Contribution of *Moraxella catarrhalis* Type IV Pili to Nasopharyngeal Colonization and Biofilm Formation[∇]

Nicole R. Luke,^{1,3} Joseph A. Jurecsek,⁴ Lauren O. Bakaletz,⁴ and Anthony A. Campagnari^{1,2,3*}

Department of Microbiology and Immunology,¹ Department of Medicine,² and the NYS Center of Excellence in Bioinformatics and Life Sciences,³ State University of New York at Buffalo, Buffalo, New York, and Columbus Children's Research Institute and the College of Medicine, The Ohio State University, Columbus, Ohio⁴

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Moraxella catarrhalis is a gram-negative mucosal pathogen of the human respiratory tract. Although little information is available regarding the initial steps of *M. catarrhalis* pathogenesis, this organism must be able to colonize the human mucosal surface in order to initiate an infection. Type IV pili (TFP), filamentous surface appendages primarily comprised of a single protein subunit termed pilin, play a crucial role in the initiation of disease by a wide range of bacteria. We previously identified the genes that encode the major proteins involved in the biosynthesis of *M. catarrhalis* TFP and determined that the TFP expressed by this organism are highly conserved and essential for natural transformation. We extended this initial study by investigating the contribution of TFP to the early stages of *M. catarrhalis* colonization. TFP-deficient *M. catarrhalis* bacteria exhibit diminished adherence to eukaryotic cells in vitro. Additionally, our studies demonstrate that *M. catarrhalis* cells form a mature biofilm in continuous-flow chambers and that biofilm formation is enhanced by TFP expression. The potential role of TFP in colonization by *M. catarrhalis* was further investigated using in vivo studies comparing the abilities of wild-type *M. catarrhalis* and an isogenic TFP mutant to colonize the nasopharynx of the chinchilla. These results suggest that the expression of TFP contributes to mucosal airway colonization. Furthermore, these data indicate that the chinchilla model of nasopharyngeal colonization provides an effective animal system for studying the early steps of *M. catarrhalis* pathogenesis.

Moraxella catarrhalis, a gram-negative mucosal pathogen of the human respiratory tract, can also reside as a nasopharyngeal commensal. *M. catarrhalis* is capable of causing multiple diseases of the upper respiratory tract, including sinusitis, laryngitis, and acute and chronic otitis media, predominantly in pediatric populations, as well as recurrent exacerbations of chronic obstructive pulmonary disease in adults with underlying lung disease (31, 41). As with many other pathogenic bacteria, adherence to host tissues and subsequent colonization of the respiratory tract mucosa are believed to be essential prior to the development of *M. catarrhalis* infections.

M. catarrhalis expresses a number of cell surface components that have been postulated to be involved in bacterial virulence (reviewed in references 29 and 41). However, in vivo studies of *M. catarrhalis* pathogenesis have been limited by the lack of an effective animal model to investigate bacterial colonization of the upper respiratory tract epithelium. Even with the most prevalently used model system, the mouse pulmonary clearance model, *M. catarrhalis* is markedly cleared from the site within 6 h and the organism is almost completely eradicated by 24 h (40). Although little information is available regarding the actual steps involved in the pathogenesis of *M. catarrhalis* infections in vivo, it is clear that this organism must attach to the mucosal surface in order to establish colonization. Therefore, the identification of bacterial colonization factors and of new vaccine and treatment targets is a major focus of current re-

search efforts. In this study, we investigated the contribution of type IV pilus (TFP) expression to the pathogenicity of *M. catarrhalis*.

TFP, filamentous surface appendages comprised primarily of a single protein subunit termed pilin, are recognized as important virulence determinants for a wide variety of bacterial pathogens. There have been numerous biological functions associated with TFP expression by various bacterial species, and these may include adherence to eukaryotic cells, biofilm formation and stability, competence for natural transformation, and flagellum-independent cell movement, termed twitching motility (15, 28, 33, 38). Many of these functions are believed to contribute to colonization of mucosal surfaces, often a critical step in the disease process.

In a previous study, we demonstrated that *M. catarrhalis* expresses TFP and identified genes involved in TFP biogenesis (26). Comparative analyses of isogenic mutants deficient in wild-type TFP expression indicated that *M. catarrhalis* clinical isolates constitutively express a single type IVA pilin, PilA, and that pili are essential for natural transformation. However, the contribution of TFP to *M. catarrhalis* virulence was not assessed.

