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Isolation and Characterization of *Bacteroides nodosus* Fimbriae: Structural Subunit and Basal Protein Antigens

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We examined the isolation of fimbriae from *Bacteroides nodosus*. It was found that the best preparations were obtained from the supernatant of washed cells cultured on solid medium, from which fimbriae could be recovered in high yield and purity by a simple one-step procedure. Analysis of such preparations by sodium dodecyl sulfate gel electrophoresis showed that greater than 98% of the protein consisted of fimbrial structural subunits whose molecular weight was ca. 17,000. These preparations also usually exhibited minor contamination with a polypeptide of ca. 80,000 molecular weight, as well as trace amounts of lipopolysaccharide. Attempts to release additional fimbriae by the traditional means of subjecting the bacterial cells to physical stress, such as shearing or heating, resulted primarily in an increase in the level of contamination, without significant gain in the yield of fimbriae. Removal of the 80,000-dalton component could not be achieved by any of a variety of techniques normally used in fimbriae purification, including isoelectric precipitation, $MgCl_2$ precipitation, and CsCl gradient ultracentrifugation, implying a direct physical association with the fimbrial strand. Electron micrographs of fractions containing this protein show cap-shaped structures attached to the ends of what appeared to be fimbrial stubs. These observations suggest that the 80,000-dalton polypeptide may actually constitute the basal attachment site which anchors the fimbria to the outer membrane, analogous to a similar protein recently described in enterotoxigenic strains of *Escherichia coli*. In *B. nodosus*, this 80,000-dalton protein is a major surface antigen, and like the fimbrial subunit, exhibited variation in electrophoretic mobility between serotypically different isolates.

Bacteroides nodosus is the essential causative agent of ovine foot rot (1, 9). Virulent isolates of this organism contain fimbrial structures (21) which have been implicated as playing a central role in both the infectivity of the bacteria and the immunological response of the host (12, 26, 27, 29, 39). The presence of fimbriae on *B. nodosus* appears to be an unstable characteristic (26). Benign isolates show altered colony morphology with reduced fimbriae; similar variants may also be generated in vitro by serial culture of virulent isolates, especially in liquid media (26, 36). The loss of fimbriae appears to be an irreversible process (26).

There are at least eight major serogroups of *B. nodosus*, defined by the K-agglutination test (3, 5, 23) and supported by patterns of cross-immunity (6). The fimbria is the major determinant involved in the K-agglutination reaction (10, 30, 39), and vaccination of sheep with fimbriate cells or isolated fimbriae confers strong protective immunity against *B. nodosus* challenge, which is generally restricted to the serogroup involved (6, 7, 12, 29, 32). Vaccination with nonfimbriate cells is much less effective (26, 32, 37). Although there have been some indications of serotype-independent immunity (6, 31, 37), the levels of protection recorded have been marginal, and the antigens involved are undefined.

It is now well established that *B. nodosus* fimbriae are composed of small polypeptide subunits of ca. 18,000 molecular weight (10), similar to those observed in other fimbriate species (21). Correspondingly, published methods for the preparation of fimbriae from *B. nodosus* (10, 20, 32) have been largely based on procedures originally developed for use with enterotoxigenic *Escherichia coli* (2, 34). These methods involve subjecting the cells to mechanical shear forces, to facilitate release of fimbriae from the cell surface, which is then followed by a lengthy series of purification steps (e.g., see reference 10). However, using these procedures, we noted substantial contaminants and, in particular, the presence and persistence of a protein of about 80,000 molecular weight. Our results indicate, first, that the current methods used for the production of fimbriae from *B. nodosus* may be inappropriate, even counterproductive, and we present a simplified protocol for the isolation of fimbriae in high purity. Second, our evidence suggests that the 80,000-dalton component may actually be physically associated with fimbriae in vivo and in vitro, and that it probably represents the basal protein which anchors the fimbrial strand to the outer surface of the cell.

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MATERIALS AND METHODS

Bacterial culture. *Bacteroides nodosus* 198, originally obtained from the Commonwealth Scientific and Industrial Research Organization McMaster Laboratory (Sydney, Australia) culture collection, was used in these studies. This strain is the prototype assigned to serogroup A, subtype 1 and has been reclassified as VCS 1001 (3). Strain VCS 1006 (serogroup B, subtype 1) was also used in some experiments. Seeding cultures were purified from freeze-dried samples by two subcultures on 4% hoof-agar (HA) medium (35). For large-scale cultures, cells were grown on 2% HA medium and harvested by being scraped into 10 mM sodium phosphate buffer (pH 7.2) containing 140 mM NaCl (PBS). For liquid cultures, cells were inoculated into trypticase-arginine-serine broth (25). All cultures were maintained at 37°C in an atmosphere of 90% H_2 and 10% CO_2.

