to affect this process (22, 108). The low sensitization by some extracts may also be the result of inhibition by other non-antigenic components, since the material used for treating the RBC's was a crude Autoclaved extract.

Despite the above discrepancies, the hemagglutination patterns within species (homology groups) were consistent with the antigenic mosaic. Strains which possessed multiple antigens or large amounts of one antigen (visualized by immunodiffusion) displayed high hemagglutination titers. Unfortunately, 30% of the *B. fragilis* strains displayed low hemagglutination activity. Over half of these strains could be detected by immunodiffusion which suggests that the negative hemagglutination tests were due to failure to adsorb antigen onto the

RBCs. The other non-reacting strains may represent different serotypes. Interspecies reactions were infrequent and generally at low levels. *B. distasonis* was the most cross reactive homology group. However, this activity was apparently non-specific in nature since cells sensitized with extracts of this group were agglutinated to some degree in normal and non-*Bacteroides* antisera and this may reflect an instability of the sensitized red blood cells. Both *B. fragilis* antisera agglutinated most of the strains to titers $\leq$ 20. Although no tests were performed, this agglutination was believed to be non-specific.

Capsulation of *Bacteroides* Strains

In the course of this investigation, it was observed that many of the *Bacteroides* strains were encapsulated. With most strains capsulated cells accounted for approximately 10% or less of the total cell number; however, a few strains such as *B. ovatus* 0038-1 were entirely encapsulated. Because the presence of such a capsule would undoubtedly affect both the serological and chemical properties of the organisms, a preliminary attempt was made to determine the factors which caused capsulation, to determine the composition of the capsule, and its relation to the serological properties of the organisms.

When *Bacteroides* were grown under optimal conditions, capsulated cells represented less than 10% of the total cell number as estimated from India ink wet mounts. Only under suboptimal conditions e.g., growth in a simple glucose salts medium, was a slight elevation (up to approximately 30%) of capsulated cells noticed. A wide range of

substances known to affect capsule production in other bacteria was tested with limited success, see table 29. Of the factors tested, only decreased levels of yeast extract and growth in a simple glucose-salts medium (see Appendix 1) seemed to stimulate capsule production. Duguid and Wilkinson (166) noted that capsule production in *Klebsiella aerogenes* was minimal when carbon and energy sources were growth limiting. However, when nitrogen, phosphorus, or sulfur sources, i.e., factors not immediately required for polysaccharide synthesis, were growth limiting capsule production was maximal. Alternatively, studies with *B. anthracis* demonstrated that capsule formation was elevated in the absence of an antagonist found in some media (101). The study on *B. anthracis* also noted that on solid media cells were grossly heterogeneous with respect to capsulation, often with capsulated cells representing less than 1% of the population. These cells were located in areas of dense growth, an observation attributed to an inactivation or acquired resistance to the inhibitor. As a result, colonies did not appear entirely mucoid. Similar observations have been made on the *Bacteroides*. Although there is no evidence supporting this, another factor that must also be considered is that *in vivo*, *Bacteroides* may be entirely encapsulated, a property which is rapidly lost when cultured *in vitro*.

In the cases described above, the capsular material is chemically distinct from the cell wall polysaccharides. Work and coworkers (80, 169) investigated a lys$^-$ *E. coli* mutant which under lysine-limiting conditions, excreted LPS which collected around the organism to form a

capsule. These authors hypothesized that during periods of lysine deprivation, LPS production remained at a normal rate whereas synthesis of the underlying layers, was much slower. Therefore, the LPS was produced in excess and accumulated.

The composition of the Bacteroides capsule was very similar to that of the cell wall polysaccharides extracted with 45% phenol, see tables 31, 21, 22, and 24. The capsules were different from one another and contained no uronic acids. The similarity of composition to that of isolated LPS causes problems of interpretation in the chemical studies. These will be discussed later.

Kasper has recently reported that only B. fragilis strains possess capsules, by demonstrating a polysaccharide layer in electron micrographs of cross sectioned B. fragilis strains stained with ruthenium red (75). It is unclear whether this material represents the capsular material demonstrated by the India ink wet mount technique, since in the present investigation, capsules were demonstrated by the latter method in representatives of all of the Bacteroides species and a capsular layer of the dimensions described by Kasper (1.5 - 2 times the thickness of the cell wall) would be difficult to distinguish when viewed in the light microscope using the India ink wet mount technique (limit is approximately 0.25 times the cell diameter). Although no detailed chemical composition was reported, Kasper inferred some chemical distinctions between capsular material and LPS (75).

The effect the capsule has on the serological properties is still unclear. Suspensions consisting of 100% capsulated cells were

agglutinated with homologous antisera but only to very low titers and antigen extracts of capsulated cells did not display precipitin lines not already present in extracts of noncapsulated cells. These observations suggest that capsular antigens may be very similar to cell wall antigens. However, capsulation of 0038-1 may be the reason why only low titer antisera could be obtained. This was supported by the failure of immunization with capsulated B. fragilis 2553 cells to generate an antiserum which reacted specifically with capsular material. The antiphagocytic properties that capsules confer on bacteria and the ability of capsules to cause "immunological paralysis" could explain the poor antibody response elicited against the capsulated organism (166).

Since strains used in the serological analyses were grown under conditions for maximum cell yield, it is unlikely that many cells were capsulated. However, it cannot be ruled out that interspecies cross reactions may be the result of the capsule. Furthermore, it is tempting to speculate that Beerens' "E" antigen, which is associated with pathogenicity, is related to the capsule.

Chemical Composition of LPS and Peptidoglycan

The interpretation of the chemical composition studies must be made in view of several unforseen factors which have resulted, in part, from the technical procedures chosen for use in this investigation. These will be discussed briefly now and in later sections where appropriate.

