

## Characterization and Evaluation of the *Moraxella catarrhalis* Oligopeptide Permease A as a Mucosal Vaccine Antigen<sup>∇</sup>

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***Moraxella catarrhalis* is a common cause of otitis media in children and of lower respiratory tract infections in adults with chronic obstructive pulmonary disease; therefore, these two groups would benefit from a vaccine to prevent *M. catarrhalis* infections. A genome mining approach for vaccine antigens identified oligopeptide permease protein A (OppA), an oligopeptide binding protein of an apparent oligopeptide transport system. Analysis of the *oppA* gene by PCR and sequence analysis revealed that OppA is highly conserved among clinical isolates of *M. catarrhalis*. Recombinant OppA was expressed as a lipoprotein and purified, and an *oppA* knockout mutant was constructed. Antiserum raised to recombinant purified OppA recognized epitopes on the bacterial surface of the wild type but not the OppA knockout mutant. Antibodies raised to purified recombinant OppA recognized native OppA in multiple strains. Intranasal immunization of mice induced systemic and mucosal antibodies to OppA and resulted in enhanced clearance of *M. catarrhalis* in a mouse pulmonary clearance model. OppA is a highly conserved, immunogenic protein that expresses epitopes on the bacterial surface and that induces potentially protective immune responses in a mouse model. OppA should be evaluated further as a vaccine antigen for *M. catarrhalis*.**

*Moraxella catarrhalis* is a Gram-negative diplococcus frequently found as a commensal of the upper respiratory tract (14, 19). However, over the past 2 to 3 decades this bacterium has shifted from being considered a harmless commensal to being recognized as a genuine respiratory tract pathogen of serious public health concern (34, 54).

Acute otitis media is the most common bacterial infection in children, with 70% experiencing at least one episode by age 3 (53). *M. catarrhalis* is the third leading cause of otitis media after *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (21, 34). *M. catarrhalis* is associated with up to 25% of acute otitis media cases by culture (37) and 46.4% of chronic middle ear effusion cases by PCR (41). In addition, the nasopharyngeal carriage rate of *M. catarrhalis* in children is high (up to 75%) and the frequency of colonization is positively related to the development of otitis media (14).

In adults, *M. catarrhalis* is the second most common bacterial cause of exacerbations of chronic obstructive pulmonary disease (COPD) after nontypeable *H. influenzae* (33, 48, 49). COPD is the fourth leading cause of death in the United States, affecting 24 million Americans (25, 26). *M. catarrhalis* causes approximately 10% of exacerbations of COPD, accounting for 2 to 4 million episodes annually (33). Furthermore, *M. catarrhalis* also colonizes the lower respiratory tract in up to 2.5 to 10% of adults with COPD at their stable states (31, 59). Lower airway colonization contributes to airway inflammation in COPD as a result of sloughing of highly inflammatory bacterial cell wall antigens into the airway (49).

The significant clinical implications of *M. catarrhalis* require a vaccine targeting both otitis media in children and exacerbations in adults with COPD. *M. catarrhalis* is a nonencapsulated bacterium and does not secrete exotoxin. The current vaccine studies have mostly focused on various outer membrane proteins (OMPs) as vaccine candidates. To date, a limited number of OMPs have been examined and are currently under different stages of evaluation as part of an effort to develop a multicomponent vaccine against *M. catarrhalis* (27, 32, 51). To search for more vaccine candidates in an efficient way, a genome mining approach generated a pool of 348 open reading frames (ORFs) as potential surface proteins (44). OppA protein is one ORF thereby identified and is further investigated in this study.

Oligopeptide permease A (OppA) is the oligopeptide binding protein of the oligopeptide transport system, composed of five subunits including OppA, OppB, OppC, OppD, and OppF. They belong to the ATP-binding cassette (ABC) family of transporters and transport peptides coupled with energy derived from ATP hydrolysis. In many Gram-negative bacteria, OppAs are thought to be soluble periplasmic proteins while the other subunits form the membrane complex (30). Bacterial OppAs are capable of binding a wide variety of peptides and play a key role in nutrient peptide uptake and muropeptide recycling (36). OppAs are also involved in a number of other functions of bacterial physiology, including competence and sporulation (4, 22, 45), biofilm formation (23), adherence to host cells (12, 20), growth and intracellular survival (7), modulation of the expression of virulence factors (56), and antibiotic resistance (1).