Purification of fimbriae. Bacterial suspensions, either as broth cultures or as PBS suspensions from plate cultures, were centrifuged at 12,000 × *g* for 30 min to remove the cells. The fimbriae left in the supernatant were then recov-

ered by isoelectric precipitation (2, 10, 34) as follows. Supernatants were adjusted to pH 4.5 with the addition of 0.1 M sodium acetate, left to stand at 4°C overnight, and then centrifuged at 12,000 × *g* for 15 min. Pelleted fimbriae were stored in portions, frozen at −20°C, and taken up in PBS as required.

To remove additional fimbriae from the cells, cell pellets were suspended in PBS and subjected to physical shearing (2, 34), by homogenization in a Virtis Blender at full speed for three intervals of 20 s, separated by 2-min cooling periods in an ice bath. Alternatively, suspended cells were subjected to heat treatment (19, 34), by incubation at 60°C for 25 min. In both cases, cells and particulate debris were removed by centrifugation, and the fimbriae were collected from the supernatants after isoelectric precipitation, as described above.

Where required, further purification of fimbriae was carried out by using 0.1 M $MgCl_2$ precipitation (2), by diluting the samples suspended in PBS with an equal volume of 0.2 M $MgCl_2$. Isopycnic CsCl gradient ultracentrifugation was performed under the conditions described by Every (10). Protein concentrations were determined by the Lowry method.

Electrophoretic analysis. Preparations were analyzed by electrophoresis on linear 6 to 15% gradient polyacrylamide gels, containing 0.15% sodium dodecyl sulfate (SDS) and 0.5 M urea, by the modification of the Laemmli system detailed previously (17). Samples were dissolved by being boiled for 3 min in a buffer containing 1.6% SDS, 0.5 M urea, 1% β-mercaptoethanol, and 50 mM Tris (pH 7.5) before application to the gel. After electrophoresis, gels were stained with 0.25% Coomassie blue R250 in acetic acid-methanol-water (7:33:60) and destained in the same solvent system. Gels were scanned with a Joyce-Loebl densitometer with a 513-nm filter. Standard protein mixtures (Bethesda Research Laboratories) were included in all gels for the calibration of molecular weights.

For Western analyses, (unstained) gel displays were electrophoretically transferred to nitrocellulose paper (BA85; Schleicher & Schuell Co.) by the method of Towbin et al. (38). In this case, a mixture of prestained protein standards (Bethesda Research Laboratories) and ^{14}C-labeled protein standards (Amersham Corp.) were included in peripheral gel lanes. After transfer, any remaining protein binding sites on the paper were blocked at 40°C for 1 h with 2.4% bovine serum albumin (BSA) and 1% bovine gelatin in a 12 mM Tris buffer (pH 7.5), containing 120 mM NaCl (Tris-saline). The papers were incubated at 20°C overnight in rabbit or sheep antiserum diluted in Tris-saline buffer containing 2.4% BSA and 0.2% gelatin. After thorough washing (38) with Tris-saline, the papers were then incubated for 3 h with ^{125}I-protein A (0.1 μCi/ml) in a 2.4% BSA–0.2% gelatin–Tris-saline solution at pH 7.4 or 7.9, depending on whether rabbit or sheep antiserum, respectively, had been used. The papers were washed again with Tris-saline (at the appropriate pH) and dried; the protein A-antibody binding sites were visualized by autoradiography.

Antisera. To obtain antisera, rabbits were immunized intramuscularly with isolated fimbriae (100 μg) in Freund complete adjuvant and boosted with fimbriae in Freund incomplete adjuvant. Sheep antiserum was obtained after subcutaneous vaccination with ca. 10^9 cells of *B. nodosus* in Freund incomplete adjuvant (two doses).

Electron microscopy. Fractions containing fimbriae and other components were analyzed by electron microscopy with uranyl acetate staining. Samples in PBS were mixed with an equal volume of 0.5% uranyl acetate in water and then transferred with a platinum loop to Formvar- and carbon-coated grids. Grids were blot dried with filter paper and examined in a JEM 100CX electron microscope at 80 kV.

RESULTS

Isolation of fimbriae. One of the primary problems facing investigators who want to prepare fimbriae from *B. nodosus* is the difficulty of culturing the organism, especially in its fimbriate form. The usual means of isolating fimbriae from *B. nodosus* is to subject the cells to shear forces (10, 20, 31). The cells may be derived from either plate or broth cultures. Plate cultures have the advantage of fimbrial expression being more easily maintained and readily assessed from colony morphology (26, 36). Liquid media are more amenable to large-scale cultures, but have the disadvantage of considerable batch-to-batch variability in the degree of fimbrial production by the cells. We examined the methods of fimbrial purification and the quality of preparations obtained from both sources of culture, using strain VCS 1001 as the prototype.

