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VACCINES

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 on 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 chronic obstructive pulmonary disease (COPD). Based on these observations, AfeA is an excellent vaccine antigen to be included in a vaccine to prevent infections caused by *M. catarrhalis*.

KEYWORDS ABC transporters, *Moraxella catarrhalis*, immunization, otitis media, pulmonary infection, surface antigens, vaccines

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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 copathogen.

Approximately 80% of children experience an episode of otitis media by the age of 3 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 of antibiotic resistance in bacteria (9, 10). A vaccine to prevent otitis media would have an enormous benefit in preventing global morbidity, reducing health care

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 in adults and is the fourth most common cause of death in the United States 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 the 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% 1-year mortality rate (30, 31). Thus, one of the most urgent areas of research to impact patients with COPD is the development of approaches to prevent 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 fluid samples. However, an increasing understanding of the role of biofilms in otitis media has revealed that reliance on culture alone detects pathogens in only a subset of episodes in otitis media (32). When middle-ear fluid samples 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* spp., 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 United States (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 prevention of 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 (i) is highly conserved among strains, (ii) induces a high-titer antibody that recognizes native protein following immunization with recombinant purified protein, (iii) expresses abundant epitopes on the bacterial surface, (iv) induces protective responses in the mouse pulmonary clearance model following aerosol challenge with *M. catarrhalis*, (v) is expressed during human respiratory tract infection, and (vi) 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 to AX067466) to identify

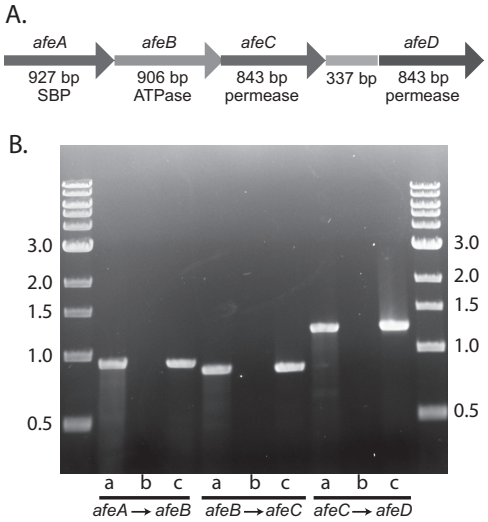

FIG 1 (A) Schematic illustration of the *afe* gene cluster in the *M. catarrhalis* genome. Arrows indicate direction of transcription, and numbers indicate size of genes in bp. (B) Results of reverse transcriptase PCR (RT-PCR) with RNA extracted from *M. catarrhalis* O35E to detect *afe* gene cluster transcript in the regions that span genes as noted at the bottom. a lanes, PCR product from genomic DNA template; b lanes, RT-PCR in the absence of reverse transcriptase; c lanes, RT-PCR product. DNA standards are noted in kilobases. Bands in c lanes indicate that the gene cluster expresses a single transcript.

open reading frames (ORFs) predicted to be exposed on the bacterial cell surface (37). Of 348 ORFs predicted to be surface exposed, 14 ORFs had homology to substrate binding proteins (SBPs) of the ABC transporter systems. This observation led to an extensive evaluation of the 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 the genes in strain BBH18 (GenBank accession number 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 identification [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) (Fig. 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 (Fig. 1B, b lanes). Figure 1B shows that the genes of the *afeABCD* gene cluster are transcribed as a single transcript (Fig. 1B, c lanes).

