The Cell-surface Antigens of *Bacteroides vulgatus*

By IAN R. POXTON* AND MADELEINE K.-Y. IP

Department of Bacteriology, University of Edinburgh, Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

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The cell surface of *Bacteroides vulgatus* was examined by electron microscopy. The outer membrane complex was removed by EDTA and mild sonication and the antigens of this complex were characterized by enzyme-linked immunosorbent assay and crossed immunoelectrophoresis. The species-specific antigen was identified and was shown to be the major outer membrane protein with a molecular weight of 100000.

**INTRODUCTION**

*Bacteroides vulgatus*, an obligately anaerobic, Gram-negative, rod-shaped bacterium is the predominant bacteroides organism in the human colon (Finegold *et al.*, 1975; Duerden, 1980). It is occasionally isolated from clinical specimens (Holland *et al.*, 1977). Recently, it was shown that the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the outer membrane (OM) polypeptides of several *B. vulgatus* strains were extremely similar (Poxton & Brown, 1979); and the antigens of the OM complex of bacteroides appear to be species- or subspecies-specific when detected by an enzyme-linked immunosorbent assay (Poxton, 1979).

It is becoming recognized that proteins of the OM of other Gram-negative bacteria may include group- and species-specific antigens that might be useful in serological identification (e.g. Hofstra & Dankert, 1980; Sippel *et al.*, 1978).

The present study characterizes the antigens of the OM complex of *B. vulgatus* and investigates the species-specific antigens. The recognition of species-specific antigens of bacteroides would greatly aid the development of more rapid methods for their identification.

**METHODS**

*Culture of bacteria and preparation of EDTA–outer membrane complex.* Bacteroides vulgatus NCTC 10583 and NCTC 11154, and six laboratory strains (GNAB 9, 25, 30, 107, 120 and WPH 116) which had been characterized in detail according to the criteria of Duerden *et al.* (1980), were cultured anaerobically from freeze-dried inocula in PPY medium (Holbrook *et al.*, 1977) and outer membrane (OM) complexes were prepared by the EDTA–heat–mild sonication treatment previously described (Poxton & Brown, 1979).

*Electron microscopy.* This was based on the method of Springer & Roth (1973). Bacteria were harvested from blood agar plates (5% human blood in Columbia base; Oxoid) or from PPY medium, and suspended in a mixture of 1 ml ruthenium red (1.5 mg ml⁻¹) in water, 1 ml 3.6% (w/v) glutaraldehyde and 1 ml 0.2 M-sodium cacodylate buffer pH 6.5 and held at 0 °C for 1 h. After three washes in 0.07 M-cacodylate buffer, the pellet was suspended in a mixture of 1 ml ruthenium red (1.5 mg ml⁻¹), 1 ml 4% (w/v) osmium tetroxide and 1 ml 0.2 M-cacodylate buffer and incubated for 3 h at 27 °C. The pellet was washed once in 0.07 M-cacodylate buffer and dehydrated in successive 10 min steps in 25%, 50%, 75% and 90% (v/v) ethanol, then in absolute ethanol for two periods of 1 h. Propylene oxide was added for two 10 min periods and the pellet was then immersed in Epon Arealdeite (1:1, v/v) mixture. The pellet was embedded in fresh Epon Arealdeite in disposable capsules and maintained at 45 °C for 24 h then at 60 °C for 48 h. Sections were cut and placed on rhodium-coated copper grids. The thin sections were stained with saturated uranyl acetate in 75% ethanol in the dark for 30 min, then washed once in...
centrifugation (6000 g). The suspension was mixed vigorously in a Waring blender for 30 s. Bacteria were removed by centrifugation (6000 g, 30 min) leaving the capsular material in solution. This was precipitated by the addition of 4 vol. acetone at −18 °C. The precipitate was recovered by centrifugation (4000 g, 30 min), washed once in acetone, dissolved in water and dialysed against distilled water. To purify the carbohydrate fraction from the crude capsular material it was extracted with aqueous phenol: an equal volume of 90% (w/w) phenol was added to the solution of capsular material and stirred vigorously for 15 min at 20 °C. The phases were separated by centrifugation (6000 g, 30 min) and the upper phase was dialysed for 16 h against running tap water, then against distilled water, and finally freeze-dried.

**Lipopoly saccharide (LPS) preparation.** LPS was extracted from freeze-dried bacteria that had been grown for 18 h in PPY medium, by the aqueous phenol procedure developed by Westphal & Lüderitz (1954). It was purified and washed by centrifugation at 100 000 g for 3 h.

