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Role of Fimbriae Expressed by Nontypeable *Haemophilus influenzae* in Pathogenesis of and Protection against Otitis Media and Relatedness of the Fimbrin Subunit to Outer Membrane Protein A

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Nontypeable *Haemophilus influenzae* is a primary pathogen in both acute otitis media (OM) and chronic OM, yet the pathogenesis of this disease is not fully understood. Although fimbriae have been observed on all clinical OM isolates examined to date, their role in pathogenesis remains unclear. Therefore, the gene which codes for the fimbrial subunit protein (fimbrin) in nontypeable *H. influenzae* 1128 was isolated, cloned, and sequenced. The nucleotide sequence of the fimbrin gene was found to contain an open reading frame of 1,077 bp which would encode a mature fimbrin protein consisting of 338 amino acids with a calculated molecular mass of 36.4 kDa. The translated amino acid sequence was found to be homologous with various OmpA proteins of other gram-negative bacteria, and algorithmic analysis predicted that this protein is organized as a coiled coil. To directly test whether fimbriae are involved in pathogenesis, the fimbrin gene was disrupted, and the biological consequences of disruption were absence of both expression of the fimbrial appendage and the specific immunogold labeling thereof with antisera directed against isolated fimbrial protein, reduced adherence to human oropharyngeal cells in vitro, augmented clearance from the tympanum post-transbullar inoculation, and significantly reduced induction of OM post-intranasal inoculation in a chinchilla model compared with the fimbriated parent strain. We additionally find that either passive immunization or active immunization against isolated fimbrial protein confers partial protection against transbullar challenge. A Western blot (immunoblot) indicated a degree of serological relatedness among fimbrin proteins of 15 nontypeable and type b isolates. These data suggest that fimbrin could be useful as a component of a vaccine to protect against OM.

Nontypeable *Haemophilus influenzae* (NTHi) is a primary pathogen in otitis media (OM) and other respiratory tract infections, and yet neither the pathogenic mechanisms of nor the host immunological response to this very heterogeneous group of microorganisms has been fully defined. A surface appendage has been observed on 100% of the NTHi isolates recovered from the middle ears and nasopharynges of children with chronic OM (8), suggesting a possible role for fimbriae in pathogenesis. Transmission electron microscopic observations indicated that fimbriae may be involved in the initial docking or adherence of NTHi to mucosal epithelial cells (8). The term "fimbriae" refers to the thin (diameter, approximately 2.4 nm), peritrichously arranged, flexible, non-hollow-core, and non-hemagglutinating filaments (Fig. 1A) which are expressed by all of the clinical OM isolates we have examined to date and which are distinct from the classic pili of type b *H. influenzae* (Hib) (4, 31) (Fig. 1B). Several NTHi strains have been shown to have a gene for pilin-like protein (3, 18); however, 95 to 100% of middle-ear isolates examined do not express this gene product, nor has its expression been successfully induced by animal passage (18).

Immunologically, fimbrin is recognized both locally and systemically by chinchillas during experimental disease (25) and also by both sera and middle-ear effusions of children with chronic OM (4, 24). Despite these observations, however, definitive evidence for involvement of fimbriae in pathogenesis of OM is lacking. In direct support of this postulated role, we report that the fimbrin gene-disrupted mutant is both less adherent and less pathogenic than the parent strain. We provide evidence that antibodies directed against fimbrial protein afford protection against NTHi-induced OM in a chinchilla model. The additional finding that the fimbrin subunit is homologous with the OmpA family of proteins and is expressed on the bacterial cell surface as a filamentous structure which is both an adhesin and a virulence factor is significant.

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MATERIALS AND METHODS

Bacterial strains. Clinical isolates 1128 and 1885 were obtained in pure culture from middle-ear effusions aspirated

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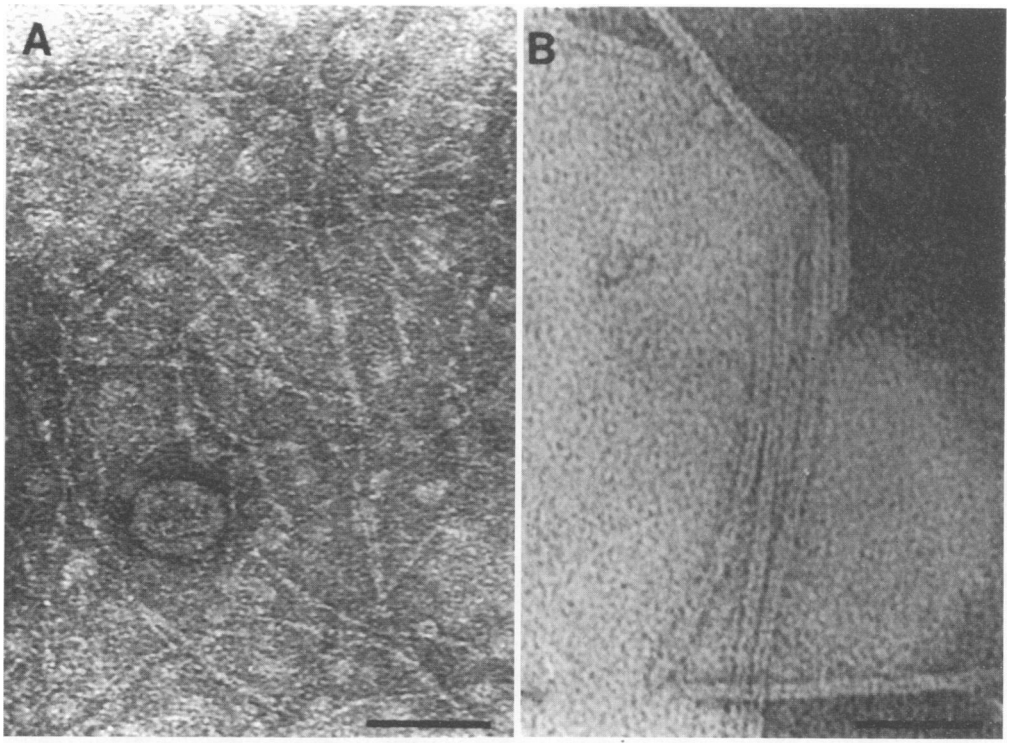


FIG. 1. Transmission electron micrographs of *H. influenzae* approximately 2.4-nm-diameter fimbriae (A) and approximately 5.0-nm-diameter hollow-core pili (B). Bars, 0.125 μm.

from children undergoing tympanostomy and tube insertion for chronic OM at The Children's Hospital (Columbus, Ohio). Both are minimally passaged, fimbriated, and maintained frozen in skim milk plus 20% glycerol. Strains were grown on chocolate agar (Baltimore Biological Laboratories, Baltimore, Md.) or in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 10 μg each of hemin and NAD (Sigma) per ml. *Escherichia coli* DH5α (Gibco BRL, Grand Island, N.Y.) was grown in Luria-Bertani medium (47).

Isolation of fimbrin and other immunogens. Preparations enriched in outer membrane proteins (OMP) were prepared essentially as described by Carlone et al. (20). Sarcosyl-insoluble proteins were maintained frozen at −70°C until use. This enriched preparation represents the immunogen referred to as "total OMP" and was further manipulated to yield selected immunogens. Individual OMPs and fimbrin were resolved via electrophoresis in 5 to 20% continuous-gradient polyacrylamide slab gels (0.01% [wt/vol] sodium dodecyl sulfate [SDS]) run at 30 mA per gel for approximately 4 h. Gel strips containing the desired protein were cut from the gel, and the proteins were electroeluted (Bio-Rad Electro-Eluter; membrane cap molecular weight cutoff, 12,000) and dialyzed against double-distilled water for 24 h (molecular weight cutoff, 10,000; Micro-ProDiCon membrane; Spectrum Houston, Tex.). Dialyzed preparations of individual OMPs and fimbrin were resolved via electrophoresis a second time and were also negatively stained and examined by transmission electron microscopy. All preparations were assessed for endotoxin content (in nanograms per milliliter) via a chromogenic *Limulus* amoebocyte lysate assay (Whittaker M. A. Bioproducts, Walkersville, Md.) (total OMP, 1.4×10^6; OMP P2, 210; strain 1128 fimbrial protein, 36; strain 1885 fimbrial protein, 30).

Amplification of fimbrin genomic sequences by PCR. Sequencing of the N terminus of the 25-kDa species of fimbrin protein (using an Applied Biosystems 475A pulsed liquid protein sequencer) yielded the sequence APQENTFYAGVK AGQGSFHD. The 37-kDa species yielded an identical N-terminal sequence of 15 residues. Both fimbrial species of protein (500 μg) were subjected to CNBr cleavage (49), the products were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon (Millipore Corp., Bedford, Mass.), and a 10-kDa internal fragment was sequenced, yielding the sequence VSKTFSLNSDVTFAF. On the basis of these sequences, two oligonucleotides were synthesized: a 20-mer with 128-fold degeneracy corresponding to Gln-3 through Ala-9 [5′ CA(AG)GA(AG)AA(CT)AC(AG TC)TT(CT)TA(CT)GC 3′] and an 18-mer with 512-fold degeneracy corresponding to Phe-15 through Asp-10 of the internal peptide [5′ AAA(AGTC)GC(AG)A(AGTC)GT(AG TC)AC(GA)TC 3′]. These nucleotides were used as sense and antisense primers, respectively, to amplify a genomic DNA fragment encoding the N-terminal region of fimbrin.

H. influenzae genomic DNA was isolated by the method of Marmur (48) followed by phenol extraction and dialysis against 50 mM Tris HCl (pH 8) containing 5 mM EDTA. The PCR mixture contained 100 ng of genomic DNA, 50 pmol of both primers, 10 nmol of each deoxynucleoside triphosphate, and 5 U of *Taq* DNA polymerase (Gibco BRL) in a final volume of 100 μl. The amplification protocol consisted of a denaturing step at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 2 min. A final elongation step was conducted at 72°C for 10 min. The amplified product was purified from an agarose gel and labeled with ^{32}P by using a random primed labeling kit (Boehringer Mannheim, Indianapolis, Ind.).

Cloning and sequencing of the fimbrin gene. Chromosomal DNA, isolated from NTHi 1128, was randomly sheared by

sonication, and fragments ranging from 2 to 5 kb were isolated by using a 1% low-melting-point agarose. The fragments were ligated to *Not*I-*Eco*RI linker-adapters and then with λgt11 arms (Stratagene) and packaged in vitro into lambda particles by using Gigapack Plus (Stratagene) to create a library which contained 6×10^6 recombinant phage.

Immunological screening of the λgt11:NTHi 1128 library was done essentially by the methods of Young et al. (77) using polyclonal rabbit and chinchilla sera raised against isolated fimbrial protein. Screening by plaque hybridization (58) was performed by using the 624-bp PCR probe described above via overnight hybridization at 42°C with standard reagents containing 50% formamide. Filters were washed for 30 min at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS prior to exposure to X-ray films. Positive plaques were identified from autoradiograms and recovered from agar plugs.

The DNA inserts from the positive phage clones were excised with *Eco*RI, subcloned in pUC19, and sequenced by the chain termination method of Sanger et al. (59) using Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio). The sequences of both strands were determined with synthetic oligonucleotide primers. All restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and calf-intestinal phosphatase were purchased from Gibco BRL or Boehringer Mannheim and used according to the recommendations of the manufacturers. DNA fragments were separated by electrophoresis using standard procedures (47).

With the 624-bp PCR product as the probe, three positive clones designated λFD1, λFD2, and λFD3 were obtained from the genomic library. The inserts from these phages were subcloned into pUC18 plasmids to yield pFD1, pFD2, and pFD3. Sequencing of these plasmids revealed that they encoded unique overlapping portions of the fimbrin gene sequence, but none of them contained the full-length gene. pFD1 and pFD3 contained an overlap of 237 bp and therefore were used to construct a plasmid carrying the complete coding sequence as well as 5′ and 3′ flanking regions of the fimbrin gene. The *Eco*RI-*Hin*dIII fragment of pFD1, containing the 5′ upstream region and the first 450 bp of the fimbrin gene, was isolated and inserted in the *Eco*RI-*Hin*dIII-digested and dephosphorylated pFD3 to create pFD, a plasmid that contains the entire open reading frame (ORF).

Fimbrin gene disruption. The 952-bp *Sfu*I fragment from pBR325, containing the gene encoding chloramphenicol acetyltransferase (*cat*) was ligated to the *Bst*EII-digested plasmid pFD. The ligation mixture was transformed into competent *E. coli* DH5α, and mutants were selected on Luria-Bertani agar containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml). Plasmids were isolated and characterized by restriction enzyme mapping. One plasmid, designated pNFM, had a single copy of the chloramphenicol cassette inserted into the *Bst*EII site of pFD. The pNFM plasmid was purified, linearized with *Bam*HI, and transformed into competent NTHi 1128. *H. influenzae* was grown to early log phase in supplemented brain heart infusion broth. Cells were made competent for natural transformation via the use of MIV medium as described by Herriot et al. (37). Competent cells were incubated in the presence of 1 μg of linearized plasmid DNA at 37°C for 30 min. After DNA uptake, cells were diluted and plated to select for disrupted mutants by an agar overlay technique (37) with an antibiotic screening concentration of 2 μg of chloramphenicol per ml.

Southern analysis of *Eco*RI-, *Eco*RI-*Hin*dIII-, and *Eco*RI-*Pst*I-digested chromosomal DNA isolated from one of the chloramphenicol-resistant transformants of strain 1128 and from strain 1128 was performed (47). *Eco*RI cuts once within the fimbrin gene downstream of the point of insertion of the chloramphenicol cassette. The 952-bp *Sfu*I fragment from pBR325 encoding the *cat* gene and the 1,077-bp *Eco*RI-*Bam*HI fragment encoding the fimbrin gene were used as ^{32}P-labeled hybridization probes.

Western blot. Western blots (immunoblots) were performed as previously described (7). Antiserum dilutions used are indicated in figure legends. Horseradish peroxidase-conjugated secondary antisera were used at dilutions of 1:200 to 1:500.

