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Identification, Purification, and Characterization of the Type 4 Fimbriae of *Pasteurella multocida*

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The presence of fimbriae on *Pasteurella multocida* has been reported, but there have been no prior studies aimed at conclusively characterizing these structures. We now report on the identification and characterization of type 4 fimbriae on serogroup A, B, and D strains of *P. multocida*. Under microaerophilic conditions *P. multocida* showed an increased expression of the fimbriae, which were observed to form bundles. Fimbriae purified by high-performance reverse-phase liquid chromatography constituted a single 18-kDa subunit, the first 21 amino acids of which shared very high similarity with the N-terminal amino acid sequence of other type 4 fimbrial subunits. Antiserum against the *P. multocida* 18-kDa protein immunostained the type 4 fimbrial subunit of *Moraxella bovis* and *Dichelobacter nodosus*. Based on these observations we conclude that *P. multocida* possesses type 4 fimbriae and have designated the *P. multocida* fimbrial subunit PtfA.

Pasteurella multocida is an important animal pathogen which causes fowl cholera in poultry, atrophic rhinitis in swine, and hemorrhagic septicemia in cattle (4) and is also a commensal of both human and animal respiratory tracts (29). Strains of *P. multocida* are grouped into 16 serotypes (1 to 16) based on somatic antigens and into five capsular groups (A, B, D, E, and F) (4, 12, 29). Serogroup A strains commonly cause fowl cholera and occasionally cause pneumonia in sheep, cattle, and pigs. Serogroup D organisms cause atrophic rhinitis, while serogroup B and E strains cause hemorrhagic septicemia (4). Surface components such as capsule and outer membrane proteins have been the subject of a number of studies designed to further elucidate the pathogenic mechanisms of *P. multocida*, but adhesins of *P. multocida* have received little investigation.

The colonization of host surfaces by bacteria is frequently mediated by adhesins such as fimbriae (pili). Thus, attachment by fimbriae to host surfaces is usually correlated with virulence (11, 37). *P. multocida* is able to colonize the nasal mucosa of swine (1) and has been shown to adhere to the mucosal epithelium of the nasopharynx of rabbits (10). The presence of fimbriae has been observed on a few strains of *P. multocida*. Recently, two types of fimbriae were identified on serogroup D strains; the first type was similar to type 1 fimbriae and the second was morphologically similar to the *Escherichia coli* "curli" pili. However, it appeared that these structures did not contribute to adhesion to either erythrocytes or immobilized mucus (17). Serogroup A strains adhere to mucosal epithelium and express fimbriae. Glorioso et al. (10) concluded that this adhesion may be due to fimbriae rather than capsular material and that fimbriae are the main surface structures involved in adhesion. Both capsulated and noncapsulated avian strains of *P. multocida* were shown to express hair-like fimbriae which did not agglutinate erythrocytes (28).

A *P. multocida* strain isolated from a human case of otitis media was shown to have spreading and corroding colonies on blood agar, and it was assumed that the mode of surface translocation was due to twitching motility (15). Both corroding of agar and twitching motility are traits common to bacteria that produce type 4 fimbriae (14, 34). Type 4 fimbriae are long appendages composed of repeating fimbrial subunits which range in molecular mass from 15 to 20 kDa. The N-terminal sequence of the mature fimbrial subunit protein is highly conserved (33, 34). Based only on the fact that *P. multocida* appeared to have spreading and corroding colonies, Dalrymple and Mattick (6) classified *P. multocida* as having type 4 fimbriae. However, definitive evidence for this conclusion is lacking.

P. multocida PBA100 (serogroup A:serotype 1) (16) and PBA101 (A:3) (30) were isolated from Australian cases of fowl cholera, while X-73 (A:1), P-1059 (A:3), and P-1662 (A:4) were kindly supplied by J. R. Glisson, University of Georgia, Athens, Ga.; American type strain (ATS) 11 (D:11) and ATS 15 (D:15) were obtained from the National Animal Disease Center, Ames, Iowa; and M1404 (B:2) was previously described (13). *P. multocida* strains were cultured at 37°C in nutrient broth No. 2 (Oxoid) or on horse blood agar (HBA) either aerobically or under microaerophilic conditions as described previously (35). *E. coli* HB101 (3) strains harboring plasmids were cultured at 37°C in Luria Bertani broth or on Luria Bertani agar (1.2% wt/vol) with the addition of ampicillin (100 μg/ml). Bacterial cell lysates of *Neisseria gonorrhoeae* MS11 (24) and *Dichelobacter nodosus* A198 (7) were kindly provided (see below).