ABC transporters have been recognized as important targets for the development of antibacterial vaccines and therapies based on their roles in bacterial virulence and antigenic com-

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TABLE 1. Oligonucleotide primer sequences

Primer	Gene <sup>b</sup>	Expt	Direction	Sequence <sup>a</sup>
P1	<i>oppA</i>	PCR/sequencing	Forward	5' ATGAAAAAACGAAGTTATTGTC 3'
P2	<i>oppA</i>	PCR	Reverse	5' TCAATTCGCTGTTGTCGTATC 3'
P3	<i>oppA</i>	RT-PCR	Forward	5' AATCAGGCATCGGTCATCTC 3'
P4	<i>oppA</i>	RT-PCR	Reverse	5' TCAGTCGTGATAGGCTGTGC 3'
P5	<i>oppA</i>	Sequencing	Forward	5' GGGATGCTGACAATGTTC 3'
P6	<i>oppA</i>	Sequencing	Forward	5' TGTCAATTGAAACCGAGACC 3'
P7	<i>oppA</i>	Cloning	Forward	5' ATCGCCATGGAAGCAATAATAGCACGACA 3'
P8	<i>oppA</i>	Cloning	Reverse	5' GGTCGGATCCATTTCGCTGTTGTCGTATC 3'
P9	F1/ <i>oppA</i> USG	Overlap extension PCR/colony PCR/sequencing	Forward	5' TCTGACACGCTATCCTCACGAA 3'
P10	F1/ <i>oppA</i> USG	Overlap extension PCR/colony PCR	Reverse	5' <b>TAGTTAGTCACTTGTGATGCTGTCGTGCTATTAT</b> 3'
P11	F2/ <i>aphA</i> -3	Overlap extension PCR/colony PCR	Forward	5' <b>GCATCACAAAGTACTAAGTACTAGGAGGAATAAATGG</b> 3'
P12	F2/ <i>aphA</i> -3	Overlap extension PCR/colony PCR	Reverse	5' <b>GCCATGCTTGACTTATTCCTCCAGGTTACTAAAAC</b> 3'
P13	F3/ <i>oppA</i> DSG	Overlap extension PCR/colony PCR	Forward	5' <b>GGGAATAATGCAAGCATGGCAAAGTGAAAAATCG</b> 3'
P14	F3/ <i>oppA</i> DSG	Overlap extension PCR/colony PCR	Reverse	5' CACAAGCCTTCTGGTGATT 3'
P15	<i>oppA</i>	Colony PCR	Forward	5' AAGACTTTGGGCAAATGGTG 3'
P16	<i>oppA</i>	Colony PCR	Reverse	5' TCAGTCGTGATAGGCTGTGC 3'
P17	<i>oppA</i> USG/F1	Sequencing	Forward	5' AAGGAGAAGTAGCAAGGAGG 3'
P18	<i>aphA</i> -3/F2	Sequencing	Forward	5' GAAGATGAACAAAGCCCTG 3'
P19	<i>oppA</i> DSG/F3	Sequencing	Forward	5' AACTTTTACCGCCTTGG 3'

<sup>a</sup> Restriction enzyme sites are underlined. Overlapping regions are shown in bold. Extended sequences complementary to the end of the adjacent gene are shown in italics.

<sup>b</sup> USG, upstream gene; DSG, downstream gene.

position (16). In fact, OppA acts as a natural immunogen of *Borrelia burgdorferi* during infection in mouse, rabbit, and human (10, 35). More importantly, immunization of mice with OppA induces protection from *Yersinia pestis* infection (52). OppA is therefore an attractive vaccine candidate. Its potential as a vaccine antigen against *M. catarrhalis* was evaluated here in terms of sequence conservation, immunogenicity, exposure of epitopes on the bacterial surface, and induction of potentially protective immune responses.