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

TABLE 1 Oligonucleotide primers used in the study

Name of primer	Expt[a]	Sequence[b]
Afe frag1 F1	Mutant fragment 1 upstream of *afeA*	TTTAAATAAAAAGCCATACG
Afe frag1 R1	Mutant fragment 1 upstream of *afeA*	TAGTTAGTCAAAATTAACCTAATTGCTTGA
Afe frag2 F1	Mutant fragment 2 kanamycin cassette	AGGTTAATTTTGACTAACTAGGAGGAATAA
Afe frag2 R1	Mutant fragment 2 kanamycin cassette	AAAAATATAACATTATTCCCTCCAGGTACT
Afe frag3 F1	Mutant fragment 3 downstream of *afeA*	GGGAATAATGTTATATTTTTAATATATTTT
Afe frag3 R1	Mutant fragment 3 downstream of *afeA*	CCTGCTGGTGTCATGTATCA
Afe lipoprotein F1	Clone AfeA gene	GTACCCATGGAGGTCAGCAGACCAAAGAAGA
Afe lipoprotein R1	Clone AfeA gene	GATCGGATCCCTTTTCAAAACCGCTGGCGA
AfeA 5	RT-PCR *afeA-afeB*	GCACTCATTAAGCAAGACCC
AfeB 3	RT-PCR *afeA-afeB*	GAGCCAAAGCCCTTGCCA
AfeB 5	RT-PCR *afeB-afeC*	GGCAAGGGCTTTGGCTCAAG
AfeC 3	RT-PCR *afeB-afeC*	CAGTCACTTGATACATATC
AfeC 5	RT-PCR *afeC-afeD*	GATATGTATCAAGTGACTG
AfeD 3	RT-PCR *afeC-afeD*	CAATCGTCGCACCTGTCGC
AfeA F1	Amplify *afeA* to assess conservation among strains	ATGAAATCAATCAAAACTTT
AfeA R1	Amplify *afeA* to assess conservation among strains	TCACTTTTCAAAACCGCTGG
Afe comp F1	Complement *afe* mutation	GATCGGATCCCAATTCATGATTAAGTGGTG
Afe comp R1	Complement *afe* mutation	GATCGAGCTCGGTCTTGAACGGTGTTTGTT
Afe PET F1	Clone nonlipidated AfeA for thermal shift assays	CACCTGCGGTCAGCAGACCAAAGA
Afe PET 2	Clone nonlipidated AfeA for thermal shift assays	TCACTTTTCAAAACCGCTGG

[a]Reverse transcriptase PCR.
[b]Underlined sequences indicate restriction enzyme sites.

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 homologies. 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 the template in a PCR 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, a size identical to that of strain O35E, was detected in all 20 strains (Fig. 2). A negative control in which the 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 genomes of all clinical isolates of *M. catarrhalis* tested to date and that the gene is highly conserved among strains.

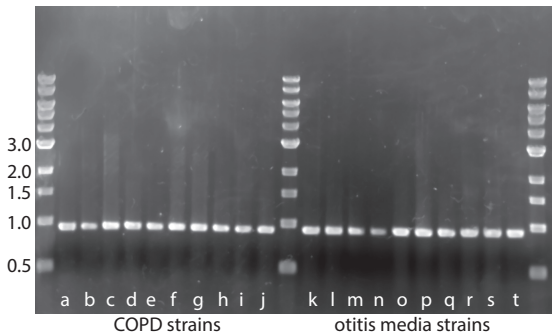

FIG 2 Ethidium bromide-stained agarose gel showing amplicons of the *afeA* gene amplified from genomic DNA of 20 clinical isolates of *M. catarrhalis*. Lane a, O35E; lanes b to j, sputum isolates from adults experiencing exacerbations of COPD (lane b, 6P29B1; lane c, 10P66B1; lane d, 14P30B1; lane e, 39P33B1; lane f, 47P31B1; lane g, M2; lane h, M3; lane i, M4; lane j, M5); lanes k to t, middle-ear fluid isolates obtained by tympanocentesis from children experiencing acute otitis media (lane k, 2015; lane l, 5193; lane m, 6955; lane n, 7169; lane o, 9483; lane p, 0701057V1L; lane q, 0701064V3L; lane r, 0702076SV4R; lane s, 0701062V1L; lane t, 0701067V3L). Molecular size markers are on the left in kilobases.

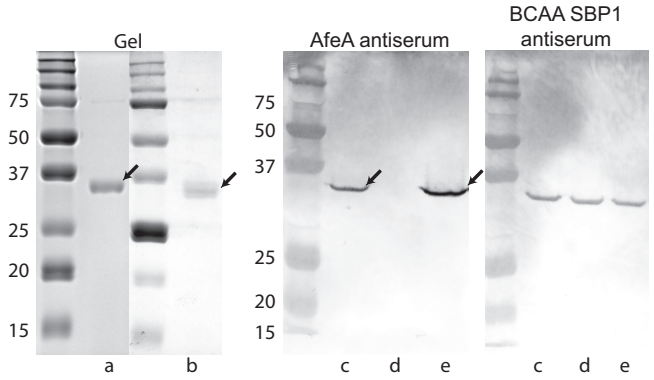

FIG 3 Left, lane a, purified AfeA in Coomassie blue-stained sodium dodecyl (SDS) gel; lane b, purified AfeA in silver-stained SDS gel. Center, immunoblot assay with rabbit antiserum to recombinant purified AfeA ($1{:}10^6$ dilution). Right, immunoblot assay with rabbit antiserum to recombinant purified BCAA SBP1 (branched-chain amino acid substrate binding protein 1). c lanes, whole-cell lysate of wild-type strain O35E; d lanes, whole-cell lysate of *afe* knockout mutant; e lanes, whole-cell lysate of *afe* complemented mutant. Molecular weight markers are shown in kilodaltons. Arrows denote AfeA.

