Immunochemistry of the cell surfaces of *Bacteroides bivius* and *Bacteroides disiens*

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Summary. Outer membranes were extracted from seven strains of *Bacteroides bivius* and six strains of *B. disiens* by the Sarkosyl method. Lipopolysaccharides (LPS) were extracted from the same strains by the Proteinase K method, and from three strains of each species by an aqueous phenol method. Analysis of the outer-membrane proteins by SDS-PAGE demonstrated that, within a species, very similar patterns with many shared or common bands were produced, but there were sufficient differences between species to allow separation. Immunoblotting with antisera raised against whole cells of each of the type strains showed that many antigens were shared between species. Smooth LPS was present in both species. By immunoblotting, the O-antigen of *B. disiens* was shown to be common to all six strains, and there was no cross-reaction between the *B. disiens* antiserum and *B. bivius* LPS. The O-antigen of *B. bivius* was not detected by immunoblotting with homologous antiserum, but antiserum to *B. bivius* reacted with a series of common low molecular mass antigens that were present in LPS preparations from strains of both species.

Introduction

Bacteroides bivius and *B. disiens* are closely related species of anaerobic, gram-negative bacteria that share the same main ecological niche, being most commonly isolated from the vagina and cervix in both health and disease and from genital infections in the male (Holdeman and Johnson, 1977; Duerden, 1980). Both species are non-pigmented, bile sensitive, weakly saccharolytic and proteolytic, and do not produce indole. They are differentiated by the ability of most *B. bivius* strains to produce acid from lactose and galactose whereas no strains of *B. disiens* ferment either of these sugars. DNA hybridisation has shown that they are distinct species (Holdeman *et al.*, 1977; Holdeman and Johnson, 1977).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell-surface proteins has been a useful aid in the identification of species and sub-types of a range of bacteria, including anaerobes (Poxton and Brown, 1979; Moore *et al.*, 1980). The aim of the present study was to use SDS-PAGE in conjunction with immunoblotting to compare the outer-membrane proteins and lipopolysaccharide (LPS) of several strains of *B. bivius* and *B. disiens* to determine any inter- or

intra-species similarities or differences. The Sarkosyl method for the preparation of outer-membrane proteins and the Proteinase K method for LPS were chosen for their simplicity and applicability to small culture volumes; these advantages make the methods particularly useful for diagnostic or taxonomic investigations. LPS extracted by the Proteinase K method was compared with that extracted by the classical aqueous phenol method.

Materials and methods

Bacterial strains

B. bivius strains VPI 6822 and VPI 6318, and *B. disiens* strains VPI 8057 and VPI 7582 were obtained from the Virginia Polytechnic Institute and State University, Blacksburg, VA, USA. Strain GNAB 12 was isolated from a high vaginal swab in this department. The MW series of strains was supplied by Dr B. Watt, City Hospital, Edinburgh. Identity of strains was confirmed by the method of Brown *et al.* (1989). Tests included bile tolerance, indole production, fermentation of glucose, lactose, sucrose and maltose, and gelatinase activity.

Preparation of outer membrane

Cultures were prepared by seeding 500-ml volumes of pre-reduced proteose peptone-yeast extract (PPY) broth (Deacon *et al.*, 1978) with 25 ml of 48-h starter cultures

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in the same medium. They were incubated anaerobically (H_2 90%, CO_2 10%) for 24 h at 37°C. The cells were harvested and washed once in 200 ml of phosphate-buffered saline (50 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl; PBS). The centrifuge tubes were drained and the pellet was suspended in 5 ml of distilled water. The cells were broken by passage through a French pressure cell (Aminco, Silver Springs, MD, USA) at 6000 psi (42 MPa) at 0°C. Unbroken cells were removed by centrifugation at 6000 *g* for 10 min at 4°C. A modification of the method of Filip *et al.* (1973) was used for Sarkosyl treatment. One volume of a 7% w/v solution of sodium N-lauroyl sarcosinate was added to nine volumes of the broken-cell suspension. The insoluble outer membrane was harvested and washed once in 5 ml of distilled water by centrifugation at 50 000 *g* at 4°C for 1 h. The pellet was resuspended in 0.5 ml of distilled water by repeated aspiration into a syringe through a 26-gauge needle. The suspension was stored at -20°C. Protein was assayed by the method of Lowry *et al.* (1951).

