A Cation-binding Surface Protein as a Vaccine Antigen to Prevent Moraxella catarrhalis Otitis Media and Infections in Chronic Obstructive Pulmonary Disease

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Abstract

Moraxella catarrhalis is an exclusively human respiratory tract pathogen that is a common cause of otitis media in children and respiratory tract infections in adults with chronic obstructive pulmonary disease. A vaccine to prevent these infections would have a major impact in reducing the substantial global morbidity and mortality in these populations. Through a genome mining approach, we identified AfeA, an ~32 kDa substrate binding protein of an ABC transport system as an excellent candidate vaccine antigen. Recombinant AfeA was expressed and purified and binds ferric, ferrous, manganese and zinc ions as demonstrated by thermal shift assays. It is a highly conserved protein that is present in all strains of M. catarrhalis.

Immunization with recombinant, purified AfeA induces high titer antibodies that recognize the native M. catarrhalis protein. AfeA expresses abundant epitopes on the bacterial surface and induces protective responses in the mouse pulmonary clearance model following aerosol challenge with M. catarrhalis. Finally, AfeA is expressed during human respiratory tract infection of adults with COPD. Based on these observations, AfeA is an excellent vaccine antigen to be included in a vaccine to prevent infections caused by M. catarrhalis.
Introduction

*Moraxella catarrhalis* is a human respiratory tract pathogen that causes a substantial global burden of disease, particularly otitis media (middle ear infections) in children and respiratory tract infections (exacerbations) in adults with chronic obstructive pulmonary disease (COPD) (1-3). While *M. catarrhalis* has been overlooked as a pathogen in both of these clinical settings, recent studies have elucidated the key role of *M. catarrhalis* both as a primary pathogen and as a co-pathogen.

Approximately 80% of children experience an episode of otitis media by the age of three years and up to 30% of children experience recurrent otitis media, which is associated with delays in speech and language development as a result of impaired hearing (4-6). An estimated 709 million cases of otitis media occur annually worldwide, including 31 million cases of chronic suppurative otitis media, a particularly devastating complication of otitis media in developing countries (7, 8). As the most common reason for infants and children to receive antibiotic therapy, otitis media is a main driver of the global crisis in antibiotic resistance in bacteria (9, 10). A vaccine to prevent otitis media would have an enormous benefit in preventing global morbidity, reducing healthcare costs, and helping to ameliorate the growing problem of antibiotic resistance. These benefits are already being realized in countries that have implemented vaccination programs with pneumococcal conjugate vaccines (11-17).
COPD is a debilitating disease of adults that is the fourth most common cause of death in the US and the world (18, 19). While death rates from heart disease and stroke are declining, the death rate from COPD has doubled since 1970 (19). The course of COPD is characterized by intermittent worsening of symptoms called exacerbations (20-23). Approximately half of exacerbations are caused by bacterial infection (20). Exacerbations result in substantial morbidity and cost, including clinic visits, emergency room visits, hospital admissions, respiratory failure and death. Exacerbations accelerate decline in lung function (24-26) and are the most important cause of the reduced quality of life in patients with COPD (27-29).

Remarkably, exacerbations of COPD that require hospital admission are associated with a 23% one-year mortality (30, 31). Thus, one of the most urgent areas of research to impact patients with COPD is the development of approaches to preventing exacerbations.

To develop vaccines to prevent otitis media in children and exacerbations in adults with COPD, it is critical to know the etiology of these infections. The gold standard for determining the etiology of otitis media has been culture of middle ear fluid obtained by tympanocentesis. Based on middle ear fluid cultures, the three most common causes of otitis media are nontypeable Haemophilus influenzae, Streptococcus pneumoniae and M. catarrhalis, which is isolated in culture from 5 to 20% of middle ear fluids. However, an increasing understanding of the role of biofilms in otitis media has revealed that relying on culture alone detects pathogens in only a subset of episodes in otitis media (32). When middle ear fluids are subjected to more sensitive molecular analysis, M. catarrhalis is detected alone or
with other pathogens in 30 to 50% of middle ear fluid samples from children with otitis media (33, 34). Analyses of middle ear fluid by PCR increased the frequency of detection of *S. pneumoniae* and nontypeable *H. influenzae* 3.2-fold compared to culture, while *M. catarrhalis* was 4.5 times more likely to be identified by PCR (35).

*M. catarrhalis* has been overlooked as a pathogen in COPD because the organism is difficult to distinguish from commensal *Neisseria*, which are part of the normal flora of the human upper respiratory tract. Thus, *M. catarrhalis* is missed in sputum cultures by many clinical microbiology laboratories. Based on a rigorous prospective study using accurate methods to identify the organism, *M. catarrhalis* is the second most common cause of exacerbation of COPD after nontypeable *H. influenzae* (36). Adults with COPD experience 2 to 4 million exacerbations caused by *M. catarrhalis* annually in the US (36).

Therefore, a vaccine to prevent *M. catarrhalis* infections has the potential to have a huge impact in preventing otitis media in children and infections in adults with COPD. An effective vaccine strategy in these clinical settings will require preventing infections caused by nontypeable *H. influenzae*, *S. pneumoniae* and *M. catarrhalis*.

In this study, we have identified and characterized a high value vaccine candidate antigen of *M. catarrhalis* using a genome mining approach. AfeA is a substrate binding protein of an ATP binding cassette (ABC) transporter that has characteristics of an excellent vaccine antigen. The present study shows that AfeA 1) is highly conserved among strains, 2) induces high titer antibody that recognizes native protein following immunization with recombinant purified protein, 3)
expresses abundant epitopes on the bacterial surface, 4) induces protective responses in the mouse pulmonary clearance model following aerosol challenge with *M. catarrhalis*, 5) is expressed during human respiratory tract infection and 6) binds ferric, ferrous, manganese and zinc ions.