Examination of the gross chemical composition of phenol-extracted LPS disclosed that the preparations were not pure. In addition to protein and nucleic acids, it is probable that an intracellular or cell wall-associated glycan was also extracted, as suggested by the detection of large amounts of glucose in chromatograms of *Bacteroides* LPS acid hydrolysates. The presence of glycan material in *Bacteroides* cell walls and "purified" LPS has been reported by several authors (57, 133, 147). This contamination was interesting because the phenol extraction was performed on cell walls believed to be void of such contaminants. An explanation for the failure of the method of cell wall preparation to remove these contaminants might reside in the fact that the initial batch cultures were treated with formalin. Although formalin does not affect immunogenicity, it reacts with free amino groups and certain side chains in proteins, resulting in extensive cross linking (72). Thus, nucleic acids and proteins can be bound to cell wall material and thereby rendered resistant to proteases, nucleases, and washings. This cross linking might also have trapped the glycan material leading to its association with "purified" cell walls and, subsequently, with the LPS material. The presence of another contaminant, capsular material, cannot be ruled out even though antigen extraction was believed to have been carried out on largely non-capsulated cells. The close relationship of the sugar composition of the capsule to that of the LPS material makes interpretation of LPS chemotypes difficult until more is known about the capsule.

Another factor which might have some bearing on the chemical studies, also concerns the method of cell wall preparation. Whole

cells were suspended in EDTA-buffer prior to disruption. Studies with *E. coli* and other facultative organisms (47, 83) have demonstrated that treating cells with EDTA can lead to the release of up to 50% of the LPS. Although this EDTA sensitivity has been shown to be very often species dependent, the fact that it can occur means that the yield values must be viewed with caution as must the compositional studies, since an unrepresentative LPS sample may have been analyzed.

Due to the interference of contaminants and the limitations of the colorimetric tests used (see table 28), chemical analysis cannot give more than a rough estimation of the quantitative composition of the *Bacteroides* LPS. Moreover, the colorimetric test accounted for only 80 to 95% of the material. Nevertheless, the studies do reveal a chemical makeup for all of the *Bacteroides* LPS similar to those found in facultative organisms. Yield values for LPS (table 28) were very similar and if contamination with nucleic acids is allowed for, the total carbohydrate levels in 2553 and 0038-1 are comparable to those of the other *Bacteroides* strains.

The compositional results are in agreement with quantitative analyses on *Bacteroides* LPS reported in the literature. Discrepancies only pertain to levels of neutral sugar, 6-deoxyhexoses, and hexosamines and these may reflect differences in extraction times. Hofstad and Kristofferson (57) demonstrated that prolonged extraction times similar to those used in the present study resulted in preparations with higher levels of neutral sugar (estimated as glucose), attributed to a contaminating glycan, and diminished levels of 6-deoxyhexoses and hexosamines.

There were only three major chemical differences between LPS of *Bacteroides* and that isolated from facultative organisms; those are the levels of total carbohydrate, phosphorus, and the core sugars, KDO and heptose. Carbohydrate levels were generally higher in *Bacteroides* than in the facultative organisms. This higher level may be attributed, in part, to the presence of the glycan material. Except for 2553 and 0038-1, phosphorus represented approximately 1.8% dry weight of the *Bacteroides* LPS. The higher values for 2553 and 0038-1 and the three facultative organisms can be explained in part by nucleic acid contamination. However, with this accounted for, the two facultative organisms, *E. coli* and *Pseudomonas aeruginosa*, still would possess slightly higher levels of phosphorus. This might imply major differences in LPS architecture since phosphorus, either in free or combined form, is known to be an important LPS component, particularly in the core region and Lipid A.

The peptidoglycan layer of the *Bacteroides* strains is believed to be similar to that found in facultative organisms, containing the basic components, glucosamine, muramic acid, D-alanine, L-glutamic acid, meso-DAP, and L-alanine. Although all of these components were identified in *Bacteroides* peptidoglycan acid hydrolysates, a purified preparation containing only these basic elements could not be prepared from the *Bacteroides* strains of the peptidoglycan preparations appeared to be heavily contaminated with extraneous protein. This was believed to be a technical problem since control preparations of *E. coli*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* processed at

the same time also gave peptidoglycan heavily contaminated with protein. Failure to obtain purified material may have been due to the formalin treatment used to kill the bacteria, since such treatment may lead to extensive cross linking and binding of protein contaminants to the peptidoglycan layer.

Presence of KDO and Heptose in *Bacteroides* LPS

It is evident from this study and from studies of several other laboratories, that KDO and heptose, if present at all in the *Bacteroides* LPS, are not at levels characteristic of facultative gram negative organisms. However it should be pointed out that the data obtained in this study does not rule out the presence of either compound. Data supporting the presence of these sugars was the detection of formaldehyde in the gel filtration profiles of *Bacteroides* polysaccharides at points similar to those observed in the profiles of the facultative organisms when the fractions were treated with periodic acid. Although periodate oxidation of heptose and KDO leads to a release of formaldehyde, the reaction is by no means specific for these sugars since formaldehyde is also released on oxidation of terminal reducing residues of oligosaccharides having carbons 5 and 6 unsubstituted and other compounds containing a primary alcohol group, e.g., serine. Therefore, additional proof was sought by chromatographic and colorimetric means. Paper chromatography and electrophoretic separation failed to detect these components in *Bacteroides* material. A very weak spot corresponding to KDO was detected in material from only one of three facultative organisms

and heptose was not detected in any. Therefore it is very likely that the techniques employed were not sensitive enough to detect lower levels of KDO or heptose should they be present in the *Bacteroides* samples. The third means of detecting KDO was the thiobarbituric acid colorimetric assay. Two methods were employed; both depend on the periodate oxidation of KDO which yields a chromogenic compound, β-formylpyruvate, that readily combines with thiobarbituric acid (TBA) to form the chromophore (145). The assays are applicable only to free KDO and numerous factors have been shown to interfere with the reaction. Because the test is dependent on the initial oxidation of KDO, any compound or condition interfering with this step would inhibit color formation. One such condition might be the exhaustion of periodic acid through the oxidation of non-KDO material. The presence of large amounts of carbohydrate, e.g., glycan, might lead to this problem. However, the periodic acid used in these assays was believed to be in excess. All of the *Bacteroides* preparations in this colorimetric test displayed a strong color, characteristic of the chromogen malonaldehyde, which is derived from numerous compounds including 2-deoxysugars. Although both assays correct for such interferring compounds, the presence of high levels of this chromogen may mask low levels of the KDO-derived chromogen. Another interferring compound, fucose, has been shown to decrease the absorbance of β-formyl pyruvate-TBA chromophore in the Warren method by 35%. This is particularly important since fucose is present in high levels in all but two *Bacteroides* LPS.

