Surface Structure of *Bacteroides nodosus* in Relation to Virulence and Immunoprotection in Sheep

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A comparative electron microscopic study was made of virulent ovine strains, benign ovine strains, bovine strains and culture variants of *Bacteroides nodosus* using negative staining, thin section and freeze-fracture etch techniques. The plasma membrane, peptidoglycan layer and outer membrane structures were similar in all the organisms, but there were marked differences in the presence of pili, diffuse polar material and additional layer. The variations in these surface structures were examined in relation to the virulence and immunoprotection of *B. nodosus* towards foot-rot in sheep. Only organisms with abundant pili caused virulent foot-rot; diffuse polar material and perhaps the additional layer may also be associated with virulence, but conclusive evidence was lacking. It appeared that pili and one or more unknown cell components, possibly diffuse polar material but not the additional layer, were necessary for immunoprotection.

INTRODUCTION

Bacteroides nodosus has been characterized from cases of virulent foot-rot in sheep (Beveridge, 1941), benign foot-rot in sheep (Thomas, 1962; Egerton & Parsonson, 1969) and interdigital dermatitis in cattle (Laing & Egerton, 1978). Variants derived by *in vitro* subculture of virulent strains of *B. nodosus* have also been described (Thorley, 1976; Skerman *et al.*, 1981) and shown to be of low or no virulence towards sheep. Comparative studies have been made on these organisms to determine whether the virulent ovine strains have any distinguishing biochemical or ultrastructural characteristics that may be associated with their pathogenicity and immunoprotective properties in sheep (Egerton & Parsonson, 1969; Short *et al.*, 1976; Depiazzi & Richards, 1979; Stewart, 1979; Skerman *et al.*, 1981; Every, 1982). Virulent strains have distinct extracellular proteinase characteristics which correlate with virulence (Egerton & Parsonson, 1969; Depiazzi & Richards, 1979; Stewart, 1979; Skerman *et al.*, 1981; Every, 1982) but apparently not with vaccine efficacy (Stewart, 1978). The only ultrastructural feature associated with virulence and/or protective immunity is the presence of pili (Short *et al.*, 1976; Stewart, 1978; Thorley & Egerton, 1981).

Several new structures within the cell envelope and on the surface of virulent strains of *B. nodosus* have been described (Every & Skerman, 1980). This paper reports the distribution of these structures on various isolates, and variants of *B. nodosus* and their possible association with virulence and immunoprotective properties.

METHODS

Organisms. *Bacteroides nodosus* strains 65 and 91 were isolated from clinical cases of ovine foot-rot in New Zealand. Strains 141 and 142 were isolated from interdigital dermatitis in New Zealand cattle. Strain ATCC 25549, an ovine isolate, was obtained from the American Type Culture Collection. Strain A198 from virulent foot-rot and A134, A178, A305 from benign foot-rot were isolated from Australian sheep and were obtained from the McMaster Laboratory, Division of Animal Health, CSIRO, Sydney, Australia. The prefix A is used to distinguish them from New Zealand strains.

Bacteroides nodosus culture variants were derived from serially passaged liquid cultures of wild-type strains as described by Skerman *et al.* (1981). The mucoid colony-type variant 91M and the circular colony-type variant 91C were derived from strain 91 which produced a beaded colony-type on agar. The mucoid colony-type variant A198M was derived from a beaded colony type strain A198.

Virulence and immunoprotection tests were performed as described previously (Skerman *et al.*, 1981).

Culture methods. Cultures were grown anaerobically at 37 °C, either in liquid trypticase/arginine/serine medium for 24 to 48 h or on agar maintenance medium for 4 to 5 d as described by Skerman (1975).

Electron microscopy. Uranyl acetate or potassium phosphotungstate were used for negative staining of intact bacteria and cell walls (Every & Skerman, 1980). Uranyl acetate was preferred for assessment of pili numbers. Cell walls were prepared by sonic disintegration and differential centrifugation (Every & Skerman, 1980). Thin sections were prepared and stained with uranyl acetate and lead citrate (Every & Skerman, 1980) or with ruthenium red (Cagle *et al.*, 1972). Bacteria were freeze-etched (Moor & Mühlethaler, 1963) in the presence or absence of glycerol (Every & Skerman, 1980). Specimens were examined in a Philips EM201C electron microscope at 60 kV with a 50 µm objective aperture.

