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Morphogenetic Expression of *Bacteroides nodosus* Fimbriae in *Pseudomonas aeruginosa*

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Type 4 fimbriae are found in a range of pathogenic bacteria, including *Bacteroides nodosus*, *Moraxella bovis*, *Neisseria gonorrhoeae*, and *Pseudomonas aeruginosa*. The structural subunits of these fimbriae all contain a highly conserved hydrophobic amino-terminal sequence preceding a variable hydrophilic carboxy-terminal region. We show here that recombinant *P. aeruginosa* cells containing the *B. nodosus* fimbrial subunit gene under the control of a strong promoter (p_L, from bacteriophage λ) produced large amounts of fimbriae that were structurally and antigenically indistinguishable from those produced by *B. nodosus*. This was demonstrated by fimbrial isolation and purification, electrophoretic and Western transfer analyses, and immunogold labeling and electron microscopy. These results suggest that type 4 fimbriated bacteria use a common mechanism for fimbrial assembly and that the structural subunits are interchangeable, thereby providing a basis for the development of multivalent vaccines.

Bacteroides nodosus is the essential causative agent of ovine footrot (4, 11). This anaerobe contains numerous surface filaments, about 6 nm in diameter and ranging up to several micrometers in length (14, 46, 50), termed fimbriae (or common pili), which play a central role in both pathogenesis and immunity (for a recent review, see reference 29). Fimbriae have adherent functions and appear to be a mechanism for the colonization of epithelial tissues in eucaryotic hosts. The properties of *B. nodosus* fimbriae (14) suggest that they belong in the category of type 4, as proposed by Ottow (38), citing *Pseudomonas aeruginosa* (6) as a prototype. Fimbriae of this type have a polar location on the cell and appear to be involved in surface translocation by a phenomenon known as twitching motility (21). The same characteristics are also observed in the fimbriae found in a broad range of gram-negative species classified within the genera *Acinetobacter*, *Alteromonas*, *Bacteroides*, *Eikenella*, *Moraxella*, *Neisseria*, and *Pseudomonas*, among others (6, 17, 20, 21).

This grouping is supported by recent protein and DNA sequence analyses of the structural subunits of the fimbriae of *B. nodosus* (12, 31), *Moraxella nonliquefaciens* (16), *Moraxella bovis* (28), *Neisseria gonorrhoeae* (22, 33, 44), *Neisseria meningitidis* (22, 36), and *P. aeruginosa* (42). These subunits, which range in size from about 145 to 160 amino acids among different species and serotypes, all share the distinctive feature of an unusual modified amino acid, methylphenylalanine (MeF), as the first residue in the mature protein, as well as a striking degree of sequence conservation throughout the amino-terminal region. This region is highly hydrophobic and exhibits at least 90% homology with the following 32-amino-acid consensus sequence:

MeF T L I E L M I V (I/V) A I (I/V) G I L A A (I/V)

A (I/L) P A Y (Q/N) D Y (I/V) (A/S) (R/K) (A/S) Q

Further pockets of homology are evident up to the glycine residue at position 54/55 (Fig. 1). In addition, the subunits also seem to share a similar and unusual 6- to 7-amino-acid positively charged leader sequence in the primary translation product, M(K/N)(S/T/—)(A/L)QKG, which is cleaved from the protein at some point prior to incorporation into the mature fimbrial strand. Interspecies divergence is seen primarily in the more hydrophilic carboxy-terminal two-thirds of the molecule. Structural and antigenic variation within species also occur in this region, especially in hypervariable domains in which the immunodominant serological epitopes appear to be located (19, 28, 32, 42, 44, 49). This variation involves not only amino acid substitutions, but also small insertions and deletions (19, 28, 32, 42) (Fig. 1). In some cases at least, variant forms appear to be actively generated by recombination between active and silent gene loci (18, 19, 45).

The genes encoding the fimbrial subunits of *N. gonorrhoeae*, *P. aeruginosa*, *M. bovis*, and *B. nodosus* appear to be located on the bacterial chromosome, but little is known about the morphogenesis of type 4 fimbriae. Studies of type 1 chromosomally encoded and plasmid-encoded fimbrial systems found in uropathogenic and enterotoxigenic strains of *Escherichia coli* (and other *Enterobacteriaceae*) have shown that biosynthesis of these fimbriae involves the participation of several genes, which are normally clustered (24, 35, 37). One of these genes codes for the structural subunit itself, another for a basal protein which appears to act as the site for attachment or extrusion of the fimbrial strand at the cell surface, and the remainder for other polypeptides whose exact functions remain to be determined, but either are required for fimbrial assembly or affect the level of fimbrial expression (24, 35, 37). An analogous basal protein appears to be associated with the fimbriae of *B. nodosus* (30). However, there is no obvious homology between the structural subunits of type 4 fimbriae and those found in *E. coli*. There are in fact significant differences, and these systems appear to be distinct. Type 4 fimbrial subunits expressed from cloned genes in *E. coli* are not assembled into mature fimbriae, but rather are found embedded in the inner membrane of the cell (1, 13, 28, 33).