Although LPS from most gram negative bacteria contain KDO and heptose, the possible absence of these sugars in *Bacteroides* is not unique as there are reports of several other bacterial species apparently lacking these components (35, 62, 151). Unfortunately, few detailed structural investigations on these LPS have been reported. If KDO is absent, an alternative acid-labile linkage must be present. One such possibility would be a furanoside linkage between the reducing terminus of the polysaccharide chain and the lipid moiety. Recently, studies on *E. coli* K-12 (95) and *Pseudomonas aeroginosa* (15) have demonstrated that L-rhamnose is present in the core region. Although its location in *P. aeruginosa* is unclear, it has been shown in *E. coli* K-12 to be a side chain substituent of KDO. In the absence of KDO, an alternative linkage although less acid labile could also be a rhamnosyl linkage.

Gel-filtration Studies

One effect of mild acetic acid hydrolysis on lipopolysaccharides from enteric bacteria is the partial degradation of this complex into fragments corresponding to O-antigenic side chains and core polysaccharide regions. These regions can readily be separated by gel-filtration techniques. Assuming *Bacteroides* LPS splits in a similar way, then gel-filtration profiles provide further evidence for a different LPS architecture in *Bacteroides* particularly in the core region. Gel filtration profiles of *Bacteroides* suggest that mild acid hydrolysis results in greater amounts of higher molecular weight polymers and very little core-type material. These profiles also parallel

the chemical studies. The two strains showing lower levels of peak I material, *B. distasonis* and *B. thetaiotaomicron*, also lacked high levels of fucose and rhamnose, two sugars known to be important side chain constituents in certain strains. Among the other *Bacteroides* strains, the presence of fucose was correlated with the amount of peak I material. The low level of core region material was also in agreement with the apparent lack of KDO and heptose and lower levels of phosphorus in the *Bacteroides* LPS.

If peak I does represent side chain material, then *B. distasonis* and *B. thetaiotaomicron* may represent semi-rough or leaky mutants, strains whose number of side chains per cell is diminished. Studies on some *Pseudomonas* (16) and *E. coli* (63) strains have demonstrated a heterogeneity of LPS in the cell walls of these organisms. In addition to the complete LPS complex, core regions are present without side chains and some are substituted with incomplete side chains. In the latter case, peaks elute at points intermediate between high molecular weight material (peak I) and core region (peak II). In profiles of *B. vulgatus*, *B. thetaiotaomicron*, and *B. ovatus*, peaks were observed in this area. Interpretation of profiles must be cautious due to the possible interference of capsular and glycan material. From the lower glucose levels of peak I material as compared with LPS, it may be assumed that glycan material in four of the strains, if present, occurs in small amounts. Amounts of peak I material can also be correlated with the degree of capsulation, e.g., 0038-1. Comparison of sugar composition of capsular, LPS, and peak I material fail to clarify this

problem since conflicting results are obtained with galactosamine, mannose, and rhamnose, see tables 21, 22, 24, and 31. As mentioned earlier, the Bacteroides for the gel-filtration studies were grown under conditions for maximum cell yield so that capsulated cells are believed to represent only a small fraction of the total cell number. Finally the lower levels of peak I in B. distasonis and B. thetaiotaomicron may also be the result of less acid labile linkages.

The profiles might also disclose aspects of the overall picture of LPS-cell wall organization and biosynthesis. It has been shown that with enteric organisms, the heterogeneity of the product of the mild acid treatment is due to the presence in the cell wall of some core region material which have not been substituted by O-specific side chains. Therefore it may be hypothesized that very little 'core' material exists in the Bacteroides cell wall that is not conjugated with 'side chains'. Under conditions that may limit core region biosynthesis, e.g., growth in simple medium, side chain material may be over-produced and subsequently, collects in the form of a capsule.

Chemotypes of the Bacteroides Reference Strains

Variations in the sugar constituents of LPS and of the other antigen extracts do imply distinct chemotypes, e.g., the simpler sugar patterns of B. distasonis and B. thetaiotaomicron. However, the extent to which these various sugars contribute to the basic LPS chemotypes of the individual strains cannot be determined until purified material has been prepared and the contaminants, particularly

the glycan and capsular material, have been removed. Variation in levels of galactosamine, galactose, and mannose in the two *B. fragilis* strains 2393 and 2553 can be attributed to differences in amounts of capsular material in the sample being examined. Similar problems exist between other strains. Despite these discrepancies, the sugar patterns of both *B. fragilis* strains were in agreement with those reported by Hofstad (57). He reported the presence of galactosamine and mannose as well as large amounts of glucose (87.3 mole per cent) in *B. fragilis* 2553 suggesting that his isolated LPS contained glycan and possibly capsular material. Furthermore, he reported similar compositions in two other *B. fragilis* strains. The high values of glucose and galactose disagree with the findings of Kasper (74) who reported the absence of both sugars. This discrepancy is believed to relate to the problem of non-LPS contaminants.

Xylose (97, 147) and sialic acids (74) have also been reported as constituents of *B. fragilis* LPS. Neither of these components were identified in this study. However, it was noted that fucose ran to a position very close to xylose in the paper chromatography solvent systems employed and may have been misidentified in the previous studies. An unidentified sugar component was observed in 5482. The compound was liberated early in short term acid hydrolysis, was aniline-pthalate reactive, and had mobility in chromatography studies similar to that of the 3,6-dideoxyhexose, colitose. The color with aniline-pthalate, although weak, appeared similar to that given by colitose.

The chemical composition of each strain did not vary significantly in the four extracts examined, although an enrichment of some neutral and amino sugars was observed in the peak I material. Components found in trace amounts in LPS were generally not detected in the whole cell extracts and this probably reflects the sensitivity of the methods used to detect the sugars.

The high levels of glycan material may explain the discrepancies observed in the hemagglutination studies. If large amounts of glycan were extracted in relation to O-antigenic material, then the phenol-sulfuric acid assay would have indicated much higher levels of carbohydrate and very little serologically active material would have been available to adsorb onto the red blood cells. Likewise, glycan material may have physically interferred with the adsorption process. Throughout this investigation, glycan material was believed to be an intracellular contaminant. However, it may be part of the cell wall complex since simple glycans, such as mannans, and galactans, linked to core polysaccharides have been reported in other bacteria (81).