Assessment of biological consequences of fimbrin gene disruption. (i) Negative staining and immunogold labeling of the parent and disrupted mutant for direct observation of fimbriae by transmission electron microscopy. Negative staining was performed as previously described (8). For immunogold labeling, bacteria (grown for 18 h on chocolate agar) were sequentially incubated at room temperature with 0.25% skim milk–0.1% bovine serum albumin in 0.01 M phosphate-buffered saline (pH 7.2) (30 min), dilutions of either a polyclonal antibody or a monoclonal antibody directed against the isolated fimbrial protein of NTHi 1128 (15 min), and gold-conjugated anti-host species serum or protein A (for chinchilla sera) (60 min) (EY Labs, Inc., San Mateo, Calif., and SPI Supplies, Westchester, Pa., respectively). After being washed, bacteria were placed on a Formvar- and carbon-coated copper grid, air dried, and stably shadow-cast with platinum and palladium (Ladd Research Industries, Inc., Burlington, Vt.) at a 6° angle. Shadow-casting imparts a sense of height to low-profile fimbriae and allows enhanced resolution of these immunolabeled structures, which, in our experience, has been unachievable with standard methodologies.

(ii) In vitro determination of the relative abilities of the parent and disrupted mutant to adhere to eukaryotic cells. The assay system used was a modified adherence enzyme-linked immunosorbent assay (ELISA) (53) wherein suspensions of washed human oropharyngeal cells were immobilized and fixed on the surface of a 96-well assay plate with poly-L-lysine (Sigma) and glutaraldehyde (Polysciences, Warrington, Pa.). Oropharyngeal cells were then incubated with biotinylated (28) NTHi 1128 and washed free of nonadherent bacteria, and the relative number of adherent cells was detected via the use of extravidin-peroxidase and ABTS (Sigma). Inhibition of adherence was calculated by extrapolation from a standard curve in which nonbiotinylated NTHi competitively inhibited the binding of biotinylated bacteria. The assay was controlled for saturability, specificity, effect of biotinylation on adherence, and nonspecific binding (5). Attempts to specifically inhibit adherence were performed either via preincubation of oropharyngeal cells with isolated and reassembled fimbrial protein (75 μg/ml) prior to incubation with the biotinylated NTHi or by preincubation of bacteria with rabbit polyclonal antiserum directed against the isolated fimbrial protein prior to their incubation with the oropharyngeal cells. Isolated P2 protein from NTHi 1128 and normal rabbit serum were used as controls in these assays, respectively. All assays were performed a minimum of three times by using fresh pools of both human oropharyngeal cells and biotinylated organisms.

(iii) Induction of OM via transbullar (TB) inoculation of chinchillas with NTHi 1128 parent and disrupted mutant. Five chinchillas (*Chinchilla laniger*) each were inoculated, into the left superior bulla, with 2.5×10^3 to 3.5×10^3 CFU of NTHi 1128 or the fimbrin gene-disrupted mutant. Sterile saline was inoculated into the right superior bulla as a control. Animals were assessed daily via otoscopy (blinded) for evidence of tympanic-membrane inflammation (scale, 0 [none] to 4+

[severe inflammation or perforation]) and other signs of OM, and an epitympanic tap was performed every 3 to 4 days for recovery of any middle-ear fluids (MEF) and semiquantitative assessment of viable bacteria per milliliter of MEF. Animals were additionally rated daily on a 0-to-3+ scale of labyrinthine involvement to indicate severity of the effect of the induced OM on the inner ear and related balance function.

(iv) Induction of nasopharyngeal colonization and OM via intranasal (IN) inoculation of chinchillas. Twelve chinchillas (six per isolate) were inoculated via passive inhalation of approximately 10^8 CFU of either the parent or the mutant delivered in 0.6 ml of sterile saline (divided equally between nares), assessed daily as described for TB inoculation, and were additionally subjected to nasopharyngeal (NP) lavage with sterile saline every 3 to 4 days for semiquantitative assessment of CFU of NTHi per milliliter of lavage fluid (6).

Preparation of antisera. For sera used in passive-protection studies, a cohort of chinchillas and a New Zealand White rabbit were immunized with 50 μg of dialyzed fimbrial protein (strain 1128) in complete Freund's adjuvant followed 3 weeks later by a 50-μg boost in incomplete Freund's adjuvant and an identical additional boost 2 weeks later. The chinchilla and rabbit titers were periodically monitored by ELISA (data not shown), and the specificity of the response was assessed by both Western blot and ELISA.

Other chinchilla antisera were prepared by immunization with 100 μg of immunogen (either saline-based sham preparation, total OMP preparation [strain 1128], dialyzed fimbrial protein [strain 1128 or 1885], or isolated P2 protein [strain 1128]) in CFA subcutaneously and boosted 30 days later with 50 μg in incomplete Freund's adjuvant.

A monoclonal antibody (4A5H6) was generated against the dialyzed fimbrial protein via standard methods (19) and recovered from ascitic fluid (QUICKMAb; Sterogene, Arcadia, Calif.) and was determined, via ELISA (Screentype; Boehringer Mannheim), to be an immunoglobulin M isotype.

Challenge and assessment of immunized chinchillas. In the passive-immunization study, chinchillas (separate cohorts of 4 or 5 each, free of middle-ear disease) were injected intracardially (5 ml/kg of body weight) with dilutions of either normal chinchilla or rabbit serum or hyperimmune chinchilla or rabbit serum directed against dialyzed fimbrial protein. Cohorts were challenged 24 h later via TB inoculation of approximately 2.5×10^3 CFU of NTHi 1128 into the left superior bulla and evaluated via otoscopy daily for 21 days. Observations were performed blindly, and tympanic-membrane inflammation was rated.

Cohorts in which active immunity was being induced were immunized as described on days 0 and 30. Animals were challenged 10 days after the final immunization via TB inoculation into the left superior bulla of 2.5×10^3 to 3.5×10^3 CFU of either strain 1128 or strain 1885 (a dosage predetermined to induce moderate [2+] tympanic-membrane inflammation in all sham-immunized chinchillas within 48 h of inoculation). Animals were assessed over a 4-week period of observation via daily otoscopy for tympanic-membrane inflammation, epitympanic tap of the inferior bulla for semiquantitative determination of viable CFU of NTHi per milliliter of MEF every 3 to 4 days, and Western blot analysis of serum both pre- and postimmunization and postchallenge against all immunogens. One animal in each cohort was predesignated for recovery of both the tympanic membrane and middle-ear mucosa from the inferior bulla for histopathological evaluation of fixed, hematoxylin-eosin-stained tissue sections.

Preliminary determination of serological relatedness among fimbrin proteins expressed by clinical NTHi and Hib isolates. Enriched OMP preparations were prepared as described above (20) from 13 fimbriated NTHi (minimally passaged clinical OM isolates) and both a piliated and a nonpiliated (both strains bear fimbriae) Hib isolates. Separated OMPs were transferred to nitrocellulose as described above and incubated with rabbit or chinchilla polyclonal antisera directed against isolated fimbrial protein (strain 1128), and reactive bands were detected via the use of an appropriate HRP-conjugated antiserum (Zymed Laboratories, Inc., South San Francisco, Calif.).

Statistical analyses. Otoscopically determined tympanic-membrane inflammation scores were compared via the nonparametric Wilcoxon's rank sum test, with a P value of ≤ 0.01 accepted as the minimal level of significance, unless otherwise indicated. Qualitative histopathological evaluations were performed in a blinded fashion by two observers. For all other analyses, values were assumed to be normally distributed, and Student's t test was used to determine differences between arithmetic means. A P value of ≤ 0.01 was accepted as the minimal level of significance.

Nucleotide sequence accession number. The GenBank nucleotide sequence data base accession number for the NTHi 1128 fimbrin gene is LO8448.

RESULTS

Isolation of fimbrin, sequencing, and immunological reactivity. Upon SDS-PAGE of the isolated 25-kDa protein, fimbrin migrated as two bands with apparent molecular masses of 25 and 37 kDa (Fig. 2A). The lower band reassembled into a filamentous structure of the same diameter as native fimbriae upon dialysis of the electroeluted protein (Fig. 2B and C) and is referred to as "fimbrial protein" to distinguish this protein from both native fimbriae and the denatured subunit fimbrin. After electroelution from the gel, the 25-kDa protein again migrated as two bands under the same preparative and electrophoretic conditions, whereas the 37-kDa protein yielded only the same species (data not shown). When the enriched OMP preparation from NTHi 1128 was subjected to solubilization at 100°C for 5 min, the 25-kDa band was barely visible while there was a compensatory increase in the intensity of the fimbrin band at 37 kDa. This result suggests that the 37-kDa protein is probably the fully denatured form of the protein that migrated at 25 kDa. In support of this conclusion, both protein preparations showed identical N-terminal amino acid sequences.

Immunological data further supported the conclusions that both bands represent the same fimbrial protein. Rabbit and chinchilla (Fig. 3A, lanes a and b) antisera as well as monoclonal antibody 4A5H6 (data not shown), raised against the electroeluted 25-kDa protein band, were reactive with both bands. Additionally, these antisera were found to indirectly immunolabel the described surface appendage on NTHi 1128 (Fig. 4B and 5A), whereas normal sera did not label the fimbriae. That the 37-kDa species did not represent contamination by the similarly migrating major OMP P2 (approximately 41 kDa) was shown by an absence of cross-reactivity with a high-titer rabbit serum directed against P2 (gift of Timothy Murphy, Division of Infectious Diseases, Buffalo Veterans Administration Medical Center, Buffalo, N.Y.) whereas OMP P2 protein of strain 1128 was cross-reactive (Fig. 3B).

Cloning of the gene encoding fimbrin. Immunological screening of the λgt11 genomic library of NTHi 1128 yielded four clones, designated λFR2, λFR7, λFR10, and λFC2, containing DNA inserts which did not hybridize to each other and were selected for further analysis. The DNA inserts were subcloned in pUC19 (pFR2, pFR7, pFR10, and pFC2) and

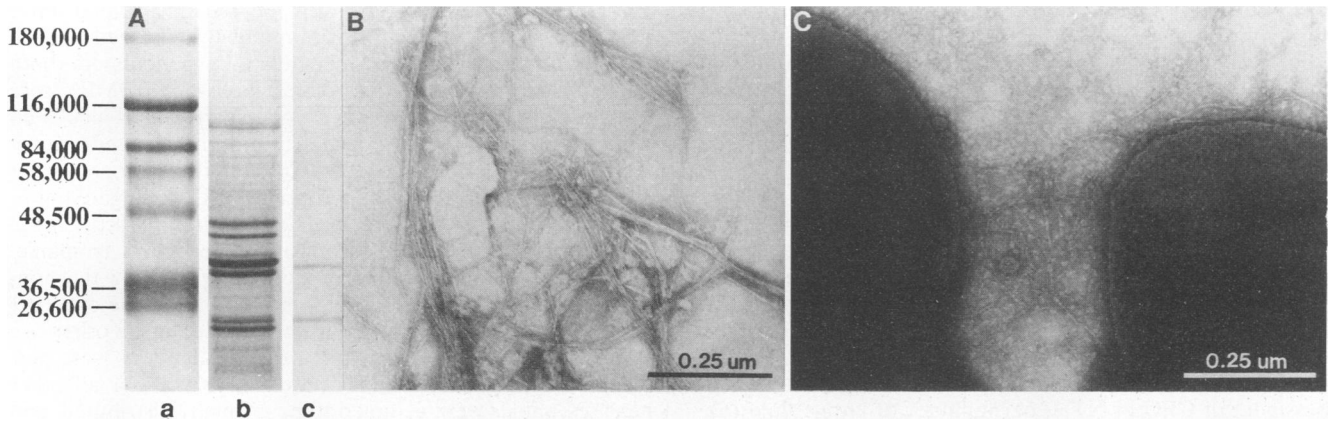


FIG. 2. (A) Coomassie brilliant blue-stained SDS-PAGE-separated molecular mass markers (in daltons) (lane a), total OMP preparation (NTHi 1128) (lane b), and isolated fimbrin (strain 1128) (lane c). (B) Transmission electron micrograph of negatively stained isolated and reassembled fimbrial protein. (C) Transmission electron micrograph of negatively stained, fimbriated NTHi 1128. Fimbriae can be seen extending between two bacterial cells.

sequenced. Clone FR2 contained an insert of 1,118 bp with a partial ORF of 274 codons which showed 99.4% identity with the amino acid sequence of the *H. influenzae e* lipoprotein (Hel) described by Green et al. (34). Clone FR7 contained a partial ORF of 206 codons that showed 98.4% identity with the Hib 28-kDa membrane protein (22). In clone FC2, an ORF of 220 codons that showed sequence similarity (54% identity) to an 891-bp region coding for an alpha-helical region within the 177-kDa MukB protein of *E. coli* (52) was found.

Clone FR10 encoded two ORFs. The 5′ part of the sequence encoded an ORF which showed significant homology with OmpA of *Klebsiella pneumoniae* (42), whereas the second ORF showed no obvious homology to any known proteins. Since immunological screening yielded several genes encoding OMPs other than fimbrin, the λgt11 library was also screened with a probe produced by PCR with two degenerate oligonucleotide primers based on the N-terminal amino acid sequence of the isolated fimbrin protein from NTHi 1128 and the sequence of a CNBr cleavage fragment of the protein with the genomic DNA as a template. The resulting 624-bp fragment was cloned into plasmid pUC19 and sequenced. That this 624-bp represents a segment of the authentic fimbrin gene was shown by the fact that the deduced amino acid sequence showed that it contained the 20- and 15-amino-acid sequences revealed by the protein sequencing of the N-terminal regions of the intact fimbrin protein and its CNBr cleavage fragment, respectively. The 5′ part of clone FR10 obtained by the immunological screening actually contained the 3′ part of this fimbrin gene.

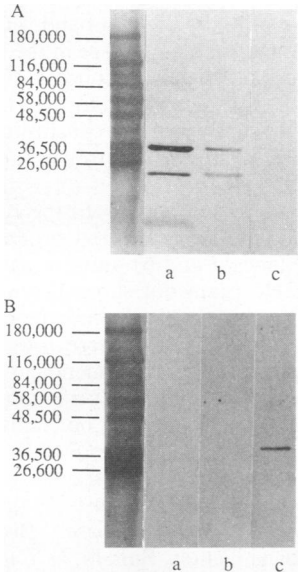


FIG. 3. (A) Western blot of chinchilla anti-isolated and reassembled fimbrial protein (strain 1128) antisera (1:50). Lanes: a, total OMP; b, isolated fimbrin; c, isolated P2. (B) Western blot of rabbit anti-P2 protein antisera (1:500). Lanes: a, isolated fimbrin (strain 1128); b, isolated 37-kDa species fimbrin (fully denatured subunit); c, isolated P2 (strain 1128). Unlabeled lanes contain molecular mass markers (in daltons).