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 *Escherichia 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 (Fig. 3).

Characterization of *afe* knockout mutant. An isogenic *afe* knockout mutant in strain O35E 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 O35E (Fig. 3, c lanes), 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 (Fig. 3, d lanes). The complemented *afe* mutant expressed AfeA, as expected (Fig. 3, e lanes). The wild type (WT), *afe* knockout mutant, and complemented mutant all expressed branched-chain amino acid (BCAA) substrate binding protein, a control result indicating that the expression of an unrelated substrate binding protein was unaffected by the genetic manipulations used to engineer and complement the *afeA* knockout mutant (Fig. 3, right).

Expression of AfeA by *M. catarrhalis*. Rabbit antiserum raised to purified recombinant AfeA was used in immunoblot assay to assess the 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 (Fig. 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 enzyme-linked immunosorbent assays (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 the outer membrane

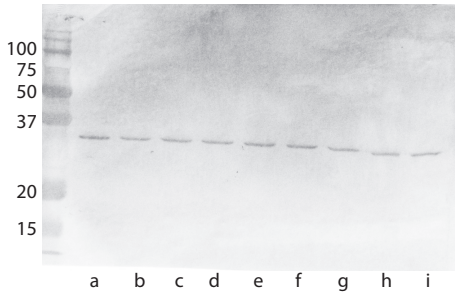

FIG 4 Immunoblot assay of whole-cell lysates of 9 clinical isolates of *M. catarrhalis* probed with rabbit antiserum to recombinant purified AfeA (1:10^6 dilution). Strains are sputum isolates in lanes as follows: lane a, 6P29B1; lane b, 10P66B1; lane c, 14P30B1; lane d, 39P33B1; lane e, 47P31B1; lane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

protein (OMP) OppA was used as a positive control (surface protein), and antiserum to the BCAA SBP1 was used as a negative control (nonsurface protein). The anti-AfeA antibody bound to the WT strain but not to the *afe* knockout mutant (Fig. 5A), while anti-OppA antibodies (positive control) bound to both strains (Fig. 5C), and anti-BCAA antibodies (negative control) bound to neither strain (Fig. 5E). The complemented *afe* mutant expressed AfeA on the bacterial surface; interestingly, the complemented *afe* mutant appears to express more surface-exposed AfeA than the WT, based on the higher optical density (OD) observed with the complemented mutant (Fig. 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 the WT, *afe* knockout mutant, and complemented mutant strains using antiserum to AfeA. Antibodies to AfeA demonstrate an increase in mean fluorescence intensity from preimmune to immune serum, as indicated by a distinct shift of the curve to the right (Fig. 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. An assay of the complemented *afe* mutant showed partial restoration of activity (Fig. 6A, right). Antiserum to OppA (surface protein as a positive control) showed binding to both the wild type and *afe* mutant (Fig. 6B), and antiserum to BCAA (nonsurface protein as a negative control) showed no binding to either strain (Fig. 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 (Fig. 7A). To assess the effect of immunization with AfeA in the induction of protective immune responses, we assessed bacterial clearance 3 h 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 the controls (Fig. 7B). Statistically significant overall group differences were observed, with a *P* value of 0.0003. Pairwise comparisons between phosphate-buffered saline (PBS) with 25 μg, 50 μg, and whole organisms (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 Fig. 7B was repeated and yielded an identical result of enhanced clearance by approximately 0.5 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,