Results

**Identification and characterization of the afeA gene.** As part of a genome mining approach to identify vaccine antigens of *M. catarrhalis*, we previously analyzed the genome of strain ATCC 43617 (accession numbers AX067426–AX067466) to identify open reading frames (ORFs) predicted to be exposed on the bacterial cell surface (37). Of 348 ORFs predicted to be surface-exposed, 14 had homology to substrate binding proteins (SBPs) of ABC transporter systems. This observation led to an extensive evaluation of SBPs of ABC transporters of *M. catarrhalis* (38) and to the identification and characterization of three SBPs as promising vaccine antigens, including oligopeptide protein A (OppA), substrate binding protein 2 (SBP2) and CysP (39-41).

Annotation of genes in strain BBH18 (GenBank NC_014147.1 GI:296112228) identified a gene cluster that has homology to genes that encode an ABC transporter system in *Actinobacillus actinomycetemcomitans*, afeABCD, that promotes cell growth under iron-chelated conditions (42, 43). *M. catarrhalis* AfeA, the predicted SBP of this ABC transporter system, is 69% identical and 79% similar to the *A. actinomycetemcomitans* AfeA and is the subject of the current study. A Protein BLAST search with AfeA of *M. catarrhalis* (Protein ID WP_003658713.1) revealed
that AfeA is 72 to 79% identical and 82 to 88% similar to predicted metal binding SBPs in other *Moraxella* species, *Pasteurella* species and *Haemophilus* species.

ABC transporters generally include one or more permeases, ATPases and substrate binding proteins. AfeA is part of a gene cluster that includes genes that encode one substrate binding protein (AfeA), one ATPase (AfeB), and two permeases (AfeC and AfeD) (Figure 1A).

To determine whether the genes of the *afeABCD* gene cluster are transcribed as a single transcript or as multiple transcripts, reverse transcriptase PCR was performed using RNA isolated from *M. catarrhalis* strain O35E grown in broth using primers designed to correspond to transcripts that span adjacent genes in the gene cluster. Control assays lacking reverse transcriptase confirmed that the purified RNA was free of contaminating DNA (Figure 1B, lanes b). Figure 1B shows that the genes of the *afeABCD* gene cluster are transcribed as a single transcript (Figure 1B lanes c).

**Conservation of AfeA among strains of *M. catarrhalis*.** A tBLASTn search of the 3 publicly available complete genomes and 46 draft whole genome sequences available in GenBank with AfeA of *M. catarrhalis* strain BBH18 revealed that the *afeA* gene was present in the genomes of all 49 strains. A total of 21 strains showed 100% amino acid identity with AfeA; 22 strains showed 99% amino acid identity due to a single amino acid difference; one strain showed 98% identity (strain ctg3), and two strains showed 87% identity and 95% similarity (strains 304 and 324). The 3 remaining strains identified as *M. catarrhalis* showed lower homology. These
strains are variant strains and, based on genome sequences, will soon be reclassified as a different species (44).

To assess the presence and conservation of the afeA gene in clinical isolates of *M. catarrhalis*, DNA purified from 20 clinical isolates was used as a template in a PCR reaction with primers that were designed to amplify the afeA gene (Table 1). The clinical isolates included 10 middle ear fluid isolates obtained by tympanocentesis from children with acute otitis media and 10 sputum isolates from adults who were experiencing exacerbations of COPD. A band of 927 bp with an identical size to that of strain O35E was detected in all 20 strains (Figure 2). A negative control in which DNA template was replaced with water showed no band (data not shown). The sequences of the afeA amplicons revealed 99.7 to 100% identity in nucleotide sequence and 100% amino acid identity in all 20 strains. We conclude that the afeA gene is present in the genome of all clinical isolates of *M. catarrhalis* isolates tested to date and that the gene is highly conserved among strains.

Characterization of purified recombinant AfeA. The *M. catarrhalis* afeA gene encodes a predicted lipoprotein with a 19 amino acid signal peptide at the amino terminus (LipoP 1.0). The mature AfeA protein, after cleavage of the signal peptide, contains 289 amino acids. The afeA gene region encoding the mature AfeA protein was inserted into the pCATCH vector to express recombinant AfeA as a lipoprotein with a C-terminal hexahistidine tag in *E. coli* BL21(DE3) as described in Materials and Methods. Following expression and affinity purification with metal affinity
resin, the purified protein separated as a single band of ~32 kDa in SDS PAGE with Coomassie Blue stain and silver stain (Figure 3).

Characterization of afe knockout mutant. An isogenic afe knockout mutant in strain 035E was constructed by replacing the afe gene cluster with a nonpolar kanamycin resistance cassette via homologous recombination. Antiserum raised to recombinant AfeA detected a single band of ~32 kDa in immunoblot assay with a whole cell lysate of wild type strain 035E (Figure 3, lanes c), confirming the specificity of the antiserum for AfeA. A whole cell lysate of the afe knockout mutant showed an absence of the ~32 kDa band, confirming the absence of expression of AfeA in the mutant (Figure 3, lanes d). The complemented afe mutant expressed AfeA as expected (Figure 3, lanes e). The WT, afe knockout mutant and complemented mutant all expressed BCAA substrate binding protein, a control result indicating that expression of an unrelated substrate binding protein was unaffected by the genetic manipulations used to engineer and complement the afeA knockout mutant (Figure 3, right panel).

Expression of AfeA by M. catarrhalis. Rabbit antiserum raised to purified recombinant AfeA was used in immunoblot assay to assess expression of AfeA by M. catarrhalis. Figure 3C shows that the antiserum recognizes a single band of the predicted size of AfeA (~32 kDa) in a whole cell lysate of M. catarrhalis and detects no band in the knockout mutant. We conclude that antiserum raised to recombinant purified AfeA recognizes the AfeA protein expressed by M. catarrhalis.
To assess the expression of AfeA by clinical isolates of *M. catarrhalis*, immunoblot assays with whole cell lysates of 20 clinical isolates (10 otitis media strains and 10 COPD exacerbation strains) were probed with AfeA antiserum. A band of ~32 kDa was present in all 20 strains (Figure 4). We conclude that clinical isolates of *M. catarrhalis* express AfeA during growth in vitro.