Analysis of plasmid pFD (described in Materials and Methods) that contains the entire ORF for the fimbrin gene revealed an ORF of 1,077 bp, beginning with an ATG codon at position 407 and ending with a TAA stop codon at position 1484 (Fig. 6). The ORF is preceded by a putative ribosome binding site AGGA (61) similar to the consensus sequence for *E. coli* and beginning 11 bp upstream of the initiation codon. One stem-loop structure consistent with a *rho*-independent transcription terminator is located downstream of the ORF (57). The ORF shows a leader peptide of 21 amino acid residues with characteristics consistent with bacterial signal sequences. Thus, the primary translation product should be a protein of 359 amino acids that is processed to yield the mature fimbrin protein consisting of 338 amino acids. The calculated molecular mass of 36.4 kDa is in agreement with the molecular mass of 37 kDa estimated from SDS-PAGE of the isolated fimbrin protein. The deduced amino acid sequence of the fimbrin gene agreed with the amino acid sequences of both the N terminus and an internal peptide derived from CNBr cleavage of the purified protein.

The fimbrin sequence showed similarities (54) to the OmpA proteins of other gram-negative bacteria (Fig. 7). The homologous proteins and their percentages of identity to the fimbrin protein are as follows: *E. coli* OmpA (10), 51.8%; *Shigella dysenteriae* OmpA (13), 51.3%; *Salmonella typhimurium* OmpA (29), 49.8%; *K. pneumoniae* OmpA (42), 49.3%; *Enterobacter aerogenes* OmpA (14), 47%; *Serratia marcescens* OmpA (15),

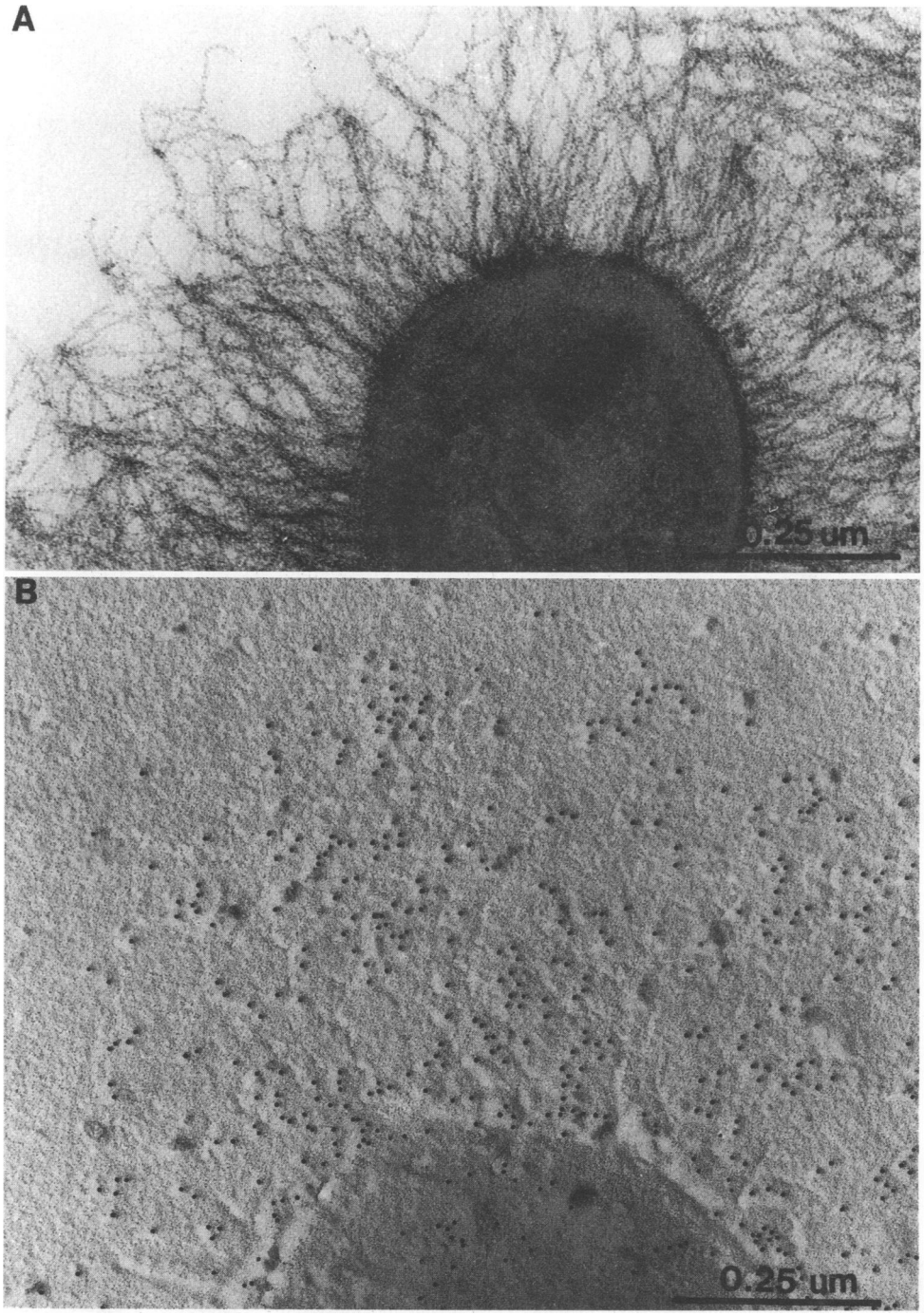


FIG. 4. (A) Transmission electron micrograph of Epon-embedded and thin-sectioned NTHi 1128. Note thin, filamentous, peritrichously arranged fimbriae. (B) Transmission electron micrograph of unfixed, unstained NTHi 1128 which has been indirectly immunolabeled with chinchilla anti-fimbrial protein antisera and gold-conjugated protein A and shadow-cast to impart a three-dimensional image to the low-profile fimbriae. Fimbriae appear as white rivulets labeled with black gold spheres.

43.2%. The deduced sequence for the leader peptide differs by only 4 amino acids from the signal sequence in OmpA of *E. aerogenes*, *S. dysenteriae*, and *S. marcescens*. The first 13 residues of the mature fimbrin protein showed 85% identity with the *Actinobacillus actinomycetemcomitans* OmpA sequence (76) and 70% with *Haemophilus ducreyi* OmpA (65). While this study was under review, Munson et al. (51a) described a Hib OMP (P5) which showed 92% identity with fimbrin. This degree of homology strongly suggests that the present gene is the P5 homolog of NTHi. If so, P5 may be involved in adherence of type b in the same way as demonstrated in the present paper for NTHi.

Examination of the amino acid sequence of the fimbrin subunit revealed the presence of three cysteine residues at

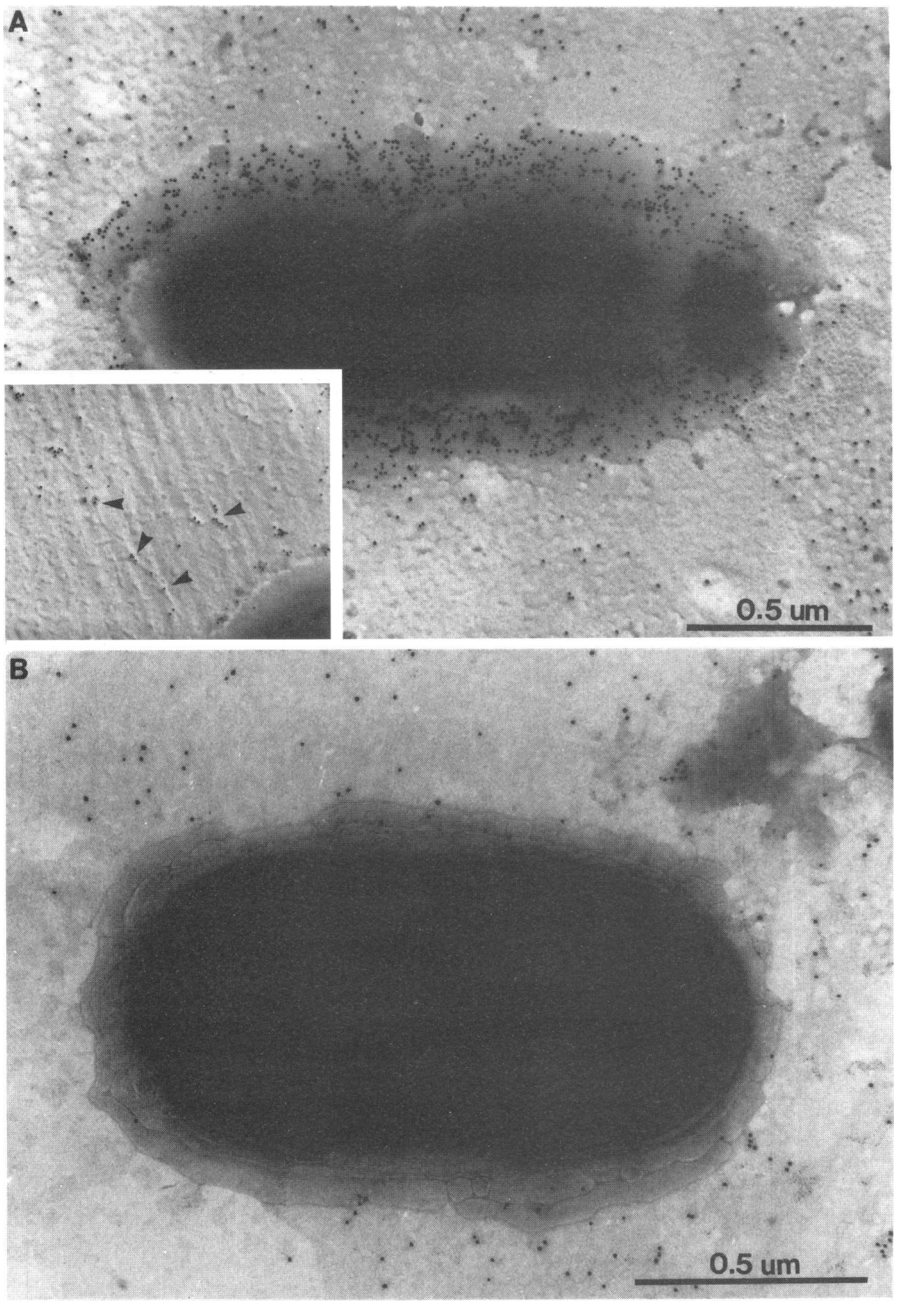


FIG. 5. Transmission electron micrographs of negatively stained and shadow-cast parental isolate (NTHi 1128) (A) and fimbrin gene-disrupted mutant indirectly labeled with monoclonal antibody 4A5H6 directed against isolated fimbrial protein of the parental strain and gold-conjugated goat anti-mouse immunoglobulin antiserum (B). Inset in panel A, a higher magnification (×32,000) of a different but identically prepared specimen; gold-labeled fimbriae are indicated (arrowheads). Fimbrin protein within the outer membrane more heavily labels with the immunogold technique because the concentration of the proteins would be high in the membrane (see Fig. 4A). Fimbriae extending from the cell in a stellate array have a tortuous character and therefore would not present a high concentration of the protein in a given plane; thus, contiguous gold labeling of the fimbriae would be infrequent.

positions 211, 332, and 344. While the deduced sequence from NTHi 1128 fimbrin is distinct from the majority of OmpA proteins which contain an 8-residue Ala-Pro repeating unit at what is considered to be the boundary between cell membrane-bound and periplasmic domains, there is an analogous highly hydrophobic region in approximately the same location (amino acid residues 204 to 210).

The fimbrin protein sequence contains a heptapeptide periodicity in which, in general, every 1st and 4th residue is hydrophobic or nonpolar. Algorithmic analysis of this translated amino acid sequence by the method of Lupas et al. (46) indicates that fimbrin is organized as a coiled-coil not unlike the conformation described for the fibrillar antiphagocytic M protein of group A streptococci (38, 39, 55). The observed very

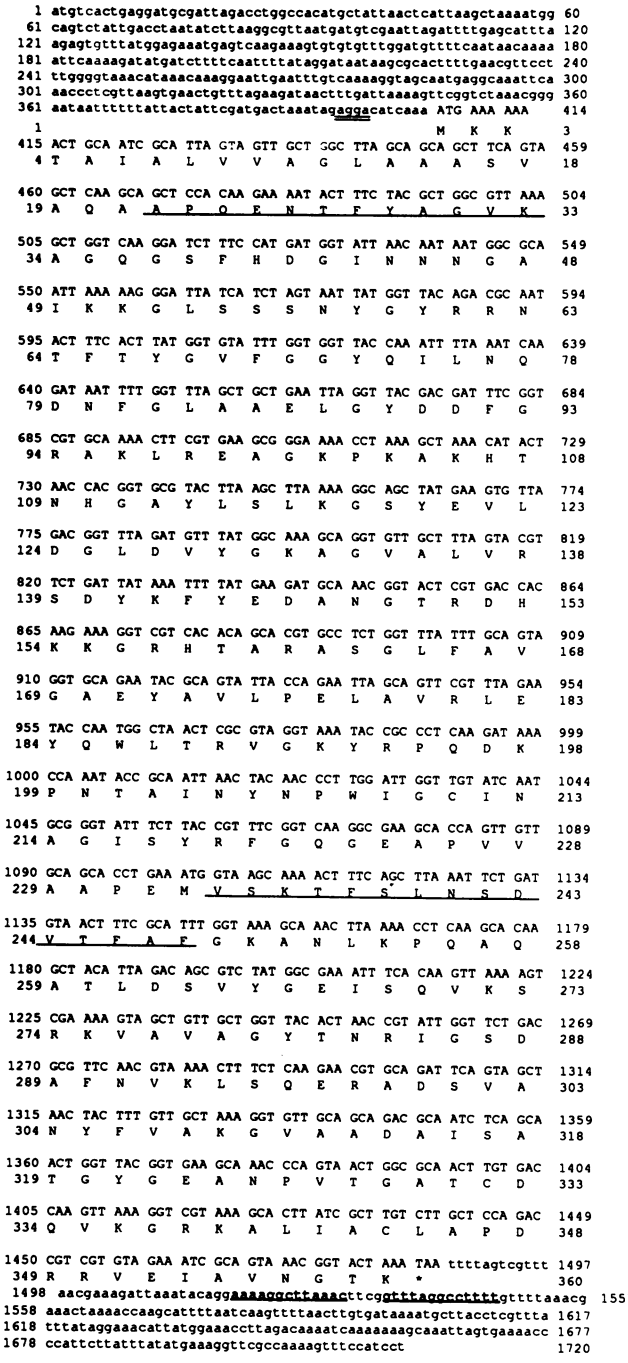


FIG. 6. Nucleotide sequence of the NTHi fimbrin gene. The deduced amino acid sequence is shown below the DNA sequence. Capital letters correspond to the ORF. Amino acid sequences of the amino terminus and an internal CNBr fragment determined by sequencing of the fimbrin protein are underlined. The ribosome binding site is doubly underlined. A stem-loop structure located downstream of the fimbrin gene is boldfaced and underlined.