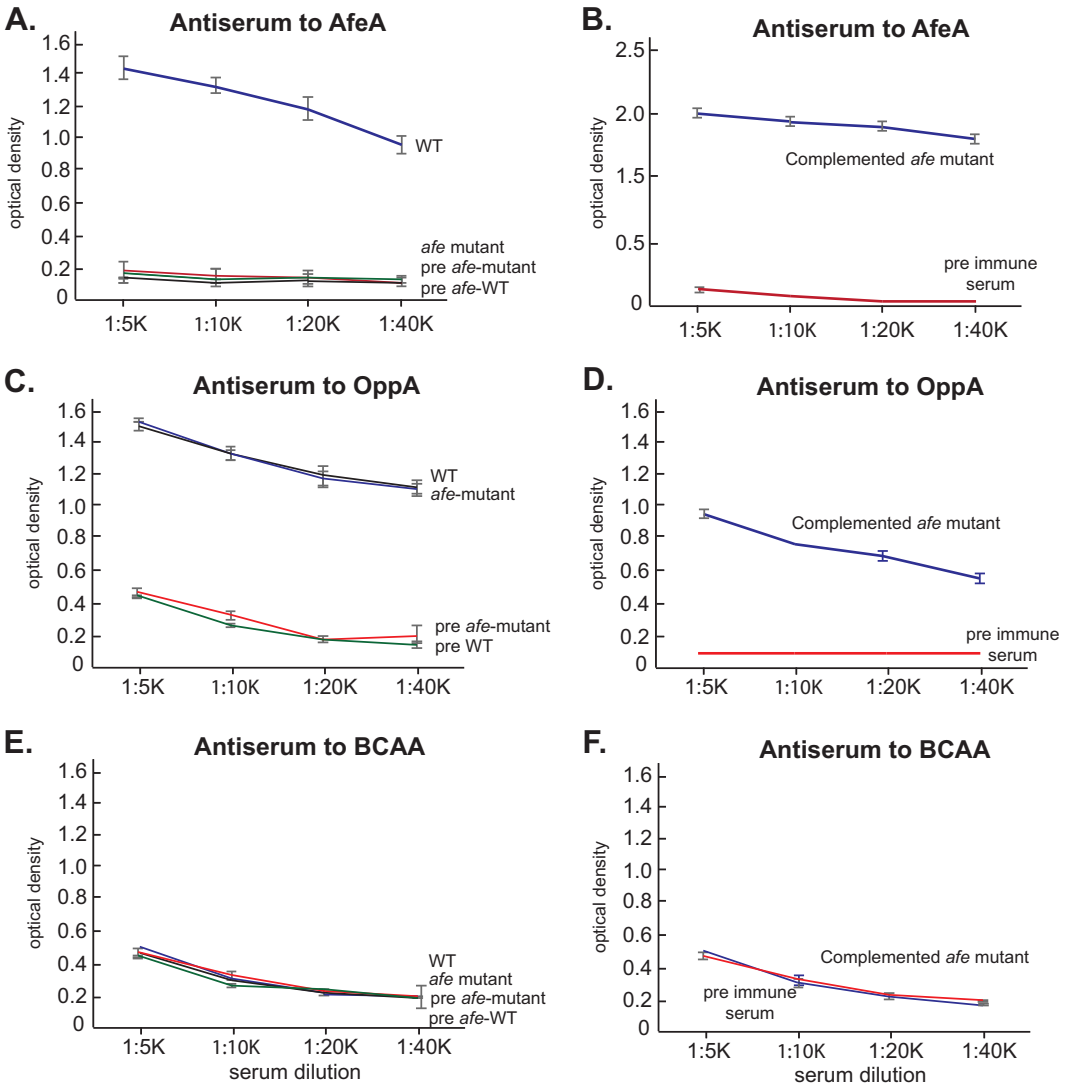

FIG 5 Results of whole-cell ELISA with *M. catarrhalis* strain O35E, *afe* knockout mutant, and complemented *afe* mutant used to coat wells and assayed with antisera as noted. *x* axes are serum dilutions, and *y* axes are optical density at 450 nm. Results are shown with preimmune and immune antisera. (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 nonsurface protein). (F) BCAA antiserum with complemented *afe* mutant. Error bars indicate the standard deviations of the results from three independent experiments.