Electron Microscopy of Bacteroides LPS

Electron micrographs of Bacteroides LPS disclose a bilayer structure similar to that commonly observed with LPS from other gram negative organisms. A less electron dense crust was associated with the surfaces of the trilaminar layers in all of the Bacteroides preparations and may represent the contaminating material known to be present in these preparations. However, the LPS samples of some of the facultative organisms, also known to contain extraneous material,

did not appear to have this crust. Therefore, this crust may represent distinct morphological differences between LPS isolated from Bacteroides and facultative bacteria or could possibly be the glycan material described in the chemical studies.

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APPENDIX 1. Reagents and Media

Table 33. Trypticase - Yeast Extract - Glucose (TYG)[a] Medium

Ingredient	Amount	Source
Trypticase	1 g	BBL
Yeast extract	1 g	Difco Laboratories
Resazurin solution[b]	0.4 ml	-
Salts solution[c]	4.0 ml	Fisher Scientific
Distilled water	100.0 ml	-
Vitamin K- heme solution[d,e]	1.0 ml	Sigma Chemical Co.
Cysteine-HCL-H_2O[e]	0.05 g	Sigma Chemical Co.
Glucose[f]	1.0 g	Fisher Scientific

[a]Dispensed into culture tubes in 25 ml volumes and autoclaved 15 minutes, 121 C, 15 lb/sq. in.

[b]Resazurin solution: Dissolve one resazurin tablet (ca. 11 mg, Allied Chemical 506) in distilled water, 44 ml

[c]$CaCl_2$ (anhydrous), 0.2 g; $MgSO_4$, 0.2 g; K_2HPO_4, 1.0 g; KH_2PO_4, 1.0 g; $NaHCO_3$, 10.0 g; NaCl, 2.0 g; Mix $CaCl_2$ and $MgSO_4$ in 300 ml distilled water until dissolved. Add 500 ml water and remaining salts slowly with stirring until all salts are dissolved. Add 200 ml distilled water, mix, and store at 4 C.

[d]Stock solutions of both menadione and hemin are prepared; 100 mg menadione in 20 ml 95% ethyl alcohol. Filter sterilize; 50 mg hemin dissolved in 1.0 ml IN NaOH; make to 100 ml with 100 ml distilled water. Autoclave at 121 C for 15 min. Add 1 ml sterile menadione stock solution to 100 ml hemin stock solution.

[e]Add after boiling.

[f]Other carbohydrate substrates may be substituted.

Table 34. High Trypticase - Yeast Extract - Glucose (hTYG) Medium[a]

Ingredient	Amount
Vitamin K - Hemin Solution[b]	10 ml
Trypticase	200 g
Glucose	120 g
Yeast extract	50 g
8 N NaOH	8.0 ml
Salts solution[c]	20.0 ml
Cysteine - HCL-H_2O	6 g
Sodium formaldehyde sulfoxalate (Eastman Kodak Co.)	6 g
Phosphate buffer[d]	1000 ml

[a]Ingredient amounts are for 20 liter batches; above mixture is dispensed in equal amounts into eight 3.0 liter flask; enough water is added to bring up to 2.5 liters per flask. Flasks are autoclaved 45 min. 121 C, 15 lbs with slow exhaust, and cooled under O_2 free N_2 gas.

[b]See TYG medium

[c]See TYG medium

[d]1.0 M, pH 7.0; stock solution: 5 parts 1M KH_2PO_4, 4 parts 1M K_2HPO_4. Store with chloroform at 4 C.

Table 35. Peptone-Yeast Extract-Glucose (PYG)[a] Medium

Ingredient	Amount
Peptone (BBL)	1.0 g
Yeast extract	1.0 g
Resazurin solution[b]	0.4 ml
Salts solution[b]	4.0 ml
Distilled water	100.0 ml
Glucose[c]	1.0 g
Cysteine-HCL-H_2O[d]	0.05 g
Vitamin K-heme solution[b,d]	1.0 ml

[a]Dispensed in 25 ml volumes and autoclaved 15 minutes, 121 C, 15 lb/sq. in.

[b]See TYG medium.

[c]Other carbohydrate substrates may be substituted.

[d]Added after boiling.

Table 36. Chopped Meat Medium (CM)[a]

Ingredient	Amount
Ground beef (fat free)[b]	500 g
Distilled water[b]	1000 ml
1N NaOH[b]	25 ml
Trypticase or peptone	30 g
Yeast extract	5 g
Potassium phosphate	5 g
Resazurin solution[c]	4 ml
Cysteine-HCl-H_2O[d]	0.05 g

[a]CM medium is dispensed in 7 ml volumes containing meat particles (1 part meat particles to 4 to 5 parts fluid) and autoclaved 15 minutes, 121 C, 15 lb/sq. in.

[b]500 g of lean beef or horsemeat, stripped of all fat and connective tissue is ground. 1 liter of distilled water and 25 mls 1 N NaOH are mixed with the meat and the mixture is heated to a boil with stirring. The medium is cooled to room temperature, the fat layer is skimmed off, and the solution filtered, retaining both meat particles and filtrate. The filtrate is restored to its original volume with distilled water and the rest of the ingredients are added.

[c]See TYG medium.

[d]Added after boiling.

Table 37. Brain Heart Infusion Medium Supplemented (BHI)[a]

Ingredient	Amount
Brain Heart Infusion broth (Difco) (dehydrated)	3.7 g
Yeast extract	0.5 g
Distilled water	100.0 ml
Resazurin solution[b]	0.4 ml
Vitamin K - heme solution[b]	1.0 ml
Cysteine-HCl-H_2O[c]	0.05 g

[a]For liquid medium BHI broth is dispensed in 5 and 25 ml amounts and autoclaved 15 minutes, 121 C, 15 lb/sq. in. BHI medium is also used for plate media (BHIA) and roll tubes (BHIA) in which case BHI broth is dispensed in 10 ml volumes into tubes containing 0.25 g agar.

[b]See TYG medium.

[c]Added after boiling.