In this study, we demonstrate that the expression of pili by *M. catarrhalis* contributes to adherence to eukaryotic cells and biofilm formation in vitro. Importantly, an *M. catarrhalis* animal model of extended nasopharyngeal colonization was developed using the chinchilla. Utilizing this chinchilla model system, we found that expression of TFP provides a biological advantage for colonization and persistence on respiratory mucosal surfaces in vivo.

MATERIALS AND METHODS

Bacterial strains and culture methods. *M. catarrhalis* strains 7169 and 7169pilAK4, the isogenic *pilA*-deficient mutant of 7169 lacking surface TFP

* Corresponding author. Mailing address: State University of New York at Buffalo, 140 Biomedical Research Bldg., 3435 Main Street, Buffalo, NY 14214. Phone: (716) 829-2673. Fax: (716) 829-3889. E-mail: AAC@buffalo.edu.

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expression, have been described previously (25, 26). *M. catarrhalis* strains were cultured at 37°C on brain heart infusion (BHI) agar or Mueller-Hinton (MH) agar in a humidified 5% CO₂ atmosphere; 7169pilAK4 was cultured with the addition of kanamycin (25 µg per ml) as necessary. BHI and MH broth cultures were grown in a 37°C shaking water bath with constant agitation.

Tissue culture adherence assays. Chang epithelial cells (human conjunctiva clone 1-5c-4, Wong-Kilbourne derivative; ATCC CCL20.2) were cultured in RPMI 1640 medium with L-glutamine plus 10% heat-inactivated fetal bovine serum at 37°C in a humidified 5% CO₂-95% air atmosphere essentially as described previously (31). The cells were seeded into 96-well plates at 10⁴ cells per well and grown to approximately 90% confluence. *M. catarrhalis* cells grown to early log phase (optical density at 600 nm = 0.2 to 0.3) in MH broth were resuspended in preequilibrated cell culture medium and inoculated in triplicate onto confluent monolayers at a multiplicity of infection (MOI) of 100:1. After the addition of the bacteria, the plates were centrifuged at 500 × g for 5 min and placed at 37°C for 30 min. For enumeration of cell-associated bacteria, each well was subjected to three washes with Dulbecco's phosphate-buffered saline (pH 7.2) to remove nonadherent bacteria, and the monolayers were detached from the wells by treatment with 0.25% trypsin-1 mM EDTA. The contents of each set of triplicate wells were pooled, serially diluted, and plated in triplicate onto MH plates for the determination of colony counts at 24 h. For each adherence assay, at least two sets of triplicate wells were inoculated per strain. Serial dilutions of controls, including wells inoculated with bacteria in the absence of eukaryotic cells to assess whether the organisms exhibited any appreciable growth or adhered to the plastic during the assay, as well as the inoculum stock, were also plated in triplicate and cultured overnight as described above. The number of recovered bacteria was compared to the number of inoculated organisms per well, and the percentage (± standard error) of adherent bacteria was determined. For the duration of the assay, no growth or adherence to the plastic well was observed. Statistical significance was determined using Student's unpaired *t* test on three independent experiments performed on separate days.

Animal colonization model. Healthy adult chinchillas (*Chinchilla lanigera*) were purchased from Rauscher's Chinchilla Ranch (LaRue, OH) and provided with food and water ad libitum. All chinchilla studies were performed using protocols approved by the Columbus Children's Research Institute Animal Care and Use Committee. Cohorts of chinchillas were challenged intranasally by passive inhalation of approximately 10⁷ CFU per animal of *M. catarrhalis* strain 7169 or its *pilA* mutant, 7169pilAK4, suspended in sterile pyrogen-free saline. The exact inoculum density was confirmed by plate counts. Nasopharyngeal lavage was performed on days 1, 4, 7, 10, 14, and 18 postchallenge to determine the relative abilities of these isolates to colonize the nasopharynx (NP) of this host. Nasopharyngeal lavage was performed by passive inhalation of 500 µl sterile saline followed by recovery of the lavage fluid as described previously (4, 27). To evaluate the adherent population of bacteria, the chinchillas were sacrificed on day 18 postinoculation, and the nasopharyngeal tissues were harvested, weighed, and homogenized. Lavage samples and homogenates were dilution plated on chocolate agar supplemented with vancomycin (10 mg/liter) and trimethoprim (5 mg/liter) to suppress normal chinchilla flora; kanamycin was added to the culture medium when isolating the *pilA* mutant. Assays were performed using cohorts of three chinchillas per strain on at least two separate occasions. Statistical analyses were conducted by the Biometrics Laboratory at The Ohio State University College of Medicine, using the exact Wilcoxon rank sum test to compare relevant groups. Differences were considered significant if the *P* value was <0.05.