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 preexacerbation serum samples obtained 1 to 2 months before the patient acquired the infecting strain and postexacerbation samples from 1 to 2 months following infection. Two of 19 patients (10.5%) developed new antibody responses to AfeA following infection (Fig. 8). This proportion of patients developing new serum antibody responses is similar to what we have observed in 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 thermal shift assays to test the hypothesis that AfeA binds cations (43, 50). Initial experiments with purified

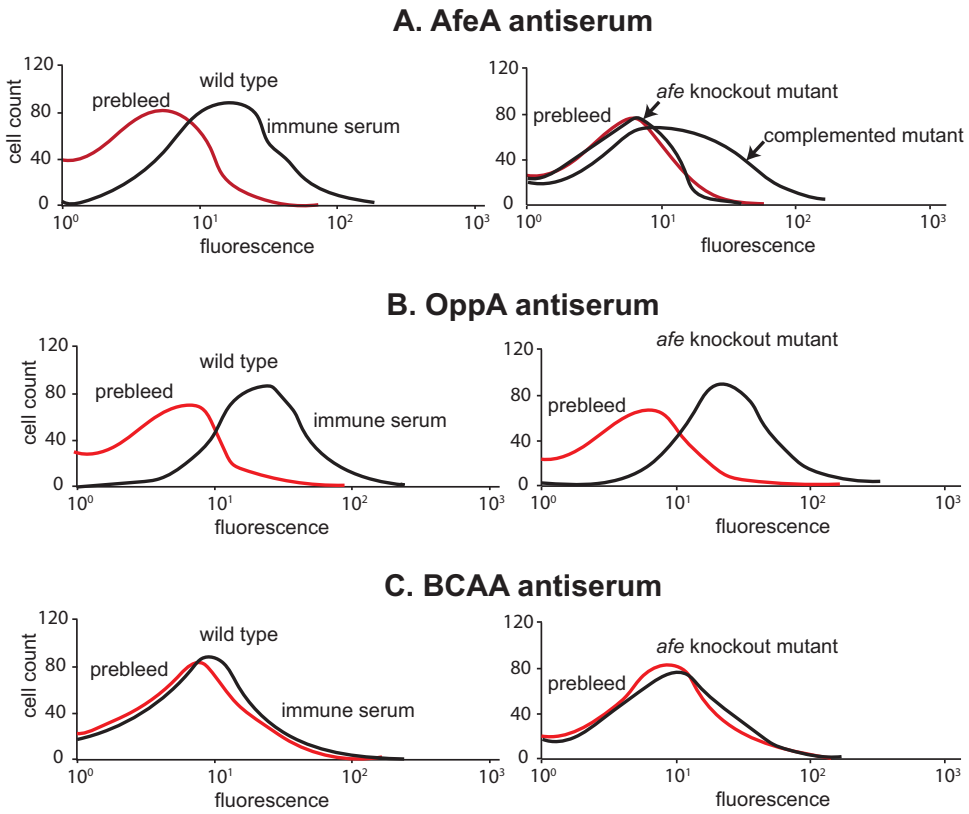

FIG 6 Results of flow cytometry with *M. catarrhalis* wild-type (WT) O35E, *afe* knockout mutant, and complemented *afe* mutant. *x* axes are fluorescence, and *y* axes are cell counts. (A) WT strain O35E, *afe* knockout mutant, and complemented *afe* mutant assayed with AfeA antiserum (1:100) and preimmune serum (1:100). (B) WT strain O35E 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 O35E and *afeA* knockout mutant assayed with BCAA antiserum (1:100) and preimmune serum (1:100) (negative control; BCAA is a nonsurface protein).

recombinant lipidated AfeA failed to produce sharp melting temperatures (T_ms), indicating that the protein did not form a stable conformation under the conditions of the assay, in spite of testing under several buffer conditions. Therefore, we engineered a construct that expressed nonlipidated AfeA and purified the protein using buffers to which the cation-chelating resin Chelex 100 (Sigma) had been added. This approach resulted in sharp biphasic melting curves, indicating that the protein formed stable conformations and that some of the protein was bound by a ligand while some was not (Table 2).

The addition of selected metals resulted in a 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 ions 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 an ~32-kDa protein with several characteristics that predict that it is a potentially effective vaccine: (i) the *afeA* gene is present in all strains of *M. catarrhalis*; (ii) it is highly conserved among clinical isolates that cause otitis media and infection in COPD; (iii) AfeA expresses abundant epitopes on the bacterial surface that are accessible to potentially protective antibodies; (iv) the protein is highly immunogenic and induces