Fimbriate cells were removed from plates by gently scraping and rinsing the plates with PBS. Under these conditions, we found that the bulk of the fimbriae had been, in fact, already released from the cells (Fig. 1, lane 1; cf., lane 2), and could be easily recovered from the supernatant (after the cells were pelleted) by isoelectric precipitation. Electrophoretic analysis showed that this precipitated fraction consist-

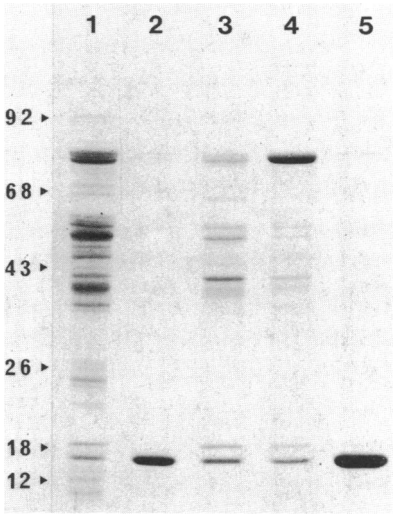

FIG. 1. Comparison of different methods for the isolation of fimbriae from *B. nodosus*. Samples were electrophoresed on SDS–linear 6 to 15% gradient polyacrylamide gels and stained with Coomassie blue R250. Lane 1 contains *B. nodosus* cells pelleted from PBS suspension after harvesting of plate cultures. The remaining lanes contain various fimbrial fractions, with the numbers in parentheses indicating the amount of cells from which the samples were derived, in relation to that loaded in lane 1 (*x*): lane 2, fimbriae in the post-cell supernatant (2*x*, 6 μg of protein); lane 3, the fraction obtained after blending the cell pellet (10*x*); lane 4, the fraction obtained after heating the cell pellet (10*x*); and lane 5, the same as lane 2, except increased loading (5*x*, 15 μg of protein). All fimbrial samples were recovered by isoelectric precipitation; see text for full experimental details. The numbers on the left of the figure refer to the electrophoretic position of molecular weight standards: phosphorylase b (92,500), BSA (68,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), and cytochrome *c* (12,300).

ed almost entirely of fimbrial subunits (molecular weight, 17,000), with faint traces of just one other component, a polypeptide of ca. 80,000 molecular weight (Fig. 1, lanes 2 and 5).

The cell pellets were suspended in PBS and subjected to shearing, by homogenization in a blender. This treatment resulted in the release of a small additional amount of fimbriae (Fig. 1, lane 3), which corresponded to about 5% of that originally obtained in the first (untreated) supernatant (cf. Fig. 1, lane 5). The shearing procedure also, however, led to a substantial increase in the range and degree of contamination of the fimbrial fraction with other bacterial components (Fig. 1, lane 3). These included major polypeptides of molecular weights ca. 19,000, 42,000, 55,000, 60,000, and 78,000, as well as the 80,000-dalton (d) protein referred to earlier. It was also evident that this fraction contained additional contaminants which are not stained by Coomassie blue, principally lipopolysaccharides (see below and Fig. 2).

Mild heat treatment is an alternative method which has been used successfully to release fimbriae from *E. coli* (19, 34). Incubation of *B. nodosus* cells at an elevated temperature (60°C, 25 min) produced a similar result to that obtained with shearing, i.e., a small amount of fimbriae with a number of contaminating components (Fig. 1, lane 4). In this case, a considerably larger amount of the 78,000-d protein was released into the supernatant.

A similar range of experiments was also undertaken with cells cultured in a liquid medium (data not shown). However, the supernatant fimbrial fraction (recovered by isoelectric precipitation of the broth) contained a large number of contaminating proteins, many of which were apparently derived from the culture medium itself. The yield of fimbriae was also consistently lower than that obtained from an equivalent volume of plate cultures, although, again, we found that the majority of the fimbriae were in fact already released into the supernatant, in the absence of any specific treatment of the cells. Shearing or heating the cells produced an outcome similar to that described above.