For electron microscopy, single colonies of *P. multocida* strains grown on HBA under aerobic or microaerophilic conditions were flooded with droplets of phosphate-buffered saline (PBS), pH 7.2. Radium copper-coated grids (400 mesh) were placed on top of the PBS-covered colony for 10 s to allow bacteria to adhere. The grids were blotted dry and then negatively stained with 0.5% phosphotungstic acid solution for 10 s. Grids were air dried and then examined with a Jeol 100 S transmission electron microscope.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) was performed by using either a Bio-Rad Protean II or a Bio-Rad Mini-Protean II gel apparatus (Bio-Rad Laboratories, Richmond, Calif.). Membrane fractions were loaded at 15 μg of protein per lane, while bacterial cultures were standardized by absorbance. SDS-15% polyacrylamide separating gels were either stained with Coomassie bril-

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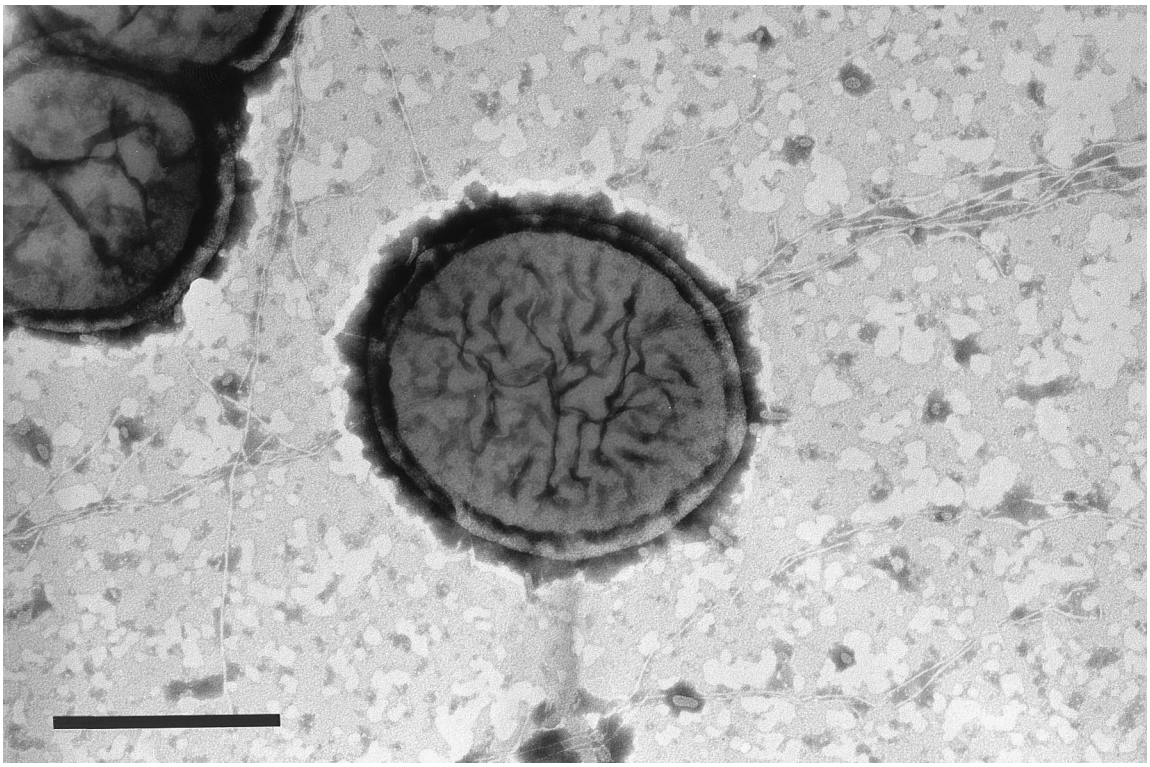

FIG. 1. Electron micrograph of negatively stained *P. multocida* PBA101 cell grown under microaerophilic conditions showing the presence of bundle-forming fimbriae. Bar = 500 nm.

liant blue or electrophoretically transferred onto nitrocellulose membranes (pore size, 0.45 μm) in a Bio-Rad TransBlot cell for Western blotting. Transferred proteins were immunostained with polyclonal antisera and peroxidase-conjugated sheep anti-rabbit immunoglobulins (Silenus Laboratories, Melbourne, Australia) as described previously (5).