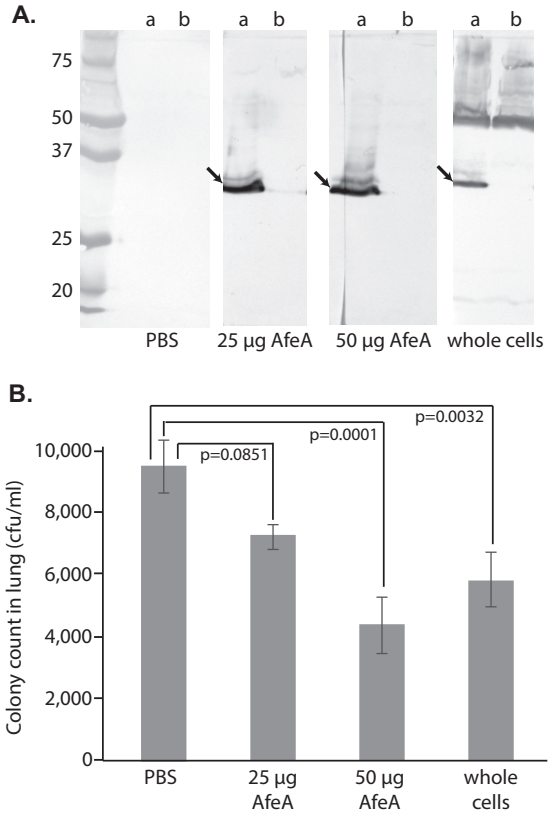

FIG 7 (A) Immunoblot assays with sera (1:2,000) pooled from mice immunized with PBS (negative control), purified recombinant AfeA (25-μg and 50-μg schedules as noted), and whole cells of *M. catarrhalis* O35E. a lanes, whole bacterial cell lysate of wild-type strain O35E; b lanes, whole-cell lysate of *afe* knockout mutant. Arrows denote AfeA. Molecular size markers are shown in kilodaltons on the left. (B) Results of pulmonary clearance 3 h after aerosol challenge with *M. catarrhalis* O35E following immunization of groups of mice with PBS (negative control), recombinant AfeA, and whole cells of *M. catarrhalis* strain O35E (positive control). *y* axis is colony count (in CFU per milliliter) in lung homogenates. Error bars represent the standard deviations ($n = 6$). 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.

protective immune responses in the mouse following aerosol challenge with *M. catarrhalis*; and (v) 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

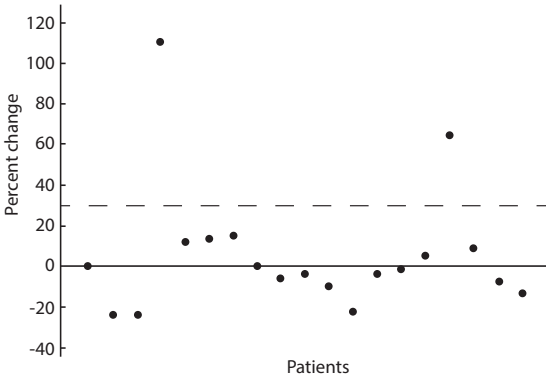

FIG 8 Results of ELISA to purified AfeA with 19 pairs of preexacerbation and postexacerbation serum samples (1:4,000) from adults with COPD followed longitudinally. *x* axis shows results from individual patients. *y* axis shows % change in optical density from preexacerbation to postexacerbation values. Dotted line represents the cutoff for a significant change based on assays with control serum pairs (see the text).

TABLE 2 Melting temperatures and results of thermal shift assays with purified nonlipidated recombinant AfeA

Sample	Cation	T_m (°C)[a]	ΔT_m (°C)[b]
Nonlipidated AfeA alone		53.1, 76.7	
+1 mM $MgCl_2$	Mg^{2+}	53.6, 76.9	0.5
+1 mM $MnCl_2$	Mn^{2+}	76.4	23.3
+1 mM $ZnCl_2$	Zn^{2+}	77.1	24.0
+1 mM $Fe(NO3)_3$	Fe^{3+}	76.8	23.7
+1 mM $FeCl_3$	Fe^{3+}	76.7	23.6
+1 mM $FeCl_2$	Fe^{2+}	76.7	23.6

[a]T_m, melting temperature. Two values indicate peaks at two temperatures due to trace cations in buffers.
[b]ΔT_m, thermal shift.