Table 38. Modified Glucose-Minimal Salts-Biotin (GMB)[a] Medium

Ingredient	Amount
Salts A[b]	25 ml
$(NH_4)_2SO_4$ B[c]	25 ml
$FeSO_4$[d]	2.5 ml
Glucose	25 g
Heme-Vitamin K Solution[e]	2.5 ml
Vitamin Solution[f]	2.5 ml
Phosphate Buffer[g]	125.0 ml
Cysteine-HCl-H_2O[h]	1.0 g
Distilled Water	2275 ml
Resazurin[i]	

[a]Recipe for 2.5 liter batch. Heat to dissolve components but do not boil. Do not pH. For 2.5 L batches (flask) autoclave 40 minutes, 121 C, 15 lb/sq. in., slow exhaust, and cool under N_2. For tubes, dispense in 20 ml volumes under CO_2. Autoclave 15 minutes, fast exhaust.

[b]10 X stock solution: $CaCl_2$, 0.2 g/L; $MgCl_2$, 0.2 g/L; KH_2PO_4, 9.0 g/L; NaCl, 9.0 g/L. Store at 4 C.

[c]100 X stock solution: $(NH_4)_2SO_4$, 100 g/L.

[d]Stock solution: 0.1 g/100 ml.

[e]See TYG medium.

[f]Stock solution, 100 ml: 20 mg each of thiamin-HCl, calcium-D-pantothenate, nicotinamide, riboflavin, and pyridoxide-HCl; 1 mg p-aminobenzoic acid, 0.25 mg each of biotin and folic acid, and 0.1 mg Vitamin B_{12}. Taken from Varel, V. H. and M. P. Bryant. 1974. Nutritional features of Bacteroides fragilis subsp. fragilis. Appl. Microbiol. 18:251-257.

[g]see hTYG medium.

[h]Add after heating.

[i]Add 10 ml of solution (see TYG medium) for tubed media only.

Table 39. Sodium Bicarbonate Solution (10%)[a]

Ingredient	Amount
Sodium bicarbonate	10 g
Distilled water	to yield 100 ml

[a]Heat to dissolve ingredients, cool, dispense into tubes in 25 ml volumes under O_2- free CO_2. Autoclave 15 minutes 121 C, 15 lb/sq. in.

Table 40. Soft Agar Medium[a]

Ingredient	Amount
Trypticase	0.2 g
Yeast extract	0.05 g
Agar (Difco)	0.4 g
Distilled water	100.0 ml

[a]Dispensed into screw capped culture tubes in 5 ml amounts and autoclaved 15 minutes, 121 C, 15 lb/sq. in.

Table 41. Trypticase Soy Agar Slants[a]

Ingredients	Amount
Trypticase Soy Agar[b] (Blood Agar Base) BBL	40 g
Distilled water	1000 ml

[a]Dispensed in 15 ml amounts in screw-capped tubes, autoclaved 15 minutes, 121 C, 15 lb/sq. in., and placed on an angle until agar has solidified.

[b]Contains following ingredients in grams per liter; Trypticase (BBL pancreatic digest of casein), 15 g; Phytone (BBL Soy peptone), 5 g; sodium chloride, 5 g; agar, 15 g.

Table 42. Phosphate - Buffered Saline (PBS)[a]

Ingredient	Amount
Sodium Phosphate Dibasic (anhydrous)[b]	1.20 g
Sodium Phosphate Primary[b]	0.22 g
Sodium Chloride	8.50 g
Distilled water	to yield 1.0 liter

[a]0.01 M Phosphate buffer, pH 7.5

[b]The potassium salt can be substituted for cold room use.

Table 43. EDTA - Saline Buffer[a,b]

Ingredient	Amount
Sodium chloride	87.0 g
Ethylenediaminetetracetic acid (EDTA)	29.0 g
Distilled water	to yield 1.0 liter

[a]0.15 M NaCl, 0.01 M EDTA

[b]Dissolve NaCl first, then add EDTA. Adjust pH to 8 with 1N NaOH.

APPENDIX 2. Colorimetric Tests and Derivatizations

Test 1. Total carbohydrate - Phenol-sulfuric acid assay

Reagents: A. 5% phenol (w/v) (crystals, Baker)

B. Conc. H_2SO_4

Protocol:
1. 0.2 ml sample
2. Make up to 1.0 ml with distilled water
3. Add 1.0 ml 5% phenol solution A
4. Add 5.0 ml conc H_2SO_4 rapidly, with stirring
5. Let stand at room temperature for 30 minutes
6. Read at 490 nm (hexoses), 480 nm (6-deoxy sugars, pentoses, uronic acids)

References:
1. DuBois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

Test 2. Total Carbohydrate - α-Napthol reaction

Reagents: A. Sulfuric acid: 89% by volume

B. α-Napthol (Sigma Chemical Co.): 2% alcoholic solution

Protocol:
1. 0.5 ml sample (5 to 25 ng sugar) cooled in an ice bath
2. Add 4.5 ml solution A with cooling in ice bath. Mix.
3. Transfer tubes to tap water, then to boiling water bath for 3 minutes.
4. Cool tubes in tap water.
5. Add 0.2 ml α-Napthol solution B, mix well.
6. Read at 560 nm after 6 hours.

References:
1. Dische, Z. 1955. In D. Glick (ed.). Methods of biochemical analysis. 2:313. Interscience, New York.
2. Williams, C. A., M. W. Chase (ed.). 1968. Method in immunology and immunochemistry. 2:285. Academic Press, New York.

Test 3. Hexosamine - Modified Rondle and Morgan

Reagents:
A. Acetylacetone solution - 0.2 ml of acetylacetone in 10 ml 0.5 N Na_2CO_3
B. Ethyl alcohol
C. Ehrlich's reagent - 0.8 g p-dimethylaminobenzaldehyde in 30 ml absolute ethanol. Add 30 ml concentrated HCl. Mix

Protocol:
1. Transfer sample containing free hexosamine (10 to 100 µg) to 10 ml volumetric flask. Total volume should be 4 ml
2. 1 ml reagent A is added. Mix by rotation
3. Stopper flasks and place in boiling water for 30 minutes
4. Cool to room temperature
5. Add 3 ml absolute ethanol. Mix
6. Add 1 ml reagent C with stirring
7. Make up to mark with absolute ethanol
8. Stopper and mix thoroughly
9. Place in 37 C bath for 30 minutes
10. Cool to room temperature and read at 540 nm

Reference:
1. Rondle, C. J. M. and W. T. J. Morgan. 1955. The determination of glucosamine and galactosamine. Biochem. J. 61:586-589.