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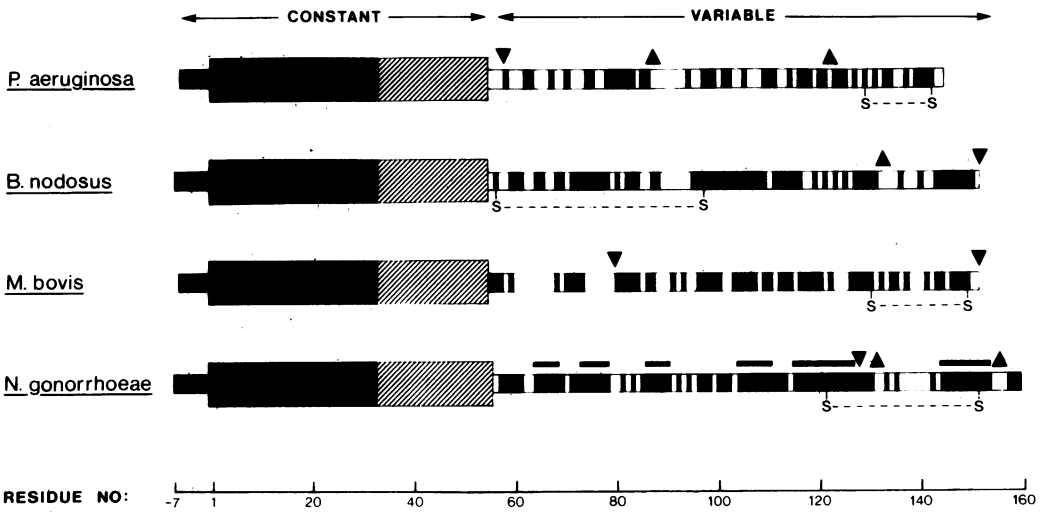

FIG. 1. Diagrammatic representation and comparison of the primary sequences of the fimbrial subunits of *P. aeruginosa*, *B. nodosus*, *M. bovis*, and *N. gonorrhoeae*. In the constant region, the thinner dark block represents the short 6- to 7-amino-acid leader sequence, and the thicker dark block is the highly conserved hydrophobic 32-amino-acid sequence (see text), beginning with the modified phenylalanine (MeF) residue at position 1 in the mature protein. The shaded area from residue 33 to the glycine at position 54/55 indicates a secondary region which is conserved within species but shows some divergence between species. Little, if any, homology is observed between species in the variable region: the dark and light areas here indicate conserved and different amino acids, respectively, between two serological variants from within the species in question, with the sites of small insertions (▼) and deletions (▲) indicated accordingly. Cysteine (disulfide) bridges are marked (S-----S). Strains compared are *P. aeruginosa* K/O (42), *B. nodosus* A/E (32), *M. bovis* β/α (28), and *N. gonorrhoeae* MS11/variant 3C (19). Several variants of *N. gonorrhoeae* have been sequenced (19), and the bars above the variable region indicate the residues which were conserved in all cases. The two variants selected for direct comparison here were those which were the least alike.

For *N. gonorrhoeae* and *P. aeruginosa*, it has been suggested that the hydrophobic amino-terminal portion of the fimbrial subunit is involved in subunit-subunit interactions within the fimbrial strand (44, 51), and this may well be the case. However, the extremely strong conservation of this sequence, including the small leader peptide, across the range of different bacterial species and genera which possess type 4 fimbriae suggested to us that this region may also contain important signals for fimbrial morphogenesis (29), in terms of the interaction of the structural subunit with other factors involved in the assembly system. If so, the fimbrial subunits containing these signals might be interchangeable throughout the type 4 group. This possibility was tested by inserting the *B. nodosus* fimbrial subunit gene into *P. aeruginosa*, and we show here that such recombinants will produce, after appropriate molecular genetic manipulation, high levels of mature *B. nodosus*-type fimbriae. *P. aeruginosa* was selected from the available type 4 fimbriated bacteria as the (prototype) recipient host simply on the basis that this species is a genetically well-characterized aerobe for which a range of plasmid shuttle vectors are available for gene cloning and transfer.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. nodosus* VCS1001 (ATCC 25549), formerly known as strain 198, was used for this study. This strain is the designated prototype of serogroup A (8). *P. aeruginosa* PAK/2Pfs (ATCC 53308), a phage-resistant, nonretractile multifimbriated mutant of *P. aeruginosa* PAK (5), was used as the host for morphogenetic expression. The plasmids used in the recombinant DNA constructions (Fig. 2) were pBA121 (1), which contains the *B. nodosus* VCS1001 fimbrial subunit gene; pPL-λ (P-L Biochemicals), which contains the bacteriophage λ promoter, p_L; and pKT240 (3), a broad-host-range vector capable of being maintained in both *E. coli* and *P. aeruginosa*. All constructions involving the p_L promoter were carried out in *E. coli* E418 (N. Murray, strain W1485, λ*cIts*857 *S*am7; obtained from B. Egan, University of Adelaide), grown at the permissive temperature of 30°C. The p_L promoter was derepressed, when required, by shifting the culture to 42°C.

Recombinant DNA constructions. Plasmid DNA was purified by cesium chloride-ethidium bromide isopycnic ultracentrifugation following either the small-scale alkali-sodium dodecyl sulfate (SDS) procedure (27) or the large-scale Triton X-100 procedure (1). All restriction endonuclease digestions and DNA ligations were carried out under standard conditions (27). Restriction digests were analyzed on 0.6 or 1% agarose gels in a Tris-borate-EDTA buffer system, containing 0.5 μg of ethidium bromide per ml (1, 27). The *Dra*I and *Bam*HI restriction fragments were gel purified prior to insertion into pPL-λ and pKT240, respectively (Fig. 2). These DNA fragments were recovered by electroelution and chromatography on NACS-52 minicolumns (Bethesda Research Laboratories).