Preparation of LPS

The Proteinase K method for LPS extraction was modified from Hitchcock and Brown (1983). Volumes (5 ml) of the cultures prepared for Sarkosyl extraction were harvested and washed twice in PBS by centrifugation at 4000 *g* at 4°C for 15 min. The pellet was suspended in PBS to an A_{525} of 0.6 and 1.5-ml volumes were centrifuged at approximately 10 000 *g* for 90 s (Microfuge Beckman Model B). The pellet was solubilised in 50 μ l of PAGE sample buffer (0.0625 M Tris-HCl, pH 6.8, containing sodium dodecyl sulphate 2% w/v, glycerol 10% v/v, 2-mercaptoethanol 1% v/v and bromophenol blue 0.001% w/v) and heated in a boiling water-bath for 10 min. Proteinase K (25 μ g) (Sigma Protease XI) in 10 μ l of sample buffer was added to each tube and then incubated at 60°C for 1 h. Samples were stored at -20°C.

The aqueous phenol method for the extraction of LPS was that of Poxton and Brown (1986).

Carbohydrate was assayed by the method of Dubois *et al.* (1956).

SDS-PAGE

SDS-PAGE was performed on 10% slab gels with the Laemmli buffer system (Laemmli, 1970) by the method of Poxton and Brown (1979). Samples (25 μ l or 100 μ l) of the Proteinase K LPS extracts, 50- μ l or 100- μ l samples of the aqueous phenol extracts containing 10 μ g or 20 μ g of carbohydrate respectively, or 50- μ l samples containing 9–25 μ g of protein of the Sarkosyl preparations were loaded on to the gels. The LPS separating gels were stained by a modification of the silver stain methods of Tsai and Frasch (1982) and Hitchcock and Brown (1983). The gel was fixed in 200 ml of propan-2-ol 25% v/v, acetic acid 7% v/v overnight, and then oxidised in freshly prepared periodic acid 1.05 g in 150 ml of distilled water

containing 4 ml of the above fixative for 5 min. The gel was then washed in at least four changes of 200 ml of distilled water over 4 h, drained and added to fresh ammoniacal silver nitrate solution—1.4 ml of ammonia solution (SG 0.88) was added to 21 ml of NaOH 0.36%, and 4 ml of $AgNO_3$ 19.4% solution was added with vigorous agitation; after the brown precipitate had disappeared, the volume was made up to 100 ml with distilled water. After 15 min, the gel was washed in at least four changes of distilled water over 40 min and then transferred to a fresh solution of citric acid 0.005% in 200 ml of formaldehyde 0.019% at 25°C. When the desired staining intensity was reached, the gel was washed repeatedly in large volumes of distilled water.

The Sarkosyl outer-membrane gels were stained with Coomassie blue by the method of Poxton and Sutherland (1976).

Immunoblot transfer

This was based on the method of Towbin *et al.* (1979) as described by Cousland and Poxton (1983) with BioRad immunoblotting reagents, but the nitrocellulose membrane was of 0.2- μ m pore size (Sartorius). For dot blotting, 2 μ l of aqueous phenol-extracted LPS was applied directly to the nitrocellulose. Antisera were used at a dilution of 1 in 100.

Preparation of antisera

Antisera were raised in rabbits against washed whole cells of the type strains—*B. bivius* VPI 6822 and *B. disiens* VPI 8057—and against *B. bivius* VPI 6318 by the method of Poxton *et al.* (1982).

Samples of the antisera raised against the type cultures were absorbed with their homologous aqueous phenol-extracted LPS. Serum (2 ml) was mixed with 1 ml of a solution containing 1 mg of LPS (0.2 ml of an aqueous solution of LPS 5 mg/ml w/v + 0.8 ml of PBS) and agitated on a blood-cell mixer at 15°C for 1.5 h. After centrifugation at 10 000 *g* at 15°C for 5 min, the supernate was re-extracted as above. Unreacted LPS and immune complexes were removed by two cycles of centrifugation at 100 000 *g* at 4°C for 3 h, and the supernate was collected and stored at -20°C. The absorbed sera were shown by dot blotting not to react at a final dilution of 1 in 100 with their homologous aqueous phenol LPS.