2. Williams, C. A. and M. W. Chase (eds.). 1968. Methods in immunology and immunochemistry. Vol. 2. Academic Press, New York, p. 296-298.

Test 4. Protein Estimation - Lowry Method

Reagents:

A. 30 g $NaCO_3$ (Fisher Scientific Co.) plus 6 g NaOH (Fisher Scientific Co.). Adjust up to 1 liter with distilled water.
B. 0.5% $CuSO_4$ (Fisher Scientific Co.) (100 ml solution)
C. 1% NaK tartrate (100 ml solution)
D. Folin Reagent: dilute 1:3 concentrate reagent (2N) (Fisher Scientific Co)

Protocol:

1. Mix 50 ml solution A to 1 ml of Solution B and Solution C (already premixed)
2. Add 5.0 ml of above mixture to 0.5 ml sample containing 1 to 200 ng protein (BSA as standard). Mix, and allow to stand 10 minutes at room temperature.
3. Add 0.5 ml diluted Folin reagent to each tube. Mix.
4. After exactly 10 minutes, read at 650 nm.

References:

1. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193:265-275

Test 5. Formaldehyde Determination

Reagents:
- A. 0.02 M periodic acid solution - 25 mls 0.04 M periodic acid + 25 mls 0.065 M sodium bicarbonate.
- B. Nash reagent - 15 g ammonium acetate + 0.3 ml acetic acid + 1.0 ml acetyl acetone; make up to 100 ml.
- C. Rhamnose solution - 410 mg in 25 ml distilled water.

Protocol:
1. 1 ml sample in 25 ml volumetric flask.
2. Add 0.5 ml of reagent A. Mix.
3. Let stand for 10 minutes at room temperature.
4. Add 0.5 ml rhamnos solution C. Mix.
5. Wait 5 minutes, then add 1 ml Nash reagent B.
6. Let stand for 15 minutes at 50 C.
7. Adjust volume to mark with distilled water.
8. Mix and read at 412 nm against reagent blank.

Reference:
1. Vaskovsky, V. E. and S. V. Isay. 1969. Quantitative determination of formaldehyde liberated with periodate oxidation. Anal. Biochem. 30:25-31.

Test 6. Ester-Bonded Fatty Acids - Hydroxylamine Reaction

Reagents:
A. Hydroxylamine reagent: 2 g Hydroxylamine (Sigma Chemical Co.) dissolved in 15 ml distilled water and 15 mls 2 N NaOH.
B. $FeCl_3$ reagent: 0.37 M $FeCl_3$ (Fisher Scientific Co.) dissolved in 0.1 N HCl.
C. 3.3 M HCl

Protocol:
1. To dried sample add 2 ml absolute ethyl alcohol.
2. Add 1 ml Hydroxylamine reagent A.
3. Incubate 20 minutes at room temperature or 2 minutes at 60° C.
4. Add 1.5 ml reagent C, mix.
5. Read at 520 nm after at least 20 minutes.

References:
Shapiro, B. 1953. Purification and properties of a lysolecithinase from pancreas. Biochem. J. 53:663-666.

Test 7. 6-Deoxyhexoses - Cysteine-sulfuric acid method

Reagents:
A. Sulfuric acid: 6 parts concentrated H_2SO_4 plus 1 part water by volume.
B. Cysteine hydrochloride: 3% solution.

Protocol:
1. To 1 ml sample solution (3 to 10 μg of 6-deoxyhexose) add 4.5 ml chilled reagent A slowly in an ice bath. Mix carefully.
2. Warm to room temperature in a water bath.
3. Place in boiling water for 3 minutes*
4. Cool to room temperature in a water bath.
5. Add 0.1 ml reagent B. Mix.
6. Measure difference between 396 nm and 429 nm after 2 hours.

*5 or 10 minutes may be substituted. A 3 minute reaction permits simultaneous determinations of hexoses. For hexose the difference is measured between 414 nm and 380 nm after 30 minutes.

Reference: 1. Dische, Z. and L. B. Shettles. 1948. A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem. 175:595-603.

Test 8. Phosphorus Estimation

Reagents:
- A. 10 N H_2SO_4
- B. 15% w/v sodium bisulfite; filter if necessary
- C. 20% w/v sodium sulfite
- D. Molybdate III: 2.5% w/v ammonium molybdate in water.
- E. 0.25% w/v 1, 2, 4, Aminonapthol sulfonic acid: Dissolve 0.5 g in 195 mls of 15% sodium bisulfite, reagent B; add 5 mls 20% sodium sulfite, if needed, to dissolve powder.

Protocol:
1. Add 2.5 ml reagent A to sample and heat until charring (performed in a micro-Kjeldahl flask). Include a glass bead when heating.
2. After charring or fumes have disappeared add 2 drops 30% peroxide.
3. Allow fumes and brown discoloration to disappear.
4. Cool.
5. Rinse flask into 50 ml volumetric flask with 35 mls distilled water.
6. Add 5.0 mls reagent D.
7. Add 2 mls reagent E.
8. Dilute to mark and mix.
9. Read after 20 min. at 820 nm.

Note:
1. Include two 80 μg P standards, one heated, one unheated, and one heated water blank.
2. Use KH_2PO_4 in 0.1 N H_2SO_4 in standard.

References:
1. Kabat, E. A. (ed.). 1961. Kabat and Mayer's Experimental Immunochemistry, 2nd ed. p. 484-487. Thomas, Springfield, Illinois.

Test 9. Determination of Nucleic Acids and Proteins (Spectrophotometrically)

Reagents: A. 0.01 N NaOH

Protocol:
1. Dissolve sample (up to 50 ng nucleic acid) in 2 ml reagent A.
2. Read at 260 nm and 280 nm.

References:
1. Kabat, E. A. (ed.). 1961. Kabat and Mayer's Experimental Immunochemistry, 2nd ed. p. 708. Thomas, Springfield, Illinois.