**Expression of AfeA epitopes on the bacterial surface.** Whole cell ELISAs were performed to determine the extent to which AfeA epitopes are expressed on the bacterial surface. Wells were coated with wild type (WT) *M. catarrhalis* O35E and the afe knockout mutant. Antiserum to OMP OppA was used as a positive control (surface protein) and antiserum to the BCAA SBP1 was used as a negative control (nonsurface protein). Anti AfeA antibody bound to the WT strain but not to the afe knockout mutant (Figure 5A), while anti-OppA antibodies (positive control) bound to both strains (Figure 5C), and anti-BCAA antibodies (negative control) bound to neither strain (Figure 5E). The complemented afe mutant expressed AfeA on the bacterial surface; interestingly, the complemented afe mutant appears to express more surface exposed AfeA than WT, based on the higher OD observed with complemented mutant (Figure 5B). The strong signal of antibodies binding to whole cells of the WT strain and the absence of binding of anti AfeA antibodies to the afe knockout mutant confirm the specificity of binding to AfeA in this assay and support the conclusion that AfeA expresses abundant epitopes on the bacterial cell surface. As a second independent method to assess AfeA surface epitopes, we performed flow cytometry with WT afe knockout mutant and complemented
mutant strains using antiserum to AfeA. Antibodies to AfeA demonstrate an increase in mean fluorescence intensity from pre immune to immune serum as indicated by a distinct shift of the curve to the right. (Figure 6A). An assay of the same antiserum with the afe knockout mutant showed no shift, indicating that the antiserum contains AfeA-specific antibodies that bind to surface epitopes. Assay of the complemented afe showed partial restoration of activity (Figure 6A, right). Antiserum to OppA (surface protein as positive control) showed binding to both wild type and afe mutant (Figure 6B) and antiserum to BCAA (non surface protein as negative control) showed no binding to either strain (Figure 6C). Based on the results of whole cell ELISA and flow cytometry, we conclude that *M. catarrhalis* expresses AfeA epitopes on the bacterial surface.

**Induction of mouse pulmonary clearance.** Immunoblot assays with pooled mouse serum showed that the mice immunized with AfeA developed antibodies to AfeA (Figure 7A). To assess the effect of immunization with AfeA in the induction of protective immune responses, we assessed bacterial clearance three hours following aerosol challenge with *M. catarrhalis* in the mouse pulmonary clearance model. Mice immunized with purified AfeA (25 µg and 50 µg doses) showed enhanced clearance of *M. catarrhalis* from the lungs following aerosol challenge with *M. catarrhalis* compared to controls (Figure 7B). Statistically significant overall group differences were observed with a p-value of 0.0003. Pairwise comparisons between PBS with 25µg, 50µg, and whole organism (positive control) had associated p-values of 0.0851, 0.0001, and 0.0032, respectively. A schedule using a 50 µg dose
of AfeA induced a level of clearance similar to that observed with other vaccine antigens with this model (40, 41, 45-47). The experiment depicted in Figure 7B was repeated and yielded an identical result of enhanced clearance by approximately one half log of bacteria. We conclude that immunization of mice with AfeA induces protective responses in the mouse pulmonary challenge model.

**Human antibody response to AfeA following infection.** To determine whether AfeA was expressed by *M. catarrhalis* during infection of the human respiratory tract, we performed ELISAs with serum samples from patients with COPD who experienced exacerbations caused by *M. catarrhalis* (36). The prospective study design enabled the use of pre-exacerbation serum samples obtained 1 to 2 months before the patient acquired the infecting strain and post-exacerbation samples from 1 to 2 months following infection. Two of 19 patients (10.5%) developed new antibody responses to AfeA following infection (Figure 8). This proportion of patients developing new serum antibody responses is similar to what we have observed to other conserved surface antigens of *M. catarrhalis* in this cohort of adults with COPD (37, 48, 49). The observation that a subset of patients who experienced exacerbations of COPD developed new serum antibody responses indicates that AfeA is expressed in these patients during human infection.

**Binding of Cations by AfeA.** Based on the observation that homologues of AfeA in other species are involved in metal transport, we performed experiments to test the hypothesis that AfeA binds cations by performing thermal shift assays (43, 50).
Initial experiments with purified, recombinant, lipidated AfeA failed to produce sharp melting temperatures ($T_m$), indicating that the protein did not form a stable conformation in the conditions of the assay, in spite of testing in several buffer conditions. Therefore, we engineered a construct that expressed non lipidated AfeA and purified the protein using buffers to which the cation chelating resin, Chelex 100 (Sigma), has been added. This approach resulted in sharp, biphasic melting curves, indicating that the protein formed stable conformations and some of the protein was bound by a ligand while some was not (Table 2).

Addition of selected metals resulted in thermal shift of the curve from a lower $T_m$ (corresponding to unbound AfeA) to a curve with an increased $T_m$ indicating binding of the added ligand to AfeA (Table 2). Specific binding of ferric, ferrous, manganese and zinc, was observed with a thermal shift of ~23°C with each of these cations. No thermal shift was observed with magnesium ions, confirming the specificity of binding in the assay. We conclude that AfeA binds ferric, ferrous, manganese and zinc ions.

**Discussion**

We have identified a promising new vaccine antigen for *M. catarrhalis*. AfeA is a ~32 kDa protein with several characteristics that predict it is a potentially effective vaccine: 1) The *afeA* gene is present in all strains of *M. catarrhalis*. 2) It is highly conserved among clinical isolates that cause otitis media and infection in COPD. 3) AfeA expresses abundant epitopes on the bacterial surface that are accessible to potentially protective antibodies. 4) The protein is highly immunogenic.
and induces protective immune responses in the mouse following aerosol challenge with *M. catarrhalis*. 5) AfeA is expressed during human infection, based on the development of antibody responses following exacerbations of COPD in selected patients.