Biofilm growth in continuous-flow chambers. *M. catarrhalis* biofilms were grown in continuous-flow chambers, using BHI broth diluted 1:10 in sterile phosphate-buffered saline (13, 17). To compare the ability of the wild-type strain to that of its *pilA* mutant to form a biofilm in a flow chamber in vitro, 18-hour plate-grown colonies were suspended in sterile saline to obtain an optical density at 490 nm of 0.60. Five hundred microliters of this bacterial suspension was inoculated into a flow chamber (Stovall Flow Cell, Greensboro, NC) and allowed to incubate at 37°C for 1 h (with the coverslip down) to promote adherence. Following this incubation period, the chamber was inverted (coverslip up), and the diluted BHI broth was pumped through the chamber at a flow rate of 180 µl per min for 72 h. The flow cell chambers and medium were maintained at 37°C until the end of the experiment, at which time the biofilms were stained with a Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, OR) by injecting 0.5 ml of stain solution into each chamber. Following a 15-minute incubation in the dark prior to being viewed, z-stack composite fluorescent images were captured using a 63× objective on a confocal microscope (510 Meta; Zeiss, Thornwood, NY). The digital three-dimensional z-stack images were generated using Zeiss LSM image browser software.

FIG. 1. Comparison of adherence levels of strain 7169 (WT 7169) and the isogenic *pilA*-deficient mutant 7169pilAK4 to monolayers of Chang cells. The percent adherence represents the portion of the inoculum that was cell associated at 30 min. Values represent means ± standard deviations.

RESULTS

Contribution of TFP to in vitro adherence. We previously demonstrated that *M. catarrhalis* clinical isolates constitutively express TFP and that TFP expression by this organism is absolutely essential for natural transformation (26). In this study, we evaluated the contribution of TFP expression to the pathogenesis of *M. catarrhalis*. A comparative adherence assay using wild-type *M. catarrhalis* strain 7169 and the *PilA*-deficient isogenic mutant 7169pilAK4, which lacks surface pili, was performed to evaluate if expression of TFP by *M. catarrhalis* contributes to the ability of the organism to adhere to Chang conjunctival epithelial cells. 7169pilAK4 was significantly impaired in adherence to Chang cells (*P* = 0.0054), as only 11.15% ± 1.59% of the bacteria with the *pilA* mutation were able to adhere to the monolayers, compared to 23.54% ± 3.12% of the wild-type bacteria (Fig. 1). These data suggest a role for TFP in the adherence of *M. catarrhalis* in vitro.

***M. catarrhalis* colonizes the chinchilla NP.** In hopes of identifying a model system that could be used to evaluate bacterial colonization and pathogenesis under conditions that more closely mimic human disease, we investigated the chinchilla model of nasopharyngeal colonization. The chinchilla model has been effective in evaluating the respiratory mucosal pathogens nontypeable *Haemophilus influenzae* and *Streptococcus pneumoniae*, which share a similar ecological niche with *M. catarrhalis*. We first performed a pilot study to investigate the ability of strain 7169 to colonize and persist in the chinchilla NP. Following intranasal inoculation with approximately 10⁷ CFU of *M. catarrhalis*, the numbers of recoverable bacteria diminished through day 4, and then a stable level of colonization (approximately 10² CFU per ml of NP lavage fluid) was achieved and maintained for a minimum of 2 weeks (Fig. 2).