**Treatments of OM complex.** Separate samples of the EDTA-released OM complex at a concentration of 0.5–1.0 mg protein ml⁻¹ were subjected to one of the following denaturing or modifying agents: (i) heat (121 °C for 15 min); (ii) formaldehyde (20% at 20 °C for 16 h); (iii) sodium periodate (0.1 M at 20 °C for 16 h); excess periodate was consumed by the addition of ethylene glycol; (iv) urea (8 M at 20 °C for 16 h); (v) pronase (0.1 mg ml⁻¹ in PBS at 20 °C for 16 h); (vi) trypsin (as for pronase). Excess formaldehyde, urea and the periodate reagents were removed by dialysis against PBS and enzymes were inactivated by the addition of ethylene glycol to a final concentration of 0.2%. Controls were included, in which the OM samples were treated as above except that the agent was omitted.

**Antiserum.** Antiserum was raised against washed live cells of *B. vulgatus* NCTC 10583 in New Zealand White rabbits as previously described (Poxton, 1979).

**Enzyme-linked immunosorbent assay (ELISA).** Both the indirect ELISA described by Poxton (1979) and an ELISA-inhibition test were used. The ELISA-inhibition was performed as for the indirect test, except that the antiserum was pre-incubated with the potential inhibitor: antiserum (50 μl), diluted to a concentration twice that of its titre, was incubated with an equal volume of doubling dilutions of treated OM complex (see above) or LPS (1 mg ml⁻¹) for 30 min in a 37 °C water bath; 50 μl volumes of this pre-incubated serum were added to each well. Inhibition was observed by comparing the test with a positive and negative control.

**SDS–PAGE.** This was performed on 10% (w/v) acrylamide slab gels, with the buffer system of Laemmli (1970), by the method described by Poxton & Brown (1979).

**Crossed immunoelectrophoresis (CIE).** EDTA–OM complexes and isolated LPS and capsular polysaccharide were examined by CIE according to the method developed by Weeke (1973), as described by Poxton & Byrne (1981). CIE was also performed, as indicated, with SDS–PAGE in the first dimension.

**Analytical techniques.** Protein concentrations were estimated by the Lowry method, and carbohydrate as glucose equivalents was estimated by the method of Dubois *et al.* (1956).

## RESULTS AND DISCUSSION

Electron microscopy of thin sections of *B. vulgatus* NCTC 10583 demonstrated the typical appearance of the cell envelope of a Gram-negative bacterium (Fig. 1). The inner or cytoplasmic membrane and the outer membrane were clearly visible, and separated by a dense layer corresponding to peptidoglycan. On the outer surface of the outer membrane there was a thin capsular layer. Electron microscopy of the bacteria after the EDTA–heat–sonication treatment did not reveal any dramatic differences from the untreated organisms, except that the capsular layer was absent. There were no obvious examples of organisms with part of the outer membrane removed as shown for *B. fragilis* by Kasper & Seiler (1975). When the material released by EDTA was examined in the electron microscope, after sedimenting at 100 000 g for 1 h, vesicles of varying size bounded by a
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Fig. 1 Thin section of B. vulgatus NCTC 10583 stained with ruthenium red. Inner (I) and outer (O) membrane, peptidoglycan (P) and capsule (C) are visible. The bar marker represents 0.1 μm.

Fig. 2 Thin section of EDTA-released outer membrane vesicles which were deposited by ultracentrifugation at 100000 g for 1 h. The bar marker represents 0.1 μm.

single trilamellate membrane were observed (Fig. 2). This suggests that after fragments of outer membrane had been removed the outer layer of the organism was re-formed.

Chemical analysis of the EDTA-released OM complex showed it to be predominantly protein with a small proportion of carbohydrate (protein:carbohydrate, approximately 14:1 by weight). SDS–PAGE revealed at least 30 polypeptides, with two major bands at 100000 and 70000 molecular weight. After ultracentrifugation (100000 g, 1 h), the pellet was very similar to the whole EDTA-released complex except that the polypeptide of 70000 molecular weight was predominantly in the supernate (Fig. 3).

When the EDTA-released OM complex from eight strains of B. vulgatus was titrated with antiserum raised against B. vulgatus NCTC 10583 by the indirect ELISA technique, titres of 2560–102400 were obtained, whereas titres of less than 400 were obtained when EDTA antigens from 17 other species of bacteroides were titrated with the B. vulgatus antiserum.