The various fimbrial fractions displayed in Fig. 1 were subjected to immunological analyses by the Western transfer technique (Fig. 2). Essentially identical results were obtained irrespective of whether the antiserum used was generated in rabbits vaccinated with isolated fimbriae or in sheep immunized with intact *B. nodosus* cells. The supernatant fimbrial fraction showed just two bands, corresponding to the fimbrial 17,000-d subunit and the 80,000-d contaminant (Fig. 2, lane 3). Since the latter polypeptide was present in only trace amounts in the vaccinating fimbrial preparation, it is evidently a strong immunogen. The observation that this protein also elicited a strong antibody response in sheep immunized with whole cells suggests that it has a surface location. In agreement with the Coomassie blue profiles (Fig. 1), both antigens were present (to different extents) in the sheared and heated preparations, as well as in displays of whole cells (Fig. 2, lanes 2, 4, and 5). These fractions also contained a number of additional antigens, in particular a series of bands arranged in a ladder formation. These bands were partially recognized by preimmune serum, whereas *B. nodosus* polypeptide antigens were not (data not shown). This pattern is typical of lipopolysaccharide chains (14, 22),

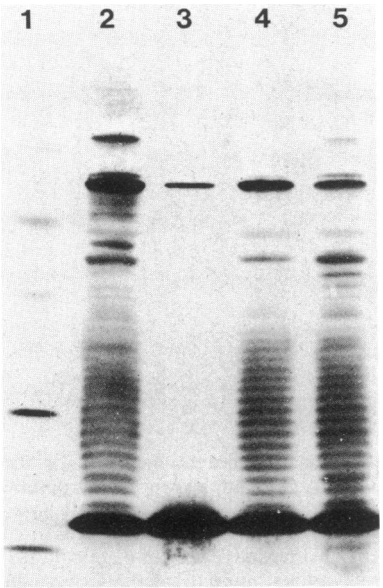

FIG. 2. Western transfer analysis of fimbrial fractions. The samples (lanes 2 to 5) were identical to those in Fig. 1 (lanes 1 to 4): lane 2, *B. nodosus* cell pellet; lane 3, supernatant fimbrial fraction; lane 4, fimbrial fraction from blended cells; and lane 5, fimbrial fraction from heated cells. The resulting gel display was electrophoretically transferred to nitrocellulose paper and incubated with rabbit anti-fimbrial antiserum (diluted 1:1000). The position of bound antibodies was visualized by autoradiography after incubation with ^{125}I-labeled protein A. Lane 1 contained ^{14}C-labeled methylated protein molecular weight standards: phosphorylase b (92,500), BSA (68,000), ovalbumin (43,000), carbonic anhydrase (29,100), and lysozyme (14,300).

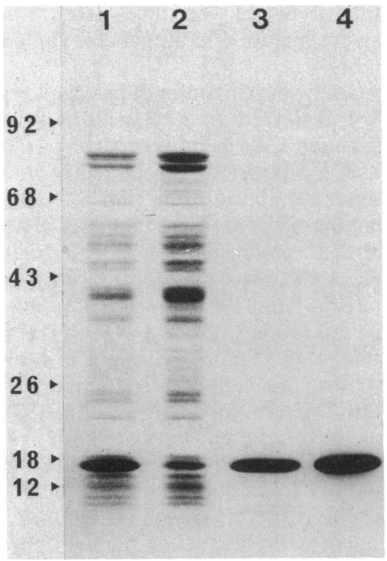

FIG. 3. Simplified protocol for rapid isolation of *B. nodosus* fimbriae in high purity. Samples from the various stages of the fractionation procedure were displayed by electrophoresis on SDS–linear 6 to 15% gradient polyacrylamide gels and stained with Coomassie blue R250. The lane contents are as follows. The numbers in parentheses indicate the relative amount of cells from which the samples were derived, compared with that loaded in lane 1 (x). Lane 1, unfractionated *B. nodosus* cells (x); lane 2, cells pelleted from phosphate-buffered saline suspension ($2x$); lane 3, the post-cell supernatant ($1.8x$); and lane 4, the fimbrial pellet recovered from the supernatant by isoelectric precipitation ($4x$). The numbers on the left of the figure refer to the positions of the protein molecular weight standards, as detailed in the legend to Fig. 1.

which are known to be a major component of the cell wall in *B. nodosus* and probably represent the O antigen group described in earlier serological analyses (5, 8, 28, 30).

In view of these results, we have developed a very simple and rapid procedure for the isolation of *B. nodosus* fimbriae in high yield and purity (Fig. 3). This procedure involves a one-step isoelectric precipitation of fimbriae from the post-cell supernatant. It is clear that the vast majority of the fimbriae were already present in this fraction in the absence of any specific treatment of the cells. Over a large number of preparations, the yield of purified fimbriae was relatively consistent, generally ranging from 2 to 3 mg of protein per g (wet weight) of cells. The isoelectric precipitation itself serves primarily as a convenient method for the recovery and concentration of the fimbriae, but it also removes traces of nonspecific (mainly low-molecular-weight) contaminants from the supernatant fraction (Fig. 3, lanes 3 and 4). The purity of the resulting fimbrial preparations, as assessed by densitometric scans of Coomassie blue-stained gel displays (e.g., Fig. 3, lane 4), ranged from 97 to 99%, with respect to the 17,000-d structural subunit. The only other visible component was the 80,000-d polypeptide antigen. Only very faint traces, if any, of lipopolysaccharide antigens were detectable in the preparations. This procedure gave similar results with other serotypes of *B. nodosus*.