RESULTS

Negatively stained whole bacteria

Pili were present in widely varying numbers on all *B. nodosus* examined (Fig. 1 and Table 1), but the pili morphology was the same in all cases. A few bacteria from some cultures of the virulent strains had more than 200 pili per cell, whereas at the other extreme, the avirulent culture variant 91C usually had only one pilus per cell on less than 2% of the bacteria and in some cultures none of the bacteria had pili.

Except for culture variant 91C, the number of pili per cell within a single population varied widely (Fig. 2). This variation did not diminish even after 40 selective transfers of a specific colony-type on agar. Also, the average number of pili per cell remained high, though variable from culture to culture, on successive agar subcultures of beaded colony-type virulent strains. In

Table 1. *B. nodosus* strains and culture variants: their surface structures, virulence and immunoprotective properties

Source of bacteria	<i>B. nodosus</i> strain	Virulence*	Immuno-protection†	Pili‡	Additional layer§	Diffuse polar material
Virulent	A198	++	++	+++	+	+
ovine	ATCC 25549	++	++	+++	+	+
foot-rot	91	++	++	+++	+	+
	65	++	++	+++	+	+
Benign	A134	+	NT	+	+	—
ovine	A178	+	NT	+	+	—
foot-rot	A305	+	NT	+	+	+
Bovine	141	+	NT	+++	+	—
interdigital dermatitis	142	+	NT	++	+	—
<i>In vitro</i> culture	A198M	+	+	+	+	+
variants	91M	+	+	+	+	+
	91C	—	+	—	—	+

* Virulence was assessed from the development of foot lesions after artificial infection of Merino sheep in heated pens (Skerman *et al.*, 1981); ++, progressive virulent foot-rot; +, non-progressive foot-rot (scald); —, no progressive foot-rot or scald.

† Immunoprotection was assessed in vaccine-challenge trials (Skerman *et al.*, 1981); ++, 80–100% sheep resistant to homologous artificial challenge; +, 30–40% sheep resistant to challenge with homologous prototype strain; NT, not tested because homologous challenge infection was not feasible.

‡ Numbers of pili per bacterium were assessed on negatively stained bacteria (see Methods); +++, average of 50 or more pili; ++, average of 20–50 pili; +, average of 1–10 pili; —, less than 2% of bacteria with 1 pilus.

§ The presence (+) or absence (—) of the additional layer was assessed on sonically disrupted bacteria, in thin sections and in freeze-etch preparations (see Methods).

|| The presence (+) or absence (—) of diffuse polar material was assessed on longitudinal thin sections.

Fig. 1. (a) *B. nodosus* virulent foot-rot strain 91 grown in liquid culture and negatively stained with uranyl acetate. Numerous pili emerge from one pole. (b) *B. nodosus* culture variant 91M grown in liquid culture and negatively stained with uranyl acetate. Only a few pili emerge from the bacteria, and rod-like structures are present (arrow). (c) *B. nodosus* culture variant 91C grown in liquid culture and stained with uranyl acetate. No pili are present. The bar markers represent 1 μ m.

Fig. 2. Frequency distribution of pili per bacterium in liquid culture populations of *B. nodosus*. Over 200 bacteria from each population were examined. □, strain 91; ▨, culture variant 91M; ■, culture variant 91C. The frequency distribution in other strains resembled strain 91.

contrast, serial passage of some strains in liquid cultures gave rise to the mucoid and circular colony variants with low numbers of pili (Table 1). The relationship between the number of pili per cell, virulence and immunoprotective properties of various strains and variants of *B. nodosus* is shown in Table 1.

Negatively stained bacterial fragments

Fragments of the cell wall in the form of rod-like structures (Fig. 1b) were observed in liquid cultures of all strains and variants except 91C, which did, however, show rod-like structures in some agar cultures.

Cell walls from all sonically disrupted *B. nodosus* showed polar ring structures (prs) (Figs 3 and 4). A regularly striated additional layer (Fig. 3) covered the prs on all strains and variants except 91C (Fig. 4).