Results

SDS-PAGE and immunoblotting of Sarkosyl outer-membrane preparations

Fig. 1 shows examples of the patterns obtained after separation and Coomassie-blue staining of Sarkosyl extracts of seven strains of *B. bivius* and six of *B. disiens*. The patterns produced by the *B.*

Fig. 1. SDS-PAGE of Sarkosyl outer-membrane preparations of *B. bivius* and *B. disiens* strains separated on acrylamide 10% slab gels stained with Coomassie blue. Track 1, *B. bivius* VPI 6822; 2, MW 1; 3, MW 3; 4, MW 6; 5, MW 8; 6, MW 15; 7, VPI 6318; 8, *B. disiens* VPI 8057; 9, MW 20; 10, VPI 7582; 11, GNAB 12; 12, MW 19; 13, MW 21. Molecular masses are in Kda.

bivius strains (tracks 1–7) show much similarity to one another with many shared or common bands and only a few strain-specific bands, and have in particular four major common bands of approximately 31, 27, 26 and 21 Kda. The patterns produced by the six *B. disiens* strains (tracks 8–13) show less similarity to each other with more strain-specific bands but still have a great many shared or common bands. One major common band of 23 Kda, and a common duplex of 32–33 Kda are seen. Several bands appeared to be shared by both species, and bands of approximately 90, 66, 53 and 41 Kda appear to be common to all strains of both species.

Three gels identical to those shown in fig. 1 were run and each was transferred to nitrocellulose membranes. One of these blots was probed with antiserum raised against whole cells of *B. bivius* VPI 6822 (fig. 2a) and the second was probed with antiserum to *B. disiens* VPI 8057 (fig. 2b). The third blot was cut to separate the two species and each was probed with its homologous antiserum which had been absorbed with its own LPS. Fig. 2a shows that all the *B. bivius* strains (tracks 1–7) have almost identical profiles except for slight quantitative differences and a few strain-specific antigens. There are many common antigens and the general profile bears some resemblance to the pattern in fig. 1, all the major protein bands being antigenic. A greatly

elongated high-molecular-mass band partially obscuring the separated protein bands and clearly seen in the upper half of the homologous reaction (track 1) and faintly in track 2 were also seen in immunoblots of Proteinase K extracts of the same two strains (see fig. 4a later). Curved bands seen clearly at the front of tracks 1, 3 and 6, and less clearly in tracks 2, 4, 5 and 7, were not seen in the Coomassie blue-stained gel. When these seven Sarkosyl preparations were probed with LPS-absorbed *B. bivius* antiserum, the latter two described bands were not seen, indicating that they were LPS antigens. Antigens of 53, 46 and 29 Kda were also not detected with the absorbed serum.

The heterologous reactions in tracks 8–13 show that there are a considerable number of *B. disiens* antigens that cross-react with *B. bivius* antiserum. Again, some of these antigens correspond to the *B. disiens* outer-membrane-protein bands in fig. 1. A few of the bands (122, 72 and 27 Kda) appear to be common to *B. bivius* and *B. disiens* but three of the four major common protein antigens of *B. bivius* appear to be species specific. The 23- and 33-Kda common protein bands were detected by the *B. bivius* antiserum.

Fig. 2b shows the same strains as in fig. 1, probed with the antiserum raised against *B. disiens* VPI 8057. The six *B. disiens* strains (tracks 8–13) share common antigens of 122, 118, 72, 52 and 33 Kda.

Fig. 2. Immunoblot of Sarkosyl outer-membrane preparations of *B. bivius* and *B. disiens* after separation as in fig. 1 and transfer to nitrocellulose membrane. Tracks were loaded with the same samples as in fig. 1. Gels were probed with (a) *B. bivius* VPI 6822 and (b) with *B. disiens* VPI 8057 whole cell antisera, followed by anti-rabbit IgG-horseradish peroxidase conjugate and colour development. Molecular masses are in Kda.

Several antigenic bands, seen most clearly in track 11, of 118, 52 and 33 Kda correspond to the protein bands in fig. 1. In the upper half of the other tracks, several extra bands, not revealed by the Coomassie blue-stained gel, have largely obscured the protein bands. These are shown to be LPS antigens below. When these six Sarkosyl preparations were probed with LPS-absorbed *B. disiens* antiserum, all tracks, with the exception of a few strain-specific bands, were identical to each other and to the pattern produced by strain GNAB 12 (track 11) in fig. 2b.

Some of the low-molecular-mass protein bands, including the major 23-Kda common protein, are not apparently antigenic.

The heterologous reactions (tracks 1–7) again reveal a considerable number of cross-reacting antigens that are identical to the corresponding tracks in fig. 2a. However, in fig. 2b many of the bands in the lower half of the gel are either absent or gave very weak reactions. The elongated high-molecular-mass band seen in tracks 1 and 2 in fig. 2a were absent in fig. 2b.