The 80,000-d antigen. In fimbrial preparations from a number of *B. nodosus* strains, we consistently found the 80,000-d antigen to be a significant contaminant. We also observed that this protein exhibited variation in electrophoretic mobility between serotypically different isolates. This is illustrated in Fig. 4, which compares the Coomassie blue (protein) and Western (antigen) profiles of isolated fimbriae and whole cells of strains VCS 1001 (serotype A1) and VCS 1006 (serotype B1) (3). In fimbrial preparations from the latter, the 80,000-d band seen in VCS 1001 was absent, and was replaced by one corresponding to about 84,000 molecular weight (Fig. 4A, lane 4; cf., lane 2). A similar change is evident in displays of whole cells (Fig. 4A, lane 3; cf., lane 1). Correspondingly, Western transfer analysis shows that the same shift occurred in the antigenic signal from this component in isolated fimbriae, as well as whole cells (Fig. 4B). This result verifies that it was the visible contaminant of fimbrial preparations which was indeed responsible for the antigen band detected in Western analyses. In other strains that we have examined so far, the apparent size of the 80,000-d antigen generally lay within the limits represented by VCS 1001 and VCS 1006. Although it was more difficult to discern under gradient gel conditions, the fimbrial subunit also showed some degree of apparent size variation among these strains (as well as others). In either case, it remains to be established whether such variation reflects actual changes in the length of the polypeptides or internal amino-acid substitution.

Several different approaches were tried in an attempt to separate the 80,000-d antigen from the 17,000-d structural subunit in our fimbrial preparations. These approaches included methods traditionally used in the purification of fimbriae (2, 32, 34), such as repeated isoelectric or $MgCl_2$

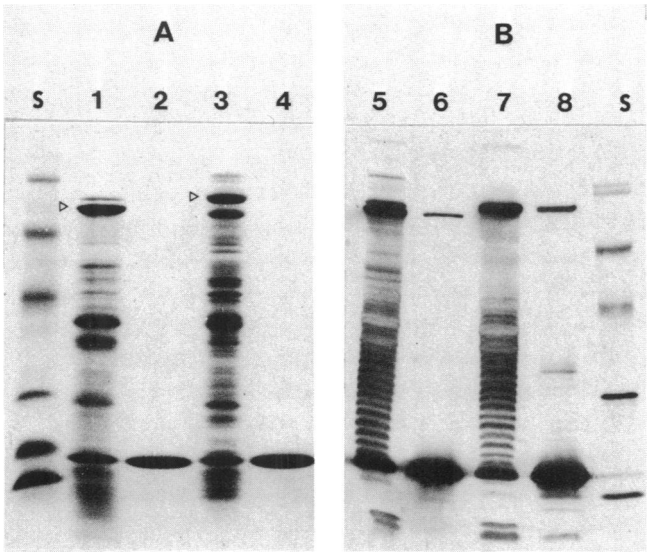

FIG. 4. Variation in the electrophoretic mobility of the 80,000-d antigen isolated with fimbriae from serotypically different *B. nodosus* cells. Samples were displayed on SDS–linear 6 to 15% gradient polyacrylamide gels and either stained for protein with Coomassie blue R250 (A) or subjected to Western transfer analysis with anti-fimbrial antiserum (B). The lane contents were as follows. Lanes 1 and 5, whole cells of *B. nodosus* VCS 1001; lanes 2 and 6, isolated fimbriae from VCS 1001; lanes 3 and 7, whole cells of VCS 1006; lanes 4 and 8, isolated fimbriae from VCS 1006. The open arrowheads in (A) indicate the position of the Coomassie blue bands which are present as the trace contaminants in fimbrial preparations from each strain. The peripheral lanes labeled *S* contain standard protein molecular weight markers: in (A), as described in the legend to Fig. 1 and in (B), as described in the legend to Fig. 2.