The AfeA protein was discovered through mining the *M. catarrhalis* genome to identify conserved proteins that were predicted to be expressed on the bacterial surface (37). This work led to the somewhat surprising observation that selected substrate binding proteins (SBPs) of ABC transporter systems express epitopes on the surface of *M. catarrhalis*. SBPs are located in the periplasm of Gram-negative bacteria and function to bind and transport ligands from the outer membrane to permeases in the cytoplasmic membrane for import (51, 52). Based on homology with SBPs of other Gram-negative bacteria, AfeA is predicted to transport ferric ions and possibly other cations, including manganese and zinc. We demonstrated with thermal shift assays that AfeA of *M. catarrhalis* binds ferric, ferrous, manganese and zinc ions (Table 2).

We report three independent lines of evidence to support the conclusion that AfeA expresses epitopes on the bacterial surface: 1) whole cell ELISA with antiserum to AfeA (Figure 5); 2) flow cytometry with antiserum to AfeA (Figure 6); and 3) induction of potentially protective immune responses by AfeA in the mouse pulmonary clearance model (Figure 7). Using this genome mining approach, we previously identified three additional SBPs of ABC transporters that express surface epitopes and are candidate vaccine antigens of *M. catarrhalis*, including OppA, SBP2 and CysP (39-41). Of interest, all four of these surface exposed SBPs are
lipoproteins. AfeA is likely present both in the periplasm and on the bacterial surface. Future studies will elucidate the precise distribution of the protein in bacterial compartments.

As part of previous work we assessed the role of SBPs in virulence mechanisms of *M. catarrhalis* infection. A knockout mutant of the *afe* gene cluster showed reduced growth rate in chemically defined media compared to wild type, and also showed reduced capacity for invasion of human respiratory epithelial cells (38). The ability to invade and survive inside host cells is a potentially important virulence mechanism because intracellular *M. catarrhalis* in host cells serves as a reservoir for the bacterium to persist in the human respiratory tract (53). AfeA is a nutritional virulence factor that mediates the uptake of required trace cations, which are present in extremely low levels intracellularly. A vaccine target that is also a virulence factor enhances its potential as a vaccine antigen because an immune response that targets a virulence factor may serve to inhibit infection in addition to binding antibodies that mediate host responses to enhance clearance of the bacterium. The inhibition of a virulence mechanism through targeting a vaccine antigen is reminiscent of inhibition of the glycerophosphodiesterase activity of protein D of nontypeable *H. influenzae*, which contributes to the protective response induced by Protein D in the 10 valent pneumococcal conjugate vaccine that contains Protein D (54, 55).

AfeA induces a new antibody response following only ~10% of exacerbations of COPD caused by *M. catarrhalis* (Figure 8). This rate is similar to several other conserved surface proteins of *M. catarrhalis* that are under consideration as vaccine
The immune response to a putative vaccine antigen following infection by a bacterial pathogen is not a reliable predictor of the value of a vaccine target based on the observation that many highly effective vaccine antigens are not the target of immune responses following infection. For example, in the pre Hib conjugate vaccine era, infants who recovered from *H. influenzae* type b meningitis did not consistently develop an antibody response to the polysaccharide capsule, yet the capsular polysaccharide is a highly effective vaccine when conjugated to an appropriate protein carrier (56). Similarly, a conserved surface protein of *Borrelia bergdorferi* that lacks detectable immune responses following infection is a promising vaccine target (57). The observation that AfeA induces a new antibody response in some patients indicates that the protein is expressed during human infection and also reflects heterogeneity of immune responses among adults with COPD.

A challenge in identifying and characterizing protective vaccine antigens for *M. catarrhalis* is the absence of a reliable correlate of protection. *M. catarrhalis* expresses surface molecules that inactivate terminal components of the complement pathway, interfering with assessing antibodies for bactericidal activity, which is a correlate of protection for other Gram-negative pathogens, including nontypeable *H. influenzae* (58, 59). As an exclusively human pathogen, *M. catarrhalis* does not persistently colonize or cause infection in experimental animals. Based on experience in developing vaccines for other bacterial infections, the induction of a protective response in an animal model by a surface exposed molecule is predictive of an effective vaccine antigen. A limitation of the mouse pulmonary clearance...
model for *M. catarrhalis* is that it is not a true model of infection. Rather, the model measures the rate of clearance of bacteria from the lungs. In spite of this limitation, the model is quantitative, is reproducible, is used by several research groups and is the most widely used model to assess vaccine antigens of *M. catarrhalis* (39, 40, 45, 60).

The 10 valent pneumococcal conjugate vaccine that contains Protein D shows ~35% efficacy in preventing otitis media caused by nontypeable *H. influenzae* (61).

Based on this experience, a successful vaccine to prevent infections by nonencapsulated Gram-negative pathogens, such as *M. catarrhalis*, will likely require more than one surface protein antigen. AfeA represents a new, high value vaccine target that has two advantages over several other antigens under consideration.

First, AfeA is highly immunogenic, inducing antibody titers that detect the protein in dilutions in the millions following a standard immunization schedule. Second, AfeA expresses abundant surface epitopes, providing more targets for potentially protective antibodies. AfeA is an excellent candidate antigen to be included in a vaccine to prevent *M. catarrhalis* infections, which would have a major benefit for children by preventing otitis media and for adults with COPD by preventing exacerbations that cause enormous morbidity and mortality.

**Materials and Methods**

**Bacterial strains and growth.** *M. catarrhalis* strain 035E was provided by Eric Hansen. Strains 2015, 5193, 6955, 7169, 9483, 0701057VIL, 0701064V3L, 0702076SV4R, 0701062V1L and 0701067V3L are middle ear fluid isolates obtained
via tympanocentesis from children with otitis media provided by Howard Faden in Buffalo NY and Janet Casey in Rochester NY. Strains 6P29B1, 10P66B1, 14P30B1, 39P33B1 and 47P31B1 are sputum isolates obtained from adults with COPD during exacerbations as part of a prospective study in Buffalo NY (36). Strains M2, M3, M4, M5 and M6 are sputum isolates from adults with COPD provided by Daniel Musher in Houston TX. Pulsed-field gel electrophoresis of genomic DNA cut with SmaI showed that the strains are genetically diverse. *M. catarrhalis* strains were grown on brain heart infusion (BHI) plates at 37°C with 5% CO₂ or in BHI broth with shaking at 37°C.