DNA transformation. *P. aeruginosa* cells were made competent for transformation by treatment with $MgCl_2$ as follows. Cells were grown overnight at 43°C (23) in KNO_3 broth (2.5% Oxoid nutrient broth no. 2, 0.5% yeast extract, and 0.4% KNO_3) and then diluted 1:25 in fresh KNO_3 broth and incubated with shaking at 37°C until the culture had reached an A_{500} of 0.5. The culture was then chilled quickly on ice, and the cells were recovered by centrifugation (6,000 × *g*, 6 min). All subsequent steps were carried out at 4°C. The cells were washed twice by suspension in one-fifth volume of 0.1 M $MgCl_2$ and then suspended in one-eighth volume of 0.15 M $MgCl_2$ and left on ice for 20 to 30 min. The cells were finally suspended in one-twentieth volume of 0.15 M $MgCl_2$ and kept on ice ready for transformation. Approximately 200 ng of DNA was used to transform 0.1 ml of competent cells. *E.*

coli cells were made competent for transformation by conventional treatment with $CaCl_2$ (27).

For both *P. aeruginosa* and *E. coli*, transformation was carried out by standard procedures (27), including the heat shock step. Transformants were selected and maintained on Luria (L) broth (27) agar plates, containing 50 μg of ampicillin per ml (for *E. coli*) or 1 mg of carbenicillin per ml (for *P. aeruginosa*) (3).

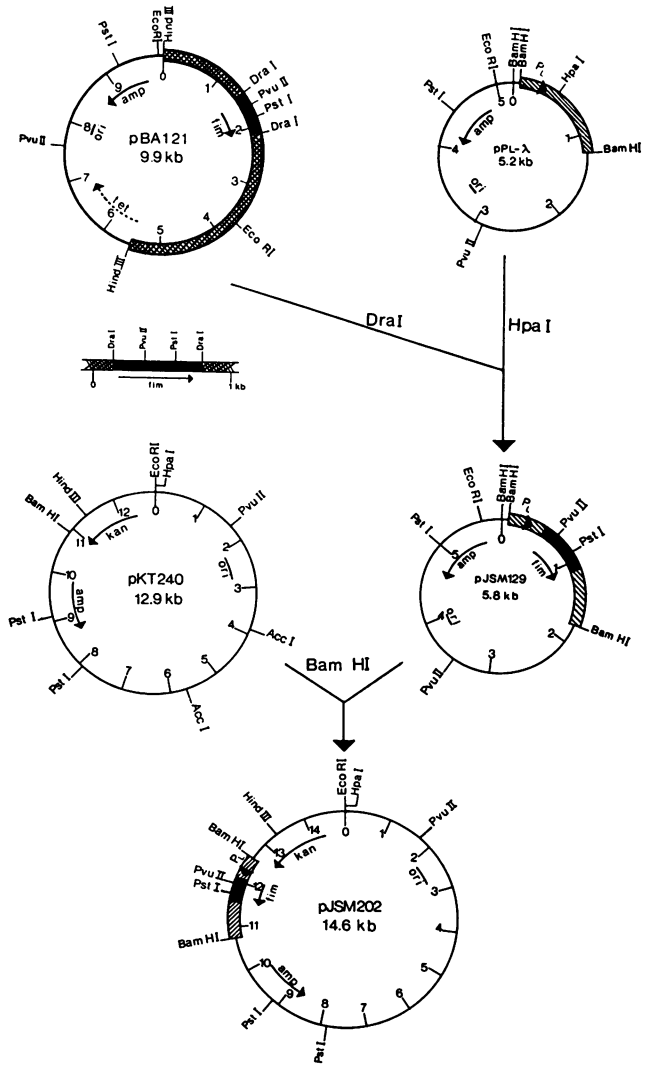

FIG. 2. Genealogy and construction of pJSM202. The cross-hatched sequences in pBA121 (30) indicate those originally cloned from *B. nodosus* VCS1001, with the internal dark segment delineating the *Dra*I fragment which contains the fimbrial subunit gene. The hatched sequences in pPL-λ, pJSM129, and pJSM202 indicate the *Bam*HI segment (originally derived from bacteriophage λ) which contains the p_L promoter and into which the *Dra*I fragment was cloned (at the *Hpa*I site). The orientation of the fimbrial subunit gene with respect to the p_L promoter in the *Bam*HI segment was determined by reference to the internal *Pst*I and *Pvu*II sites and verified by overproduction of the protein under appropriate conditions. The orientation of the *Bam*HI insert in pJSM202 was not determined. The thin line in pJSM202 represents sequences derived from pKT240 (3). Various restriction endonuclease sites are indicated. The numbers refer to the distance in kilobases (kb) around each plasmid. The arrows indicate the direction of transcription and approximate boundaries of the structural genes in question. Abbreviations: *fim*, fimbrial subunit gene; *amp*, ampicillin (or carbenicillin) resistance; *tet*, tetracycline resistance (nonfunctional in pBA121); *kan*, kanamycin resistance; *ori*, origin of replication.

Isolation of fimbriae. *B. nodosus* VCS1001 cells were grown on hoof agar plates (47) for 4 days at 37°C in an atmosphere of 90% H_2–10% CO_2 and harvested by scraping into cold phosphate-buffered saline (PBS) (140 mM NaCl, 10 mM sodium phosphate, pH 7.2) (30). *B. nodosus* fimbriae were purified from the supernatant of PBS-suspended cells by isoelectric precipitation with 0.1 M sodium acetate, pH 4.5, as described previously (30).

Normal *P. aeruginosa* fimbriae were isolated from PAK/2Pfs cells cultured aerobically at 37°C overnight on nutrient (L broth) agar plates. The cells were harvested by scraping into cold PBS, and the resulting suspension was blended for 1 min with a Silverson mixer-emulsifier. All steps were carried out at 4°C. The cells and cell debris were removed by centrifugation (24,000 × *g*, 30 min), and the supernatant was adjusted to pH 4.5 by the addition of acetic acid. After standing overnight, the fimbriae were collected by centrifugation (24,000 × *g*, 30 min). The fimbriae were then further purified by a second round of dissolution in PBS and precipitation with sodium acetate, pH 4.5.

