Demonstration of Type IV Pilus Expression and a Twitching Phenotype by *Haemophilus influenzae*

Lauren O. Bakaletz,* Beth D. Baker, Joseph A. Jurcisek, Alistair Harrison,
Laura A. Novotny, James E. Bookwalter, Rachna Mungur,
and Robert S. Munson, Jr.*

Department of Pediatrics, Center for Microbial Pathogenesis, Columbus Children's Research Institute,
The Ohio State University College of Medicine and Public Health, Columbus, Ohio

Received 17 August 2004/Returned for modification 29 September 2004/Accepted 12 November 2004

Haemophilus influenzae is considered a nonmotile organism that expresses neither flagella nor type IV pili, although *H. influenzae* strain Rd possesses a cryptic pilus locus. We demonstrate here that the homologous gene cluster *pilABCD* in an otitis media isolate of nontypeable *H. influenzae* strain 86-028NP encodes a surface appendage that is highly similar, structurally and functionally, to the well-characterized subgroup of bacterial pili known as type IV pili. This gene cluster includes a gene (*pilA*) that likely encodes the major subunit of the heretofore uncharacterized *H. influenzae*-expressed type IV pilus, a gene with homology to a type IV prepilin peptidase (*pilD*) as well as two additional uncharacterized genes (*pilB* and *pilC*). A second gene cluster (*comABCDEF*) was also identified by homology to other *pil* or type II secretion system genes. When grown in chemically defined medium at an alkaline pH, strain 86-028NP produces approximately 7-nm-diameter structures that are near polar in location. Importantly, these organisms exhibit twitching motility. A mutation in the *pilA* gene abolishes both expression of the pilus structure and the twitching phenotype, whereas a mutant lacking ComE, a *Pseudomonas* PilQ homologue, produced large appendages that appeared to be membrane bound and terminated in a slightly bulbous tip. These latter structures often showed a regular pattern of areas of constriction and expansion. The recognition that *H. influenzae* possesses a mechanism for twitching motility will likely profoundly influence our understanding of *H. influenzae*-induced diseases of the respiratory tract and their sequelae.

Haemophilus influenzae is a common commensal of the human nasopharynx; however, it is also responsible for multiple diseases of the upper and lower respiratory tract, including sinusitis, otitis media (OM), and bronchitis as well as exacerbations of chronic obstructive pulmonary disease, bronchiectasis, and cystic fibrosis (CF), among others (17, 32). Our understanding of the pathogenesis of *H. influenzae*-induced diseases is still incomplete; however, adherence to and the colonization of mucosal epithelial surfaces are acknowledged first steps in the disease process. As such, many strains of *H. influenzae* express adhesins including hemagglutinating (or long, thick, and positive for hemagglutination [LKP]) pili, fimbriae, and nonfimbrial adhesins (20, 21, 41). However, none of these structures are associated with a motility function, and *H. influenzae* does not express flagella.

Twitching motility is a flagellum-independent form of bacterial translocation over moist surfaces and occurs by extension, tethering, and then retraction of polar structures known as type IV pili, or Tfp (6, 29, 43, 51). Tfp are typically 5 to 7 nm in diameter, several micrometers in length, and comprised of a single protein subunit assembled into a helical conformation with five subunits per turn (6, 44). Tfp play a significant role in the pathogenesis of disease caused by *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Neis-*

seria gonorrhoeae, and numerous other gram-negative pathogens. There are two classes of pilin subunits, type IVa and type IVb, which are distinguished from one another by the average length of both the leader peptide and the mature subunit, by which an N-methylated amino acid occupies the N-terminal position of the mature protein, and by the average length of the D region (for disulfide region) (10). Most of the respiratory pathogens express class IVa pilins, whereas the enteropathogens more typically express class IVb pilins. Regardless of the class of Tfp, expression of these structures has been found to be universally important for both adherence and biofilm formation by many bacteria (24, 27, 29, 35), as well as for virulence of *Neisseria* species, *Moraxella bovis*, *V. cholerae*, enteropathogenic *Escherichia coli* and *P. aeruginosa*, among others (26, 27, 35, 42).

Tfp expression is a complex and highly regulated bacterial function. In *P. aeruginosa*, the biogenesis and function of Tfp are controlled by over 40 genes (42). To date, only a subset of the vast number of related Tfp genes (12, 43) has been found in several members of the HAP (*Haemophilus*, *Actinobacillus*, and *Pasteurella*) family (7, 13, 14, 40), and the expression of Tfp, or a twitching phenotype, has not been described for any *H. influenzae* isolate. In fact, *H. influenzae* is classically described as a bacterium that does not express these structures (1, 8, 18, 19), despite the presence of a cryptic gene cluster within the strain Rd genome (16).

Recently, it was recognized that many nontypeable *H. influenzae* (NTHI) strains form a biofilm (15, 33). Murphy and Kirkham (33) demonstrated that the *hif* locus, required for the expression of LKP pili (30), was important for biofilm formation by NTHI strain M37. However, strain 86-028NP (the

* Corresponding author. Mailing address: Department of Pediatrics, Columbus Children's Research Institute, Center for Microbial Pathogenesis, The Ohio State University College of Medicine and Public Health, 700 Children's Dr., Columbus, OH 43205-2696. Phone: (614) 722-2915. Fax: (614) 722-2818. E-mail for Lauren O. Bakaletz: BakaletL@pediatrics.ohio-state.edu. E-mail for Robert S. Munson, Jr.: MunsonR@pediatrics.ohio-state.edu.