For purification of fimbriae, PBA101 cells grown under microaerophilic conditions were separated into whole-membrane and cytoplasmic fractions as described previously (30). The fimbriae were purified from the whole-membrane fraction by high-performance reverse-phase liquid chromatography with a Bio-Rad HRLC 800 apparatus. Whole-membrane fractions containing fimbriae were diluted fivefold with 50 mM Tris-HCl buffer, pH 7.4, and loaded onto a Phenomenex W-Porex 5 C-4 column (250 by 4.6 mm internal diameter). Elution of fimbriae was achieved with a continuous gradient of acetonitrile in water (5 to 80%) containing 0.01% trifluoroacetic acid. Fimbrial proteins were well separated from contaminating membrane proteins and were eluted with 65% acetonitrile. Purified fimbriae were dissolved in Tris-HCl buffer and tested for homogeneity by SDS-PAGE. Purified fimbrial proteins (approximately 200 pmol) were subjected to direct N-terminal amino acid sequencing by using an Applied Biosystems model 476A protein sequencer system (Applied Biosystems Inc., Foster City, Calif.).

Whole-membrane fractions were separated by SDS-PAGE, and Coomassie Brilliant Blue-stained fimbrial protein was excised from the gel as described previously (2). Gel fragments were macerated and emulsified with incomplete Freund's adjuvant (1:1.5 vol/vol). New Zealand White female rabbits were injected intramuscularly with the gel-adjuvant emulsion, which contained approximately 20 μg of total protein. After 14 days rabbits were immunized intradermally in five sites with macerated gel containing the fimbrial protein without adjuvant. Sera were collected 26 days later and the presence of antifimbrial antibodies was assessed by Western blotting.

Agar corrosion was observed when *P. multocida* PBA101 and PBA100 were grown on HBA. Accordingly, *P. multocida* strains grown on HBA at 37°C under aerobic or microaerophilic conditions were examined by transmission electron microscopy. All strains examined had surface appendages that appeared to be fimbriae. The fimbriae of PBA101 were typical and varied in length but were usually longer than 20 μm and were 7 to 7.5 nm in diameter (Fig. 1). The proportion of cells that produced fimbriae differed depending on the growth conditions. Under aerobic conditions less than 40% of cells produced fimbriae, whereas more than 70% of cells grown under microaerophilic conditions produced fimbriae, a finding supported by Western blot analysis (see below). Furthermore, cells grown aerobically produced only one to two fimbriae per cell, in contrast to cells grown microaerophilically which appeared to produce four to five fimbriae per cell; an increased number of unattached fimbriae were also found surrounding the latter cells. However, in the case of cells grown microaerophilically it was difficult to estimate the number of fimbriae per cell since the fimbriae formed bundles containing multiple fimbrial strands (Fig. 1). The fimbriae of PBA101 cells grown under aerobic conditions did not appear to form such bundles.

Western blot and SDS-PAGE analyses of *P. multocida* grown under microaerophilic conditions were undertaken to identify the fimbrial subunit by using polyclonal antiserum raised against the type 4 fimbriae of *Moraxella bovis* Dalton 2d (8). The antiserum immunostained an 18-kDa protein in whole-cell lysates of PBA101, X-73, P-1059, and P-1662 and the whole-membrane fraction of PBA101 as well as recombi-

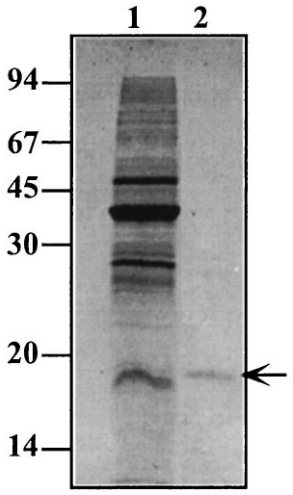

FIG. 2. Coomassie blue-stained SDS-PAGE gel showing whole-membrane fraction of PBA101, which contained fimbrial protein (lane 1) and purified PtfA protein used for amino acid sequencing (lane 2). Arrow indicates PtfA protein. The positions of standard molecular mass markers (kDa) are shown on the left. The originals were scanned with an Epson GT-8000 scanner and the Adobe Photoshop 2.5.1 LE program.