Fimbriae were isolated from recombinant *P. aeruginosa* PAK/2Pfs cells following overnight culture at 37°C on L broth agar plates supplemented with carbenicillin (1 mg/ml). The cells were harvested in PBS and subjected to mechanical blending as described above (see Fig. 4). The fimbriae were recovered from the (cell-free) supernatant by either isoelectric precipitation with sodium acetate, pH 4.5, as described above, or by the independent method of precipitation with 0.1 M $MgCl_2$ (30).

Electrophoretic display. Samples were analyzed by electrophoresis on SDS-urea 8 to 15% polyacrylamide gradient gels with the modified Laemmli buffer system described previously (30). Protein bands were visualized by staining with Coomassie blue R250.

Western transfer. Unstained gel displays were electrophoretically transferred to nitrocellulose paper (48), which was then incubated with antifimbrial antiserum (diluted 1:1,000), followed by ^{125}I-protein A (0.1 μCi/ml), as detailed in Anderson et al. (2).

Antisera. Antisera specific for *B. nodosus* VCS1001 fimbriae and *P. aeruginosa* PAK/2Pfs fimbriae were raised in rabbits following vaccination with purified fimbrial preparations, as described previously (30).

Immunogold labeling and electron microscopy. Whole cells of *P. aeruginosa* PAK/2Pfs, with or without pJSM202, were diluted in H_2O and looped onto parlodion-carbon-coated gold grids. The grids were dried, treated with 5% bovine serum albumin in PBS for 2 min, and then incubated for 1 h with either anti-*P. aeruginosa* PAK/2Pfs fimbrial antiserum or anti-*B. nodosus* VCS1001 fimbrial antiserum, diluted 1:500 and 1:100 in PBS, respectively. The grids were washed and then incubated for 1 h with protein A labeled with 15-nm colloidal gold particles (Janssen Pharmaceutica, Belgium) diluted 1:10 in 140 mM NaCl–10 mM Tris hydrochloride, pH 8.2, containing 0.1% bovine serum albumin. The grids were again washed and then negatively stained with 2% sodium phosphotungstate (pH 7.0) and examined in a Jeol 100CX electron microscope at 60 kV.

RESULTS

Genetic constructions. The *B. nodosus* fimbrial subunit gene used in this study was derived from strain VCS1001, which is the designated prototype of serogroup A (8). The

gene is located within a 5.5-kilobase *Hin*dIII genomic DNA fragment and is expressed in *E. coli* from an associated promoter within the cloned sequence (1). In our initial experiments, this *Hin*dIII fragment was subcloned directly from pBA121 into the corresponding site of the broad-host-range plasmid vector pKT240 (Fig. 2) to yield pJSM125. This plasmid was then transformed into the multifimbriated *P. aeruginosa* strain PAK/2Pfs. Immunological analyses of the resulting transformants showed that the *B. nodosus* fimbrial subunit gene was expressed poorly in these cells. Nevertheless, there were clear indications that at least some of the *B. nodosus* fimbrial subunits produced in these recombinants were assembled into mature fimbriae, since the antigenic signal copurified with the fimbriae and was retained in the most highly purified fractions (data not shown). However, these fractions were composed almost entirely of the host *P. aeruginosa* subunit, and it was not possible to determine the exact distribution of the trace amounts of *B. nodosus* antigen in the fimbriae.

The problem of the low expression of the *B. nodosus* fimbrial subunit gene in *P. aeruginosa* was overcome by placing this gene under the transcriptional control of the strong promoter, p_L, from bacteriophage λ as follows. pBA121 was digested with the restriction endonuclease *Dra*I, which cleaves at convenient sites flanking the fimbrial subunit gene, one 30 nucleotides upstream from the initiation codon and the other 69 nucleotides downstream from the termination codon, to yield a blunt-ended 576-base-pair gene cartridge. This cartridge, which includes the Shine-Dalgarno sequence as well as a downstream region of hyphenated dyad symmetry which may function as a transcription termination signal (12), was inserted into the *Hpa*I site located downstream from the p_L promoter in pPL-λ (Fig. 2). The entire gene-promoter construction was then transferred as a *Bam*HI fragment into the corresponding site of pKT240 to generate pJSM202 (ATCC 40203) (Fig. 2). All constructions involving the p_L promoter were carried out in an *E. coli* λ lysogen (E418) containing the temperature-sensitive *c*I857 repressor gene, grown at 30°C. Viable transformants could not be obtained in *E. coli* cells lacking a functional λ *c*I repressor, presumably because these cells are unable to cope with the large amount of *B. nodosus* fimbrial subunit being produced and deposited into the membrane (1). On the other hand, we found that pJSM202 was not lethal for *P. aeruginosa*, even though the p_L promoter was active in this host (see below). We also found that there was no advantage to be gained from regulation of the p_L promoter in *P. aeruginosa* by inserting the *c*I857 repressor gene into pJSM202, although this system was functional (data not shown).

Morphogenetic expression. Analysis of *P. aeruginosa* PAK/2Pfs transformants containing pJSM202 showed that not only were large amounts of the *B. nodosus* fimbrial subunit produced, but also that these subunits were assembled into mature fimbrial structures on the surface of the cells. Although *B. nodosus*-type fimbriae are physically very similar to those naturally produced by the *P. aeruginosa* host strain, they may be distinguished immunologically and by electrophoretic differences between the structural subunits. The apparent molecular weight of the *B. nodosus* VCS1001 subunit in SDS-polyacrylamide gels is about 17,000, whereas that of the *P. aeruginosa* PAK/2Pfs subunit is about 16,000; their actual molecular weights (calculated from the sequence) are 16,218 (151 amino acids) (12) and 15,082 (145 amino acids) (42), respectively.