TABLE 1. Primers used in this study

Primer	Sequence
1.....	TGTGACACTTCCGCAAAA
2.....	TAATAAAAGGAAATGAATGA
3.....	ACGGCTAAAATCTAATCCAATCTG
4.....	AACCGCACTTTTCACATTCAA
5.....	ATGATTAATTTCCACACCTC
6.....	AACGATCCTTTTACTCCTT
7.....	CATTAATAGAGCTAAGGATCCTGATTGCAATTATTGC
8.....	GCAATAATTTGCAATCAGGATCCTTAGCTCTATTAATG
9.....	TTAACAACAGGAAGTGGATCCTTGTCTTCTCTGCG
10.....	CGCAGAAGAAAGCAAGGATCCACTTCTGTTGTAA
11.....	GGAAGATCTGGGACGGCGGCTTTGTTG
12.....	GGAAGATCTCACCCCTTGTAATTACTGTTATGT
13.....	GCGCGTCGACAACCAATAAGGAAATA
14.....	CCGGAATTCAGCTGATGATGGCGAAAC
15.....	CGCGGATCCTACAGCGTTTATAGAGGCGGTTGAT
16.....	CCGGAATTCGCGATTGCTGGGTTGCTTGT

strain used in the present study), like the majority of NTHI isolates (30), does not possess an LKP pilus locus, yet this isolate forms biofilms both in vitro and in vivo (our unpublished observation). Thus, we reasoned that if a pilus-like structure was indeed important to biofilm formation by NTHI, as has been shown in many other bacterial systems (23, 26, 27, 29, 35), there might be another type of pilus responsible for this phenotype. Herein, we identify genes required for Tfp expression, demonstrate that expression of Tfp is dependent on the products of the *pilA* and *comE* genes, and demonstrate for the first time twitching motility by *H. influenzae*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. NTHI strain 86-028NP is a low-passage isolate from a child with chronic otitis media (25) whose genome has recently been sequenced (<http://www.microbial-pathogenesis.org/>) (31). Thirteen additional low-passage clinical isolates of NTHI that were recovered from patients undergoing tympanostomy and tube insertion for chronic otitis media, a clinical isolate recovered from a patient with CF, and strain Rd were also used. The clinical isolates were designated by the following strain numbers: 86-028NP, 1728MEE, 1729MEE, 1714MEE, 214NP, 1236MEE, 165NP, 1060MEE, 1128MEE, 10548MEE, 3224A, 3185A, 1885MEE, and 27W11679INI.

Haemophilus strains were grown on chocolate agar or brain heart infusion (BHI) agar supplemented with NAD and heme at a final concentration 2 µg/ml or in a chemically defined medium (9). Media were supplemented with kanamycin at a concentration of 20 µg/ml or spectinomycin at a concentration of 200 µg/ml as appropriate. *E. coli* strains were grown on Luria-Bertani plates or in Luria-Bertani broth. Where appropriate, media were supplemented with kanamycin at 20 µg/ml and/or with ampicillin at 100 µg/ml.

Recombinant DNA and DNA sequencing methodologies. Restriction enzymes were purchased from New England Biolabs (Boston, Mass.). DNA ligase was purchased from Invitrogen (Carlsbad, Calif.). PCR amplifications were performed with *Pfu* Turbo (Stratagene, La Jolla, Calif.). Plasmids were purified with QIAGEN (Valencia, Calif.) kits. DNA sequence was determined with an Applied Biosystems 3100 automated sequencer using dye terminator chemistries. Standard methodologies were employed for plasmid constructions. *H. influenzae* plasmid transformations were performed by electroporation, and mutant constructions were performed by transformation with linear DNA using a modified M-IV method in which cyclic AMP was added to a final concentration of 1 mM after 70 min of incubation in M-IV medium (5), and incubation continued for an additional 30 min to increase transformation efficiency. Sequence comparisons were performed by using the BLAST algorithm (3).

For Southern hybridization, genomic DNA was purified from multiple clinical and laboratory isolates of *H. influenzae* by using a PUREGENE DNA isolation kit (Gentra Systems, Minneapolis, Minn.). Two micrograms of genomic DNA was digested with MfeI, and the fragments were resolved on a 0.8% agarose gel and then blotted onto a NYTRAN SuPerCharge membrane using a Turbo Blotter kit (Schleicher & Schuell, Keene, N.H.). Membranes were hybridized to a probe generated by PCR amplification of the coding sequence of the 86-028NP *pilA* gene by using the primers 1 and 2 (Table 1).

The amplicon was purified with a QIAquick PCR purification kit (QIAGEN). One hundred nanograms of purified PCR product was labeled with horseradish peroxidase using the ECL Direct Nucleic Acid Labeling and Detection system (Amersham Biosciences UK Ltd., Little Chalfont, Bucks, United Kingdom) according to the manufacturer's directions. Developed blots were exposed to Super Rx X-ray film (Fuji Photo Film Co., Tokyo, Japan).

Construction of *pilA* and *comE* mutants. Mutants deficient in expression of either the *pilA* or *comE* gene product were constructed in NTHI strain 86-028NP. To accomplish this, the coding sequences of *pilA* and *comE* plus an additional 1 kb upstream and downstream were amplified from 86-028NP genomic DNA by PCR using primers 3, 4, 5, and 6, respectively, (Table 1). These products were TA cloned into pGEM-T Easy (Promega Corp., Madison, Wis.) and transformed into *E. coli* DH10B cells (Invitrogen). Due to the lack of useful restriction sites, it was necessary to introduce an appropriate restriction site into *pilA*. A BamHI site was introduced into *pilA* by using a QuikChange Site-Directed Mutagenesis kit (Stratagene Corp.), according to the manufacturer's directions, with primers 7 and 8 (Table 1). The mutagenized plasmid was characterized by sequencing and then digested with BamHI, ligated into a BamHI fragment containing the ΩKm-2 element (36), and transformed into One Shot TOP 10 chemically competent *E. coli* (Invitrogen). A plasmid with the correct restriction map was identified, linearized with MluI, and then transformed into strain 86-028NP by using a modified M-IV transformation protocol (5, 28). Transformants were selected on chocolate agar supplemented with kanamycin, and allelic exchange was verified by Southern analysis.

Similarly, a BamHI site was constructed in the *comE* gene by using primers 9 and 10 (Table 1), employing the methodology described for the *pilA* construction. A *comE* mutant in strain 86-028NP was then constructed by using the strategy described above for the construction of the *pilA* mutant.