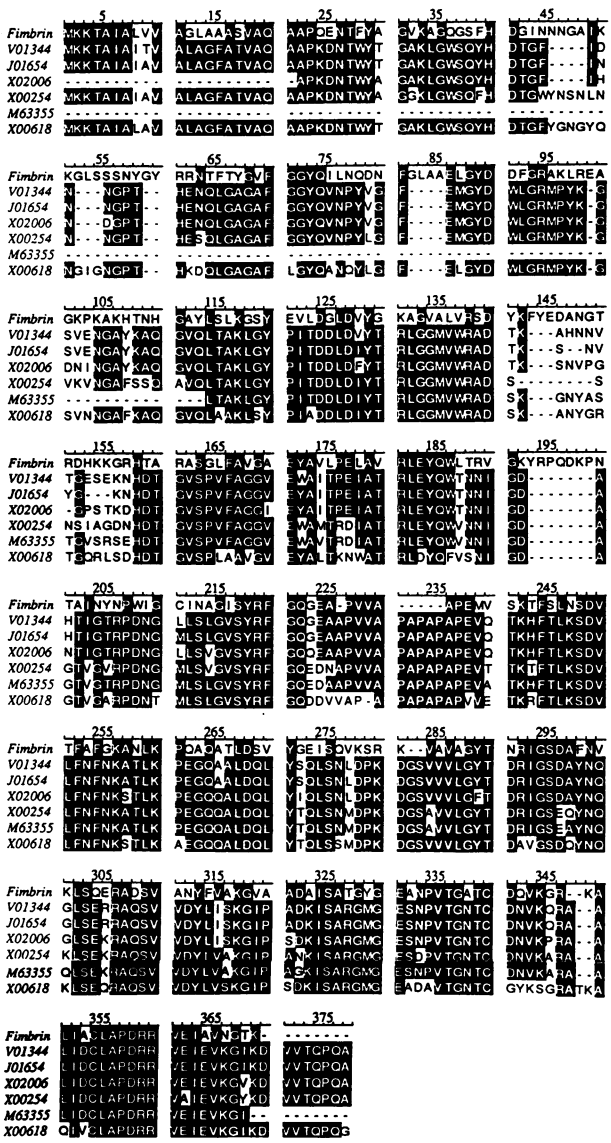


FIG. 7. Comparison of the amino acid sequences of the fimbrin protein and OmpA proteins from *S. dysenteriae* V01344, *E. coli* J01654, *S. typhimurium* X02006, *E. aerogenes* X00254, *K. pneumoniae* M63355, and *S. marcescens* X00618. Identical amino acids are indicated (black background). −, a gap in the sequence compared with the other aligned sequences. Partial sequences are shown for *K. pneumoniae* and *S. typhimurium*. Sequences were aligned with the Clustal V Seqapp program (37a).

long native fimbriae might be formed by a near-linear array of monomeric fimbrin proteins. However, the nature of the interactions involved with polymer formation remains to be elucidated.

Fimbrin gene disruption. The fimbrin gene was disrupted by insertion of a *cat* gene at the unique *Bst*EII site (Fig. 8). After verification that a single *cat* gene was inserted in the appropriate site within the fimbrin gene, the disrupted gene was introduced into the NTHi 1128 genome by homologous recombination. Southern hybridization of the *Eco*RI-, *Eco*RI-*Hin*dIII-, *Eco*RI-*Pst*I-, and *Taq*I-digested genomic DNA from the gene-disrupted mutant and the wild type with *cat* and fimbrin gene probes verified that the fimbrin gene was disrupted (Fig. 9).

The disrupted mutant was also compared with the parent strain 1128 by Western analysis of both whole-cell extracts and total OMP preparations by using polyclonal antifimbrial pro-

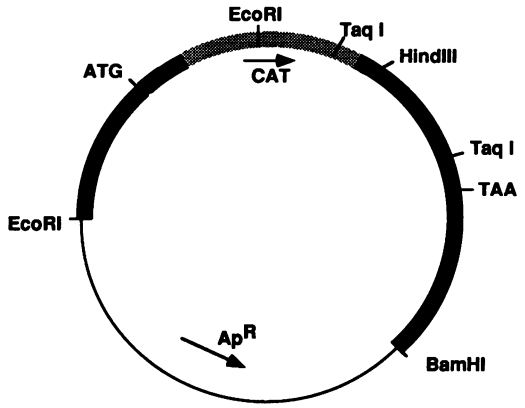


FIG. 8. Schematic representation of plasmid pNFM, which contains the disrupted NTHi fimbrin gene obtained by introducing the chloramphenicol acetyltransferase gene in the *Bst*EII site of the fimbrin gene. ■, NTHi fimbrin gene and flanking regions. The fimbrin ORF shows the start codon ATG and the stop codon TAA. ▩, *cat* gene; —, pUC19.

tein serum prepared against strain 1128 to detect immunoreactive proteins. Both the 37- and the 25-kDa bands in the cell extracts from the parent strain showed reactivity (Fig. 10A, lanes a to c); however, there was also reactivity with a 26-kDa band in the cell extracts from the fimbrin gene-disrupted mutant (lanes d and e). This reactivity could be attributable to a comigrating OMP which was a contaminant in the originally isolated fimbrial preparation used to generate the polyclonal

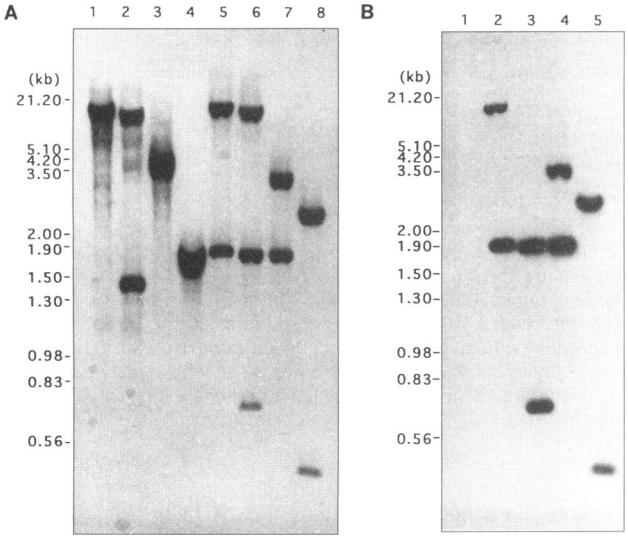


FIG. 9. Southern hybridization analysis. Genomic DNAs from the NTHi 1128 parent strain (lanes 1 to 4 in panel A and lane 1 in panel B) and fimbrin gene-disrupted mutant (lanes 5 to 8 in panel A and lanes 2 to 5 in panel B) were digested to completion with *Eco*RI (lanes 1 and 5 in panel A and lanes 1 and 2 in panel B), *Eco*RI-*Hin*dIII (lanes 2 and 6 in panel A and lane 3 in panel B), *Eco*RI-*Pst*I (lanes 3 and 7 in panel A and lane 4 in panel B), and *Taq*I (lanes 4 and 8 in panel A and lane 5 in panel B); electrophoresed on a 1% agarose gel, transferred to a nitrocellulose membrane, and probed with ^{32}P-labeled fimbrin gene (A) and ^{32}P-labeled chloramphenicol acetyltransferase gene (B).

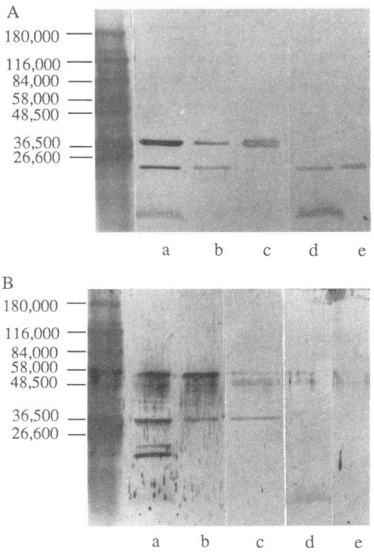


FIG. 10. Western blot of total OMP (strain 1128) (lanes a), fimbrin (strain 1128) (lanes b), isolated 37-kDa species fimbrin (fully denatured subunit) (lanes c), total OMP (fimbrin gene-disrupted mutant) (lanes d), and isolated ~26-kDa OMP (fimbrin gene-disrupted mutant) using chinchilla anti-isolated and reassembled fimbrial protein antisera (1:50) (A) or chinchilla anti-37-kDa species fimbrin (fully denatured subunit) (1:50) (B). Unlabeled lane, molecular mass markers (in daltons).

antisera. Alternatively, it could represent an additional fimbrial component (accessory protein) or another unrelated protein that associates with the true fimbrin protein. To distinguish between these possibilities, antiserum was generated in chinchillas against the 37-kDa species and used in an immunoblot against both total OMP preparations and isolated individual OMPs (Fig. 10B). The 26-kDa band from the fimbrin gene-disrupted mutant did not cross-react with this antiserum (lanes d and e), whereas there was reactivity with both the 37- and the 25-kDa bands of the wild-type (lanes a to c). Therefore, it is concluded that the fimbrin gene product is the 37-kDa protein and the partially unfolded fimbrin at 25 kDa whereas the 26-kDa band in the gene-disrupted mutant represents a comigrating protein.

To test for the possibility that the 26-kDa protein may be integral to fimbrial assembly function, mixtures of the 26-kDa species from the disrupted mutant or the 25-kDa species from the parent strain and equimolar amounts of the 37-kDa species from the parent strain were examined by transmission electron microscopy (Fig. 11). Only the isolated wild-type proteins were clearly shown to be capable of reassembling into a filamentous structure of the appropriate width and morphology (Fig. 11C and D). Furthermore, immunogold labeling of both the parent strain and the disrupted mutant with chinchilla antisera directed against the 26-kDa protein from the gene-disrupted mutant did not label fimbriae but rather generally labeled the bacterial outer membrane (data not shown). Amino acid sequencing of two peptides obtained by CNBr treatment of the 26-kDa protein showed that this protein contained 15- and 16-residue segments which were identical to those contained within Omp *e* (34). Thus, this OMP, expressed by both the wild type and the disrupted mutant, is a contaminant present in isolated fimbrin and reassembled fimbrial protein preparations.

Examination of the fimbrin gene-disrupted mutant by neg-

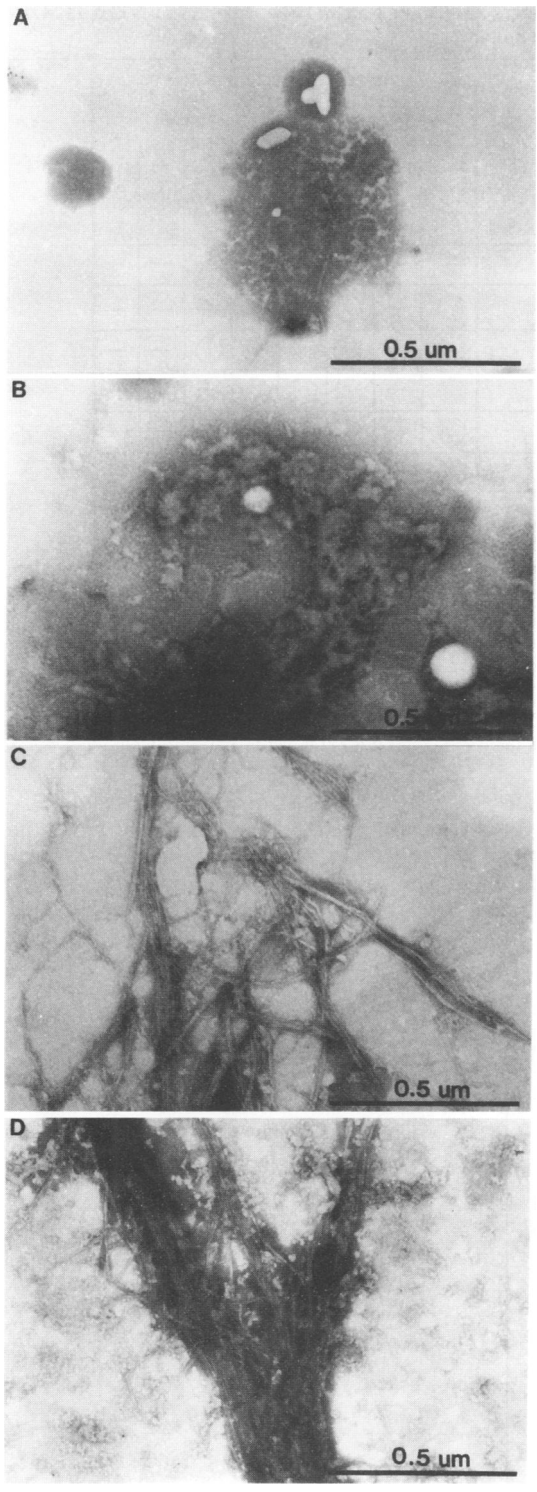


FIG. 11. Transmission electron micrographs of negatively stained isolated ~26-kDa OMP (mutant) (A), isolated 37-kDa species fimbrin (fully denatured subunit; parent) (B), isolated 25-kDa species fimbrin (partially denatured subunit; parent) (C), and an equimolar mixture of 25- and 37-kDa species fimbrin (parent) (D). Note the amorphous appearance of isolated OMPs in panels A and B. The fine twisted filamentous bundle barely visible in panel A is a rare finding in this preparation; individual filaments are approximately half the width of fimbriae. Reassembled fimbrial protein filaments in panels C and D are of the same width as native fimbriae.