Morphogenetic expression of *B. nodosus*-type fimbriae in recombinant *P. aeruginosa* cells was first indicated by the presence of substantial amounts of this antigen in the extracellular environment. In many fimbriated bacteria, including *B. nodosus* and *P. aeruginosa*, a proportion of the fimbriae appear to be shed into the medium (or suspension buffer) and remain there after removal of the cells by centrifugation. Additional fimbriae may be removed by mechanical blending of the cell suspension. Figure 3 shows a Western transfer analysis of the cell pellet and derived supernatant fractions from recombinant cells cultured on solid medium. First, the *B. nodosus* fimbrial subunit was transcribed very actively from the p_L promoter in *P. aeruginosa* cells containing pJSM202, at a level at least two orders of magnitude higher than that in the same host containing pJSM125, in which the gene is transcribed from an associated promoter within the original cloned segment (1) of *B. nodosus* DNA. The activity of the p_L promoter in *P. aeruginosa* seemed to be similar to that in *E. coli* (Fig. 3). Second, a large proportion of the fimbrial subunit antigen was found in the supernatant fraction obtained from recombinant *P. aeruginosa* cells, in contrast to *E. coli* E418 cells containing pJSM202, in which the antigen was entirely located in the cell (pellet) fraction. A substantial amount of *B. nodosus* fimbrial subunit also remained with the recombinant *P. aeruginosa* cells, but it is not known to what extent this represents fimbriae not dislodged from the cell (cf. Fig. 4) or (as yet) unassembled subunits accumulated within the cell (52). Third, the fimbrial subunit found in *E. coli* appeared to be slightly larger than that found in *P. aeruginosa* or in *B. nodosus* itself. This reflects the inability of *E. coli* to cleave the short leader sequence from the primary translation product (12), which presumably is part of the normal process of morphogenetic expression.

The assembly of *B. nodosus* fimbrial subunits into mature fimbriae in recombinant *P. aeruginosa* cells was also dem-

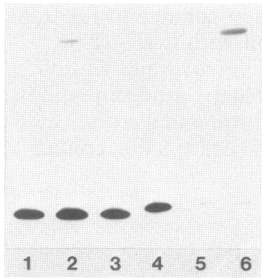

FIG. 3. Extracellular expression of fimbrial antigen in recombinant cells. Lanes: 1, purified fimbriae from *B. nodosus* VCS1001; 2 and 3, samples of the cell pellet and supernatant material, respectively, from *P. aeruginosa* PAK/2Pfs transformed with pJSM202; 4 and 5, equivalent amount of cell pellet and supernatant material, respectively, from *E. coli* E418 transformed with pJSM202 and cultured at the restrictive temperature; 6, *P. aeruginosa* PAK/2Pfs cells transformed with pJSM125 (see text). Lane 6 contained approximately four times the amount applied in lanes 2 to 5. Recombinant *P. aeruginosa* cells were grown at 37°C overnight on L broth agar plates containing carbenicillin. *E. coli* cells were grown on L broth agar plates containing ampicillin at 30°C for 8 h and then shifted to 42°C for a further 4 h to induce expression of the fimbrial subunit from the (derepressed) p_L promoter. The cells were harvested in cold PBS and, after mechanical blending, were pelleted by centrifugation. Equal amounts of the resulting cell pellet and supernatant fractions were then displayed on an SDS-urea 8 to 15% polyacrylamide gradient gel and subjected to Western transfer analysis with anti-*B. nodosus* fimbrial antiserum. The position of bound antibodies was visualized by autoradiography. The amount loaded from different preparations was standardized by reference to the optical density of the original cell suspension.

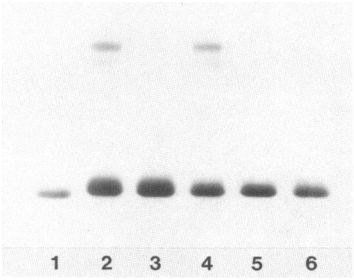

FIG. 4. Isolation of fimbriae from *P. aeruginosa* cells containing pJSM202. Recombinant cells were cultured on nutrient agar plates and harvested in PBS, as described in the legend to Fig. 3. The cells were removed by centrifugation, and the supernatant fraction was reserved. The cells were suspended in the same volume of PBS, subjected to mechanical blending, and again pelleted by centrifugation to yield a blended supernatant fraction. Fimbriae were purified from these fractions by isoelectric or $MgCl_2$ precipitation, as described in the text. Samples were then displayed on SDS-urea 8 to 15% polyacrylamide gels and stained for protein with Coomassie blue R250. Lanes 2 and 3 contain fimbriae recovered from the original supernatant fraction by isoelectric or $MgCl_2$ precipitation, respectively. Lanes 4 and 5 contain fimbriae recovered from the blended supernatant fraction by isoelectric or $MgCl_2$ precipitation, respectively. Lanes 2 to 5 all contain an amount of material equivalent to 3.5% of that isolated from a single standard (85-mm) petri dish culture of the recombinant *P. aeruginosa* cells. Lane 1 contains purified *P. aeruginosa* PAK/2Pfs fimbriae (about 5 μg). Lane 6 contains purified *B. nodosus* VCS1001 fimbriae (about 10 μg).

onstrated by the presence of this antigen in purified fimbriae isolated under different conditions. Fimbriae may be recovered from cell-free supernatants either by isoelectric precipitation at pH 4.5 or by precipitation with $MgCl_2$ (30). These are standard procedures applicable to the isolation of fimbriae from both *P. aeruginosa* and *B. nodosus*. With either method, high yields of relatively pure fimbriae were obtained from *P. aeruginosa* cells containing pJSM202 (Fig. 4). This was confirmed by electron microscopy of negatively stained samples. Electrophoretic analysis showed that these fimbriae were composed primarily of a structural subunit which comigrated in the gel with that obtained from *B. nodosus* (Fig. 4). These fimbriae could also be further purified by isopycnic banding in CsCl gradients (14, 30). Quite unexpectedly, there was little or no evidence of the fimbrial subunit characteristic of the host strain, PAK/2Pfs.