Test 10. Heptose

Reagents: A. 5 ml H_2O + 30 ml concentrated H_2SO_4

B. 600 mg L-cysteine-HCl dissolved up to 20 ml with H_2O (3%)

Protocol: NOTE: Prepare a duplicate of both standards and samples

1. 1.0 ml sample (up to 20 μg heptose)
2. Cool in an ice bath
3. 4.5 ml reagent A added; mix well at 4 C
4. After 3 minutes place in a 20 C water bath for 3 minutes
5. Place sample in boiling water bath for 10 minutes
6. Cool to room temperature
7. To one set of samples add 0.1 ml reagent B. To the other set add 0.1 ml H_2O
8. Store in dark for 2 hours at room temperature
9. Measure the difference between 505 and 545 nm
10. Substract the readings of the samples with water from the samples containing reagent B

Reference:

1. Osborn, M. J. 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of Salmonella typhimurium. Proc. Natl. Acad. Sci. U.S. 50:499-506.

Test 11. 2-Keto-3-deoxyoctonate (KDO) - Method of Warren

Reagents:
- A. Sodium periodate (meta) 0.2 M in 9 M phosphoric acid
- B. Sodium arsenite 10% in a solution of 0.5 M sodium sulfate - 0.1 N H_2SO_4.
- C. Thiobarbituric acid (Eastman Kodak Co.), 0.6% in 0.5 M sodium sulfate.

NOTE: Prepare all solutions without warming.

Protocol:
1. Sample containing up to 0.05 μ mole of free N-acetylneuraminic acid in 0.2 ml.
2. Add 0.1 ml reagent A. Mix.
3. Let stand for 20 minutes at room temperature.
4. Add 1 ml reagent B.
5. Shake until brown color disappears.
6. Add 3 ml reagent C. Mix.
7. Place in boiling water for 15 minutes.
8. Remove and place in cold water bath for 5 minutes.
9. Extract mixture with 4.3 ml cyclohexanone (Eastman Kodak Co.).
10. Centrifuge 3 minutes and aspirate off sample for readings.
11. Read at 549 and 532 nm and find difference.

Reference: 1. Warner, L. 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234:1971-1975.

Test 12. KDO - Method of Waradarsky and Saslow

Reagents:
- A. 2.28 g H_5IO_6 dissolved up to 100 ml with H_2O.
- B. 4.0 g $NaAsO_2$ dissolved up to 100 ml with 0.5 N HCl (4.0%).
- C. 600 mg 2-thiobarbituric acid dissolved up to 100 ml in boiling water (0.6%).
- D. 5 ml concentrated HCl + 95 ml n-butanol.

Protocol:
1. 0.2 ml sample.
2. 0.2 ml 0.5 N H_2SO_4 is added with mixing.
3. Place in boiling water for 8 minutes.
4. Cool to room temperature.
5. Add 0.2 ml reagent A. Mix.
6. Let stand for 10 minutes at room temperature.
7. Add 0.8 ml reagent B. Mix.
8. Add 3.2 ml reagent C. Mix.
9. Place in boiling water bath for 10 minutes.
10. Cool to room temperature in a water bath.
11. Add 4.0 ml reagent D. Mix.
12. Centrifuge gently for 10 minutes.
13. Aspirate 3.2 ml butanol layer.
14. Measure the difference between 552 and 508 nm.

Reference:
1. Waradarsky, V. S. and L. D. Saslow. 1959. A sensitive colorimetric method for the estimation of 2-deoxy sugars with the use of the malonaldehyde-thiobarbituric acid reaction. J. Biol. Chem. 234:1945-1950.

Test 13. Alditol Acetate Derivatization

Protocol:
1. Sample (10 to 20 mg LPS) hydrolyzed in 2 N H_2SO_4 for 2 hours at 100 C.
2. Hydrolysate cooled to room temperature.
3. Internal standard (xylose, 200 mg) was added to hydrolysate.
4. Hydrolysate applied to an ion exchange column of Dowex I - acetate form.
5. Wash with distilled water until a constant pH (3-4) is recorded.
6. Freeze dry (or rotor evaporate) to dryness the column eluate.
7. Transfer to a microflex vial (Pierce, Rockford, Ill.) with 0.3 to 0.5 ml 0.8 M $NaBH_4$.
8. Reduce sugars for 3 hours at room temperature.
9. Neutralize $NaBH_4$ with 4 M acetic acid i.e., until fizzing stops.
10. Extract solution with chloroform three times to remove lipid, hydrolyphopic polymers, etc.
11. Freeze dry solution.
12. Add methanol: acetic acid (200:1) to dried material and blow down with nitrogen. Repeat 2 to 3 times.
13. Add 0.3 ml acetic anhydride (under-hood) and 1 mg sodium acetate. Shake gently.
14. Heat at 100 C for 4 hours.
15. Cool mixture.
16. Add 1 ml toluene. Mix.
17. Evaporate with nitrogen gas to dryness.
18. Repeat steps 16 and 17 three times.
19. Redissolve residue in 1 ml chloroform.
20. Add 1 ml distilled water. Mix.
21. Draw off chloroform layer and retain.
22. Shake chloroform layer with 1 ml 0.5 N $NaHCO_3$. Centrifuge, retain chloroform layer.
23. Add 1 ml distilled water to chloroform layer. Mix. Centrifuge and retain chloroform layer.
24. Add 1 mg Na_2SO_4 (or $MgSO_4$) to chloroform layer. Centrifuge and aspirate off chloroform layer.
25. Wash Na_2SO_4 with 1 ml chloroform and combine with original chloroform layer (step 24).
26. Evaporate chloroform layer to dryness with nitrogen.
27. Redissolve residue with chloroform (0.3 ml) transfer to microflex vial, store at -20 C until use.

APPENDIX 3. Solvent Systems

I. Paper Chromatography Solvent Systems

Solvent A: Sugar Solvent: Ethylacetate + pyridine + water, (80:20:10)

Solvent B: Hexosamine Solvent: Butanol + pyridine + glacial acetic acid + water, (60:35:0.1:25)

Solvent C: Butanol + pyridine + 0.1 N HCl, (5:3:2,v/v)

Solvent D: 95% (v/v) acetone-water

Solvent E: DAP solvent: Methanol + water + concentrated HCl + pyridine, (80:17.5:2.5:10)

Solvent F: n-Butanol + glacial acetic acid + water, (120:30:50)

Solvent G: Phenol + water, (90:10), with 1 ml NH_4OH (sp. gr 0.880) per 200 ml of solvent.