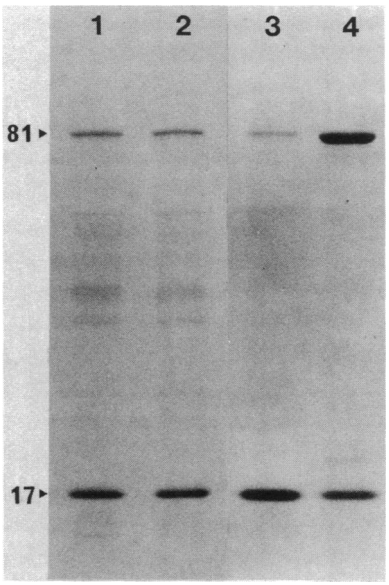

FIG. 5. Copurification of the 80,000-d polypeptide with the 17,000-d structural subunit of the fimbriae. Samples taken after different purification procedures were electrophoresed on SDS–linear 6 to 15% gradient polyacrylamide gels and stained with Coomassie blue R250. The starting material for these analyses was the isoelectrically precipitated fimbrial fraction derived from heated cells (lane 4). In this initial sample, the 80,000-d polypeptide is obscured by the substantial band of the 78,000-d protein which contaminates such fractions (cf., Fig. 1; see text). Lanes 1 and 2 contain fimbriae after one and three rounds, respectively, of 0.1 M $MgCl_2$ precipitation. Note the absence of the 78,000-d polypeptide. Lane 3 contains fimbriae isopycnically banded in a CsCl gradient (10).

precipitations, as well as the isopycnic banding of the fimbriae in CsCl density gradients (10). For reasons of visual clarity, the results were best demonstrated by the preparation depicted in Fig. 5, which was obtained from heated cells and which contained a far greater proportion than usual of the 80,000-d protein. Although outer major contaminants, such as the 78,000-d polypeptide referred to earlier, were removed by conventional procedures used for fimbrial purification, it is clear that the 80,000-d component was not (Fig. 5, lanes 1 and 2). Furthermore, a substantial amount of this protein also copurified with the fimbriae in CsCl gradients (Fig. 5, lane 3). In this case, the fimbriae formed a clearly visible and quite discrete band in the gradient. This band occurs at the relatively high density of about 1.285 g/ml (10).

These observations suggested that the 80,000-d polypeptide may be, in fact, physically associated with the fimbrial strand. The most logical origin of such a component would be the basal protein which links the fimbriae to the surface of the bacterial cell. The various fimbrial fractions were therefore examined by electron microscopy (Fig. 6 and 7). Highly purified fimbriae obtained from the supernatant of washed cells showed primarily a population of long filaments of various lengths (Fig. 6), and whose diameters measured ca. 8 ± 1 nm, in agreement with previous reports (10, 16, 24, 27, 39). On the other hand, fractions containing substantial amounts of the 80,000-d protein, such as that depicted in Fig. 5, showed a significant number of what may be described as cap-shaped structures, attached to one end of shorter filaments (Fig. 7). These filaments appeared to be truncated fimbriae, since their diameters, (measured as 9.5 ± 1 nm, against a background of negative staining) were similar to those of purified fimbriae (Fig. 6B) and indistinguishable from those of the longer fimbrial strands present in the same fraction (e.g., Fig. 7F). The presence of fimbrial stubs in such fractions would not be surprising, in view of the fact that the bulk of the fimbriae had already been separated from the cells as a result of being washed (see above).

These fractions also contained other, more amorphous structures (Fig. 7A), possibly lipopolysaccharide-cell wall fragments, which were removed by further purification of the material, e.g., by $MgCl_2$ precipitation. In this case (Fig. 5, lane 2), the only visible components remaining were fragments of fimbriae and the cap-shaped structures attached to fimbrial stubs, correlating with the co-retention of the 17,000- and 80,000-d polypeptides. The terminal cap structures were also occasionally observed to be attached to the longer fimbriae present in supernatant-derived preparations, at a frequency consistent with the low level of 80,000-d protein in this fraction. Although some free cap structures were also seen, it is significant that in all fractions the majority of such structures appeared to be associated with one end of a fimbrial strand.

Although there may be some ambiguity in the interpretation of electron micrographic data, these results provide supportive evidence for the existence of a terminal, probably basal, structure which is an integral feature of *B. nodosus*