Complementation was performed by using a derivative of the shuttle vector pGZRS-39A (46), in which the kanamycin resistance gene was replaced by the spectinomycin resistance gene from pSPECR (48). The pGZRS-39A plasmid backbone, without the kanamycin resistance gene, was amplified by using primers 11 and 12 (Table 1). Both primers had BglII sites at their 5' ends. The amplicon was digested with BglII and ligated into the spectinomycin gene that had been isolated from pSPECR after BglII digestion. After transformation into *H. influenzae* strain Rd, spectinomycin-resistant clones were selected, plasmids were characterized, and a plasmid with the correct restriction map was saved as pSPEC1. We then amplified the *pilABCD* gene cluster using primers 13 and 14 and the *comEF* genes using primers 15 and 16 (Table 1). The amplicons were cloned into pCR Blunt II TOPO (Invitrogen), and plasmids with the correct inserts were saved. The *pilABCD* fragment was released as an EcoRI fragment and cloned into pSPEC1 that had been digested with EcoRI. The *comEF* fragment was cloned as a BamHI-EcoRI fragment into pSPEC1 that had been digested with the same enzymes. After transformation of the ligation mixtures into *H. influenzae* strain Rd, plasmids with the correct restriction maps were saved as pPIL1 and pCOM1 and transformed into strain 86-028NP and subsequently into strains 86-028NP *pilA::kan* and 86-028NP *comE::kan*, thus generating the complemented strains 86-028NP *pilA::kan*(pPIL1) and *comE::kan*(pCOM1), respectively.

Negative staining and transmission electron microscopy (TEM). NTHI strain 86-028NP and its *pilA* or *comE* mutant were inoculated onto either chocolate agar, supplemented BHI agar, or chemically defined agar plates (at pH 7.2 or 8.5 to 9.0) and incubated for 24 h at 37°C and 5% CO₂. Bacteria were scraped from agar plates, suspended in 10 µl of sterile water, and then negatively stained with an equal volume of a Whatman-filtered solution containing 2.0% (wt/vol) ammonium acetate (Sigma, St. Louis, Mo.) and 2.0% (wt/vol) ammonium molybdate (Sigma) in sterile water (4). Formvar- and carbon-coated copper grids (300 mesh; Electron Microscopy Sciences, Hatfield, Pa.) were placed under the droplet. After 5 min, grids were blotted and allowed to air dry overnight prior to viewing with a Hitachi model H-600 transmission electron microscope with an attached video monitor (Gatan, Inc., Pleasanton, Calif.) and digital imaging system (Gatan, Inc.).

For blinded TEM evaluation of the 86-028NP parental isolate and its *pilA* mutant, negative stains were prepared as described above, and multiple unlabeled grids were provided (to L.O.B.). For each grid, a minimum of 15 individual grid squares were evaluated at $\geq 25,000\times$ magnification. Within each of these 15 grid squares, a minimum of eight bacterial cells were scanned around their entire periphery for the presence of Tfp-like structures. Thus, a minimum of 120 individual NTHI isolates were evaluated per grid. Grids were also evaluated for the presence of bundles of free pili.

Motility assay. NTHI strain 86-028NP and its *pilA* or *comE* mutant were inoculated onto chemically defined 1% agar slabs (3 mm in depth), pH 9.0, and placed onto sterile microscope slides. A 0.2-µl droplet containing 10⁹ CFU of

NTHI/ml of saline was dropped onto the agar slab, covered with a sterile glass coverslip, and incubated at 37°C, 5% CO₂, in a humidified atmosphere (11). The outermost edge of the inoculum was examined hourly for up to 24 h with a Zeiss Axioskop 40 microscope under a ×40 objective. Images were recorded with a Zeiss Axiocam MRC digital camera, and the assay was repeated a minimum of four times on different days.

The complemented strains 86-028NP *pilA::kan*(pPIL1) and 86-028NP *comE::kan*(pCOM1) were evaluated in an identical manner.

Nucleotide sequence accession numbers. The *pil* and *com* gene cluster sequences from strain 86-028NP have been submitted to GenBank with accession numbers AY816324 and AY816325, respectively, and the *pilA* gene sequences have been submitted to GenBank with accession numbers AY818310 to AY818319.

RESULTS

Identification of the type IV pilin gene cluster in strain 86-028NP. As we characterized the contig set from our genomic sequencing efforts, we identified a four-gene cluster in the strain 86-028NP genome that was highly homologous to gene loci identified in *H. influenzae* strain Rd, *Actinobacillus pleuropneumoniae*, and *Pasteurella multocida* (14, 38, 40, 52). *A. pleuropneumoniae* and *P. multocida* are known to express Tfp, whereas Tfp have not been observed in *H. influenzae*. In strain Rd, the four genes are encoded by HI0299 to HI0296. The Institute for Genomic Research has annotated HI0299 as a prepilin peptidase-dependent protein D, HI0296 as a type IV prepilin-like leader peptidase, and HI0297 and HI0298 as protein transport proteins. We designated the 86-028NP homologues of these genes *pilABCD*. The derived amino acid sequence of the 86-028NP *pilA* gene is 55% identical to the derived amino acid sequence of the *apfA* gene of *A. pleuropneumoniae* (GenBank accession number AF302997) and 59% identical to the derived amino acid sequence of the *ptfA* gene of *P. multocida* (GenBank accession number AF154834). The products of the *pilBCD* genes of strain 86-028NP are also homologous to the products of the corresponding genes in *A. pleuropneumoniae* and *P. multocida*. When the derived amino acid sequence of the strain 86-028NP *pilB* gene was compared to those other proteins in the National Center for Biotechnology Information nonredundant database with the BLASTP algorithm, significant homology to numerous Tfp and type II secretion pathway proteins was observed. With respect to the Tfp proteins from *P. aeruginosa*, the putative PilB protein of strain 86-028NP is 38% identical over 496 amino acids to the PilB protein (BLAST E score of 7e-90), 39% identical over 227 amino acids to the PilT protein (BLAST E score of 3e-28), and 38% identical over 149 amino acids to PilU (BLAST E score of 3e-15). In *P. aeruginosa* and *Neisseria meningitidis*, the PilT protein is required for pilus retraction and twitching motility (1, 19). The strain 86-028NP *pilC* gene product is a member of COG1459.1 (PulF), a group of proteins also involved in type II secretion. Similarly, the product of the strain 86-028NP *pilD* gene is a member of COG1989.1 (PulO), a group of prepilin peptidases.