These results were confirmed by Western transfer analysis of the fimbriae produced by the recombinant cells in comparison with those obtained from *B. nodosus* VCS1001 and *P. aeruginosa* PAK/2Pfs (Fig. 5). This experiment clearly showed that not only did the fimbriae produced by *P. aeruginosa* cells containing pJSM202 consist of a structural subunit which had the same electrophoretic mobility as that found in *B. nodosus* itself (Fig. 5A), but also that this protein was recognized by antibodies directed against *B. nodosus* fimbriae (Fig. 5C). The reciprocal Western assay with anti-*P. aeruginosa* fimbrial antiserum confirmed that the fimbriae produced by these recombinant cells contained little, if any, of the structural subunit naturally expressed by the host (Fig. 5B).

The production of *B. nodosus*-type fimbriae in the recombinant *P. aeruginosa* cells was also independently verified by immunogold labeling and electron microscopy (Fig. 6). Antibody binding was indicated by the presence of electron-dense spherical gold particles, as well as by clumping together of the fimbrial strands. As expected, the fimbriae produced by the host strain PAK/2Pfs reacted strongly with the homologous antiserum (Fig. 6A), but not with antiserum against *B. nodosus* fimbriae (Fig. 6B). This pattern was completely reversed in the recombinant *P. aeruginosa* cells containing pJSM202. Fimbriae produced by these cells were not recognized by antibodies directed against *P. aeruginosa* fimbriae (Fig. 6C), but rather reacted strongly with those against *B. nodosus* fimbriae (Fig. 6D). This was the same profile exhibited by *B. nodosus* itself.

Several other criteria were also used to verify the production of *B. nodosus* fimbriae by *P. aeruginosa* cells containing pJSM202. These included demonstrations that antisera raised against the fimbriae prepared from recombinant cells agglutinate (8, 10) *B. nodosus* VCS1001 cells and reciprocally that antisera raised against *B. nodosus* VCS1001 cause agglutination of the recombinant *P. aeruginosa* cells. These agglutination reactions were (*B. nodosus*) serotype specific. Control experiments showed that anti-*B. nodosus* antisera did not agglutinate host *P. aeruginosa* cells and that antisera raised against normal *P. aeruginosa* fimbriae did not agglutinate *B. nodosus* cells. Similar results were obtained in standard solid-phase antibody-antigen binding assays, such as enzyme-linked immunosorbent assays (data not shown).

These results demonstrate that *P. aeruginosa* PAK/2Pfs cells containing the recombinant plasmid pJSM202 produce mature fimbrial structures which are physically, structurally, and antigenically indistinguishable from those produced by the *B. nodosus* strain from which the fimbrial subunit gene was originally derived. *B. nodosus*-type fimbriae were also obtained when other fimbriated *P. aeruginosa* strains were

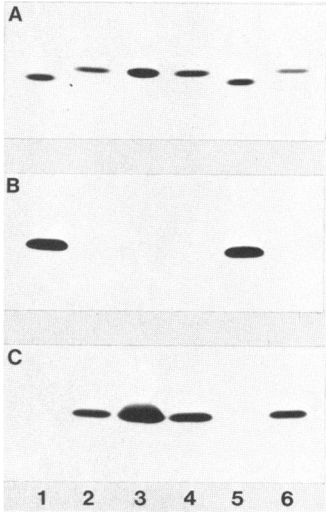

FIG. 5. Electrophoretic and immunological analysis of fimbriae produced by *P. aeruginosa* PAK/2Pfs(pJSM202). Fimbriae were purified (by two rounds of isoelectric precipitation) from two independent primary clones of *P. aeruginosa* PAK/2Pfs transformed with pJSM202 (lanes 3 and 4) and compared with fimbriae obtained from the host *P. aeruginosa* PAK/2Pfs (lanes 1 and 5) and from *B. nodosus* VCS1001 (lanes 2 and 6). These fimbriae (about 2 μg in each case) were displayed on SDS-urea 8 to 15% polyacrylamide gradient gels and stained for protein with Coomassie blue R250 (A). Duplicate (unstained) displays were subjected to Western transfer analysis with either anti-*P. aeruginosa* PAK/2Pfs fimbrial antiserum (B) or anti-*B. nododus* VCS1001 fimbrial antiserum (C). In each case only the lower portion of the gel (containing the fimbrial subunit) is shown.