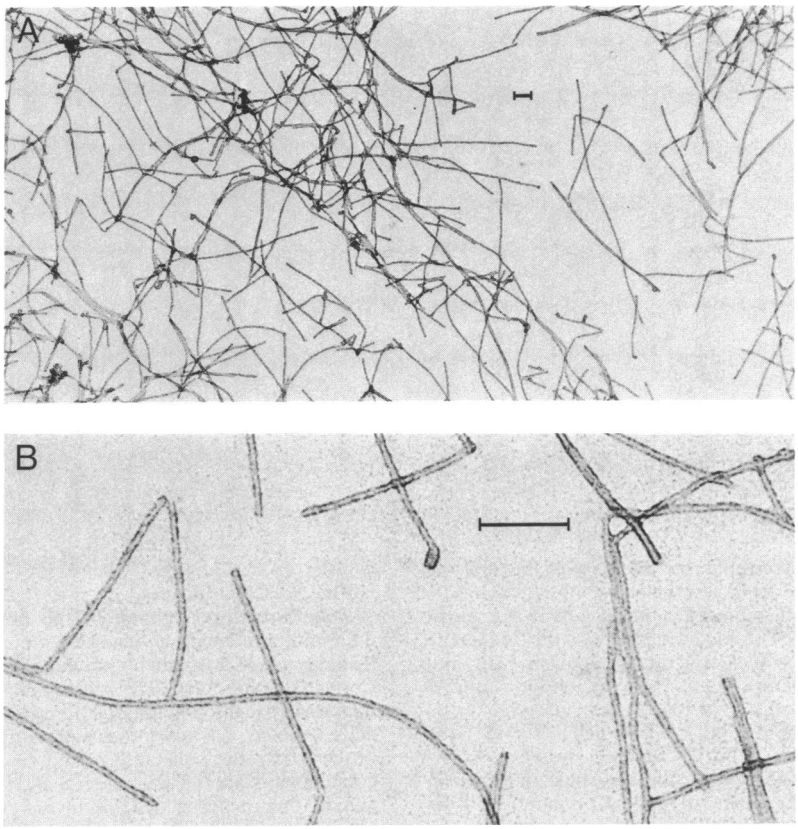

FIG. 6. Electron microscopy of purified fimbriae. Fimbriae were prepared by the rapid protocol outlined in Fig. 3 and positively stained with uranyl acetate. (A) represents a typical field; (B) is a higher (×5) magnification. For (A) and (B), the bar is 100 nm.

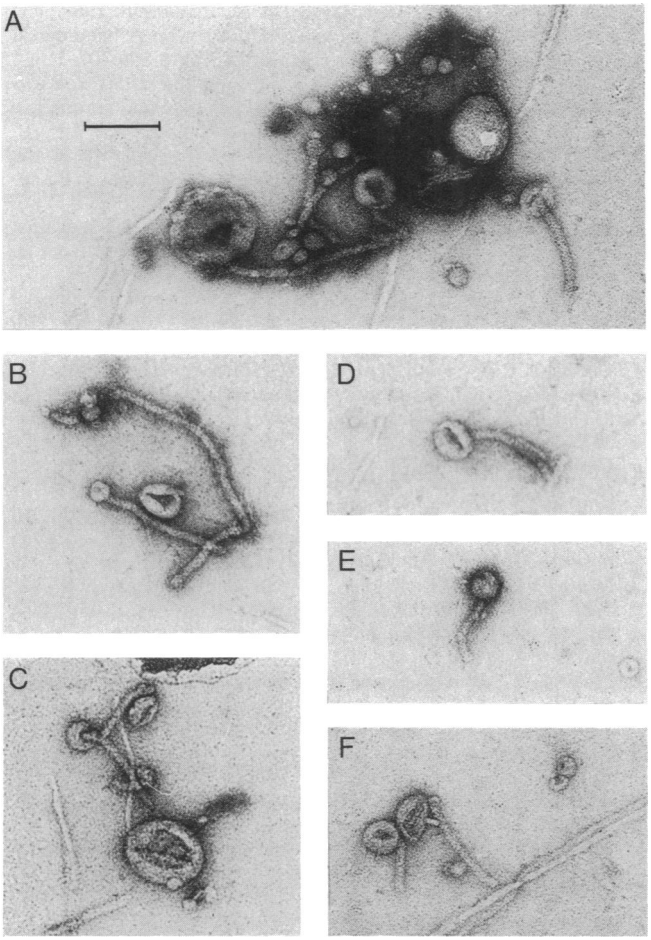

FIG. 7. Electron micrographic examination of fimbrial fractions containing significant amounts of the 80,000-d polypeptide. (A) shows a representative field of the range of components found in the crude fimbrial fraction derived from heated cells. (B) to (F) show isolated examples of fimbrial strands in association with cap-shaped structures. In this case, the samples were negatively stained with uranyl acetate. The magnification is the same in each panel; bar, 100 nm.

fimbriae in vitro and in vivo. The attendant electrophoretic and immunological data suggest that these structures do, in fact, correspond to the 80,000-d antigen which copurified with the 17,000-d subunit of the fimbrial strand.

DISCUSSION

Two important and interrelated findings have emerged from this study: the development of a more appropriate protocol for the isolation of fimbriae from *B. nodosus* and the recognition that the fimbrial structure may in fact contain two significant polypeptide components, the 17,000-d subunit of the strand itself and an 80,000-d basal protein. Since both polypeptides are major antigens in vivo, these findings have substantial implications in the area of the serology and immunology of ovine foot rot.