In other Tfp systems, proteins designated as secretins have been characterized as those that form gated channels in the outer membrane through which the pilus is assembled. The Tfp secretin in *P. aeruginosa* is the PilQ protein (29). We employed the BLAST algorithm to identify the homologue of PilQ in the 86-028NP genome. PilQ is 31% identical to a putative 445-amino-acid protein produced by strain 86-028NP.

FIG. 1. Open reading frame maps of the *pil* and *com* gene clusters from *H. influenzae* strain 86-028NP.

In strain 86-028NP, this protein is the homologue of the ComE protein of *H. influenzae* strain Rd (HI0435), a protein known to be required for transformation. In both strains 86-028NP and Rd, the *com* gene cluster contains six genes. We have retained the strain Rd nomenclature, thus designating strain 86-028NP genes as *comABCDEF*. A map of the *pil* and *com* gene clusters from strain 86-028NP is shown in Fig. 1.

Southern blot hybridization and analysis of *pilA* gene sequences. A single *pilA* gene is present in the genome of *H. influenzae* strain Rd, in the CF isolate, and in 13 of 13 (100%) low-passage clinical NTHI isolates recovered from patients with chronic otitis media (Fig. 2). We PCR amplified the *pilA* gene of 10 NTHI strains and determined the DNA sequence. The derived amino acid sequence of these genes was highly conserved (Fig. 3). The *pilA* genes of all isolates encoded a 12-residue leader peptide that is largely invariant, save a Q-to-L substitution at position 6 in two isolates as well as in strain Rd. Mature PilA contains 137 residues and is predicted to contain a characteristic methylated phenylalanine at position +1. Tyrosine residues at positions +24 and +27, believed to be involved in subunit-subunit interactions, are highly conserved,

FIG. 2. Southern blot demonstrating that a single *pilA* gene was contained within the genomes of *H. influenzae* strain Rd, the clinical CF isolate, and 13 of 13 low-passage clinical NTHI isolates recovered from patients with chronic otitis media. The chromosomal DNA digests were from the following strains: lane 1, 86-028NP; lane 2, 1728MEE; lane 3, 1729MEE; lane 4, 1714MEE; lane 5, 214NP; lane 6, 1236MEE; lane 7, 165NP; lane 8, 1060MEE; lane 9, 1128MEE; lane 10, 10548MEE; lane 11, 3224A; lane 12, 3185A; lane 13, 1885MEE; lane 14, 27W116791N1.

FIG. 3. ClustalW alignment of the *pilA* gene products from 11 NTHI isolates and strain Rd. Arrowheads indicate the locations of four conserved cysteine residues. Braces denote two regions of sequence diversity wherein there appear to be two major variants among the 12 isolates analyzed.

as are four Cys residues at positions +50, +60, +119, and +132.

Visualization of Tfp on *H. influenzae*. Examination of negatively stained NTHI strain 86-028NP for Tfp expression by TEM after growth on either chocolate agar, BHI agar plates supplemented with NAD and heme (pH 7.2), or chemically defined medium (pH 7.2) showed that when grown under any of these conditions, very few bacteria expressed Tfp-like structures, with the majority of cells expressing no pili. Conversely, when grown under defined nutrient conditions at an alkaline pH (pH 8.5 to 9.0), the majority of cells (>80%) expressed structures of approximately 6 to 7 nm in diameter (Fig. 4A). There were approximately 4 to 6 pili per bacterial cell, and these were exclusively just slightly off-polar in location (Fig. 4A, inset). Many of these structures were also found free on the grid surface, where they were uniformly observed to form large bundles of parallel fibers (Fig. 4B).

In order to demonstrate that the structures we observed when strain 86-028NP was grown under alkaline conditions on defined medium were Tfp, we constructed a mutant deficient in the expression of PilA. This strain was designated 86-028NP *pilA::kan*. When the *pilA* mutant was similarly blindly evaluated for expression of Tfp after growth under conditions that induced expression of Tfp in the parental isolate (i.e., chemically defined medium and alkaline pH), we found no evidence of either cell-associated or free Tfp (Fig. 4C), confirming our hypothesis that the *pilA* gene product (and/or the *pilBCD* gene products, since the mutation is likely polar) is required for pilus expression. Similarly, no cell-associated or free bundles of Tfp were observed when the *pilA* mutant was grown in chemically defined medium at a neutral pH.

The *pilABCD* gene cluster was amplified and cloned into the shuttle vector pSPEC1 to form the plasmid pPIL1, and strain 86-028NP *pilA::kan*(pPIL1) was constructed as described in Materials and Methods. When grown in chemically defined medium at alkaline pH, this strain produces Tfp, verifying that

the mutation in the *pilA* gene in strain 86-028NP *pilA::kan* is responsible for the pilus-negative phenotype (Fig. 5A and B).

Since ComE has homology to the *Pseudomonas* PilQ secretin and is a recognized member of the secretin family, we also constructed a mutant deficient in the expression of ComE. This mutant was designated 86-028NP *comE::kan*. Cells of the 86-028NP *comE* mutant, grown on defined medium prepared at pH 8.5, were thus similarly examined by TEM. Unlike the *pilA* mutant, which expressed no surface structures resembling Tfp, the *comE* mutant expressed very large, predominantly polar appendages that appeared to be membrane bound and to contain a fibrous material (Fig. 4D). These structures typically terminated in a slightly bulbous tip (Fig. 4D and E) and often showed a regular repeating pattern of areas of constriction and expansion (Fig. 4D, inset). These large structures were also found free on the grid surface (Fig. 4E), most typically as single appendages; however, at times, there were two or more associated structures. The structures we observed extending from the surface of our *comE* mutant are highly analogous to those described previously by Wolfgang et al. (51) for a *pilQ/pilT* double mutant constructed in *N. gonorrhoeae*, wherein the formation of these extremely curious structures was attributed to the forceful extrusion of the Tfp fiber through the bacterial outer membrane in the absence of both a PilQ-comprised pore and a PilT-mediated retraction-disassembly function. When grown in chemically defined medium at pH 7.2, no Tfp or membrane-bound structures were seen.