Contribution of TFP to nasopharyngeal colonization. Once we had established that *M. catarrhalis* readily colonizes the NP of the chinchilla, we investigated the contribution of TFP expression to this process. *M. catarrhalis* strains 7169 and 7169pilAK4 were introduced into separate cohorts of chinchillas via passive inhalation. The animals in each group were subjected to nasopharyngeal lavage at defined time intervals and were sacrificed on day 18. The nasopharyngeal tissues from these chinchillas were harvested, and homogenates were analyzed for the presence of *M. catarrhalis*.

FIG. 2. Dynamics of nasopharyngeal colonization of the chinchilla following intranasal inoculation with *M. catarrhalis* 7169. The mean numbers of CFU per milliliter of lavage fluid $\pm$ standard deviations (error bars) for five chinchillas are depicted.

On days 1, 4, and 7 following intranasal challenge, more of the *pilA*-deficient mutant than the parent was recovered via nasopharyngeal lavage (Fig. 3A). Importantly, this difference was highly significant ($P = 0.004$) 1 day after challenge, a time point when *M. catarrhalis* predominated as a nearly pure culture in the lavage fluids from chinchillas (data not shown). In fact, 7169pilAK4 was more readily harvested from the NP than the wild type was until day 4 postchallenge; after this time point, no difference in colonization levels was observed, indicating that the lack of TFP did not affect the ability of this organism to survive in the chinchilla host. Conversely, the homogenized mucosae from the nasoturbinate, ethmoid turbinates, and NP of the challenged chinchillas showed that significantly more wild-type than *pilA* mutant cells were recovered ($P = 0.017$, 0.026 , and 0.004 , respectively) (Fig. 3B). Thus, the *pilA* mutant predominated in terms of the population of *M. catarrhalis* in the chinchilla NP obtained by lavage, whereas 7169 predominated in terms of the adherent population. Collectively, these data suggest that expression of TFP by *M. catarrhalis* confers an advantage for the colonization of airway mucosa in this host and perhaps also stabilizes interbacterial interactions, thus reducing the ability of bacteria to be recovered from the NP by gentle lavage.

The wild-type strain that expressed TFP exhibited 7.67 ± 2.5 -fold and 9.50 ± 3.4 -fold (means $\pm$ standard deviations) increased recovery from the NP and the nasoturbinate, respectively, in these animals compared to the mutant lacking *PilA* expression, whereas for the ethmoid turbinates the increased recovery of the parental isolate was only 2.56 ± 0.1 -fold. These results are likely due to the specific nature of the mucosal epithelium present within each of these three anatomical microenvironments. Within the NP of the chinchilla host, the mucosa consists primarily of ciliated columnar epithelium with some goblet cells, and these cell types are also present in the nasoturbinate, which contain predominantly squamous or nonciliated columnar epithelium, with some ciliated columnar cells and a few goblet cells. In contrast, the mucosa of the ethmoid turbinates is principally olfactory epithelium with a few ciliated columnar cells (18). Collectively, these data suggested that *M. catarrhalis* TFP likely adheres to a receptor(s) expressed by the classic respiratory epithelium, as opposed to those present on olfactory epithelial cells. Moreover, our data suggested that the chinchilla may be an effective rodent host for studying the early steps in *M. catarrhalis* colonization and

FIG. 3. Evaluation of NP colonization dynamics for strains 7169 (open squares) and 7169pilAK4 (closed squares) by culture of NP lavage fluids (A) and mucosal tissue homogenates (B). Each datum point represents CFU obtained from an individual chinchilla, and bars represent means. Comparative P values of <0.05 were considered significant.

pathogenesis and indicated that expression of TFP contributes to bacterial persistence in the upper respiratory tract mucosa in vivo.

Role of *M. catarrhalis* TFP in biofilm formation. It has been documented that bacterial biofilms formed during chronic human infections, including recurrent otitis media, are often less susceptible to eradication by antimicrobial agents (9, 11, 22). Moreover, TFP have been shown to be important for the initial attachment and microcolony formation at the onset of biofilm formation by several gram-negative bacteria (20, 32–34, 43). To investigate if *M. catarrhalis* TFP contribute to biofilm formation, we compared the abilities of 7169 and 7169pilAK4 to form biofilms in continuous-flow chambers.