The methods traditionally used for the isolation and purification of *B. nodosus* fimbriae (10, 20, 32) have been adopted, with little modification, from those originally developed for use with enterotoxigenic *E. coli* (2, 21, 34), whose fimbriae are evidently difficult to dislodge. Our results suggest that these methods are, in the case of *B. nodosus*, unsuitable and unnecessary. The vast majority of the fimbriae are already detached at cell harvest, and may be recovered from the supernatant in high yield and purity by a single, rapid, isoelectric precipitation step. Procedures designed to release (additional) fimbriae by subjecting the cells to physical stress (10, 20, 32) serve mainly to introduce a substantial range of protein and lipopolysaccharide contaminants, which then necessitate a lengthy series of purification steps (see reference 10). The failure to detect the prominent ladder of lipopolysaccharide antigens in a previous Western transfer analysis of *B. nodosus* fractions (20) possibly reflects an inability of APT-paper to bind these molecules, rather than any real difference in the preparations.

The apparent ease with which *B. nodosus* fimbriae are shed from the cell is a significant feature of this organism, and one which has been recognized by previous workers (10, 30, 32). Whether this occurs because of fragility in the fimbrial structure itself or because of loose attachment to the cell surface is difficult to ascertain. Nevertheless, since the fimbriae are considered to be important protective antigens against foot rot (12, 29, 32), an important corollary to our present observations is that whole-cell vaccines which do not include the supernatant (i.e., fimbrial) fraction may have reduced efficacy.

It is well known that the fimbriae of *B. nodosus* are composed of small, repeated, polypeptide subunits (10, 30), analogous to those found in other fimbriate bacteria (13, 21). Our results indicate that there is another significant antigen, a protein of about 80,000 molecular weight, which exists in close association with the fimbrial strand in *B. nodosus* and which possibly represents the basal site for the attachment of fimbriae to the cell. This relationship was first predicated by the copurification of the 80,000-d polypeptide with 17,000-d structural subunits in a variety of fimbrial preparations. The 80,000-d polypeptide itself appears to have a surface location, since animals exposed to whole cells, either by infection (20) or vaccination, respond strongly to this antigen. Furthermore, this protein is the only other cellular component, apart from the fimbrial strand, which appears to be released in any significant amount into the supernatants of washed, but otherwise untreated, cells (see Fig. 1; cf., lanes 1 and 5). Subsequent examination of isolated fimbriae by electron microscopy clearly showed a cap-like structure attached to one end of what appeared to be fimbrial stubs, whose incidence correlated both qualitatively and quantitatively with the presence of the 80,000-d antigen in the preparation.

Further evidence that this cap structure might represent the base plate of the fimbriae is provided by an earlier ultrastructural study of *B. nodosus* (16), which identified numerous pores in the surface of the cell. These pores appear to be the channels through which fimbriae emerge, since their diameter (about 6 nm) was the same as that of the fimbrial strand, and their frequency and peritrichous distribution corresponded to the (original) degree and pattern of cell fimbriation. The significant feature of this study was the description of collars or "grommets" surrounding the pores, which the authors speculated may function as local reinforcement for the fimbriae at the cell wall (16) and whose visual and dimensional characteristics match closely match those of the cap structures observed in our preparations. Similar pores and ring structures have also been observed in other electron micrographical studies of *B. nodosus* (11, 33), as well as in other fimbriate bacteria (see reference 15).

Our supposition that the 80,000-d antigen corresponds to

the basal protein in *B. nodosus* is supported by a number of recent molecular and genetic studies of fimbrial biosynthesis in *E. coli*, where a similar protein has been described (see reference 13). This protein, whose molecular weight estimates range from 70,000 to 80,000 in different enterotoxigenic strains, is encoded by one of a cluster of genes involved in fimbrial expression, and is known to be required for the normal assembly of the fimbriae (13). It is located in the outer membrane of the cell and appears to function as the attachment-extrusion site for the fimbrial strand (4, 13, 18).

In *B. nodosus*, both the 17,000-d fimbrial subunit and the 80,000-d protein are prominent natural antigens. This fact raises some important questions concerning their individual roles in the generation of protective immunity, especially since the fimbrial preparations used in vaccination trials would, in hindsight, have contained both antigens and have elicited a dual response similar to that obtained with whole cells. This is certainly the case in the one other example that is possible to assess (20). In the past it has been assumed that it is the fimbria itself (i.e., the structural subunit) which is the primary protective and serotypic antigen (e.g., see reference 12). This is probably the case, but it is worth recalling that within the eight major serogroups of *B. nodosus* defined by Claxton et al. (3), a number of distinct subtypes exist, implying that there may be more than one antigenic variable. Our results raise the possibility that the 80,000-d protein is the second variable, particularly in view of our observation that this polypeptide, like the fimbrial subunit, shows some degree of size variation between serologically differentiable *B. nodosus* isolates. We are currently attempting to catalogue the variation in the 17,000- and 80,000-d antigen groups, with respect to the serotypic classification of *B. nodosus*, and to establish their relative contributions to the generation of protective immunity against foot rot.

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