TABLE 1. Viable bacteria in epitympanic tap fluids post-TB challenge with NTHi 1128 or the fimbrin gene-disrupted mutant

NTHi strain and animal	CFU/ml of MEF[a]
Parent	
1	$>10^8$
2	$>10^8$
3	6.7×10^6
4	2.0×10^7
5	Dry
Mutant	
6	Dry
7	Dry
8	4.8×10^4
9	4.1×10^4
10	2.5×10^4

[a] The tap was performed 4 days postinoculation via the left inferior bulla.

ative staining and immunogold labeling. Fimbriae were resolvable on approximately 25% of bacterial cells of strain 1128, whereas none were observed on the fimbrin gene-disrupted mutant. Both isolates demonstrated equivalent growth and consisted of individual cells of approximately the same size and shape; however, the disrupted mutant characteristically demonstrated more extensive membrane blebbing (Fig. 5B). Native fimbriae expressed by strain 1128 showed indirect immunogold labeling with both a pool of chinchilla sera and a rabbit serum collected from animals immunized with isolated and reassembled fimbrial protein (Fig. 4B) as well as a similarly generated monoclonal antibody (Fig. 5A), whereas the disrupted mutant did not.

Antisera generated against the 37-kDa band only weakly labeled the parent strain and did not label the disrupted mutant (not shown). The fully denatured fimbrin protein of 37 kDa probably does not refold and reassemble into fimbrial filaments under the preparative conditions used, whereas the partially denatured fimbrin (migrating at 25 kDa) retains the secondary structure necessary for reassembly into fimbrial filaments. Thus, only antibodies directed against the partially denatured moiety but not the fully denatured protein recognized native fimbriae.

Biological consequences of fimbrin gene disruption. (i) Adherence ELISA demonstrated that the fimbrin gene-disrupted mutant was 35% ± 7% ($n = 8$) less adherent than the parent strain ($P \leq 0.001$). Isolated fimbrial protein inhibited adherence by 18% ± 2% ($n = 3$) at a concentration of 75 μg/ml when oropharyngeal cells were preincubated with the fimbrial protein prior to incubation with the parent strain, whereas adherence by the disrupted mutant was not affected (0%; $n = 3$) ($P \leq 0.001$). Isolated OMP P2 protein had no effect on adherence of either the parent or the fimbrin gene-disrupted mutant. Preincubation of bacteria with rabbit polyclonal antisera directed against isolated fimbrial protein resulted in an inhibition of 36% ± 9% ($n = 3$) for the parent and 1% ± 1% ($n = 3$) ($P \leq 0.001$) for the disrupted mutant, whereas normal rabbit serum had no effect on either isolate.

(ii) In the TB inoculation model, differences in tympanic-membrane inflammation over time between cohorts were not statistically significant (data not shown) and may reflect contribution to inflammation attributable to lipooligosaccharide (26); however, the rates of clearance of bacteria from the tympanic cavity were notably different between the two strains as demonstrated by epitympanic tap culture data (Table 1).

TABLE 2. Comparison of labryinthine involvement in chinchillas receiving a TB (A) or an IN (B) inoculation of NTHi 1128 or the fimbrin gene-disrupted mutant[a]

A

Day Post-Inoculation	Strain Received: Parent (n=5) Severity of involvement: None	Mild	Moderate	Severe	Mutant (n=6) Severity of involvement: None	Mild	Moderate	Severe
3	●	—	—	—	●	—	—	—
4	◍*	—	○	○	●	—	—	—
5	○	—	○	◍	◍	○	—	—
6	○	—	○	◍	◍	—	○	—
7	○	—	—	◍	◍	○	—	—
8	○†	—	○	○	◍	○	—	—
9	○	—	—	◍	◍	○	—	—
10	○	—	—	◍	◍	○	○	—
11	○	—	—	◍	◍	○	—	○
12	○	—	—	◍	◍	—	○	○
13	○	—	○	○	◍	—	○	○

B

Day Post-Inoculation	Strain Received: Parent (n=5) Severity of involvement: None	Mild	Moderate	Severe	Mutant (n=6) Severity of involvement: None	Mild	Moderate	Severe
3	●	—	—	—	●	—	—	—
4	◍*	—	○	○	●	—	—	—
5	○	—	○	◍	◍	○	—	—
6	◍	—	○	○	◍	—	○	—
7	◍	—	○	—	◍	○	—	—
8	◍	—	—	◍	◍	○	—	—
9	○†	—	—	◍	◍	○	○	—
10	○	—	—	◍	◍	○	○	—
11	○	—	—	◍	◍	—	—	○
12	○	—	—	◍	◍	—	—	○
13	○	—	—	◍	◍	—	—	○
17	●‡	—	—	—	§●	—	—	—

[a] ●, all animals; ◍, more than one animal; ○, one animal; —, no animals. *, $n = 4$ (with the death in panel B attributed to a technical difficulty); †, $n = 3$; ‡, $n = 1$; §, $n = 5$.

Four days postinoculation, average values of CFU per milliliter of MEF for the parent and fimbrin gene-disrupted mutant as cultured on brain heart infusion agar (without or with chloramphenicol, respectively) indicate a 2- to 4-log-unit fold difference between these strains. A comparison of labyrinthine involvement (effect on the inner ear; balance disorder) between the cohorts indicated a marked difference between cohorts as well (Table 2), with chinchillas receiving the parent strain demonstrating much greater severity of involvement throughout the period of observation.

(iii) Assessment of tympanic-membrane inflammation scores of IN-inoculated animals indicated significantly reduced induction of OM ($P \leq 0.05$) in animals inoculated with the fimbrin gene-disrupted mutant on days 4, 5, 7, 9, 10, and 13 postinoculation (Fig. 12). Labyrinthine involvement was similarly markedly reduced in animals receiving the disrupted mutant (Table 2), correlating with increased survivability in this cohort. The majority of deaths in the cohort receiving the parent strain was attributed to severity of induced disease. Prior to death (or sacrifice), these animals had severe labyrinthine involvement, were unable to right themselves, and were typically highly irritable, whereas none of these signs were seen in the cohort receiving the disrupted mutant. The one animal in the disrupted-mutant cohort which developed severe labyrinthine involvement late in the study was an exception to the rule. Only three animals in the parent cohort survived until day 13 after inoculation, whereas all six animals in the disrupted-mutant cohort survived to this time.

NP lavage fluids obtained on day 3 yielded counts in the range of 4.2×10^6 to 1.2×10^8 CFU/ml for three of five remaining chinchillas inoculated with the parent strain, whereas two fluids of the six obtained from the cohort inoculated with the mutant yielded countable plates with CFU values in the range of 3.2×10^5 to 6.5×10^6/ml. NP lavage fluids obtained on day 7 yielded two countable plates in the range of 4.5×10^3 to 2.2×10^6 CFU/ml from the four remaining chinchillas in the cohort receiving the parent, whereas there were six lavage fluids yielding countable plates in the range of 9.8×10^2 to 2.5×10^6 CFU/ml obtained from

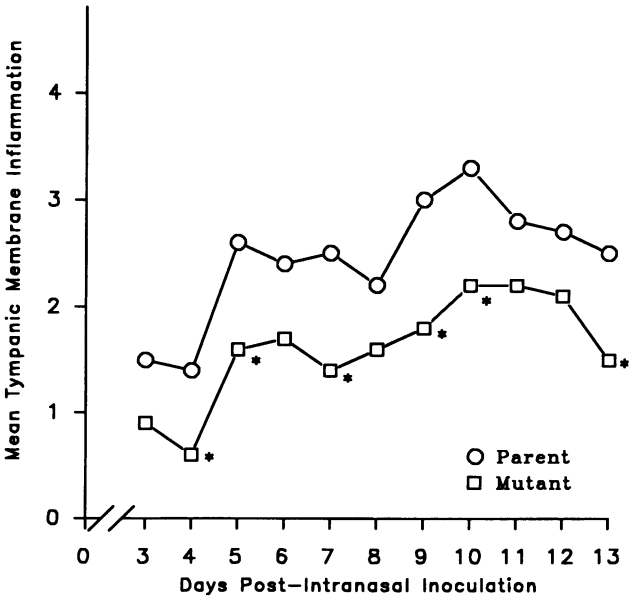


FIG. 12. Comparison of mean tympanic-membrane inflammation over a 13-day period of observation between cohorts receiving an IN inoculation of NTHi 1128 or the fimbrin gene-disrupted mutant. Asterisks, values significantly different ($P \leq 0.05$) from the corresponding values for the parent strain.

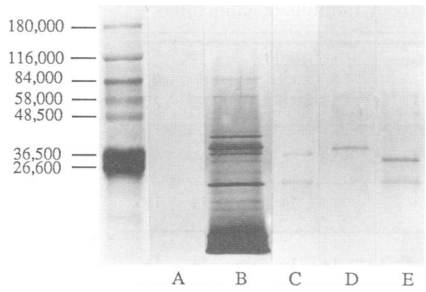


FIG. 13. Silver-stained SDS-PAGE-separated immunogen preparations. Lanes: A, Sham; B, total OMP (strain 1128); C, isolated fimbrin (strain 1128); D, isolated P2 protein (strain 1128); E, isolated fimbrin (strain 1885). Unlabeled lane, molecular mass markers (in daltons). Both fimbrin preparations are composed of two bands which are representative of fimbrin in its fully and partially denatured conformations.

the cohort inoculated with the mutant. On day 17 post-IN inoculation, only one animal remained in the parental cohort, and an NP lavage yielded 3.3×10^4 CFU/ml, whereas the mutant cohort NP lavage fluids were similarly in the range of 2.8×10^3 to 1.0×10^5. In summary, NP colonization appeared to be independent of the expression of this gene product throughout the period assessed in this study, and this phenomenon is likely attributable to other NTHi adhesins.

Epitympanic tap data, however, indicated a marked difference in ability to gain access to and/or multiply within the tympanum between these two cohorts. Taps performed 3 days post-IN inoculation yielded no countable plates for either cohort; however, a tap performed on day 7 postinoculation (n = 4 and 6 for the parent and mutant cohorts, respectively) yielded large-volume (≥100-μl) effusions in five of eight ears (63%) in the parental cohort, with counts of 10^7 to 10^9 CFU/ml of MEF. All six animals in the cohort receiving the mutant remained, there were four recoverable effusions (4 [33%] of 12 ears), of which one was sterile, and counts determined from the remaining three MEFs were 10^5 to 10^8 CFU/ml. A tap performed 12 days postinoculation (n = 3 and 6 for the parent and disrupted-mutant cohorts, respectively) yielded effusions in 4 (67%) of 6 ears in the parental cohort, with counts of 10^6 to 10^9 CFU/ml, whereas only 3 (25%) of 12 ears yielded effusions in the disrupted-mutant cohort. Plate counts of 10^5 to 10^7 CFU/ml were obtained.

Passive immunization. Administration of undiluted hyperimmune but not normal rabbit serum was lethal to chinchillas. Those receiving dilutions of anti-fimbrial protein serum of either rabbit or chinchilla origin, followed by TB challenge with the homologous NTHi strain (strain 1128), demonstrated significantly reduced tympanic-membrane inflammation compared with those receiving normal rabbit serum ($P \leq 0.05$ and 0.001, respectively) throughout the period of observation (data not shown). Increasing the dilution of either rabbit or chinchilla antiserum resulted in increased mean tympanic-membrane inflammation scores and prolonged recovery. In addition, MEFs were present in five of five challenged ears from day 1 to 14 post-TB inoculation in control animals, whereas there were no MEFs noted after day 3 in animals receiving undiluted chinchilla anti-fimbrial protein serum.

Active immunization. Immunogen preparations used to induce active immunity by parenteral injection are depicted in Fig. 13. All of the immunized cohorts responded strongly and

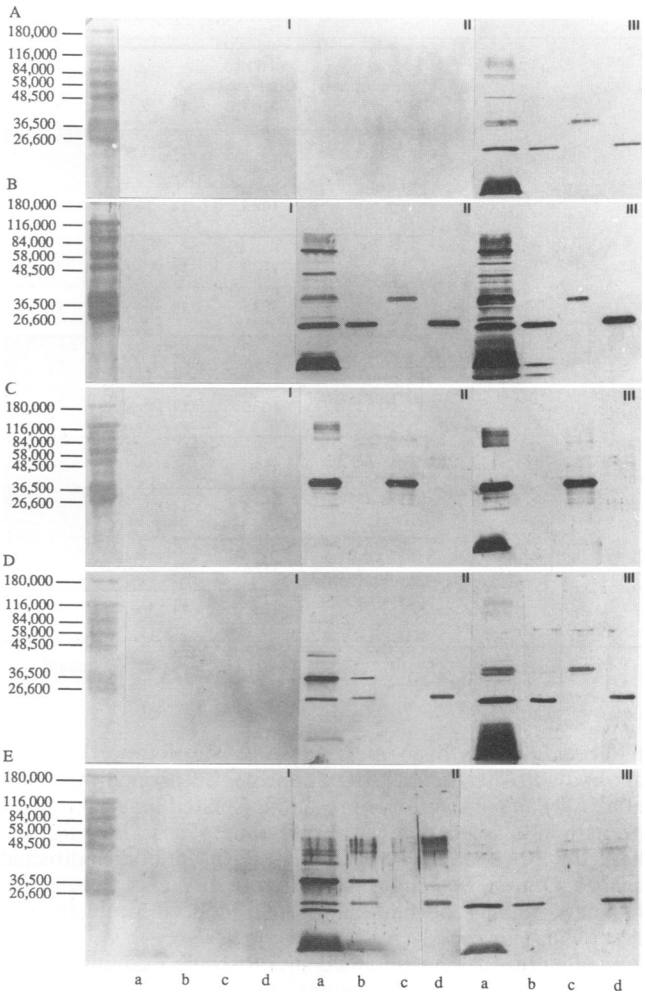


FIG. 14. Western blot using pooled chinchilla sera (1:100) obtained preimmunization (I), postimmunization (II), and postchallenge with the homologous isolate (III). Lanes: a, total OMP (strain 1128); b, fimbrin (strain 1128); c, P2 protein (strain 1128); d, fimbrin (strain 1885). Antisera were obtained from animals immunized with a saline-based sham preparation (A), total OMP (strain 1128) (B), P2 protein (strain 1128) (C), or fimbrial protein (strain 1128 [D] or 1885 [E]). Unlabeled lanes, molecular mass markers (in daltons). (The stained material in several lanes of panels II and III that appears above ~55 kDa is present in the SDS-PAGE solubilizing buffer.).