We directly visualized the biofilm formation phenotype of the TFP-defective strain 7169pilAK4 compared to that of its parent strain, 7169, by using light microscopy. As shown in Fig. 4, the difference between the ability of the parent to adhere to the glass slide and that of the *pilA* mutant was evident even at the earliest time point (1 h). Throughout the monitored time course, aggregates of 7169 formed into characteristic microcolonies that became increasingly dense and exhibited three-dimensional pillar formation. In contrast, 7169pilAK4 appeared to have defects in initial cell attachment and subsequently exhibited delayed microcolony formation and diminished three-dimensional expansion.

In 3-day-old *M. catarrhalis* biofilms, multicellular mushroom-shaped structures separated by well-demarcated water-filled channels were clearly visible (Fig. 5). It was previously shown that in *Pseudomonas aeruginosa* biofilms, the development of the prototypical biofilm structures of mushroom-like stalks, subsequently followed by the formation of the mushroom caps, is a process dependent on functional TFP (19, 20).

FIG. 4. Direct visualization of biofilm formation in a continuous-flow cell chamber over time by strains 7169 (A) and 7169pilAK (B). Representative images of each strain were taken from the center of the flow cell placed on a heated stage for nondestructive image acquisition by phase-contrast microscopy at a magnification of $\times 40$.

7169pilAK4 exhibited less pronounced biofilms with an average depth of 72 μm , in contrast to the wild type, which produced more abundant biofilms with an average thickness of 104 μm . These data suggest a contributory role for *M. catarrhalis* TFP in biofilm development and maturation.

DISCUSSION

Several previous reports of other TFP-expressing bacteria demonstrated not only that TFP are important factors in bacterial pathogenesis and adherence to eukaryotic cells but also that a functional overlap in bacterial components involved in attachment to abiotic and biotic surfaces exists (6, 12, 33, 34). We have previously shown that *pilA* and *pilQ* are transcribed by a panel of geographically diverse *M. catarrhalis* clinical isolates, implying that TFP expression by this organism is a critical factor for virulence (26). Additionally, we determined that the expression of TFP is essential for DNA uptake via natural transformation, and as a result, we were technically unable to generate wild-type revertants in *cis* or stable complemented mutants in *trans* in the 7169pilAK4 background (24, 26, 30, 42).

In this study, we observed that the lack of PilA and therefore the loss of surface pili negatively affected the ability of *M. catarrhalis* to adhere in vitro and in vivo. Our data demonstrate that TFP are likely important nasopharyngeal colonization factors for *M. catarrhalis*. The *pilA*-deficient mutant 7169pilAK4 was significantly impaired compared to the wild-type strain in attachment to cell culture monolayers in vitro and to nasopharyngeal tissues in the chinchilla host in vivo. In addition, the mutant strain was impaired in the ability to form a biofilm in flow cell chambers, implying that pilus expression is essential for biofilm maturation.

TFP represent just one of a number of cell surface adhesins that have been implicated in contributing to epithelial attachment by *M. catarrhalis* in vitro, although there have been no other studies assessing the roles of these surface components in colonization in vivo. It was previously reported that expression of UspA1 and Hag is important for *M. catarrhalis* attachment to Chang cells, a human epithelial cell line frequently used in adherence experiments with respiratory pathogens (1, 7, 16, 21, 39). Our data demonstrate that the loss of TFP

FIG. 5. Representative biofilms formed by strains 7169 (A and C) and 7169pilAK4 (B and D) grown in flow chambers for 72 h and examined by confocal laser scanning microscopy after being stained with a BacLight Live/Dead kit. Panels A and B show three-dimensional z-stacked composites, while panels C and D illustrate the comparative height difference of each biofilm. Average biofilm depths are shown.

expression reduced binding of the isogenic TFP-deficient strain 7169pilAK4 to Chang monolayers compared to the wild type, thereby implicating pili as an attachment factor for these cells as well. Both the wild-type and mutant strains produce UspA1, UspA2, and Hag, as detected by immunoblot analyses using monoclonal antibodies 24B5, 17C7, and 5D2, respectively (2, 3, 8, 35; data not shown), which supports our finding that TFP are directly involved in bacterial association with these eukaryotic cells.