Fig. 3. Cell wall fragment from *B. nodosus* bovine strain 141 prepared by sonication and differential centrifugation and stained with potassium phosphotungstate. Polar ring structures (prs) are present on the pole of the bacterium and a striated layer (sl) covers the whole surface. The bar marker represents 0.1 μ m.

Fig. 4. Cell wall fragment from *B. nodosus* culture variant 91C prepared as for Fig. 3. Polar ring structures but no striated layer, are present on the surface. The bar marker represents 0.1 μ m.

Fig. 5. (a) Part of a longitudinal thin section of *B. nodosus* benign foot-rot strain A305 from a liquid culture showing the plasma membrane (pm), peptidoglycan layer (pl), outer membrane (om), additional layer (a) and diffuse polar material (DPM). (b) Part of a longitudinal thin section of *B. nodosus* bovine strain 142 showing pm, pl, om, a, but no DPM. (c) Part of a longitudinal thin section of *B. nodosus* culture variant 91C showing pm, pl, om, DPM but no additional layer. The bar markers represent 0.1 μm .

Fig. 6. *B. nodosus* culture variant 91M obliquely freeze-fractured and etched in the presence of glycerol. The convex and concave views of the cell envelopes show details of the internal fracture faces of the plasma membrane ($\overline{pm}$ and $\underline{pm}$) and the outer membrane ($\overline{cw3}$ and $\underline{cw2}$). Fracture edges corresponding to an additional layer ($\underline{cw1}$) and the outer portion of the outer membrane bilayer ($\underline{cw2}$) can be distinguished. Only two fracture edges can be distinguished corresponding to the inner portion of the outer membrane bilayer ($\overline{cw3}$), the peptidoglycan layer ($\underline{cw4}$) and the outer portion of the plasma membrane ($\underline{r}$). Fractures through the cytoplasm ($\underline{c}$) are also present. The terms for the various structures are those used by Sleytr *et al.* (1974). In all the freeze-fracture etch preparations the arrow in the lower right hand corner denotes the shadowing direction and the marker bar represents 0.1 μ m.

Thin sections

Sections of all *B. nodosus* showed an outer membrane, peptidoglycan layer and plasma membrane and all except variant 91C showed an additional layer (Fig. 5). A diffuse polar material (DPM, Fig. 5a) was present on some strains and variants (Table 1), and its extension from the cell envelope varied from 70 nm in strain ATCC 25549 to 25 nm in strain A198. There appeared to be less DPM in stationary phase bacteria. No capsular material was detected surrounding any of the bacteria using ruthenium red stain.

Freeze-etched preparations

Freeze-etched preparations were made only of strains 141, 91, ATCC 25549, A198, A134, A305 and variants 91M and 91C. Preparations obliquely fractured and etched in the presence of glycerol all showed the same morphological features on the internal fracture faces of the outer membrane and plasma membrane (Figs 6 and 7). Fracture edges corresponding to additional layer, outer and inner parts of the outer membrane bilayer, peptidoglycan layer and outer and inner parts of the plasma membrane were present except for variant 91C which lacked a fracture edge corresponding to the additional layer. Cross-fractures of the cell envelope of variant 91C also lacked an edge corresponding to the additional layer (Fig. 8).

All the strains and variants examined had large pits in the polar region of the internal concave fracture face of the outer membrane. A good example of these structures hexagonally arranged and with the appearance of craters with raised edges and central domes is shown in Fig. 7(b).

Fig. 7. (a) *B. nodosus* culture variant 91C freeze-fractured and etched in the presence of glycerol. Two bacteria were obliquely fractured through their polar regions. The concave view of the outer portion of the outer membrane (cw2) has hexagonally arranged pits or ring-shaped depressions (rd) and small ring structures (srs) on its surface. The convex view of the inner portion of the outer membrane (cw3) has raised polar ring structures (prs) and small pits (arrow) on its surface. The terms for these structures on the fracture faces are those used by Every & Skerman (1980). (b) *B. nodosus* culture variant 91C freeze-fractured and etched in the presence of glycerol. Hexagonally arranged crater-like structures with central domes (cls) are present on cw2.