II. Electrophoresis Solvent Systems

Solvent H: Pyridine + Acetate, (100 ml pyridine, 10 ml glacial acetic acid, 1000 ml water), pH 6.5.

Solvent I: Acetate + Formate, (150 ml glacial acetic acid, 50 ml formic acid, 1000 ml water), pH 1.9.

APPENDIX 4. Paper Chromatography Stains

A. Reducing sugars - Aniline phthalate Method

Reagents: A. 1.6 g pthalic acid (Eastman Kodak Co.) dissolved in 95% (v/v) acetone. Add 1 ml aniline; mix.

Protocol:
1. Dip paper through reagent A.
2. Place in oven at 110 C until color develops.

Reference: 1. Partridge, S. M. 1949. Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature 164:443.

B. Reducing Sugars - Alkaline Silver Method

Reagents:
- A. Silver nitrate solution - 0.1 ml saturated silver nitrate per 25 ml acetone
- B. Alkaline KOH - 0.5 N KOH in 95% ethyl alcohol (2.8 g KOH per 100 mls 95% ethyl alcohol)
- C. 1% (w/v) sodium thiosulfate; aqueous solution

Protocol:
1. Dip paper through reagent A
2. Dry 5 minutes
3. Dip paper through reagent B
4. Fix by dipping in reagent C until background has faded.

Reference: 1. Trevelyan, W. E., D. P. Procter, J. S. Harrison. 1950. Detection of sugars on paper chromatograms. Nature 166:444-445.

C. Amino Compounds - Ninhydrin

Reagents: A. Dissolve 0.2 g ninhydrin (Sigma Chemical Co.) in 100 ml of 95% (v/v) acetone (95 ml acetone + 5 ml distilled water)

Protocol:
1. Dip paper through reagent A.
2. Place in oven at 110° C until color develops.

Sensitivity: Approximately 0.5 μg

D. Sialic acids and related compounds - Thiobarbiturate spray reagent.

Reagents: A. 0.02 M sodium periodate - dissolve 0.214 g sodium periodate per 50 ml 50% (v/v) acetone (50 ml acetone + 50 ml distilled water)

B. TBA solution - dissolve 1.5 g 2-thiobarbituric acid (Eastman Kodak Co.) in 25 ml 50% (v/v) acetone

C. Ethylene glycol solution - Mix ethylene glycol, acetone, and concentrated H_2SO_4 in following proportions: 50:50:0.3

Protocol:
1. Dip (or spray) paper through reagent A.
2. After 15 minutes, dip paper through reagent C. Lay paper flat.
3. After 10 minutes, dip paper through reagent B.
4. Heat paper for 5 minutes at 100 C.
5. Visualize under UV lamp.

Reference: Warren, L. 1960. Thiobarbituric acid spray reagent for deoxy sugars and sialic acids. Nature 186:237.

E. Uronic acids

Reagents: A. 0.2 g napthoresorcinol/100 ml 95% ethanol (0.2%)

B.
1. 18 ml from solution A.
2. Add 2 ml water
3. Add 2 ml H_3PO_4. Shake.

Protocol:
1. Dip paper in reagent B.
2. Air dry.
3. Heat 3 to 4 minutes at 100 C.

Reference: Partridge, S. M. 1948. Biochem. J. 42:238.

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SEROLOGICAL AND CHEMICAL STUDIES ON THE ANTIGENS
OF BACTEROIDES FRAGILIS AND RELATED SPECIES

by

James L. Babb

(ABSTRACT)

The serological and chemical properties of antigens extracted from strains of Bacteroides fragilis and related species belonging to several different DNA homology groups were investigated. Antisera prepared against formalin-treated whole cells suspensions of representative strains were tested against cell suspensions, cell wall preparations, and extracts of homologous and heterologous strains using agglutination, immunodiffusion, and hemagglutination techniques. Serological results indicated that the species were antigenically distinct, although minor cross reactions were observed across species boundaries. However, the serological properties did not appear to distinguish genetic heterogeneity at levels down to approximately 65% homology. Homology groups, including the two B. fragilis subgroups, were relatively homogeneous, although the presence of serotypes within each homology group was suggested. Immunodiffusion tests demonstrated, however, that the "homogeneity" was not always represented by a single "common" antigen but rather implied a mosaic antigen composition for each strain. A minimum of ten and six different antigenic factors were demonstrated on B. fragilis 2393 and 2553, respectively. Similar mosaics were observed with members of the other species. Hemagglutination patterns using crude antigen extracts were also consistent with the antigenic mosaic.

Many of the strains were found to be capsulated. Preliminary studies demonstrated a similar sugar composition in the capsular material to that in lipopolysaccharide extracted from the same strain with aqueous phenol. Studies also suggested that the capsule influenced the serological properties of the cell.

The chemical make-up of all of the Bacteroides LPS was found to be similar to that of LPS from facultative organisms, although KDO and heptose were not detected in any of the Bacteroides preparations. Electron microscopy of Bacteroides LPS demonstrated a trilaminar structure characteristic of LPS from other gram negative organisms. However, gel-filtration experiments suggested that the Bacteroides LPS may be of a different architecture, particularly regarding the core region. When hydrolyzed by weak acid, Bacteroides LPS behaved differently to LPS from facultative bacteria yielding large amounts of high molecular weight polymers and very little core-type material. B. distasonis and B. thetaiotaomicron displayed lower levels of high molecular weight material, as compared with the other strains, and may represent semi-rough strains.

A comparison of the sugar patterns of the antigen extracts from one member of each homology group implied distinct differences among the strains. However, the sugar patterns of two B. fragilis strains which are genetically more related to each other than to the other species, were very similar. The constituents identified in the Bacteroides LPS were glucosamine, galactosamine, glucose, galactose, mannose, fucose, and rhamnose. An unidentified aniline pthalate

reacting compound with similar mobility to colitose in chromatography studies was observed in the *B. thetaiotaomicron* 5482 LPS. It was not thought justified to assign definite chemotypes to individual homology groups due to the possible contamination of LPS preparation with non-LPS material, e.g. capsules or a glycan polymer.