The homologous antigen–antibody reaction was visualized by crossed immunoelectrophoresis (CIE). At least seven precipitin lines, including line 3, were visible (Fig. 4). When this CIE profile was compared with a CIE profile of the ultracentrifuged pellet, only lines 2 and 5 appeared not to be membrane bound. When antigens from the other seven strains, which strongly react with the NCTC 10583 antiserum in ELISA, were reacted with this antiserum in CIE, line 3 was produced by all; in addition, antigens prepared from two of them (NCTC 11154 and GNAB 120) produced line 7. No other precipitin lines, either anodic or cathodic, were produced by these seven strains. This indicates that the cross-reaction demonstrated by ELISA is due to one major species-specific antigen associated with precipitin line 3 in CIE.

Several approaches were made to determine the identity of the antigens of the OM complex of strain NCTC 10583 and the major species-specific antigen which forms precipitin line 3 in CIE. Initially, a crude screening determination was made by ELISA-inhibition (see Methods). Complete inactivation of the antigens could only be demonstrated by heating the OM complex for 15 min at 121 °C. Treatment with 8 M-urea resulted in almost complete
inactivation, whereas formaldehyde, trypsin, pronase and periodate produced only slight or partial inactivation. Inhibition by isolated LPS was minimal under circumstances that we cannot claim to be quantitative.
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Fig. 5. SDS–PAGE/crossed immunoelectrophoresis of OM complex (50 μg protein) from B. vulgatus GNAB 25. Outer membrane complex, after SDS–PAGE on a 10% acrylamide gel in the first dimension, was run into 3.5 ml 1% (w/v) agarose containing 0.25 ml whole cell antiserum of B. vulgatus NCTC 10583 and 1% (w/v) Triton X-100. The original first dimension gel has been removed and replaced by a stained duplicate (a). Note the single precipitin arc produced by the 100,000 mol. wt polypeptide. No other precipitin arc was detected (see drawing, b).

Fig. 6. SDS–PAGE of four pooled excised replicates of the single precipitin line produced by CIE of B. vulgatus GNAB 25 OM complex. The arrows indicate (i) 100,000 mol. wt major OM polypeptide; (ii) 94,000 mol. wt product of IgG; (iii) 55,000 mol. wt IgG H-chain; (iv) 23,000 mol. wt IgG L-chain.

Visualization of some of the inactivated antigen preparations that reacted with antiserum was performed by CIE. Heat treatment removed all but one precipitin line (line 1). Periodate treatment removed line 1; the other lines remained, but the peak heights of lines 4 and 5 were reduced. Purified LPS gave rise to a double peak that co-precipitated with line 1. There appears to be no cross-reaction between the LPS of B. vulgatus NCTC 10583 and the LPS of the other B. vulgatus strains examined, i.e. line 1 is produced only by NCTC 10583 antigen. This is in agreement with the observations of Elhag & Tabaqchali (1978) who showed that the O antigens of the B. fragilis group (which includes B. vulgatus) are extremely heterogeneous within a species. The phenol-extracted capsular polysaccharide produced a small line that appeared to be similar to line 6.

To determine the molecular weight of the species-specific antigen, CIE was performed on B. vulgatus GNAB 25 OM complex with SDS–PAGE for the first dimension. GNAB 25 produced only one precipitin line (line 3) in the CIE described earlier in which agarose was the material of the first dimension. By reference to a stained duplicate SDS–PAGE profile, it
was seen that the single line was produced from the major membrane-bound protein of molecular weight about 100,000 which was described earlier (Fig. 5). To confirm that the antigen which was demonstrated by CIE was in fact the same antigen that was detected by SDS–PAGE/CIE, the single precipitin line that was produced by GNAB 25 OM complex in CIE was excised from the gel and examined by SDS–PAGE. The immunoprecipitate in agarose from four gels was dissolved in 100 µl SDS–PAGE sample buffer by heating at 100 °C for 5 min and applied molten to a well in an SDS–PAGE gel slab. This produced four major bands, of molecular weights 23,000, 55,000, 94,000 and 100,000 (Fig. 6). Bands (iv) and (iii) correspond, respectively, to the L and H chains of IgG. Band (i), of molecular weight 100,000, is the major OM protein and band (ii), of molecular weight 94,000, is a degradation product of IgG which is produced from purified IgG under the electrophoresis conditions used and is probably a dimer of H chains. It was not derived from the antigen as there was no corresponding band in the GNAB 25 OM complex. From these observations it appears that the species-specific antigen is heat-labile, periodate-stable and is the major outer membrane protein with a molecular weight of about 100,000.

Our studies with B. vulgatus suggest that it might be possible to define OM protein antigens of other Bacteroides species which might be used in serological identification. Such protein antigens would probably be much more constant characters than the capsular antigens that are currently being investigated for B. fragilis (e.g. Kasper et al., 1979).

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REFERENCES


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