The *comEF* genes were amplified and cloned into pSPEC1 to form the plasmid pCOM1, and strain 86-028NP *pilA::kan*(pCOM1) was constructed as described in Materials and Methods. When grown in chemically defined medium at alkaline pH, this strain produced Tfp (Fig. 5C and D), which verifies that the mutation in the *comE* gene in strain 86-028NP *comE::kan* was responsible for the altered pilus phenotype described above.

FIG. 4. Transmission electron micrograph composite of negatively stained preparations demonstrating Tfp produced by strain 86-028NP and derivatives. (A) Parent strain grown under inducing conditions. The inset shows an image at a reduced magnification of the parent strain grown under inducing conditions. (B) Free Tfp bundles. (C) The *pilA* mutant of strain 86-028NP grown under inducing conditions. (D) The *comE* mutant of strain 86-028NP expressing very large, predominantly polar appendages that appeared to be membrane bound and to contain a fibrous material. The inset shows a regularly repeating pattern of areas of constriction and expansion often noted in appendages expressed by the *comE* mutant when grown under inducing conditions. (E) Higher magnification of structures similar to those described above (D), demonstrating the characteristic slightly bulbous tip.

Characterization of twitching phenotype. In addition to demonstrating that NTHI expresses Tfp under defined growth conditions, we have also generated data to demonstrate that the Tfp expressed by NTHI are functional. After 7 h, the outermost edge of the zone of growth surrounding a point of inoculation of the parental isolate demonstrated a rugous and ruffled appearance (Fig. 6A) that was highly characteristic of the motile rafts of twitching described for *P. aeruginosa* (11), whereas the outermost area of bacterial growth surrounding the point of inoculation of the *pilA* mutant was smooth (Fig. 6B). The *comE* mutant demonstrated an intermediate phenotype characterized by an edge with a slightly ruffled appearance (Fig. 6C). These growth phenotypes remained constant after 24 h, with the parental isolate demonstrating a rugous appearance with motile rafts of cells at the leading edge (Fig. 6F). The *pilA* mutant remained relatively smooth along the outermost edge from the point of inoculation (Fig. 6G), and the *comE* mutant maintained an intermediate phenotype (Fig. 6H). These observations were readily reproducible and were thus a highly characteristic growth phenotype for these bacteria.

When we examined the two complemented strains 86-028NP *pilA::kan*(pPIL1) and 86-028NP *comE::kan*(pCOM1) grown under identical conditions at 7 and 24 h of incubation, we

found that the outermost leading edge of growth for both strains (Fig. 6D and I or E and J, respectively) was highly analogous to that of the parental isolate. Characteristic motile rafts of cells were seen as the leading edge of growth for both complemented strains.

DISCUSSION

H. influenzae, *A. pleuropneumoniae*, and *P. multocida* are all members of the *Pasteurellaceae*, yet only the latter two microorganisms are known to express Tfp (38, 52). In these bacteria, the homologue of the *H. influenzae* HI0299 gene is known to encode type IV pilin; however, Tfp expression has not been shown for any *H. influenzae* isolate. Dougherty and Smith (13) employed cassette mutagenesis to construct a series of mutants in the strain Rd background that were defective for transformation. Several mutants in which the HI0300 (*ampD*) gene was interrupted were constructed. These mutants also had deletions extending into, or through, the HI0299 gene (designated *pilA* by Dougherty and Smith). Those investigators found that this four-member gene cluster, which they designated *pil*, was necessary for competence. They also predicted that in strain Rd, the same operon should be sufficient for expression of the

FIG. 5. Transmission electron micrograph composite of negatively stained whole bacteria. (A) Complemented *pilA* mutant expressing pilus-like structures (magnification, $\times\sim 72,000$). (B) Aggregate of non-cell-associated pilin fibers present in the complemented *pilA* mutant preparation that appear highly similar to those shown in Fig. 4B as expressed by the parental isolate. (C) Complemented *comE* mutant expressing pilus-like structures (magnification, $\times\sim 72,000$). (D) Complemented *comE* mutant expressing near-polar pili (bracket) (magnification, $\times\sim 72,000$).

pilus subunit, processing of the pilin subunit, and export of pilin to the bacterial surface. However, these latter conclusions seemed counterintuitive, since while *pilA* might encode the pilin subunit, the products of *pilB-pilD* would not be predicted to be sufficient for export of the pilin subunit through the outer membrane, nor would they be sufficient for uptake of DNA (6, 29, 43, 51). Wang et al. (45) suggested that while the *pil* locus is indeed associated with competence in *A. actinomycetem-comitans*, it is not associated with the expression of a type IV pilus. Furthermore, twitching motility, a non-flagellar-based Tfp-dependent motility, has never been observed among the *Pasteurellaceae*. Moreover, in a recent review, Chen and Dubnau stated, "Other competent organisms, such as *H. influenzae* or the gram-positive bacteria *B. subtilis* and *S. pneumoniae*, require similar genes for DNA uptake, but do not possess filamentous structures that extend from the cell surface." Nevertheless, we reasoned that if NTHI was forming a biofilm in the middle ear of animals during experimental OM (15, 37), and perhaps also doing so in humans during natural disease, it was likely to require some form of motility and that this motility might be dependent on Tfp.

As mentioned in Results, we investigated this hypothesis further and found a single *pilA* gene in the genome of all clinical *H. influenzae* strains analyzed. Interestingly, the NTHI PilA proteins appear to represent a new class of Tfp. At 12 residues, the leader peptide is larger than that characteristic for type IVa pilins (typically 5 to 6 residues in length) and yet shorter than the typical IVb leader peptide (15 to 30 residues). Similarly, at 137 residues, the mature NTHI pilin is shorter than either class IVa or IVb pilins (150 and 190 residues, respectively). Since the NTHI PilA proteins begin with an N-methylated phenylalanine, they are more like class IVa pilins; however, in electron micrographs, free NTHI Tfp always

appear in laterally associated bundles (Fig. 4B), a phenotype more classically associated with class IVb pilins due to their ability to self-associate through antiparallel interactions (10).