SDS-PAGE and immunoblotting of aqueous phenol and Proteinase K LPS extracts

Fig. 3 shows examples of patterns obtained after separation and silver staining of aqueous phenol extracts of three strains of *B. bivius* and two of *B. disiens*. With the exception of *B. bivius* strain MW8 (track 3) even when overloaded, smooth LPS ladder patterns, resembling those found in some Enterobacteriaceae, were seen. A considerable amount of material was seen at the front of all tracks. The *B. bivius* tracks had in common a series of four or so low-molecular-mass bands which may be a low-molecular-mass ladder pattern, and an elongated high-molecular-mass band. The *B. disiens* tracks had two common bands in addition to the ladder pattern. The first was a high-molecular-mass dumb-bell shaped band, and the other was a low-molecular-mass band (both arrowed).

When *B. bivius* aqueous phenol extracts were probed with antisera raised against *B. bivius* strains VPI 6822 and VPI 6318, no smooth LPS ladder pattern was seen. All other bands seen in the silver-stained gel were antigenic; at least three of the series of four low-molecular-mass bands were common antigens and the front material and elongated high-molecular-mass material appeared to be strain specific. Ladder patterns common to all three *B. disiens* aqueous phenol extracts were demonstrated when probed with antiserum raised

against *B. disiens* VPI 8057 (data not shown). These results are identical to those described below for Proteinase K extracted LPS (see fig. 4a and b).

Proteinase K extracts of all strains belonging to both species were separated by SDS-PAGE, transferred to nitrocellulose and probed with homologous and heterologous antisera. With *B. bivius* VPI 6822 antiserum (fig. 4a), all seven extracts of *B. bivius* (tracks 1–7) showed reactions with the series of low-molecular-mass LPS antigens. An elongated high-molecular-mass band in tracks 1 and 2, and an antigen at the front in tracks 1, 3 and 6 correspond to antigens seen in the immunoblot of the Sarkosyl extracts of the same strains probed with the same serum (fig. 2a). Marked cross-reactions are seen with the series of low-molecular-mass antigens from five of the six Proteinase K extracts of *B. disiens* (tracks 8–13); strain GNAB 12 (track 11) showed no heterologous reaction. Faint high-molecular-mass smooth LPS ladder patterns are visible in tracks 8 and 10 (VPI 8057 and VPI 7582 respectively).

In the reaction between *B. disiens* Proteinase K extracts and homologous antiserum (fig. 4b), all six strains were seen to have a smooth LPS ladder pattern (tracks 8–13); the bands in track 11 (GNAB 12) were fainter and more widely spaced than in the other tracks. The low-molecular-mass bands that cross reacted strongly with the *B. bivius* antiserum have reacted only weakly with homo-

Fig. 3. SDS-PAGE of aqueous phenol extracts of *B. bivius* and *B. disiens* strains stained with silver. Tracks 1 and 6, *B. bivius* VPI 6822; 2, VPI 6318; 3, MW 8; 4, *B. disiens* VPI 8057; 5, VPI 7582. Tracks 1–5 were loaded with 10 µg of carbohydrate. Track 6 was loaded with 20 µg to demonstrate ladder pattern.

Fig. 4. Immunoblots of Proteinase K extracts of *B. bivius* and *B. disiens* strains, arranged as in fig. 1, probed with (a) *B. bivius* VPI 6822 and (b) *B. disiens* VPI 8057 whole cell antisera.

gous serum. A strain-specific dumb-bell-shaped antigen is seen near the top of track 8. The *B. bivius* Proteinase K extracts have not cross-reacted with the *B. disiens* antiserum.

Discussion

The Sarkosyl method for the preparation of outer membranes, when applied to *B. bivius* and *B. disiens*, gives protein patterns on SDS-PAGE that are similar to those produced by most gram-negative

bacteria. Many different bands of protein are visualised and there are a few strongly staining bands which are usually classified as the major outer-membrane proteins. The patterns produced by strains belonging to the same species are sufficiently similar to each other but different from other species to allow identification to species level. Gram-negative bacteria that are closely related have cross-reactive outer-membrane antigens (Hofstra and Dankert, 1980) and these two closely related *Bacteroides* spp. have several protein antigens in common.

Immunoblotting of LPS with two *B. bivius* antisera detected a series of low-molecular-mass antigens that were common to that species and that also cross-reacted with a similar series of antigens of slightly different molecular mass in strains of *B. disiens*. These *B. bivius* antisera reacted with their homologous high-molecular-mass antigens but no clear ladder pattern was revealed. They did however, react weakly with high-molecular-mass ladder patterns in two strains of *B. disiens*. Antiserum raised against the type strain of *B. disiens* detected smooth LPS ladder patterns in all strains of that species, but did not cross-react with *B. bivius* LPS.