specifically to the immunogens (Fig. 14, panels II). Blots obtained with sera collected after heterologous challenge were equivalent. Reactivity against lipooligosaccharide of strain 1128 was noted in postimmunization sera of those cohorts immunized with either the total OMP preparation or isolated and reassembled fimbrial protein of either challenge strain, despite the fact that endotoxin contamination was ≤0.1% of the fimbrial immunogens. Any contribution to noted protection was considered negligible because of the observation that sera obtained from cohorts immunized with either strain 1128 fimbrial protein or strain 1885 fimbrial protein were reactive in Western blot against the heterologous strain's lipooligosaccharide (Fig. 14E, panel II), yet they were not protected upon subsequent challenge with the heterologous isolate. This phenomenon has been reported by others (9, 40), and to date no protective role for antilipooligosaccharide antibody in NTHi-

TABLE 3. Average tympanic-membrane inflammation in actively immunized chinchillas[a]

Immunogen	NTHi Challenge Strain*	Days Post-Transbullar Challenge														
		1†	2	3†	4	7†	8	9	10†	11	14†	15	16	17†	18	28
Sham	1128	2+	2+	2+	3+	2+	3+	3+	3+	3+	3+	2+	2+	2+	3+	2+
	1885	1+	1+	2+	1+	2+	2+	2+	2+	1+	2+	2+	1+	2+	1+	1+
Total OMP (NTHi #1128)	1128	1+	2+	2+	2+	2+	2+	2+	2+	2+	1+	1+	1+	1+	1+	1+
	1885	1+	2+	2+	1+	1+	1+	1+	1+	1+	1+	1+	1+	0	0	0
P2 protein (NTHi #1128)	1128	2+	3+	4+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
	1885	1+	2+	3+	2+	3+	3+	3+	3+	3+	3+	3+	2+	2+	2+	2+
Fimbrial protein (NTHi #1128)	1128	2+	2+	1+	2+	2+	1+	1+	1+	1+	1+	1+	2+	1+	1+	0
	1885	1+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	1+
Fimbrial protein (NTHi #1885)	1128	2+	3+	3+	3+	3+	3+	4+	3+	3+	3+	3+	3+	2+	2+	1+
	1885	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	0

[a] $n = 5$. *, dose = 2.5×10^3 to 3.5×10^3 CFU. †, day of epitympanic tap.

induced OM has been ascribed. Any contributory effect of antibody directed against the lipoprotein described by Green et al. (34) and shown to be present in isolated fimbrial protein preparations is not known at this time. These investigators have described the bactericidal nature of antibodies directed against Omp *e*, yet culture-negative or dry ears were not a hallmark finding of fimbrial protein-induced protection as described below.

Evaluation of data obtained by otoscopic examination indicated that parenteral immunization with the total OMP preparation and that with isolated fimbrial protein from strain 1128 were equally effective in significantly reducing tympanic-membrane inflammation ($P \leq 0.001$) compared with sham-immunized controls post-homologous challenge (Table 3). The total OMP preparation additionally protected against heterologous challenge (strain 1885) ($P \leq 0.001$) and was more likely to render middle ears effusion free or culture negative than the isolated fimbrial protein (63 to 90% versus 30 to 70% over the period of observation). Immunization with fimbrial protein isolated from strain 1885 was marginally less protective against homologous challenge ($P \leq 0.01$) than was immunization with strain 1128 fimbrial protein but was similarly not protective against heterologous challenge. Immunization with P2 protein, as prepared, was not protective against TB challenge with either strain, and indeed, animals receiving this preparation demonstrated significantly worse tympanic-membrane inflammation as determined via otoscopy ($P \leq 0.005$) compared with sham-immunized and challenged controls.

Upon epitympanic tap, the incidence of either dry ears or sterile MEFs in remaining cohorts was again found to correlate with tympanic-membrane inflammation scores. While strain 1885 was less virulent over the entire disease course than strain 1128, in both challenge cohorts, immunization with the total OMP preparation resulted in the greatest incidence of dry ears or sterile MEFs over the period of observation (40 to 80% for strain 1128 and 80 to 100% for strain 1885). Fimbrial protein-immunized and homologously challenged cohorts were ranked second in incidence of dry ears or sterile MEFs in both fimbrial protein-immunized cohorts. Mean CFU-per-milliliter values in those ears which yielded culture-positive MEFs were highly variable within any given cohort, reflecting animal-to-animal variability, and thereby no statistically significant trends were noted in this parameter of assessment.

Pooled serum from animals immunized with fimbrial protein from strain 1128 was found to specifically label the native fimbrial appendage via indirect immunogold labeling (Fig. 4B) and, in addition, was found to be reactive with two similarly migrating bands within the enriched OMP profile of each of 15 additional clinical NTHi and Hib isolates (Fig. 15). The relative electrophoretic mobility of the partially denatured fimbrin species in these isolates appears to be more heterogeneous than that of the true subunit.

Histopathological findings were consistent with other assessments of disease progression and indicated that sham-immunized and subsequently challenged animals demonstrated moderate histopathology of both tissues examined. Tympanic membranes were notably thickened with an edematous fibrous layer (Fig. 16A), whereas middle-ear mucosa specimens demonstrated moderate thickening of the mucosa, evidence of osteoneogenesis, and the presence of both erythrocytes (RBCs) and inflammatory cells in the subepithelial space (Fig. 17A). A dense polymorphonuclear leukocyte exudate was present in the middle-ear cavity. Animals immunized with either total OMP or isolated fimbrial protein prior to homologous challenge demonstrated notable reduced tympanic-membrane histopathology (Fig. 16B and C) compared with sham-immunized animals. Immunization with total OMP did, however, additionally result in the absence of a polymorphonuclear leukocyte-composed exudate overlying the middle-ear mucosa (Fig. 17B and C). Animals immunized with P2 and subsequently challenged demonstrated extensive histopathological changes to both the tympanic membrane and middle-ear mucosa (Fig. 16D and 17D) which were markedly

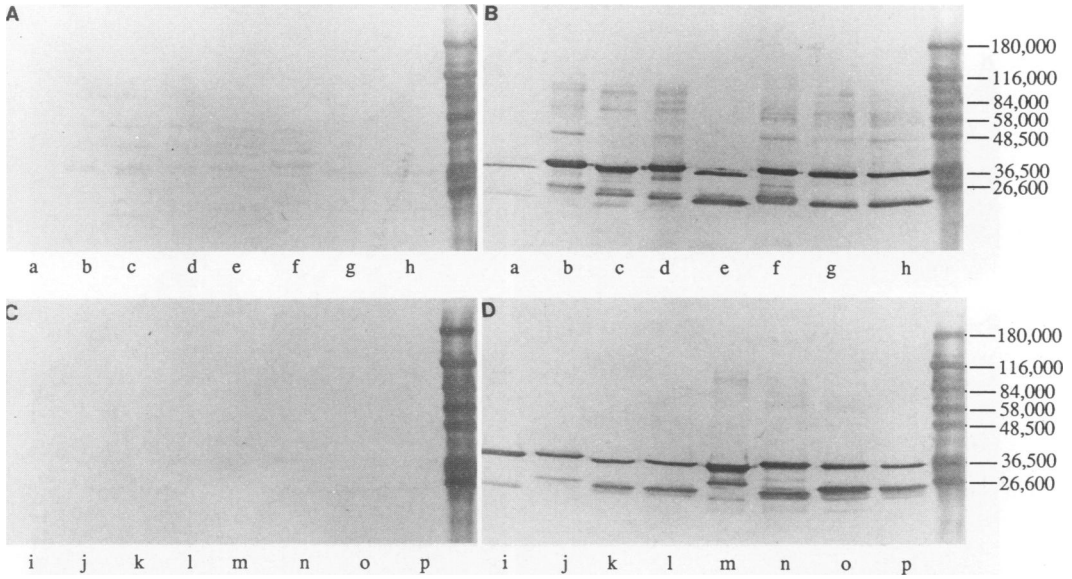


FIG. 15. Western blot of a normal chinchilla serum pool (A and C) or that obtained postimmunization with isolated fimbrial protein (strain 1128) (B and D) versus total OMP preparations from clinical isolates NTHi 86-042 (lane a), 86-043 (lane b), 1667 MEE (lane c), 1128 (lane d), 1885 MEE (lane e), 169 p^+ (lane f), 90-100 L (lane g), 90-100 R (lane h), 90-111 L (lane i), 90-112 R (lane j), 90-114 NP (lane k), 90-114 L (lane l) Mr 13 p− (lane m), and Mr 13 p^+ (lane n); and Hib Eagan p^+ (lane o) and Eagan p− (lane p). Unlabeled lanes, molecular mass markers (in daltons).

greater than those in sham-immunized and challenged animals.

DISCUSSION

To directly test whether fimbriae are integral to pathogenesis of NTHi-induced OM, the effect of fimbrin gene disruption on pathogenesis was determined. We cloned and sequenced the fimbrin gene and obtained a fimbrin gene-disrupted mutant. The cloned gene that contained an ORF for a 37-kDa protein contained the amino acid sequences of the fimbrin proteins isolated from NTHi 1128. The mutant was unable to produce the 37-kDa fimbrin protein. Indirect immunogold labeling did not detect any fimbriae on the gene-disrupted mutant, whereas fimbriae were detected on approximately 25% of the wild-type cells. While approximately 5-nm-diameter "pili," flagella, and other large bacterial structures are relatively easy to detect, the finer fimbriae are much harder to detect because they cling to the grid surface, get lost in the grain of the negative stain, and go in and out of a given focal

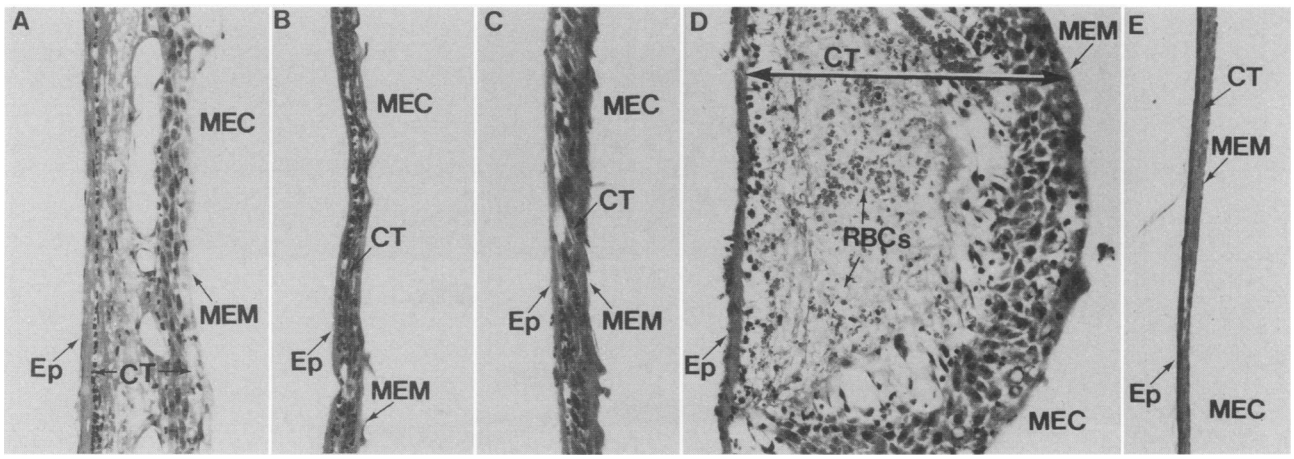
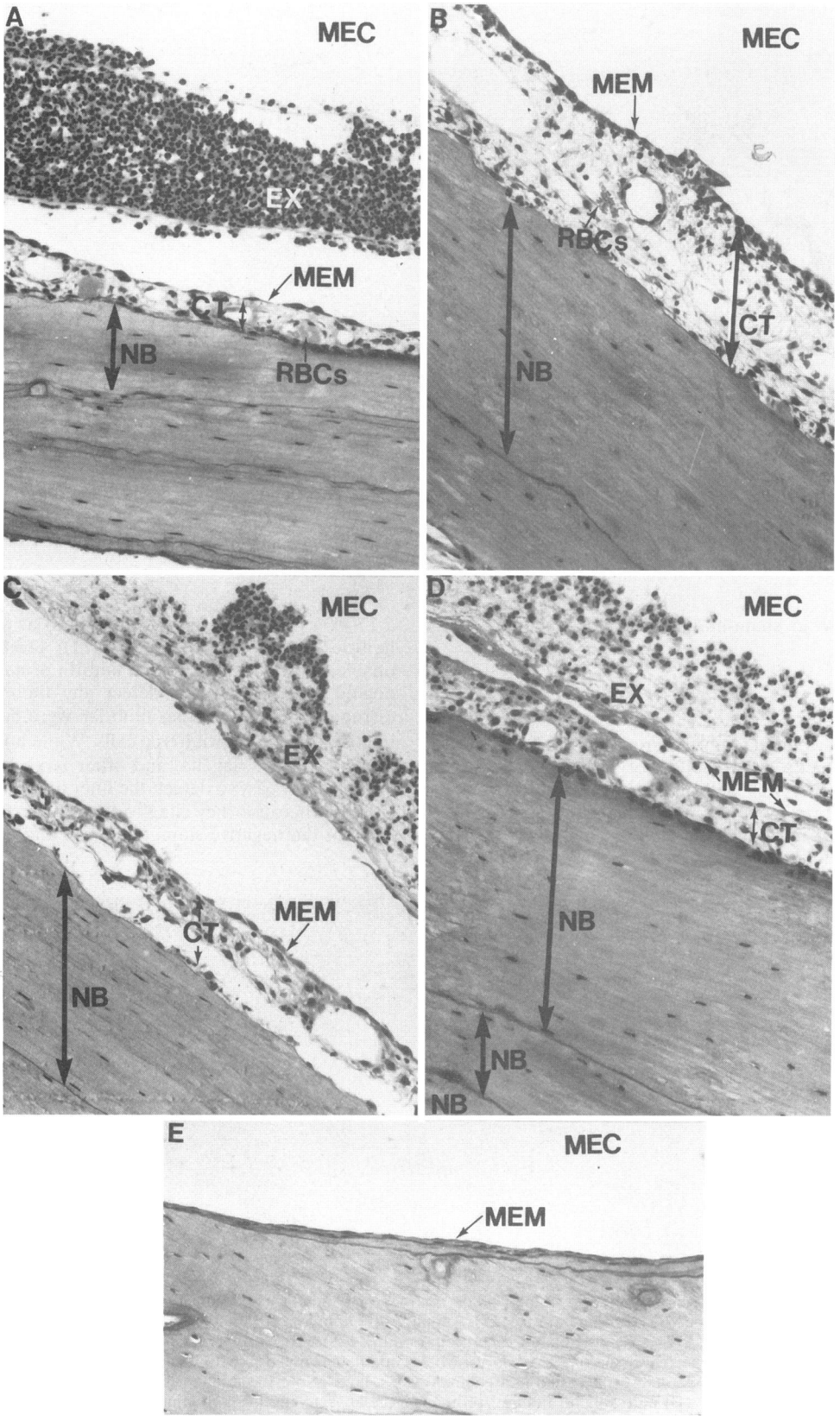