In terms of NTHI PilA sequence diversity, despite areas wherein one or two amino acids are variant, these sequences are highly homologous overall. Two areas of potentially important diversity exist at positions 55 to 64 and 79 to 87. Within the first region, among the clinical isolates, there appears to be two major variants, one representing the majority (7 of 11 isolates [64%]) and characterized by the sequence NET/IT-NCT/MGGK, and the other representing the minority (4 of 11 isolates [36%]) and characterized by the sequence GKP/LST/SCSGS. There are, however, some additional minor variations at positions +57 and +61 in the majority grouping and at positions +57 and +59 in the minority grouping. The diversity noted at position +61 has been seen only in one isolate to date (strain 1885MEE), wherein there is a T-to-M substitution. Within the second focused region of diversity (positions 79 to 87), there appears to be two equally distributed variants among the clinical NTHI isolates. The sequence ASVKTSQSGG is present in 5 of 11 clinical isolates ($\sim 45\%$), whereas the sequence KSVTTSNGA is present in 6 of 11 clinical isolates ($\sim 55\%$).

In addition to characterizing the product of the *pilA* gene, we also interrogated our contig set (generated during our efforts to sequence the genome of strain 86-028NP [see <http://www.microbial-pathogenesis.org>]) with the sequences for both the Tfp retraction protein, PilT, and the secretor protein, PilQ, as well as other Tfp proteins of *P. aeruginosa* in order to identify homologues of the genes encoding these proteins in the 86-028NP genome. We found that PilB expressed by strain 86-028NP is a homologue of PilB, PilT, and PilU. PilT is required for twitching motility and is the pilus retraction protein of both *N. meningitidis* (1, 19) and *N. gonorrhoeae* (50, 51). The product of the *comE* gene, a protein required for transformation in *H. influenzae*, was found to have homology to PilQ of *P. aeruginosa* (29). PilQ is a member of the pIV/PilD family, which in multimeric form acts as a gated channel through which macromolecules such as pilin subunits pass (29, 50, 51). Recognition that ComE has homology to secretin proteins suggests that the products of the *com* gene cluster may be involved in the biogenesis of the type IV pilus we have described here.

Collectively, these data suggested to us the tremendous likelihood that NTHI could not only express functional Tfp, it could also potentially twitch. This was an extremely exciting hypothesis, as historically, while it is well known both that *H. influenzae* is naturally competent and that Tfp are classically associated with competence, type IV pili had heretofore never been demonstrated in *H. influenzae*. Transmission electron microscopy provided direct evidence that strain 86-028NP did indeed express intact type IV pilus-like structures on its surface when grown in a defined RPMI 1640-based medium at alkaline pH. The relative size and distribution of type IV pili by individual bacterial cells were highly analogous to those described for the closely related family member *P. multocida* (38). These structures were not observed when NTHI was grown in rich supplemented BHI medium or in chemically defined medium at neutral pH. A mutant deficient in PilA did not produce these surface structures, whereas a ComE mutant produced large appendages that appeared to be membrane bound and to contain a fibrous material, terminating in a bulbous tip and often demonstrating a regular pattern of

FIG. 6. Light microscopy composite of the outermost leading edge of bacterial growth from a point inoculation site. All images were captured with a $\times 40$ objective on a Zeiss Axioskop 40. Scale bar, 10 μm . (A to E) Growth after 7 h of incubation. (F to J) Growth after 24 h of incubation. A and F, NTHI strain 86-028NP; B and G, *pilA* mutant; C and H, *comE* mutant; D and I, *pilA* mutant complemented with *pilA*; E and J, *comE* mutant complemented with *comEF*. All strains were grown on chemically defined medium (1% agar, 3-mm thickness, pH 9.0). Note the formation of rafting growth (A and F), typical of twitching motility. This rafting phenotype is absent from the *pilA* mutant (B and G), whereas the *comE* mutant (C and H) demonstrates an intermediate growth phenotype suggesting inhibited motility. The complemented *pilA* mutant demonstrated a restored rafting phenotype (D and I), as did the complemented *comE* mutant (E and J).

constriction and expansion. These structures were highly analogous to those produced by an *N. meningitidis pilQ/pilT* mutant as shown by Wolfgang and colleagues (51).

We also present several pieces of data that support the assertion that NTHI twitches. When grown on defined medium at an alkaline pH, NTHI strain 86-028NP demonstrated classic rafts of cells at the leading edge of bacterial growth from a point of inoculation, similar to that reported for *P. aeruginosa* (39), when grown under conditions of nutrient depletion. The *pilA* and *comE* mutants did not form these motile rafts, whereas the complemented mutants were shown to regain the parental phenotype.

In summary, we have identified and characterized genes that encode expression of functional type IV pili, and we have presented both direct and indirect data in support of the expression of functional Tfp by *H. influenzae*. The fact that Tfp were expressed under alkaline conditions is particularly intriguing since the pHs of both serous and mucoid effusions recovered from patients with chronic OM are uniformly alkaline (47). Alkalinity within middle ear effusions may thus represent an environmental cue that contributes to the regulation of expression of virulence factors, such as Tfp, by NTHI. In addition to providing a form of motility, type IV pili are in-

involved in transformation competence in many bacteria (1, 18, 19, 22, 45) including *H. influenzae* and may be important for biofilm formation by NTHI as well. Importantly, Tfp have been shown to play a role in pathogenesis, as they are essential for adherence to human epithelial cells by *N. gonorrhoeae* (49) and *P. aeruginosa* (2) as well as for meningeal invasion by *N. meningitidis* (34). We know that NTHI forms biofilms in the middle ear during experimental OM (15, 37), a phenotype that is often associated with twitching motility (24, 26, 29, 35). Therefore, studies designed to define the role of Tfp in competence and the pathogenesis of NTHI-induced OM and/or biofilm formation in the middle ear are under way.

ACKNOWLEDGMENTS

This study was funded by grants R01 DC03915 (to L.O.B.) and R01 DC005980 (to R.S.M.) from the NIH/NIDCD.

We thank Brian Quist and Cindy McAllister for excellent technical assistance and Jennifer Neelans for manuscript preparation. We also thank Susan West (University of Wisconsin) and Daniel Morton (University of Oklahoma Health Sciences Center) for the plasmids pG2RS-39A and pSPECR, respectively.