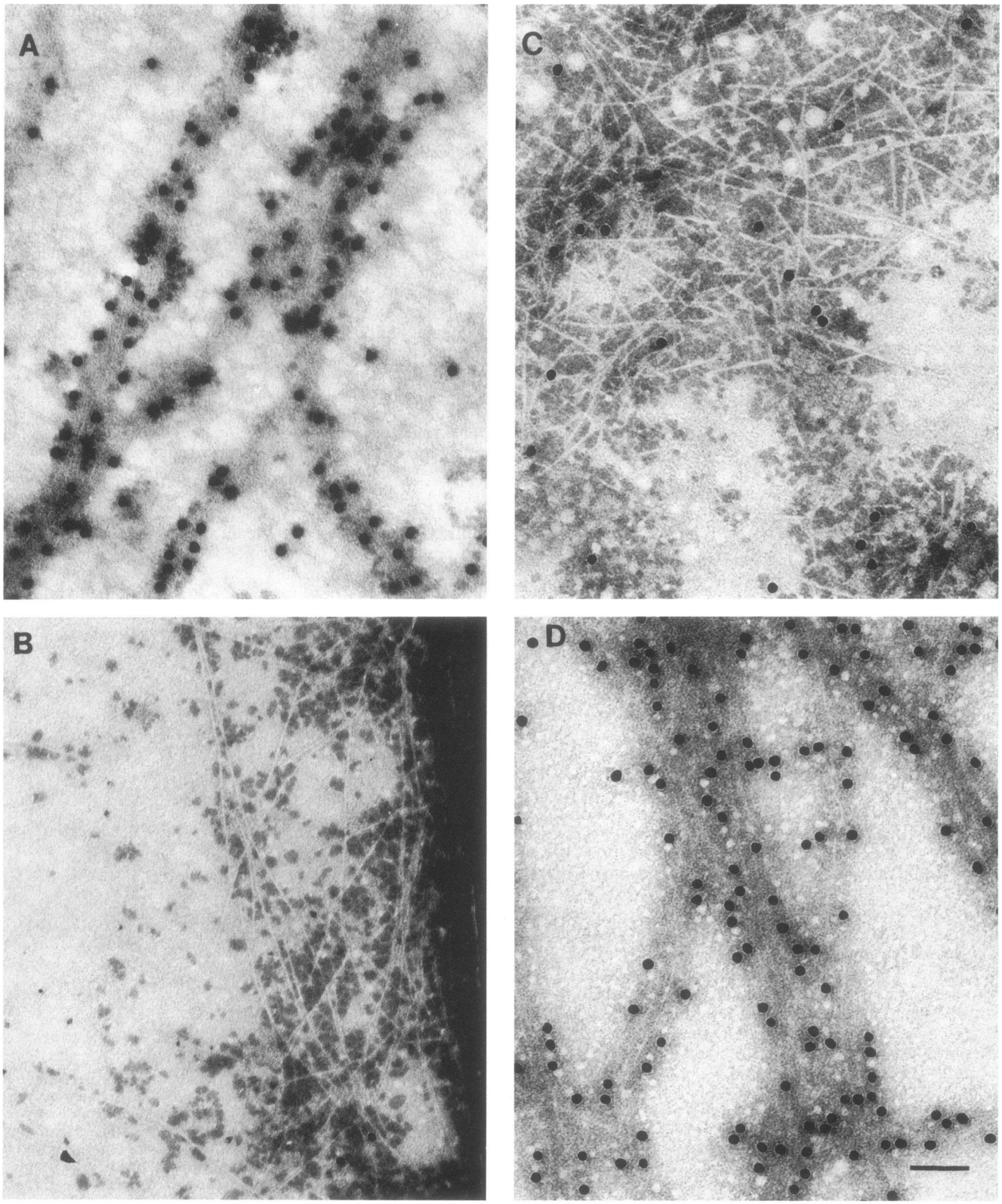

FIG. 6. Immunogold labeling of host and recombinant fimbriae. Whole cells of the host *P. aeruginosa* PAK/2Pfs (A and B) or the same strain containing pJSM202 (C and D) were placed onto parlodion-carbon-coated gold grids and treated with either anti-*P. aeruginosa* PAK/2Pfs fimbrial antiserum (A and C) or anti-*B. nodosus* VCS1001 fimbrial antiserum (B and D) and then with protein A labeled with 15-nm colloidal gold particles. The grids were then negatively stained and examined in an electron microscope at 60 kV. Each micrograph is shown at the same magnification. Bar, 0.1 μm.

used as the host for pJSM202 (data not shown). These included *P. aeruginosa* PAK (ATCC 25102), the parent of PAK/2Pfs, as well as *P. aeruginosa* PAO1 (ATCC 25247) and its derived multifimbriated mutant DB-2 (25), which normally expresses a form of fimbrial subunit structurally and serologically distinct from that found in PAK strains.

DISCUSSION

The insertion of pJSM202 into a fimbriated *P. aeruginosa* strain effectively resulted in complete substitution of the *B. nodosus* fimbrial subunit for that normally produced by the *P. aeruginosa* cells. This seemed to be largely due to the relatively high level of expression of the *B. nodosus* fimbrial subunit, but it also suggests that these cells have little, if any, preference for assembly of the homologous over the heterologous subunit into fimbrial strands. We also found, however, in control experiments (data not shown) that the level of expression of the endogenous fimbrial subunit was substantially reduced in recombinant cells containing either pJSM202 or pKT240, apparently as a consequence of the physiological effects of antibiotic selection or the expression of genes on the vector plasmid itself.