Chemically competent *Escherichia coli* strains Top10 and BL21(DE3) were obtained from Invitrogen (Carlsbad, CA) and were grown at 37°C on Luria-Bertani (LB) plates or in LB broth.

**Genomic DNA and RNA purification.** Genomic DNA of *M. catarrhalis* strains was purified with the Wizard genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer’s instructions. RNA from strain 035E was isolated with the Qiagen RNeasy minikit (Qiagen, Valencia, CA) and DNA contamination was removed with the RQ1 RNase-Free DNase kit (Promega).

**Reverse transcriptase PCR (RT-PCR).** RT-PCR was performed with the Qiagen One-Step RT-PCR kit according to the manufacturer’s instructions, with 50 ng of RNA per reaction mixture.
Cloning the *afeA* gene. The *afeA* gene from *M. catarrhalis* strain O35E was cloned into the plasmid pCATCH, which allows for expression of recombinant lipoproteins in *E. coli*, using previously described methods (39, 41, 62). Oligonucleotide primers corresponding to the 5’ end starting after the predicted cysteine codon and the 3’ end of the genes were designed with *Ncol* and *BamH1* restriction sites (Table 1). The genes were amplified by PCR from genomic DNA of *M. catarrhalis* strain O35E. The resultant PCR product was ligated into pCATCH and transformed into *E. coli* TOP10 cells. Colonies were picked, grown in broth, and plasmids were purified. PCR and sequencing confirmed the insertion of the gene into the plasmid called pAfeA.

Expression and purification of recombinant AfeA. Following growth of 50 ml of culture in LB broth with 50 µg kanamycin to an *OD*\textsubscript{600} of 0.6, AfeA expression was induced with 4 mM IPTG (isopropyl-ß-D-thiogalactopyranoside) for 4 hours at 37°C. The bacteria were then harvested by centrifugation at 4,000g for 15 minutes at 4°C. The pellet was suspended in 10 ml of lysis buffer (0.020 M sodium phosphate, 0.5 M NaCl, 1 mg/ml lysozyme, 1× Protease Arrest, pH 7.4) and mixed with a nutator for 30 minutes at room temperature. The suspension was then sonicated on ice with a Branson Sonifier 450 at setting 6, using an 80% pulsed cycle of four 30-second bursts with 2-minute pauses. The sonicated bacterial lysate was centrifuged at 10,000g for 20 minutes at 4°C. The pellet was suspended in 5 ml of Urea Lysis Buffer containing 0.05 M NaH\textsubscript{2}PO\textsubscript{4}, 0.01 M tris [pH 8], 6 M urea, 0.1 M NaCl, pH 7.5 plus 25
µl of Protease Arrest and mixed on a nutator for 20 minutes until the lysate became clear.

AfeA was purified by affinity chromatography using BD talon resin (BD Biosciences, Palo Alto CA) through the 6-histidine tag, which is on the carboxy terminus of the recombinant AfeA, using a modification of previously described methods (39, 40). An aliquot of 2 ml of BD talon resin was centrifuged at 750g for 5 minutes at 4°C, suspended in Urea Lysis Buffer, incubated for 10 minutes and centrifuged again. The resin was suspended in 5 ml of cleared bacterial lysate and mixed by nutation for 30 minutes at room temperature. The suspension was centrifuged at 750g for 5 minutes at 4°C. The resin, containing bound protein was washed in Urea Lysis Buffer twice for 10 minutes. To elute recombinant protein, the washed resin was suspended in 1 ml of the same buffer containing 0.15 M imidazole and mixed by nutation for 10 minutes at room temperature. The resin was removed by centrifugation and the supernatant containing purified recombinant protein was collected. The elution was repeated and the eluates were pooled. AfeA was refolded by sequential dialysis in buffers that contained decreasing concentrations of arginine (0.5 M to 0.005 M). Protein concentration was measured by the method of Lowry (Sigma). The purity of the protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and Coomassie Blue staining.

To express and purify recombinant AfeA that lacks the amino terminal linked lipoprotein for thermal shift assays, the afeA gene encoding the mature AfeA protein was amplified by PCR from genomic DNA of strain 03SE using primers noted in Table 1 and ligated into plasmid pET 101 D-TOPO (Invitrogen). The ligation mixture
was transformed into the chemically competent *E. coli* strain Top10 and grown on BHI plates containing 50 µg/ml carbenicillin. The AfeA protein was expressed as described above and the recombinant protein was found in the supernatant following sonication. Nonlipidated recombinant AfeA was purified from the supernatant using the same method as for the recombinant lipidated AfeA protein described above.

**Development of antiserum to AfeA.** To develop antiserum to AfeA, purified recombinant AfeA was sent to Covance (Denver, PA) for antibody production in New Zealand White rabbits using a 59-day protocol. Briefly, 250 µg purified AfeA was emulsified 1:1 in complete Freund’s adjuvant for initial subcutaneous immunization. Subsequent immunization followed a 3-week cycle of boosts with 125 µg AfeA emulsified 1:1 in incomplete Freund’s adjuvant. Serum was collected 2 weeks after the second boost.

**Construction of afe knockout mutant.** A knockout mutant in which the entire gene cluster in which the *afeA* gene is located was engineered by using overlap extension PCR and homologous recombination as we have described previously with several *M. catarrhalis* genes (38-40). Briefly, the transforming DNA for the mutant was composed of 3 overlapping fragments that included ~1 kb upstream of the *afe* gene cluster (fragment 1), the nonpolar kanamycin resistance cassette amplified from plasmid pUC18K (fragment 2), and ~1 kb downstream of the gene cluster. A mutant was constructed by transformation of *M. catarrhalis* strain O35E with a fragment.
composed of fragments 1, 2, and 3 and selection on brain heart infusion (BHI) plates containing 50 µg/ml of kanamycin. The insert and surrounding sequences of the mutant were confirmed by sequence analysis.