ADDENDUM IN PROOF

Since acceptance of the manuscript, we have obtained video microscopy images of the complemented strain designated *comE::kan(pCOM1)* twitching. The bacterial inoculum of 0.5 μ l (~8e9 CFU of nontypeable *Haemophilus influenzae*) was placed in the center of a slab of chemically defined agar (pH 9.0) and overlaid with a sterile glass coverslip. After incubation for 24 h at 37°C in a humidified atmosphere of 5% CO₂, video images were captured at 4 frames/s with a 63 $\times$ objective with a 2:1 digital zoom. The video clip is available at <http://www.microbial-pathogenesis.org/H.influenzae.86028/>.

REFERENCES

- Aas, F. E., M. Wolfgang, S. Frye, S. Dunham, C. Lovold, and M. Koomey. 2002. Competence for natural transformation in *Neisseria gonorrhoeae*: components of DNA binding and uptake linked to type IV pilus expression. *Mol. Microbiol.* **46**:749–760.
- Alm, R. A., and J. S. Mattick. 1997. Genes involved in the biogenesis and function of type-4 fimbriae in *Pseudomonas aeruginosa*. *Gene* **192**:89–98.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Bakaletz, L. O., B. M. Tallan, T. Hoepf, T. F. DeMaria, H. G. Birck, and D. J. Lim. 1988. Frequency of fimbriation of nontypeable *Haemophilus influenzae* and its ability to adhere to chinchilla and human respiratory epithelium. *Infect. Immun.* **56**:331–335.
- Barcak, G. J., M. S. Chandler, R. J. Redfield, and J. F. Tomb. 1991. Genetic systems in *Haemophilus influenzae*. *Methods Enzymol.* **204**:321–342.
- Bardy, S. L., S. Y. Ng, and K. F. Jarrell. 2003. Prokaryotic motility structures. *Microbiology* **149**:295–304.
- Boekema, B. K., J. P. Van Putten, N. Stockhofe-Zurwieden, and H. E. Smith. 2004. Host cell contact-induced transcription of the type IV fimbria gene cluster of *Actinobacillus pleuropneumoniae*. *Infect. Immun.* **72**:691–700.
- Chen, L., and D. Dubnau. 2004. DNA uptake during bacterial transformation. *Nat. Rev. Microbiol.* **2**:241–249.
- Coleman, H. N., D. A. Daines, J. Jarisch, and A. L. Smith. 2003. Chemically defined media for growth of *Haemophilus influenzae* strains. *J. Clin. Microbiol.* **41**:4408–4410.
- Craig, L., M. E. Pique, and J. A. Tainer. 2004. Type IV pilus structure and bacterial pathogenicity. *Nat. Rev. Microbiol.* **2**:363–378.
- Darzens, A. 1993. The *pilG* gene product, required for *Pseudomonas aeruginosa* pilus production and twitching motility, is homologous to the enteric, single-domain response regulator CheY. *J. Bacteriol.* **175**:5934–5944.
- Darzens, A., and M. A. Russell. 1997. Molecular genetic analysis of type-4 pilus biogenesis and twitching motility using *Pseudomonas aeruginosa* as a model system—a review. *Gene* **192**:109–115.
- Dougherty, B. A., and H. O. Smith. 1999. Identification of *Haemophilus influenzae* Rd transformation genes using cassette mutagenesis. *Microbiology* **145**:401–409.
- Dougherty, S. W., C. G. Ruffolo, and B. Adler. 2000. The type 4 fimbrial subunit gene of *Pasteurella multocida*. *Vet. Microbiol.* **72**:79–90.
- Ehrlich, G. D., R. Veeh, X. Wang, J. W. Costerton, J. D. Hayes, F. Z. Hu, B. J. Daigle, M. D. Ehrlich, and J. C. Post. 2002. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA* **287**:1710–1715.
- Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, et al. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**:496–512.
- Foxwell, A. R., J. M. Kyd, and A. W. Cripps. 1998. Nontypeable *Haemophilus influenzae*: pathogenesis and prevention. *Microbiol. Mol. Biol. Rev.* **62**:294–308.
- Friedrich, A., J. Rumszauer, A. Henne, and B. Averhoff. 2003. Pilin-like proteins in the extremely thermophilic bacterium *Thermus thermophilus* HB27: implication in competence for natural transformation and links to type IV pilus biogenesis. *Appl. Environ. Microbiol.* **69**:3695–3700.
- Fussenegger, M., T. Rudel, R. Barten, R. Ryll, and T. F. Meyer. 1997. Transformation competence and type-4 pilus biogenesis in *Neisseria gonorrhoeae*—a review. *Gene* **192**:125–334.
- Gilsdorf, J. R., K. W. McCrea, and C. F. Marrs. 1997. Role of pili in *Haemophilus influenzae* adherence and colonization. *Infect. Immun.* **65**:2997–3002.
- Gilsdorf, J. R., M. Tucci, and C. F. Marrs. 1996. Role of pili in *Haemophilus influenzae* adherence to, and internalization by, respiratory cells. *Pediatr. Res.* **39**:343–348.
- Graupner, S., N. Weger, M. Sohni, and W. Wackernagel. 2001. Requirement of novel competence genes *pilT* and *pilU* of *Pseudomonas stutzeri* for natural transformation and suppression of *pilT* deficiency by a hexahistidine tag on the type IV pilus protein PilA1. *J. Bacteriol.* **183**:4694–4701.
- Hentzer, M., G. M. Teitzel, G. J. Balzer, A. Heydorn, S. Molin, M. Givskov, and M. R. Parsek. 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J. Bacteriol.* **183**:5395–5401.
- Jesaitis, A. J., M. J. Franklin, D. Berglund, M. Sasaki, C. I. Lord, J. B. Bleazard, J. E. Duffy, H. Beyenal, and Z. Lewandowski. 2003. Compromised host defense on *Pseudomonas aeruginosa* biofilms: characterization of neutrophil and biofilm interactions. *J. Immunol.* **171**:4329–4339.