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: (i) whole-cell ELISA with antiserum to AfeA (Fig. 5), (ii) flow cytometry with antiserum to AfeA (Fig. 6), and (iii) induction of potentially protective immune responses by AfeA in the mouse pulmonary clearance model (Fig. 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 a reduced growth rate in chemically defined media compared to the wild type and also showed a 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* (Fig. 8). This rate is similar to those of several other conserved surface proteins of *M. catarrhalis* that are under consideration as vaccine antigens (37, 48, 49). 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-*H. influenzae* type b (pre-Hib) conjugate vaccine era, infants who recovered from Hib 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 burgdorferi* 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 the assessment of 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 O35E was provided by Eric Hansen. Strains 2015, 5193, 6955, 7169, 9483, 0701057V1L, 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% CO_2 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 O35E was isolated with the Qiagen RNeasy minikit (Qiagen, Valencia, CA), and DNA contamination was removed with the RQ1 RNase-free DNase kit (Promega).

RT-PCR. Reverse transcriptase PCR (RT-PCR) was performed with the Qiagen OneStep 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 the 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 NcoI and BamHI 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 and 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 the growth of 50 ml of culture in LB broth with 50 μg of kanamycin to an optical density at 600 nm (OD_{600}) of 0.6, AfeA expression was induced with 4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. The bacteria were then harvested by centrifugation at 4,000 $\times$ *g* for 15 min 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$\times$ ProteaseArrest [pH 7.4]) and mixed with a nutator for 30 min at room temperature. The suspension was then sonicated on ice with a Branson sonifier S-450 at setting 6, using an 80% pulsed cycle of four 30-s bursts with 2-min pauses. The sonicated bacterial lysate was centrifuged at 10,000 $\times$ *g* for 20 min at 4°C. The pellet was suspended in 5 ml of urea lysis buffer containing 0.05 M NaH_2PO_4, 0.01 M Tris (pH 8), 6 M urea, 0.1 M NaCl (pH 7.5), and 25 μl of ProteaseArrest and mixed on a nutator for 20 min until the lysate became clear.

AfeA was purified by affinity chromatography using BD Talon resin (BD Biosciences, Palo Alto, CA) through the hexahistidine 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 750 $\times$ *g* for 5 min at 4°C, suspended in urea lysis buffer, incubated for 10 min, and centrifuged again. The resin was suspended in 5 ml of cleared bacterial lysate and mixed by nutation for 30 min at room temperature. The suspension was centrifuged at 750 $\times$ *g* for 5 min at 4°C. The resin, containing bound protein, was washed in urea lysis buffer twice for 10 min. 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 min 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 using the modified Lowry method (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-terminally linked lipoprotein for thermal shift assays, the *afeA* gene encoding the mature AfeA protein was amplified by PCR from genomic DNA of strain O35E using the 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 of 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 of 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 (fragment 3). 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 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 the 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 OD_{600} 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 an immunoblot assay with an 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, a 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 OD_{600} 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 Immulon MicroTiter 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 h at room temperature, after which the wells were washed 3 times with PBST. Paired rabbit antisera (preimmune and immune) were diluted 1:5,000, 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 h 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, and 3% heat-inactivated goat serum was added. After another 1 h of incubation at room temperature, the wells were washed 3 times with PBST, and color-developing reagent was added. The reaction was allowed to proceed for 15 min and was stopped with the addition of 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. The 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 of 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_2PO_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 the bacteria. Ten milliliters 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 of preheat, 40 min of nebulization, 30 min of cloud decay, 10 min of decontamination, vacuum flow meter at 60 cubic feet/h, and compressed airflow meter at 10 cubic feet/h. With this system, all mice are challenged simultaneously with an identical number of bacteria.

Three hours postchallenge, the mice were euthanized by inhalation of isoflurane. Lungs were then harvested and homogenized on ice in 5 ml of PCGM buffer using a tissue homogenizer. Aliquots of 50 μl of lung homogenate undiluted and diluted 1:10 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 preexacerbation serum samples were obtained 1 to 2 months prior to exacerbation, and postexacerbation 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 purified AfeA, and serum samples were assayed at a dilution of 1:4,000. These conditions were determined to yield a linear curve between the OD_{450} and serum dilution in preliminary experiments. The pre- and postexacerbation serum pairs were always tested in the same assay on the same plate. The percent change in antibody level from the preexacerbation to postexacerbation serum samples was calculated with the following formula: [(OD of postexacerbation sample − OD of preexacerbation sample)/OD of preexacerbation sample] × 100. A cutoff of 30% for a significant change between pre- and postexacerbation 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. The temperature was raised from 25°C to 98°C in 0.5°C intervals over the course of 45 min, 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_ms) using GraphPad Prism, version 5.0, as previously described (68).

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