Sometimes the central dome was not visible so that the structures looked like raised particles or craters. They had the same centre-to-centre spacing (approx. 20 nm) as the complementary shaped polar ring structures shown in Fig. 7(a) which were also seen on all strains and variants examined.

Freeze-etching of *B. nodosus* in the absence of glycerol revealed the hexagonally arranged particles of the additional layer (Fig. 9a) on all the strains and variants examined except 91C (Fig. 9b). The etched outer convex surface of the outer membrane was only seen on variant 91C because in the other specimens it was always obscured by the additional layer.

Fig. 8. Cross-fractured cell envelope of *B. nodosus* culture variant 91C showing the fractured edges of the plasma membrane bilayer (double arrow), the edge of the peptidoglycan layer (cw4), and the double edges of the outer membrane (cw2 + cw3). Note the lack of a fracture edge for additional layer.

DISCUSSION

This study has shown that in all strains and culture variants of *B. nodosus*, the structure of the plasma membrane, peptidoglycan layer and outer membrane were similar. However, those strains and variants of low immunoprotective activity and/or virulence were distinguished from strains of high virulence and immunoprotective activity by their lack or reduction of one or two of the three surface structures: additional layer, DPM and pili. Hence the possible involvement of these three structures as virulence factors or protective immunogens can be considered. In addition to these structures, the role of proteinase in virulence (Depiazzi & Richards, 1979; Stewart, 1979; Every, 1982) should also be taken into account. In this respect, the low virulence variant A198M is of particular interest because it has the same proteinase characteristics as the highly virulent strains (Every, 1982) and the only structural distinction of this variant from virulent strains is the low number of pili. Thus abundant pili on *B. nodosus* may be essential for full virulence of the organism to be expressed.

The bovine isolates have abundant pili and an additional layer, and their low virulence could be attributed to either lack of DPM (Table 1) or their distinctive proteinase characteristics (Every, 1982). The total lack of any virulence in variant 91C could be attributed to three features: its very low proteinase activity (Skerman *et al.*, 1981; Every, 1982), its almost complete lack of pili or its lack of an additional layer.

The function of *B. nodosus* surface structures in virulence is not known. However, Every (1979) has shown that *B. nodosus* pili are most like the group 4 type pili defined by Ottow (1975) which promote non-flagellar surface translocation of bacteria (MacRae *et al.*, 1977). This supports the suggestion (Walker *et al.*, 1973) that pili aid the spread of bacteria within the

Fig. 9. (a) Convex view of the cell envelope of *B. nodosus* culture variant 91M obliquely fractured and etched in the absence of glycerol. The etched outer surface of the additional layer (cw1) has hexagonally arranged subunits. (b) Convex view of the cell envelope of *B. nodosus* variant 91C obliquely fractured and etched in the absence of glycerol. Note the absence of a regularly arranged surface layer and an edge corresponding to additional layer. The etched outer surface is that of the outer membrane (cw2).

epidermal matrix of the sheep hoof. It appears that *B. nodosus* pili do not have an adhesive function (Stewart, 1975) as in some other bacteria (Ottow, 1975). However, the additional layer may have a role in adhesion of *B. nodosus* to sheep or cattle epidermis. In the fish pathogen, *Aeromonas salmonicida*, the presence of an additional layer was correlated directly with virulence and adhesion to fish tissue. Udey (1978) and Costerton *et al.* (1974) suggested that the additional layer may function in adhesion of Gram-negative rumen bacteria to a surface.

The low numbers of pili on the *B. nodosus* colony variants could explain their weak immunoprotective activity in challenged sheep, and is consistent with sheep being protected against foot-rot by vaccines containing partially purified pili (Stewart, 1978) or pili preparations of greater than 99% purity (unpublished data). Although most sheep vaccinated with the culture variants were susceptible to foot-rot challenge there was still a significant reduction in the severity of the infection (Skerman *et al.*, 1981). This could not be attributed to pili or the additional layer because these components were not found in variant 91C. Nevertheless, unassembled subunits of pili or additional layer might still occur as antigens in the cell envelope of variant 91C. Whether such subunits or any of the structures known to be retained by variant 91C, such as DPM, prs, small ring structures and other outer membrane components, can account for the partial protection observed with variant 91C needs further investigation.

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