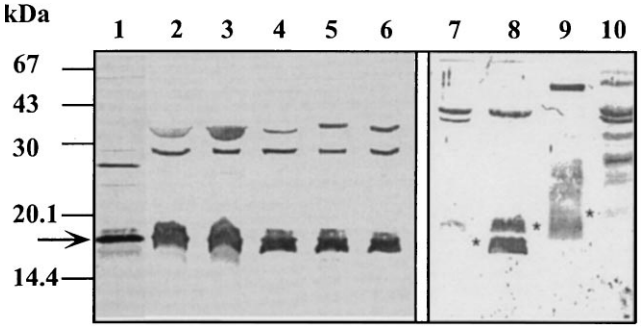

FIG. 4. Western blot analysis of whole-cell lysates (WC) and membrane fractions (MF) immunostained with antiserum against *P. multocida* PtfA. Lane 1, PBA101, aerobically grown WC; lane 2, PBA101, microaerophilically grown WC; lane 3, PBA101, microaerophilically grown MF; lane 4, X-73, microaerophilically grown WC; lane 5, P-1059, microaerophilically grown WC; lane 6, P-1662, microaerophilically grown WC; lane 7, *E. coli* control, HB101 (pBR322) WC; lane 8, *E. coli* HB101 harboring pMxB12, expressing recombinant *M. bovis* β pilin WC; lane 9, *D. nodosus* A198 WC; lane 10, *N. gonorrhoeae* MS11 WC. Arrow indicates PtfA protein, while asterisks to the left of the lanes indicate the fimbrial subunits of the other species. The positions of standard molecular mass markers (kDa) are shown on the left. The originals were scanned with an Epson GT-8000 scanner and the Adobe Photoshop 2.5.1 LE program.

nant *M. bovis* β pilin protein expressed by HB101(pMxB12) (data not shown).

The immunological data thus suggested that an 18-kDa protein was the *P. multocida* fimbrial subunit. The 18-kDa protein was therefore purified from the PBA101 whole-membrane fraction (Fig. 2, lane 1). SDS-PAGE analysis of the purified material revealed a single protein band of 18 kDa (Fig. 2, lane 2), and direct amino acid sequencing of the purified 18-kDa protein identified the first 21 amino acids of the mature protein. Sequence alignment (Fig. 3) showed that the amino acid sequence of the *P. multocida* protein shared very high similarity with the N-terminal amino acid sequence of the type 4 fimbrial subunits of *Pseudomonas aeruginosa*, *D. nodosus*, *N. gonorrhoeae*, *M. bovis*, and *Haemophilus influenzae*. The first amino acid of the 18-kDa protein could not be identified but may be phenylalanine which is modified to a N-methylphenylalanine, as is the case with many type 4 fimbrial proteins. As in *M. bovis* fimbriae, this amino acid migrated on the sequencing high-performance liquid chromatography column as a hydrophobic species. The sequenced 21 amino acids of the *P. multocida* protein were identical to the first 21 amino acid residues of the *P. aeruginosa* PilA (32) and *D. nodosus* FimA (22) fimbrial subunit proteins (Fig. 3). The first eight residues after the phenylalanine were identical to PilE (23), β pilin (21), and HtfA (31), as were residues 11, 12, 14 to 18, and 20. Therefore, the 18-kDa protein was designated PtfA, *P. multocida* type four fimbrial subunit.

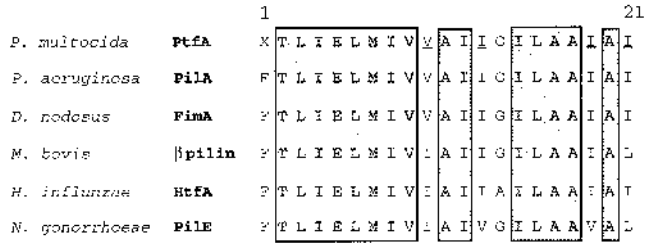

FIG. 3. Alignment of the first 21 amino acid residues of PtfA of *P. multocida* with the conserved N-terminal amino acid residues of classical type 4 fimbrial proteins. The shaded residues indicate identical amino acids while underlined residues indicate conserved amino acid changes.