FIG. 16. Light micrographs of hematoxylin-eosin-stained tissue sections of tympanic membrane from actively immunized chinchillas 48 h post-TB challenge with the homologous NTHi strain (strain 1128). Animals were immunized with a sham preparation (A), total OMP (strain 1128) (B), isolated fimbrial protein (strain 1128) (C), or isolated P2 protein (strain 1128). (E) Normal chinchilla tympanic membrane. Magnification, ×160. Ep, epidermal layer; CT, connective tissue or fibrous layer; MEM, middle-ear mucosa; MEC, middle-ear cavity. Tympanic membranes of sham-immunized animals (A) demonstrated a thickened and edematous CT layer. Note the minimal thickening of the tympanic membrane in animals immunized with total OMP (B) and isolated fimbrial protein (C) relative to the normal chinchilla tympanic membrane (E). Animals immunized with the isolated P2 protein of strain 1128 (D) demonstrated marked thickening of the tympanic membrane with evidence of bleeding (RBCs) and edema in the fibrous layer (CT).


A
MEC
EX
MEM
CT
NB
RBCs
B
MEC
MEM
RBCs
CT
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C
MEC
EX
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CT
NB
D
MEC
EX
MEM
CT
NB
NB
E
MEC
MEM

FIG. 17. Light micrographs of hematoxylin-eosin-stained tissue sections of middle-ear mucosa from actively immunized chinchillas 48 h post-TB challenge with the homologous NTHi strain (strain 1128). Animals were immunized with a sham preparation (A), total OMP (strain 1128) (B), isolated fimbrial protein (strain 1128) (C), or isolated P2 protein (strain 1128) (D). (E) Normal chinchilla middle-ear mucosa. Magnification, ×200. EX, exudate; MEC, middle-ear cavity; MEM, middle-ear mucosa; NB, new bone (osteoneogenesis); CT, connective tissue. Note the minimal thickening of the mucosal layer in sham-immunized animals (A) relative to the mucosa of a normal animal (E). There is a dense polymorphonuclear leukocyte exudate present in the middle-ear cavity. Animals immunized with total OMP (B) demonstrate significant thickening of the connective-tissue layer of the mucosa with evidence of bleeding into the subepithelial space (RBCs) and some new bone formation. Animals immunized with fimbrial protein (C) are similar to those immunized with total OMP (B) with the addition that there is a predominantly polymorphonuclear leukocyte-composed exudate in the middle-ear cavity. Those animals immunized with the isolated P2 protein (D) demonstrated inflamed middle-ear mucosae (similar to all NTHi-challenged animals); however, there was the additional finding of extensive osteoneogenesis, a more predominant mononuclear character to the exudate, and evidence of focal desquamation of the epithelial layer (middle-ear mucosa), the severity of which was not noted for any other cohort.

plane. In spite of these limitations, we could have detected fimbriae if the gene-disrupted mutant had them in view of the fact that fimbriae were detected on all of the 60 clinical isolates examined in this laboratory (8). The labeling of the entire length of the fimbriae by our antisera prepared against the fimbrin protein strongly supports our conclusions that the cloned and disrupted gene codes for the fimbrin protein.

We have also shown that fimbrin is highly homologous with OmpA. This homology with OmpA proteins of several genera of gram-negative bacteria indicates the high level of conservation and, by inference, the relative importance of this particular surface-exposed OMP. OmpA is a very abundant constituent of the outer membrane of many gram-negative bacteria including other *Haemophilus* species such as *Haemophilus somnus* (71) and *H. ducreyi* (65), and it has been extensively investigated (10, 11, 17, 23, 36, 45). The role of OmpA as a porin (68), while generally accepted, remains somewhat controversial. Nonphysiological roles such as colicin and phage receptor function have been demonstrated (23, 35), as has that for a stabilizing effect on F-pilus-mediated conjugation (1, 60, 62, 72). All known functions have, to date, been attributable to residues contained within the N-terminal region exposed at the cell surface (15, 16, 36). The highest degree of homology of fimbrin to the OmpA proteins is found in the C-terminal half of the molecule. Since the function of this region of OmpA proteins is not known, the functional significance of this homology remains obscure. The possibility that fimbrin is a surface-exposed filamentous protein is supported by the shadow-cast and indirectly immunogold-labeled specimens (Fig. 4B and 5A), in which native fimbriae are recognized along their length by various antisera directed against isolated and reassembled fimbrial protein.

The gene-disrupted mutant was found to be significantly less adherent than the parent in an in vitro assay and, unlike the fimbriated parent, was not affected by attempts to inhibit its adherence to human oropharyngeal cells with either isolated fimbrial protein or antiserum directed against it. Isolated fimbrial protein, but not the P2 protein of strain 1128, has been shown to be inhibitory in a dose-dependent fashion to adherence of a heterologous OM isolate to human oropharyngeal cells (5). The gene-disrupted mutant, which could not form fimbriae and had a reduced ability to adhere to the eukaryotic target cell, also showed reduced virulence in two chinchilla models of experimental OM. Weiser and Gotschlich (75) similarly demonstrated reduced virulence in an OmpA-deficient *E. coli* K-12 isolate compared with its parent strain using two models of infection. Other OmpA-deficient mutants have been identified or created and investigated for the biological consequences of this disruption (17, 63), but none of the disrupted functions noted to date had served to explain the unusual degree of conservancy of this particular OMP (11).

Heretofore, the association between OmpA, adherence, virulence, and a specific bacterial surface appendage has not been made. There are, however, many examples in the literature of an association between the presence of specific OMPs (often of variable or modifiable electrophoretic mobility) and adherence, virulence, or the presence of a surface appendage, and most of these observations have been of mucosal pathogens. Adherence capability is generally considered to confer a selective advantage for any mucosal pathogen and is integral to both colonization and subsequent disease induction. A nonpiliated, clinical isolate of NTHi which adhered to and entered human conjunctival epithelial cells in culture was found to express several OMPs that either were not expressed or were expressed to a lesser degree than nonadherent variants of the same strain (67). One of these OMPs had an apparent molecular mass of 36 kDa and may prove to be analogous to fimbrin, as perhaps will that noted in the 30- to 45-kDa range for an adherent *H. ducreyi* isolate. The latter bacteria adhered to cultured human foreskin cells and, while lacking pili, did appear to express a fibrillar matrix (2) on the cell surface which may also prove to be analogous to the thin, nonhemagglutinating "pili" described by Spinola et al. (64). The association of *Pseudomonas cepacia* fimbriae with adherence to A549 cells, hemagglutination, and three OMPs (16, 20, and 40 kDa) has been demonstrated by Kuehn et al. (41), and fimbrial preparations from *Porphyromonas* (*Bacteroides*) *gingivalis* strains were found to contain two major OMP components (43 and 75 kDa) with marked size, sequence, and antigenic heterogeneity (43, 44). A distinct nonfimbrial surface protein complex with erythrocyte-binding and adhesin capacity which migrates with apparent molecular masses of 33 and 38 kDa has also been described for the latter group of microorganisms and has recently been epitope mapped to a novel cell-bound structure that is filamentous in appearance (21, 27, 50, 51).

Another category of OMP adhesins are the Opa proteins described for *Neisseria gonorrhoeae* and *Neisseria meningitidis*, which have been demonstrated to be responsible for noted increases in colonial opacity and are also associated with differential adherence to various cell types, extensive interbacterial adhesions, invasion, and most notably, serum resistance (12, 32, 56, 66, 69, 70, 73, 74). Neisserial Opa proteins, like many of those discussed, demonstrate variable temperature-dependent migration when separated by SDS-PAGE (25 to 28 and 29 to 35 kDa) (66, 70) and show homology with OmpA (33).

It remains to be determined whether any of the discussed OMP adhesins, which have homology with OmpA, will similarly be demonstrated to compose a filamentous surface appendage and whether the fimbriae expressed by NTHi have functional or immunological similarities described for other OmpA proteins. The potential usefulness of fimbrin as a vaccine component will be dependent upon relative heterogeneity of this protein, and thus, the demonstrated lack of

protection afforded to animals immunized with isolated fimbrial protein against TB challenge with a heterologous NTHi strain is important to consider. It would, however, be premature to speculate as to whether heterologous protection can be induced until more is known about the serological diversity of this surface appendage as well as the mechanism(s) behind the demonstrated efficacy. The noted lack of heterologous protection may well be attributable, in part, to inappropriate aspects of the model. Heretofore-existing models of experimental OM either circumvent the early colonization and ascension steps of disease progression (TB model) or are highly strain dependent in terms of consistent induction of disease (IN model). If fimbriae are integral to adherence and colonization of the nasopharynx, which precedes ascension into the tympanum, then TB challenge might not readily demonstrate any induced protection.

Preliminary evidence has indicated that there was a degree of serological relatedness among the fimbrin proteins of 15 additional NTHi and Hib isolates. An extensive assessment of both the serological relatedness and the degree of genetic conservation of both the native structures expressed by these strains and the gene which codes for their expression, respectively, will, however, need to be conducted. Finally, consideration must be given to the method by which immunity would be optimally induced. Several novel delivery systems for preferential induction of mucosal immunity are being investigated by many laboratories with various degrees of success. The recent demonstration of the inability of a single dose of a vaccine consisting of viable recombinant *S. typhimurium* which expressed a highly immunogenic 21-kDa OmpA-like protein isolated from *Bordetella avium* (30) to protect against tracheal and thymus colonization in turkeys challenged with the wild-type stain indicates that this will likely be a complex issue to resolve.

In conclusion, we have demonstrated that the gene which encodes the subunit of a filamentous surface appendage, referred to as fimbriae, expressed by NTHi 1128 and which appears to be similarly expressed by all examined OM isolates of NTHi is homologous with those which encode the OmpA proteins of many gram-negative bacteria. The disruption thereof leads to reduced virulence in two chinchilla models. In addition, this structure has been shown to be an adhesin, and the demonstrated ability to afford partial protection against OM in an experimental model via immunization against isolated fimbrial protein indicates its potential usefulness as a vaccine component. The demonstrated link between a highly conserved bacterial OMP and a filamentous surface appendage, shown to be both an adhesin and a virulence factor, may have far-reaching implications for other mucosal pathogens.

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REFERENCES

1. **Achtman, M. S., R. Schwuchow, R. Helmuth, G. Morelli, and P. A. Manning.** 1978. Cell-cell interactions with conjugating *Escherichia coli*: con$^-$ mutants and stabilization of mating aggregates. Mol. Gen. Genet. **164:**171–183.
2. **Alfa, M. J., P. Degagne, and T. Hollyer.** 1993. *Haemophilus ducreyi* adheres to but does not invade cultured human foreskin cells. Infect. Immun. **61:**1735–1742.
3. **Bakaletz, L. O., M. A. Ahmed, L. J. Forney, P. E. Kolattukudy, and D. J. Lim.** 1992. Cloning and sequence analysis of a pilin-like gene from an otitis media isolate of nontypable *Haemophilus influenzae* (strain #1128). J. Infect. Dis. **165:**S201.
4. **Bakaletz, L. O., T. Hoepf, P. Hoskins, T. F. DeMaria, and D. J. Lim.** 1991. Serological relatedness of fimbriae expressed by NTHi isolates recovered from children with chronic otitis media, abstr. 77, p. 73. Abstr. Fifth Int. Symp. Recent Advances in Otitis Media.
5. **Bakaletz, L. O., T. M. Hoepf, and D. J. Lim.** 1991. Inhibition of adherence of NTHi to human oropharyngeal cells—an ELISA assay, abstr. 145, p. 126. Abstr. Fifth Int. Symp. Recent Advances in Otitis Media.
6. **Bakaletz, L. O., T. M. Hoepf, D. J. Lim, and B. Tallan.** 1990. Colonization of the chinchilla middle ear and nasopharynx by fimbriated isolates of nontypable *Haemophilus influenzae*, abstr. B-66, p. 37. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990.
7. **Bakaletz, L. O., B. M. Tallan, W. J. Andrzejewski, and T. F. DeMaria.** 1989. Immunological responsiveness of chinchillas to outer membrane and isolated fimbrial proteins of nontypeable *Haemophilus influenzae*. Infect. Immun. **57:**3226–3229.
8. **Bakaletz, L. O., B. M. Tallan, T. M. Hoepf, T. F. DeMaria, H. G. Birck, and D. J. Lim.** 1988. Frequency of fimbriation of nontypable *Hemophilus influenzae* and its ability to adhere to chinchilla and human respiratory epithelium. Infect. Immun. **56:**331–335.
9. **Barenkamp, S. J.** 1986. Protection by serum antibodies in experimental nontypable *Haemophilus influenzae* otitis media. Infect. Immun. **52:**572–578.
10. **Beck, E., and E. Bremer.** 1980. Nucleotide sequence of the gene OmpA coding the outer membrane protein II of *Escherichia coli* K-12. Nucleic Acids Res. **8:**3011–3024.
11. **Beher, M. G., C. A. Schnaitman, and A. P. Pugsley.** 1980. Major heat-modifiable outer membrane protein in gram-negative bacteria: comparison with the OmpA protein of *Escherichia coli*. J. Bacteriol. **143:**906–913.
12. **Bessen, D., and E. C. Gotschlich.** 1986. Interactions of gonococci with HeLa cells: attachment, detachment, replication, penetration, and the role of protein II. Infect. Immun. **54:**154–160.
13. **Braun, G., and S. T. Cole.** 1982. The nucleotide sequence encoding for major outer membrane protein OmpA of *Shigella dysenteriae*. Nucleic Acids Res. **10:**2367–2378.
14. **Braun, G., and S. T. Cole.** 1983. Molecular characterization of the gene coding for major outer membrane protein OmpA from *Enterobacter aerogenes*. Eur. J. Biochem. **137:**495–500.
15. **Braun, G., and S. T. Cole.** 1984. DNA sequence analysis of the *Serratia marcescens* ompA gene: implications for the organisation of an enterobacterial outer membrane protein. Mol. Gen. Genet. **195:**321–328.
16. **Bremer, E., S. T. Cole, I. Hindennach, U. Henning, E. Beck, C. Kurz, and H. Schaller.** 1982. Export of a protein into the outer membrane of *Escherichia coli* K12. Eur. J. Biochem. **122:**223–231.
17. **Bremer, E., T. J. Silhavy, M. Maldener, and S. T. Cole.** 1986. Isolation and characterization of mutants deleted for the sulA-ompA region of the *Escherichia coli* K-12 chromosome. FEMS Microbiol. Lett. **33:**173–178.
18. **Brinton, C. C., Jr., M. J. Carter, D. B. Derber, S. Kar, J. A. Kramarik, A. C. C. To, S. C. M. To, and S. W. Wood.** 1989. Design and development of pilus vaccines for *Haemophilus influenzae* diseases. Pediatr. Infect. Dis. J. **8:**S54–S61.
19. **Campbell, A. M.** 1984. Antibody production and purification, p. 166–185. *In* R. H. Burdon and P. H. van Knippenberg (ed.), Laboratory techniques in biochemistry and molecular biology, vol. 13. Monoclonal antibody technology. Elsevier, New York.
20. **Carlone, G. M., M. L. Thomas, H. S. Rumschlag, and F. O. Sottnek.** 1986. Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. J. Clin. Microbiol. **24:**330–332.
21. **Chandad, F., and C. Mouton.** 1990. Molecular size variation of the hemagglutinating adhesin HA-Ag2, a common antigen of *Bacteroides gingivalis*. Can. J. Microbiol. **36:**690–696.
22. **Chanyangam, M., A. L. Smith, S. L. Moseley, M. Kuehn, and P. Jenny.** 1991. Contribution of a 28-kilodalton membrane protein to the virulence of *Haemophilus influenzae*. Infect. Immun. **59:**600–608.