The experiments described herein support the proposition that type 4 fimbrial subunits from different bacterial species are assembled by a common mechanism and may be interchanged among compatible hosts. This is certainly the case for *B. nodosus* and *P. aeruginosa* and should be equally true for other type 4 species retaining the conserved amino-terminal region of the fimbrial subunit (see below). From sequence data, *B. nodosus* and *P. aeruginosa* appear to be no more or less closely related in this respect than other members of the group. Moreover, the large differences in the carboxy-terminal two-thirds of these proteins do not appear to have affected the process of fimbrial assembly in the heterologous host. These differences include almost complete divergence in primary structure, as well as changes in the location and span of the internal disulfide loop, which occurs in the middle of the molecule in *B. nodosus* and at the end in *P. aeruginosa*. In this respect *B. nodosus* would appear to be less related to *P. aeruginosa* than are *N. gonorrhoeae* and *M. bovis*, which also have disulfide bridges near the carboxy terminus of the subunit, although the lengths of the loops are different (Fig. 1). In any case it would seem that there are few constraints imposed on variation in the latter part of the subunit, except perhaps for the overall size of the molecule. On the other hand, the facts that fimbriae from different type 4 species are physically very similar and that one subunit may be successfully substituted for another in the same host suggest that these polypeptides must have similar tertiary and quaternary structures. In support of this, there appear to be similarities in the secondary structure (B. Dalrymple and J. S. Mattick, manuscript in preparation). These subunits are probably arranged with the conserved hydrophobic amino-terminal regions stacked in an overlapping helical array around the long axis of the fimbrial strand (15) and the variable carboxy-terminal regions displayed outwards. The exposed faces of the latter include the immunodominant epitopes, which divert attention from more conserved surface binding sites (41), possibly for glycoproteins on the host cell. These sites, which are not well defined, may themselves vary from species to species, depending on the target host and tissue. For example, *B. nodosus* colonizes the epidermis of the ungulate hoof, *M. bovis* the conjunctiva of cattle, and *N. gonorrhoeae* the human urogenital tract. *P. aeruginosa* itself is an opportunistic pathogen of epithelial surfaces in a variety of compromised hosts. In one case at least, two isolates of the same species (*N. gonorrhoeae*) have been reported to express variant fimbriae with altered binding properties (25). It is also worth noting that one species which appears to have type 4 fimbriae (*P. solanacearum*) is in fact a plant pathogen (17).

The results presented above add weight to the idea that type 4 fimbriae, despite the diversity of species which appear to possess them, represent a cohesive group, which are structurally, functionally, and evolutionarily related. Type 4 fimbriae may be characterized by their polar location on the cell, the twitching-motility phenotype, and the conserved amino-terminal sequence of the structural subunit. The latter characteristic has been detailed in only a few cases thus far, but it would seem relatively safe to predict that this conserved sequence will be found in most, if not all, species which exhibit the former characteristics. Assuming that this holds true, type 4 fimbriae would appear to be very widely distributed among gram-negative bacteria and presumably represent a relatively ancient mechanism for the colonization of eucaryotic tissue. This system is clearly distinct in both sequence and organization from the type 1 and related fimbrial systems found in members of the *Enterobacteriaceae* (9, 24) which probably have a similar biological role. Although the structural subunits of the various families of enterobacterial fimbriae may exhibit greater overall sequence homology among themselves (24, 35, 40) than is observed among type 4 proteins, substitution appears to be possible only between closely related variants (9, 35, 39). This suggests that a significant degree of morphogenetic drift has occurred in the enterobacterial systems, which also implies that their assembly and structure are fundamentally different from that of type 4. In view of the large evolutionary distances between different species which express type 4 fimbriae, the strong conservation of the amino-terminal sequence of the subunit is striking indeed. This would appear to be the consequence of powerful constraints related to the assembly, the structure, and, ultimately, the function of the system. The corollary, substantiated by the present study, is that there should be few, if any, barriers to the morphogenetic expression of type 4 fimbrial subunits in heterologous hosts, and this (i.e., genetic exchange) is one possible route by which variation could be generated in natural populations. In this context there may be no real distinction between intra- and interspecific variants of the type 4 fimbrial subunit, apart from differences in antigenic profile and target specificity.

Vaccine development. Fimbriae are major surface antigens, and antibodies against fimbriae generally reduce or prevent infection. This is well documented in the case of *B. nodosus*, in which the fimbriae are known to be the primary serological and protective antigens against footrot (29). Preliminary evidence suggests a similar situation with *N. gonorrhoeae* (7, 41) and *M. bovis* (26). The development of practical vaccines against these pathogens has been hampered by the difficulty in culturing the organisms and the range of serological variants. Again the best-characterized case is *B. nodosus*, of which field isolates have been grouped into eight or nine major serogroups for the purposes of immunological coverage (8). Conventional multivalent whole-cell vaccines against footrot are available, but are relatively crude and expensive. The production of *B. nodosus*-type fimbriae in *P. aeruginosa* provides an alternative route for vaccine production. Vaccination trials have shown that the fimbriae produced by *P. aeruginosa* containing pJSM202 are just as

effective as either natural fimbriae or whole cells of *B. nodosus* in eliciting protective immunity against homologous footrot challenge (J. R. Egerton, P. T. Cox, B. J. Anderson, C. Kristo, M. Norman, and J. S. Mattick, Vet. Microbiol., in press).

This system has several biotechnological advantages for the production of type 4 fimbrial vaccines. Serotypic variants may be produced simply by substituting the relevant structural subunit gene into a host-vector expression system as described here. Such genes are relatively easy to clone, and, in the case of *B. nodosus*, most have already been obtained (29). At this stage there is no a priori reason to expect that this system will not be more generally applicable to the production of similar vaccines against other type 4 fimbriated pathogens. The fimbriae may be produced in higher yields than obtained in the natural host, which in many cases have fastidious growth requirements or exhibit unstable or low fimbrial expression. Consequently, representatives for inclusion in a vaccine may be selected solely on their immunological profile rather than on the growth yield properties of the original host strain. Finally, since the variation in the carboxy-terminal region of the structural subunit does not appear to interfere with the process of fimbrial assembly or the characteristics of the fimbrial strand, there is the attractive possibility that coinsertion of two or more type 4 fimbrial subunit genes (under appropriate promoter controls) into the same compatible host cell would result in the formation of mixed or hybrid fimbriae, which may then provide a multivalent preparation active against more than one serotype or even perhaps against more than one species.

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