Antiserum raised against PtfA, anti-PtfA, immunostained the *P. multocida* 18-kDa protein of all serogroup A strains (Fig. 4). In addition, anti-PtfA immunostained serogroup B and D strains (data not shown), the recombinant *M. bovis* β pilin, and the native *D. nodosus* fimbriae, but the antiserum reacted only very weakly with the gonococcal fimbrial subunit (Fig. 4). In agreement with the electron microscopy results, PBA101 grown under aerobic conditions produced less fimbrial protein compared with cells grown under microaerophilic conditions (Fig. 4).

The presence of surface appendages on *P. multocida* has been reported previously (10, 28, 36). The present study has identified bundle-forming fimbriae on avian serogroup A strains of *P. multocida* and confirmed by N-terminal sequence analysis that an 18-kDa protein, PtfA, was the type 4 fimbrial subunit of *P. multocida*. In addition, serogroups B and D were also found to possess type 4 fimbriae. Assembled type 4 fimbriae are 6 to 7 nm in diameter, up to 25 μm long, and are composed of repeating fimbrial subunits (33, 34). The morphology of the *P. multocida* type 4 fimbriae was similar to that of other type 4 fimbriae, with respect to their diameter and length. However, the type 4 fimbriae of serogroup A *P. multocida* appeared different from fimbriae previously identified on the serogroup D strains which have been putatively classified as type 1 (17). The presence of type 4 fimbriae on serogroup D strains suggests that these strains may express more than one type of fimbriae.

The first 21 amino acid residues of PtfA shared a high degree of similarity (75% sequence identity) with the N-terminal sequences of other classical type 4 fimbrial subunits (Fig. 3). The 32-amino-acid N-terminal hydrophobic domain of type 4 fimbrial subunits is highly conserved and is thought to be involved in subunit-subunit interactions that allow the structural integrity of the fimbrial strand to be maintained (9, 25). The first residue of the mature protein is usually a methylated phenylalanine. The first residue of the PtfA protein could not be sequenced, but due to the high sequence similarity with other classical type 4 fimbriae, it is most likely that the first

residue of PtfA is a methylated phenylalanine. The glutamate residue at position 5 has been identified as being critical for assembly and efficient methylation of fimbriae (19, 26), and notably this residue was present in the PtfA subunit (Fig. 3).

The identified molecular mass of PtfA was consistent with that of type 4 fimbrial subunits of other bacterial species, which are generally between 15 kDa and 20 kDa. Antiserum against *M. bovis* Dalton 2d pili immunostained the PtfA protein (data not shown), while *P. multocida* fimbrial antiserum was able to immunostain recombinant *M. bovis* β pilin and the native *D. nodosus* fimbrial subunit and reacted weakly with the gonococcal fimbriae (Fig. 4). This cross-reactivity among type 4 fimbrial subunits has been observed previously (27). The shared antigenicity and immunogenicity of type 4 fimbriae is believed to be due largely to the conserved amino-terminal domains of the subunit proteins (27, 34). However, cross-reactive epitopes beyond the conserved N-terminal region cannot be excluded.

Type 4 fimbriae are thought to mediate adherence to host epithelial tissue, thereby facilitating the infectious process. *P. multocida* has been shown to colonize mucosal host surfaces. However, it is not yet known if this colonization is important for the progression of pasteurellosis or whether fimbriae are involved in mediating the attachment to mucosal surfaces. Fimbriated serogroup A strains were able to adhere to mucosal epithelium of the nasopharynx and to HeLa cells (10). Bacteria that colonize mucosal surfaces in vivo generally encounter microaerophilic environments. When cultured under microaerophilic conditions, *P. multocida* cells showed an increased expression of fimbriae compared with *P. multocida* cells grown aerobically. The significance of this difference is not fully understood, but it may indicate that attachment to mucosal surfaces by *P. multocida* is facilitated by type 4 fimbriae. Recently, the type 4 bundle-forming fimbriae of *N. meningitidis* were found to be invariably associated with increased adhesion to human epithelial tissue (20, 37). In order to elucidate further the role of type 4 fimbriae in the pathogenicity of *P. multocida*, molecular and immunological studies are being undertaken with recombinant PtfA.

We are grateful to the laboratory staff of J. K. Davies for the *N. gonorrhoeae* MS11 bacterial lysates and that of J. I. Rood for the *D. nodosus* bacterial lysates and the recombinant *D. nodosus* fimbrial antiserum. We are grateful to Khim Hoe, Ian McPherson, and Vicki Vallance for their valued technical assistance. We also acknowledge S. J. Billington for his useful comments.

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