Complementation of the afe mutant. Complementation was accomplished with plasmid pWW115 using previously described methods (63, 64). Briefly, a fragment containing the afe gene cluster and 300 bp upstream to include the promoter of the afe operon and 300 bp downstream was amplified from genomic DNA of strain O35E and ligated into pWW115 using primers that included a BamHI site and a SacI site (Table 1). After confirming the insert sequence of the resulting plasmid construct, the afe mutant was transformed with the plasmid onto a BHI agar plate inoculated with 100 µl of O35E at an OD₆₀₀ of 0.2 and incubated for 5 h at 37°C. Spots were then spread onto BHI agar plates that contained 100 µg of spectinomycin and incubated overnight. The resulting colonies were picked and the afe operon and surrounding regions were confirmed with sequencing and immunoblot assay with antibody to AfeA. This complemented mutant was grown in the presence of spectinomycin for all experiments.

Whole cell ELISA. To assess binding of antibodies to epitopes on the bacterial surface, whole-cell enzyme-linked immunosorbent assay (ELISA) was performed. M. catarrhalis wild type strain O35E and the corresponding afe knockout mutant were grown in BHI broth to an OD₆₀₀ of 0.2, harvested by centrifugation, and resuspended in PBS. A volume of 100 µl of the suspension was added to each well of a 96-well
Microtiter Immunolon 4 plate (Thermo Labsystems, Franklin, MA) and incubated overnight at 4°C to coat the wells with bacterial cells. Wells with PBS alone were included as controls. Wells were washed once with 0.05% Tween 20 in phosphate buffered saline (PBST) and blocked with 3% nonfat dry milk in PBS for 1 hour at room temperature, after which wells were washed 3 times with PBST. Paired rabbit antisera (pre-immune and immune) were diluted 1:5000, 1:10,000, 1:20,000 and 1:40,000 in diluent buffer (1% nonfat dry milk in PBST) and added to the sham-coated control wells and whole bacterial cell sample wells in parallel. After incubation for 2 hours at 37°C, wells were washed 3 times with PBST and a 1:3,000 dilution of peroxidase-labeled secondary antibody, anti-rabbit IgG (KPL, Gaithersburg, MD) diluted in PBST plus 3% heat-inactivated goat serum was added. After another 1 hour of incubation at room temperature, wells were washed 3 times with PBST and color developing reagent was added. The reaction was allowed to proceed for 15 minutes and was stopped with 2 M sulfuric acid. The absorbance at 450 nm was determined using a Bio-Rad model 3550-UV microplate reader (Hercules, CA).

**Mouse pulmonary clearance model.** All animal studies were reviewed and approved by the University at Buffalo Institutional Animal Care and Use Committee. Systemic immunization was accomplished with groups of 6 Balb/c mice that were immunized subcutaneously with 25 µg or 50 µg of purified recombinant AfeA emulsified in incomplete Freund's adjuvant. Additional controls were immunized with either PBS plus adjuvant (negative control, n=6) or formalin-killed M.
catarrhalis O35E emulsified in incomplete Freund’s adjuvant (positive control, n=6). Injections were repeated at 14 and 28 days after the initial immunization. Mice were challenged on day 35 as described below. To determine if immunization with AfeA induces potentially protective responses in vivo, the mouse pulmonary clearance model was performed as described previously. An overnight culture of *M. catarrhalis* O35E was inoculated into 100 ml BHI broth to an OD$_{600}$ of ~0.05 and grown to an OD$_{600}$ of ~0.3. Bacteria were collected by centrifugation and resuspended in 10 ml PCGM buffer (4.3 mM NaHPO$_4$, 1.4 mM KH$_2$PO$_4$, 137 mM NaCl, 2.7 mM KCl, 5 mM CaCl$_2$, 0.5 mM MgCl$_2$, 0.1% gelatin, pH 7.3). An aliquot of suspension was diluted and plated to determine the starting concentration of bacteria. Ten ml of the bacterial suspension (~10$^9$ cfu/ml) was placed in the nebulizer of a Glas-Col Inhalational Exposure System model 099C A4212 (Glas-Col, Terre Haute, IN). Mice were challenged using this inhalation system with the following settings: 10 min preheat, 40 min nebulization, 30 min cloud decay, 10 min decontamination, vacuum flow meter at 60 cubic feet/h, compressed air flow meter at 10 cubic feet/h. With this system, all mice are challenged simultaneously with the identical number of bacteria. Three hours post-challenge, the mice were euthanized by inhalation of isoflurane. Lungs were then harvested and homogenized on ice in 5 ml PCGM buffer using a tissue homogenizer. Aliquots of 50 μl of undiluted and 1:10 diluted lung homogenate were plated in duplicate and incubated at 35°C in 5% CO$_2$ overnight. Colonies were counted the following day.
The statistical assessment of colony counts was based on a standard analysis of variance (ANOVA) model. In addition to testing for overall group differences, pairwise comparisons between PBS and each of the other three groups were made in conjunction with Dunnett’s adjustment for multiple comparisons performed at a 0.05 family-wise error rate. Standard diagnostic plots were used to assess model fit with no violations of model assumptions observed. Analyses were carried out using SAS version 9.4 statistical software (Cary, NC).