The LPS of gram-negative bacteria, especially the O antigenic part, is usually considered to be highly variable within a species. The core, however, is often much more conserved and there are regions, especially in the inner core, that are common within a species and even among species. This may explain the shared series of low-molecular-mass LPS antigens of these two species which consist of LPS molecules with a relatively higher proportion of core oligosaccharide than O antigen repeating units. The *B. bivius* strains appear to have produced

a strong anti-core antibody response, whereas the *B. disiens* has produced a strong anti-O response.

In summary, *B. bivius* and *B. disiens* are closely related in respect to outer-membrane proteins although differences permit species identification. The two species are related serologically (Poxton *et al.*, 1982) and we have demonstrated in this study that cross-reactivity is due to shared outer-membrane proteins and low-molecular-mass LPS material, suggesting that they have common core structures. In this study, both species were shown to have smooth LPS; the typical O-antigen ladder pattern of *B. disiens* reacted well with homologous antiserum and appeared to be common to all strains with the possible exception of GNAB 12. A high-molecular-mass ladder pattern of *B. bivius* was not detected by immunoblotting but a strain-specific high-molecular-mass antigen, not resolved into a ladder pattern, was observed.

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REFERENCES

- Brown R, Collee J G, Poxton I R, Fraser A G 1989 *Bacteroides* and related genera. In: Collee J G *et al.* (eds) Mackie and McCartney's Medical Microbiology, vol 2, 13th edn. Churchill Livingstone, Edinburgh.
- Cousland G, Poxton I R 1983 Analysis of lipopolysaccharides of *Bacteroides fragilis* by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electroblot transfer. *FEMS Microbiology Letters* **20**: 461–465.
- Deacon A G, Duerden B I, Holbrook W P 1978 Gas-liquid chromatographic analysis of metabolic products in the identification of *Bacteroidaceae* of clinical interest. *Journal of Medical Microbiology* **11**: 81–99.
- Dubois M, Gilles K A, Hamilton J K, Rebers P A, Smith F 1956 Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**: 350–356.
- Duerden B I 1980 The isolation and identification of *Bacteroides* spp. from the normal human vaginal flora. *Journal of Medical Microbiology* **13**: 79–87.
- Filip C, Fletcher G, Wulff J L, Earhart C F 1973 Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauroyl sarcosinate. *Journal of Bacteriology* **115**: 717–722.
- Hitchcock P J, Brown T M 1983 Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *Journal of Bacteriology* **154**: 267–277.
- Hofstra H, Dankert J 1980 Major outer membrane proteins: common antigens in Enterobacteriaceae species. *Journal of General Microbiology* **119**: 123–131.
- Holdeman L V, Cato E P, Moore W E C 1977 Anaerobic Laboratory Manual, 4th edn. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA.
- Holdeman L V, Johnson J L 1977 *Bacteroides disiens* sp. nov. and *Bacteroides bivius* sp. nov. from human clinical infections. *International Journal of Systematic Bacteriology* **27**: 337–345.
- Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Lowry O H, Rosebrough N J, Farr A L, Randall R J 1951 Protein measurement with the folin-phenol reagent. *Journal of Biological Chemistry* **193**: 265–275.
- Moore W E C, Hash D E, Holdeman L V, Cato E P 1980 Polyacrylamide slab gel electrophoresis of soluble proteins for studies of bacterial floras. *Applied and Environmental Microbiology* **39**: 900–907.
- Poxton I R, Brown R 1979 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of cell-surface proteins as an aid to the identification of the *Bacteroides fragilis* group. *Journal of General Microbiology* **112**: 211–217.
- Poxton I R, Brown R 1986 Immunochemistry of the surface carbohydrate antigens of *Bacteroides fragilis* and definition of a common antigen. *Journal of General Microbiology* **132**: 2475–2481.
- Poxton I R, Sutherland I W 1976 The butanol-soluble proteins of *Klebsiella aerogenes*. *Microbios* **15**: 93–103.
- Poxton I R, Brown R, Collee J G 1982 Detection of species-specific and cross-reactive cell-surface antigens of *Bacteroides* species by an indirect enzyme-linked immunosorbent assay. *Journal of Medical Microbiology* **15**: 223–231.
- Towbin H, Staehelin T, Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the USA* **76**: 4350–4354.
- Tsai C M, Frasch C E 1982 A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**: 115–119.