To further extend this observation, we explored the nature of TFP-mediated interactions of *M. catarrhalis* with the nasopharyngeal mucosa in vivo, using the chinchilla model of NP colonization. Research efforts focusing on the pathogenesis of *M. catarrhalis* have been hampered largely by the lack of an effective animal model. The system used most often is the mouse pulmonary model, and while this has provided some important data, it is strictly limited to assessing bacterial clearance over a short time frame (5, 23, 36, 40). We have provided data to demonstrate that *M. catarrhalis* 7169 can colonize the chinchilla NP and persist in these animals for >2 weeks. In addition to establishing that the chinchilla NP colonization model is a relevant animal model with which to investigate the pathogenesis of *M. catarrhalis*, our studies demonstrate that TFP expressed by *M. catarrhalis* are involved in NP colonization. Our data demonstrated that significantly more 7169pilAK cells than wild-type cells could be recovered by lavage on day 1 after challenge of the chinchilla nares, suggesting that the TFP-deficient organisms are less capable of associating with NP mucosa. This trend was observed for up to 4 days postinoculation. We further investigated the contribution of *M. catarrhalis* TFP to NP persistence by quantitatively examining tissue homogenates isolated 18 days after intranasal challenge. The number of 7169 cells adherent to the NP mucosal tissues was significantly greater at every anatomic location than the number of 7169pilAK cells recovered, consistent with our hy-

pothesis that TFP promote *M. catarrhalis* adherence to the upper respiratory tract epithelium.

Importantly, the development of the chinchilla model of NP colonization for this respiratory pathogen provides a biological system with which we can now directly evaluate the significance of antibody development in bacterial clearance from nasopharyngeal surfaces. These future studies not only will provide the data necessary to further test our hypothesis that TFP are a critical factor for colonization during the natural infection process but also will have a broad range and important impact on the overall ability to study *M. catarrhalis* pathogenesis in an animal model system.

Biofilm formation is thought to be a mechanism by which mucosal pathogens exhibit long-term colonization of the host. The recent detection of biofilms on pediatric middle ear mucosa specimens obtained from children with recurrent otitis media supports the hypothesis that bacterial biofilms play a key role in chronic middle ear disorders (14). Furthermore, a recent study by Yokota et al. reported that compared to non-prone control groups, otitis-prone children tend to be colonized by the organisms associated with the development of otitis media, including *M. catarrhalis*, and they are unable to eradicate these pathogens from the upper respiratory tract mucosa despite antibiotic treatment (44). We have now performed microscopic analyses demonstrating that *M. catarrhalis* can form biofilms in continuous-flow chambers and have determined the ultrastructure of these biofilms. Our analyses indicate that the biofilms produced by *M. catarrhalis* under flow cell conditions resemble the complex structures composed of microcolony pillars interspersed with water channels produced by *P. aeruginosa* and several other bacterial species (reviewed in references 10 and 37). Moreover, we observed a difference in biofilm development between the wild type and the *pilA* mutant, implicating the involvement of TFP in biofilm formation by *M. catarrhalis*. In the early stages of biofilm formation, 7169pilAK exhibited a delayed ability to form biofilm structures compared to the wild type. It is important that there is no detectable difference in growth rates between the wild-type and mutant strains (26), and therefore these observations were not the result of growth defects. The less-robust biofilm phenotype exhibited by 7169pilAK4 demonstrates a direct role for TFP expression in biofilm structure and depth. However, these studies show that a loss of TFP does not completely abrogate biofilm formation by *M. catarrhalis*, suggesting that functional TFP are not strictly required but instead contribute to the overall process of biofilm development in a flow cell environment.

Taken together, the experimental results presented in this study suggest that *M. catarrhalis* TFP are involved in colonization of the nasopharyngeal mucosa and contribute to biofilm formation. Furthermore, this report describes the development of an in vivo respiratory colonization model using the chinchilla, which signifies a substantial advance in the field of *M. catarrhalis* research. Additional investigations of the contributions of TFP to *M. catarrhalis* pathogenicity will provide important insights into the mechanisms by which this organism causes disease. Therapeutic or vaccine-based interventions that prevent or diminish nasopharyngeal colonization will likely decrease acute and recurrent *M. catarrhalis* infections in prone populations. Thus, additional studies investigating the

contribution of *M. catarrhalis* TFP to colonization in vivo are clearly warranted and are currently under way.

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