**ELISA with human serum samples.** Serum samples were obtained from adults with COPD who were part of a 20-year prospective, observational study conducted at the Buffalo Veterans Affairs Medical Center that has been described previously (36, 65). The study was approved by the Veterans Affairs Western New York Healthcare System Human Studies Subcommittee and the University at Buffalo Institutional Review Board. Patients with COPD were seen monthly and at times when an exacerbation was suspected. At each visit expectorated sputum samples and blood samples were collected and clinical criteria were used to determine whether patients were experiencing an exacerbation or whether they were clinically stable. An exacerbation strain was defined as a strain of *M. catarrhalis* that was isolated from sputum and that was acquired simultaneous with the onset of symptoms of an exacerbation using previously described methods (65). Nineteen paired pre exacerbation serum samples were obtained 1 to 2 months prior to exacerbation and post exacerbation serum samples were obtained 1 to 2 months...
following the same exacerbation. The serum samples were used to analyze the human antibody response to purified recombinant AfeA. ELISAs were performed using previously described methods (37). Wells were coated with 0.1 µg/ml of purified AfeA and serum samples were assayed at dilutions of 1:4000. These conditions were determined to yield a linear curve between the OD_{450} and serum dilution in preliminary experiments. The pre and post exacerbation serum pairs were always tested in the same assay on the same plate. The per cent change in antibody level from the pre exacerbation to post exacerbation serum samples was calculated with the following formula: \[
\left( \frac{\text{OD of post exacerbation sample} - \text{OD of pre exacerbation sample}}{\text{OD of pre exacerbation sample}} \right) \times 100.
\] A cutoff of 30% for a significant change between pre and post exacerbation was set based on control assays performed in previous studies of 6 proteins (37, 39, 48, 49). In these assays, paired control serum samples obtained 2 months apart (the same time interval used for the experimental samples) from COPD patients whose sputum cultures were negative for *M. catarrhalis* and who were clinically stable and free of exacerbation were assayed and used to determine the cutoff value. **Thermal shift assay.** Thermal shift assays were performed using a Stratagene Mx3005P real-time PCR instrument (Stratagene, La Jolla, CA) as previously described (41, 66, 67) Briefly, purified, recombinant, nonlipidated AfeA was studied at a concentration of 10 µg in a 30-µl volume in buffer (0.01 M tris, 0.15 NaCl, pH 7.4) to which metal salts were added to a final concentration of 1 mM. SYPRO
Orange (Sigma) was added as a fluorescence reporter at a 1:1,000 dilution from its stock solution. The change in fluorescence was monitored using a Cy3 filter, with excitation and emission wavelengths of 545 nM and 568 nM, respectively.

Temperature was raised from 25°C to 98°C in 0.5°C intervals over the course of 45 minutes, and fluorescence readings were taken at each interval. The fluorescence data were plotted and normalized, and the first derivative of the curve was calculated to provide the melting temperatures (T_m) using GraphPad Prism, version 5.0, as previously described (68).

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Table 1. Oligonucleotide primers used in the study

| Name of primer | Experiment | Sequencea |
|----------------|------------|-----------|
| Afe frag1 F1   | Mutant fragment 1 upstream of afeA | TTTAAATAAAAAGCCATACG |
| Afe frag1 R1   | Mutant fragment 1 upstream of afeA | TAGTTAGTCAAATTAACCTAATTGCTTGA |
| Afe frag2 F1   | Mutant fragment 2 kanamycin cassette | AGGTTAAATTTGACTAAGGAGGAATAA |
| Afe frag2 R1   | Mutant fragment 2 kanamycin cassette | AAAATATAACATTATTTCCCTCCAGGTACT |
| Afe frag3 F1   | Mutant fragment 3 downstream of afeA | GGAATATGTATATTATTTATATATTTT |
| Afe frag3 R1   | Mutant fragment 3 downstream of afeA | CCTGCTGGTGTCATGTATCA |
| Afe lipoprotein F1 | Clone AfeA gene | GTACCAGATGGTAGCAGAACACCAAGAGAAGA |
| Afe lipoprotein R1 | Clone AfeA gene | GATCGGATCCCTTTTCAAACGGCCTGCGA |
| AfeA 5         | RT PCR afeA-afeB | GCACTCATTAAGGAAGCC |
| AfeB 3         | RT PCR afeA-afeB | GAGCCAAAGCCCTGCCA |
| AfeB 5         | RT-PCR afeB-afeC | GGGAAGGGCTTTGGCTCAAG |
| AfeC 3         | RT-PCR afeB-afeC | CAGTCACCTGACATATAC |
| AfeC 5         | RT-PCR afeC-afeD | GATATGTATCAGTGACTG |
| AfeD 3         | RT-PCR afeC-afeD | CAATCGTCGACCTGTCGC |
|                      | Amplify *afeA* to assess conservation among strains | ATGAAATCAATCAAAACTTT |
|----------------------|--------------------------------------------------|-----------------------|
| **AfeA F1**          | Amplify *afeA* to assess conservation among strains | TCACCTTTCAAAACCGCTGG |
| **AfeA R1**          | Complement *afe* mutation                         | GATCGGATCCCAATTCATGATTAAGTGTTG |
| **Afe comp F1**      | Complement *afe* mutation                         | GATCGAGCTCGGTCTTTGAACGGTGTGTGTT |
| **Afe comp R1**      | Complement *afe* mutation                         | Afe Pet F1 CACCTGCGGTCTAGCAACAAAGA |
| **Afe PET F1**       | Clone non-lipidated *AfeA* for thermal shift assays | Afe Pet R1 TCACCTTTCAAAACCGCTGG |
| **Afe PET 2**        | Clone non-lipidated *AfeA* for thermal shift assays | Afe Pet R1 TCACCTTTCAAAACCGCTGG |

*Underlines indicate restriction enzyme sites*

*Reverse transcriptase PCR*
Table 2. Melting temperatures and results of thermal shift assays with purified non-lipidated recombinant AfeA.

| Sample                  | Cation     | \(T_m (°C)^a\) | \(\Delta T_m (°C)^b\) |
|-------------------------|------------|-----------------|------------------------|
| Non lipidated AfeA alone| -          | 53.1, 76.7      | -                      |
| + 1mM MgCl\(_2\)       | Mg\(^{++}\) | 53.6, 76.9      | 0.5                    |
| + 1mM MnCl\(_2\)       | Mn\(^{++}\) | 76.4            | 23.3                   |
| + 1 mM ZnCl\(_2\)      | Zn\(^{++}\) | 77.1            | 24.0                   |
| + 1 mM Fe(NO\(_3\))\(_3\) | Fe\(^{+++}\) | 76.8            | 23.7                   |
| + 1 mM FeCl\(_3\)      | Fe\(^{+++}\) | 76.7            | 23.6                   |
| + 1 mM FeCl\(_2\)      | Fe\(^{++}\) | 76.7            | 23.6                   |