23. **Datta, D. B., B. Arden, and U. Henning.** 1977. Major proteins of the *Escherichia coli* outer cell envelope membrane as bacteriophage receptors. J. Bacteriol. **131:**821–829.
24. **DeMaria, T. F., C. McPherson, L. O. Bakaletz, and K. A. Holmes.** 1991. Isotype specific antibody response against OMPs and fimbriae of nontypable *Haemophilus influenzae* isolated from patients with chronic otitis media, abstr. 134, p. 119. Abstr. Fifth Int. Symp. Recent Advances in Otitis Media.
25. **DeMaria, T. F., T. Yamaguchi, L. O. Bakaletz, and D. J. Lim.** 1992. Serum and middle ear antibody response in the chinchilla during otitis media with effusion induced by nonviable nontypable *Haemophilus influenzae*. J. Infect. Dis. **165:**S196–S197.
26. **DeMaria, T. F., T. Yamaguchi, and D. J. Lim.** 1989. Quantitative cytological and histological changes in the middle ear after the injection of nontypable *Haemophilus influenzae* endotoxin. Am. J. Otolaryngol. **10:**261–266.
27. **Deslauriers, M., and C. Mouton.** 1992. Epitope mapping of hemagglutinating adhesin HA-Ag2 of *Bacteroides* (*Porphyromonas*) *gingivalis*. Infect. Immun. **60:**2791–2799.
28. **Forney, L. J., J. R. Gilsdorf, and D. C. L. Wong.** 1992. Effect of pili-specific antibodies on the adherence of *Haemophilus influenzae* type b to human buccal cells. J. Infect. Dis. **165:**464–470.
29. **Freudl, R., and S. T. Cole.** 1983. Cloning and molecular characterization of the OmpA gene from *Salmonella typhimurium*. Eur. J. Biochem. **134:**497–502.
30. **Gentry-Weeks, C. R., A. L. Hultsch, S. M. Kelly, J. M. Keith, and R. Curtiss III.** 1992. Cloning and sequencing of a gene encoding a 21-kilodalton outer membrane protein from *Bordetella avium* and expression of the gene in *Salmonella typhimurium*. J. Bacteriol. **174:**7729–7742.
31. **Gilsdorf, J. R., H. Y. Chang, K. W. McCrea, and L. O. Bakaletz.** 1992. Comparison of hemagglutinating pili of *Haemophilus influenzae* type b with similar structures of nontypeable *H. influenzae*. Infect. Immun. **60:**374–379.
32. **Gorby, G. L., and G. B. Schaefer.** 1992. Effect of attachment factors (pili plus Opa) on *Neisseria gonorrhoeae* invasion of human fallopian tube tissue *in vitro*: quantitation by computerized image analysis. Microb. Pathog. **13:**93–108.
33. **Gotschlich, E. C.** 1986. Conserved gonococcal surface antigens, p. 415–426. *In* A. Tagliabue, R. Rappuoli, and S. E. Piazzi (ed.), Bacterial vaccines and local immunity. Ann. Sclavo, Siena, Italy.
34. **Green, B. A., J. E. Farley, T. Quinn-Dey, R. A. Deich, and G. W. Zlotnick.** 1991. The *e* (P4) outer membrane protein of *Haemophilus influenzae*: biologic activity of anti-*e* serum and cloning and sequencing of the structural gene. Infect. Immun. **59:**3191–3198.
35. **Havekes, L. M., and W. P. M. Hoekstra.** 1976. Characterization of an *Escherichia coli* K-12 F^- Con^- mutant. J. Bacteriol. **126:**593–600.
36. **Henning, U., S. T. Cole, E. Bremer, I. Hindennach, and H. Schaler.** 1983. Gene fusions using the ompA gene coding for a major outer-membrane protein of *Escherichia coli* K12. Eur. J. Biochem. **136:**233–240.
37. **Herriot, R. M., E. M. Meyer, and M. Vogt.** 1970. Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. J. Bacteriol. **101:**517–524.

37a. **Higgins, D. G., A. J. Bleasdi, and R. R. Suchs.** 1992. CLUSTAL V improved software for multiple sequence alignment. Comp. Appl. Biosci. **8:**189–191.

38. **Hollingshead, S. K., V. A. Fischetti, and J. R. Scott.** 1986. Complete nucleotide sequence of type 6 M protein of the group A streptococcus. J. Biol. Chem. **261:**1677–1686.
39. **Hollingshead, S. K., V. A. Fischetti, and J. R. Scott.** 1987. A highly conserved region present in transcripts encoding heterologous M proteins of group A streptococci. Infect. Immun. **55:**3237–3239.
40. **Karasic, R. B., C. E. Trumpp, H. E. Gnehm, P. A. Rice, and S. I. Pelton.** 1985. Modification of otitis media in chinchillas rechallenged with nontypable *Haemophilus influenzae* and serological response to outer membrane antigens. J. Infect. Dis. **151:**273–279.
41. **Kuehn, M., K. Lent, J. Haas, J. Hagenzieker, M. Cervin, and A. L. Smith.** 1992. Fimbriation of *Pseudomonas cepacia*. Infect. Immun. **60:**2002–2007.
42. **Lawrence, J. G., H. Ochman, and D. L. Hartl.** 1991. Molecular and evolutionary relationships among enteric bacteria. J. Gen. Microbiol. **137:**1911–1921.
43. **Lee, J. Y., H. T. Sojar, G. S. Bedi, and R. J. Genco.** 1991. *Porphyromonas* (*Bacteroides*) *gingivalis* fimbrillin: size, amino-terminal sequence, and antigenic heterogeneity. Infect. Immun. **59:**383–389.
44. **Lee, J. Y., H. T. Sojar, G. S. Bedi, and R. J. Genco.** 1992. Synthetic peptides analogous to the fimbrillin sequence inhibit adherence of *Porphyromonas gingivalis*. Infect. Immun. **60:**1662–1670.
45. **Lugtenberg, B., and L. van Alphen.** 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta **737:**51–115.
46. **Lupas, A., M. Van Dyke, and J. Stock.** 1991. Predicting coiled coils from protein sequences. Science **252:**1162–1164.
47. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3:**208–218.
49. **Matsudaira, P.** 1987. Sequence from picomole quantities electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:**10035–10038.
50. **Mouton, C., D. Bouchard, M. Deslauriers, and L. Lamonde.** 1989. Immunochemical identification and preliminary characterization of a nonfimbrial hemagglutinating adhesin of *Bacteroides gingivalis*. Infect. Immun. **57:**566–573.
51. **Mouton, C., D. Ni Eidhin, M. Deslauriers, and L. Lamy.** 1991. The hemagglutinating adhesin HA-Ag2 of *Bacteroides gingivalis* is distinct from fimbrilin. Oral Microbiol. Immunol. **6:**6–11.

51a. **Munson, R. S., Jr., S. Grass, and R. West.** 1993. Molecular cloning and sequence of the gene for outer membrane protein P5 of *Haemophilus influenzae*. Infect. Immun. **61:**4017–4020.

52. **Niki, H., A. Jaffe, R. R. Imamura, T. Ogura, and S. Hizaga.** 1991. The new gene muk B codes for a 177 kDa protein with coiled-coil domain involved in chromosome partitioning of *E. coli*. EMBO J. **10:**183–193.
53. **Ofek, I., H. S. Courtney, D. M. Schifferli, and E. H. Beachey.** 1986. Enzyme-linked immunosorbent assay for adherence of bacteria to animal cells. J. Clin. Microbiol. **24:**512–516.
54. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
55. **Phillips, G. N., Jr., P. F. Flicker, C. Cohen, B. N. Manjula, and V. A. Fischetti.** 1981. Streptococcal M protein: α-helical coiled-coil structure and arrangement on the cell surface. Proc. Natl. Acad. Sci. USA **78:**4689–4693.
56. **Rice, P. A., H. E. Vayo, M. R. Tam, and M. S. Blake.** 1986. Immunoglobulin G antibodies directed against protein III block killing of serum-resistant *Neisseria gonorrhoeae* by immune serum. J. Exp. Med. **164:**1735–1748.
57. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of DNA transcription. Annu. Rev. Genet. **13:**319–353.
58. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
59. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
60. **Schweizer, M., and U. Henning.** 1977. Action of a major outer cell envelope membrane protein in conjugation of *Escherichia coli* K-12. J. Bacteriol. **129:**1651–1652.
61. **Shine, J., and L. Dalgarno.** 1975. Determinant of cistron specificity in bacterial ribosomes. Nature (London) **254:**34–38.
62. **Skurray, R. A., R. E. W. Hancock, and P. Reeves.** 1974. Con^- mutants: class of mutants in *Escherichia coli* K-12 lacking a major cell wall protein and defective in conjugation and adsorption of a bacteriophage. J. Bacteriol. **119:**726–735.
63. **Sonntag, I., H. Schwarz, Y. Hirota, and U. Henning.** 1978. Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J. Bacteriol. **136:**280–285.
64. **Spinola, S. M.** 1990. Characterization of pili expressed by *Haemophilus ducreyi*. Microb. Pathog. **9:**417–426.

65. **Spinola, S. M., G. E. Griffiths, K. L. Shanks, and M. S. Blake.** 1993. The major outer membrane protein of *Haemophilus ducreyi* is a member of the OmpA family of proteins. Infect. Immun. **61:**1346–1351.
66. **Stern, A., P. Nickel, T. F. Meyer, and M. So.** 1984. Opacity determinants of *Neisseria gonorrhoeae*: gene expression and chromosomal linkage to the gonococcal pilus gene. Cell **37:**447–456.
67. **St. Geme, J. W., III, and S. Falkow.** 1990. *Haemophilus influenzae* adheres to and enters cultured human epithelial cells. Infect. Immun. **58:**4036–4044.
68. **Sugawara, E., and H. Nikaido.** 1992. Pore-forming activity of OmpA protein of *Escherichia coli*. J. Biol. Chem. **267:**2507–2511.
69. **Swanson, J.** 1983. Gonococcal adherence: selected topics. Rev. Infect. Dis. **5:**S678–S684.
70. **Swanson, J., S. Bergström, O. Barrera, K. Robbins, and D. Corwin.** 1985. Pilus gonococcal variants. J. Exp. Med. **162:**729–744.
71. **Tagawa, Y., M. Haritani, H. Ishikawa, and N. Yuasa.** 1993. Characterization of a heat-modifiable outer membrane protein of *Haemophilus somnus*. Infect. Immun. **61:**1750–1755.
72. **van Alphen, L., L. Havekes, and B. Lugtenberg.** 1977. Major outer membrane protein d of *Escherichia coli* K-12. FEBS Lett. **75:**285.
73. **Virji, M., C. Alexandrescu, D. J. P. Ferguson, J. R. Saunders, and E. R. Moxon.** 1992. Variations in the expression of pili: the effect on adherence of *Neisseria meningitidis* to human epithelial and endothelial cells. Mol. Microbiol. **6:**1271–1279.
74. **Virji, M., K. Makepeace, D. J. P. Ferguson, M. Achtman, J. Sarkari, and E. R. Moxon.** 1992. Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by *Neisseria meningitidis*. Mol. Microbiol. **6:**2785–2795.
75. **Weiser, J. N., and E. C. Gotschlich.** 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. Infect. Immun. **59:**2252–2258.
76. **Wilson, M. E.** 1991. The heat-modifiable outer membrane protein of *Actinobacillus actinomycetemcomitans*: relationship to OmpA proteins. Infect. Immun. **59:**2505–2507.
77. **Young, R. A., and R. W. Davis.** 1983. Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. USA **80:**1194–1198.