- Kennedy, B. J., L. A. Novotny, J. A. Jurcisek, Y. Lobet, and L. O. Bakaletz. 2000. Passive transfer of antiserum specific for immunogens derived from a nontypeable *Haemophilus influenzae* adhesin and lipoprotein D prevents otitis media after heterologous challenge. *Infect. Immun.* **68**:2756–2765.
- Klausen, M., A. Aes-Jorgensen, S. Molin, and T. Tolker-Nielsen. 2003. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol. Microbiol.* **50**:61–68.
- Klausen, M., A. Heydorn, P. Ragas, L. Lambertsen, A. Aes-Jorgensen, S. Molin, and T. Tolker-Nielsen. 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol. Microbiol.* **48**:1511–1524.
- Macfadyen, L. P., C. Ma, and R. J. Redfield. 1998. A 3',5' cyclic AMP (cAMP) phosphodiesterase modulates cAMP levels and optimizes competence in *Haemophilus influenzae* Rd. *J. Bacteriol.* **180**:4401–4405.
- Mattick, J. S. 2002. Type IV pili and twitching motility. *Annu. Rev. Microbiol.* **56**:289–314.
- Mhlanga-Mutangadura, T., G. Morlin, A. L. Smith, A. Eisenstark, and M. Golomb. 1998. Evolution of the major pilus gene cluster of *Haemophilus influenzae*. *J. Bacteriol.* **180**:4693–4703.
- Munson, R. S., Jr., A. Harrison, A. Gillaspay, W. C. Ray, M. Carson, D. Armbruster, J. Gipson, M. Gipson, L. Johnson, L. Lewis, D. W. Dyer, and L. O. Bakaletz. 2004. Partial analysis of the genomes of two nontypeable *Haemophilus influenzae* otitis media isolates. *Infect. Immun.* **72**:3002–3010.
- Murphy, T. F., and M. A. Apicella. 1987. Nontypeable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the human immune response to infection. *Rev. Infect. Dis.* **9**:1–15.
- Murphy, T. F., and C. Kirkham. 2002. Biofilm formation by nontypeable *Haemophilus influenzae*: strain variability, outer membrane antigen expression and role of pili. *BMC Microbiol.* **2**:7.
- Nassif, X., S. Bourdoulous, E. Eugene, and P. O. Couraud. 2002. How do extracellular pathogens cross the blood-brain barrier? *Trends Microbiol.* **10**:227–232.
- O'Toole, G. A., and R. Kolter. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* **30**:295–304.
- Perez-Casal, J., M. G. Caparon, and J. R. Scott. 1991. Mry, a *trans*-acting positive regulator of the M protein gene of *Streptococcus pyogenes* with similarity to the receptor proteins of two-component regulatory systems. *J. Bacteriol.* **173**:2617–2624.
- Post, J. C. 2001. Direct evidence of bacterial biofilms in otitis media. *Laryngoscope* **111**:2083–2094.
- Ruffolo, C. G., J. M. Tennent, W. P. Michalski, and B. Adler. 1997. Identification, purification, and characterization of the type 4 fimbriae of *Pasteurella multocida*. *Infect. Immun.* **65**:339–343.
- Semmler, A. B., C. B. Whitchurch, and J. S. Mattick. 1999. A re-examination of twitching motility in *Pseudomonas aeruginosa*. *Microbiology* **145**:2863–2873.
- Stevenson, A., J. Macdonald, and M. Roberts. 2003. Cloning and characterization of type 4 fimbrial genes from *Actinobacillus pleuropneumoniae*. *Vet. Microbiol.* **92**:121–134.
- St. Geme, J. W., III. 2002. Molecular and cellular determinants of nontypeable *Haemophilus influenzae* adherence and invasion. *Cell. Microbiol.* **4**:191–200.
- Strom, M. S., and S. Lory. 1993. Structure-function and biogenesis of the type IV pili. *Annu. Rev. Microbiol.* **47**:565–596.
- Tonjum, T., and M. Koomey. 1997. The pilus colonization factor of pathogenic neisserial species: organelle biogenesis and structure/function relationships—a review. *Gene* **192**:155–163.
- Wall, D., and D. Kaiser. 1999. Type IV pili and cell motility. *Mol. Microbiol.* **32**:1–10.
- Wang, Y., W. Shi, W. Chen, and C. Chen. 2003. Type IV pilus gene homologs *pilABCD* are required for natural transformation in *Actinobacillus actinomycetecomitans*. *Gene* **312**:249–255.
- West, S. E., M. J. Romero, L. B. Regassa, N. A. Zielinski, and R. A. Welch. 1995. Construction of *Actinobacillus pleuropneumoniae*-*Escherichia coli* shuttle vectors: expression of antibiotic-resistance genes. *Gene* **160**:81–86.
- Weyk, M. T., and A. Makowski. 2000. pH of fluid collected from middle ear in the course of otitis media in children. *Otolaryngol. Pol.* **54**:131–133. (In Polish.)
- Whitby, P. W., D. J. Morton, and T. L. Stull. 1998. Construction of antibiotic resistance cassettes with multiple paired restriction sites for insertional mutagenesis of *Haemophilus influenzae*. *FEMS Microbiol. Lett.* **158**:57–60.
- Winther-Larsen, H. C., F. T. Hegge, M. Wolfgang, S. F. Hayes, J. P. van Putten, and M. Koomey. 2001. *Neisseria gonorrhoeae* PilV, a type IV pilus-

- associated protein essential to human epithelial cell adherence. Proc. Natl. Acad. Sci. USA **98**:15276–15281.
50. **Wolfgang, M., P. Lauer, H. S. Park, L. Brossay, J. Hebert, and M. Koomey.** 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. Mol. Microbiol. **29**:321–330.
51. **Wolfgang, M., J. P. van Putten, S. F. Hayes, D. Dorward, and M. Koomey.** 2000. Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. EMBO J. **19**:6408–6418.
52. **Zhang, Y., J. M. Tennent, A. Ingham, G. Beddome, C. Prideaux, and W. P. Michalski.** 2000. Identification of type 4 fimbriae in *Actinobacillus pleuropneumoniae*. FEMS Microbiol. Lett. **189**:15–18.

Editor: D. L. Burns