\(^a\) \(T_m (°C)\), melting temperature

\(^b\) \(\Delta T_m (°C)\), thermal shift
Figure Legends

**Figure 1.** A. Schematic illustration of the *afe* gene cluster in the *M. catarrhalis* genome. Arrows indicate direction of transcription and numbers indicate size of genes in base pairs (bp). B. Results of reverse transcriptase (RT) PCR with RNA extracted from *M. catarrhalis* 035E to detect *afe* gene cluster transcript in the regions that span genes as noted at the bottom. Lanes: a, PCR product from genomic DNA template; lanes b. RT-PCR reaction in the absence of reverse transcriptase; lanes c, RT-PCR product. DNA standards are noted in kilobases. Bands in lanes c indicate that the gene cluster expresses a single transcript.

**Figure 2.** Ethidium bromide-stained agarose gel showing amplicons of the *afeA* gene amplified from genomic DNA of 20 clinical isolates of *M. catarrhalis*. Lane a, 035E. Lanes b through *j* contain sputum isolates from adults experiencing exacerbations of COPD as follows: b, 6P29B1; c, 10P66B1; d, 14P30B1; e, 39P33B1; f, 47P31B1; g, M2, h, M3; i, M4; j, M5. Lanes k through *t* contain middle ear fluid isolates obtained by tympanocentesis from children experiencing acute otitis media as follows: k, 2015; l, 5193; m, 6955; n, 7169; o, 9483; p, 0701057VIL; q, 0701064V3L; r, 0702076SV4R; s, 0701062V1L; t, 0701067V3L. Molecular size markers are on the left in kilobases.
Figure 3. Left Panel. Lane a: purified AfeA in Coomassie blue-stained sodium dodecyl (SDS) gel. Lane b: purified AfeA in silver stained SDS gel. Center Panel: immunoblot assay with rabbit antiserum to recombinant purified AfeA (1:10^6 dilution). Right Panel immunoblot assay with rabbit antiserum to recombinant purified BCAA SBP1 (branched chain amino acid substrate binding protein 1). Lanes c: whole cell lysate of wild type strain O35E, lanes d: whole cell lysate of afe knockout mutant, lanes e: whole cell lysate of afe complemented mutant. Molecular mass markers are shown in kilodaltons. Arrows denote AfeA.

Figure 4. Immunoblot assay of whole cell lysates of 9 clinical isolates of *M. catarrhalis* probed with rabbit antiserum to recombinant purified AfeA (1:10^6 dilution). Strains are sputum isolates in lanes as follows: a, 6P29B1; b, 10P66B1; c, 14P30B1; d, 39P33B1; e, 47P31B1; f, M2, g, M3; h, M4; i, M5; Molecular mass markers are shown in kilodaltons on the left.

Figure 5. Results of whole cell ELISA with *M. catarrhalis* strain O35E, afe knockout mutant, and complemented afe mutant coated onto wells and assayed with antisera as noted. X-axes are serum dilutions and Y-axes are optical density at 450 nm. Results are shown with pre-immune and immune antisera. A. AfeA antiserum with WT and afe knockout mutant. B. AfeA antiserum with complemented afe mutant. C. OppA antiserum with WT and afe knockout mutant (positive control- OppA is a surface protein). D. OppA antiserum with complemented afe mutant. E. BCAA antiserum with WT and afe knockout mutant (negative control- BCAA is a non
Figure 6. Results of flow cytometry with *M. catarrhalis* wild-type (WT) 035E, *afe* knockout mutant and complemented *afe* mutant. Error bars indicate the standard deviation of three independent experiments.

A. WT strain 035E, *afe* knockout mutant and complemented *afe* mutant assayed with AfeA antiserum (1:100) and preimmune serum (1:100). B. WT strain 035E and *afeA* knockout mutant assayed with OppA antiserum (1:100) and preimmune serum (1:100) (positive control- OppA is a surface protein). C. WT strain 035E and *afeA* knockout mutant assayed with BCAA antiserum (1:100) and preimmune serum (1:100) (negative control- BCAA is a non surface protein).

Figure 7. A. Immunoblot assays with sera (1:2,000) pooled from mice immunized with PBS (negative control), purified recombinant AfeA (25 μg and 50 μg schedules as noted), and whole cells of *M. catarrhalis* 035E. Lanes contain whole bacterial cell lysate of lane a, *M. catarrhalis* strain 035E and lane b, *afe* knockout mutant. Arrows denote AfeA. Molecular mass markers are shown in kilodaltons on the left. B. Results of pulmonary clearance three hours after aerosol challenge with *M. catarrhalis* 035E following immunization of groups of mice with PBS (negative control), recombinant AfeA, and whole cells of *M. catarrhalis* strain 035E (positive control). Y-axis is colony count (colony forming units/ml) in lung homogenates. Error bars represent the standard deviation (n=6). Statistically significant overall group differences were observed with a p-value of 0.0003. Results of pairwise
comparisons with the PBS group (negative control) and associated p-values are shown.

**Figure 8.** Results of ELISA to purified AfeA with 19 pairs of pre exacerbation and post exacerbation serum samples (1:4,000) from adults with COPD followed longitudinally. X-axis shows results from individual patients. Y-axis shows % change in optical density from pre exacerbation to post exacerbation value. Dotted line represents the cutoff for a significant change based on assays with control serum pairs (see text).
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A.  

\[ afeA \rightarrow afeB \rightarrow afeC \rightarrow afeD \]

927 bp SBP  
906 bp ATPase  
843 bp permease  
337 bp permease

B.  

[Image of gel electrophoresis showing bands corresponding to the genes mentioned]
A. AfeA antiserum

B. OppA antiserum

C. BCAA antiserum

Cell count vs. fluorescence for prebleed, wild type, immune serum, afe knockout mutant, and complemented mutant.