#### MATERIALS AND METHODS

**Bacterial strains and growth.** *Moraxella catarrhalis* strain ATCC 43617 was obtained from the American Type Culture Collection (Manassas, VA). Isolate 035E was provided by Eric Hansen. Strains 5P34B1, 10P58B2, 12P15B1, 14P25B1, 29P24B1, 33P25B2, 39P29B2, 55P18B3, 63P62B1, and 96P9B1 were sputum isolates obtained from adults with COPD. Strains 135, 238, 555, 2901, 3614, 3584, 5488, 8184, 9483, and 6952 were middle ear fluid isolates obtained via tympanocentesis. 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<sub>2</sub> 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.

**Nucleotide sequence analysis.** *M. catarrhalis* genes encoding putative surface proteins, including lipoproteins and outer membrane proteins with characteristic signal sequences, were identified based on the genome sequence of strain ATCC 43617 using a genome mining approach as described previously (44). The *oppA* gene was defined by homolog analysis using a BLAST search at the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences of *oppA* genes amplified from 10 clinical isolates of *M. catarrhalis* and the ATCC 43617 strain were determined at the Roswell Park Cancer Institute (RPCI) DNA sequencing facility with sequencing primers P1, P5, and P6 listed in Table 1. These sequences were assembled with the Sequencher program and aligned with the ClustalW alignment function of the MacVector program.

**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. The total RNA of *M. catarrhalis* 035E was isolated with the Qiagen RNeasy minikit (Qiagen, Valencia, CA) followed by DNase I treatment by using RQ1 RNase-free DNase I from Promega

and finally cleaned up with the Qiagen RNeasy minikit according to the protocols provided with the kits.

**RT-PCR.** Reverse transcriptase PCR (RT-PCR) was performed using the Superscript II reverse transcriptase from Invitrogen (Carlsbad, CA). The first-strand cDNA was synthesized with 2 pmol of the reverse primer P3 (Table 1) and 1.5 µg of total RNA extracted from the *M. catarrhalis* 035E strain. A 10% cDNA reaction mixture was then used in the second-step PCR with both forward (P2) and reverse (P3) primers (Table 1) and Hotmaster mix (5 Prime Inc., Gaithersburg, MD) to amplify a 223-bp fragment of the *oppA* gene. A parallel reaction without reverse transcriptase was performed as a negative control to exclude amplification from contaminating DNA. One hundred nanograms of genomic DNA extracted from the *M. catarrhalis* 035E strain was used in PCR as a positive control.

**Overlap extension PCR.** Overlap extension PCR is a PCR-based strategy to create chimeric genes by fusion of independent fragments in the absence of restriction sites (50). We utilized overlap extension PCR to generate the linear double-stranded DNA for *oppA* mutant construction via homologous recombination. The transforming DNA for an *oppA* mutant was composed of three fragments: F1/*oppA*USG (*oppA* upstream gene sequence), F2/*aphA*-3 gene (kanamycin resistance gene), and F3/*oppA*DSG (*oppA* downstream gene sequence). The locations of these three fragments in relation to the *oppA* gene are depicted in Fig. 1A. Primers (P9 through P14) (Table 1) for each fragment were designed to include a 10-nucleotide (nt) 5' extension with sequence complementary to the end of the adjacent fragment. The resulting F1, F2, and F3 fragments would have 20-nt complementary overlapping regions at adjacent ends, which are necessary for the fusion. The first-step PCR was carried out to amplify the three fragments individually with a high-fidelity DNA polymerase, *Pfu* (Stratagene, Cedar Creek, TX). Genomic DNA extracted from *M. catarrhalis* strain 035E was used as template for F1 and F3 PCR. Plasmid pUC18K containing the nonpolar kanamycin resistance cassette (29) was used as template for F2 PCR. F1, F2, and F3 were then purified by the Qiaquick PCR purification kit (Qiagen, Valencia, CA). Ten nanomoles of each purified fragment was mixed in the absence of additional primers in a second-step PCR with *Pfu*. The PCR program consisted of 10 repetitive cycles with a denaturing step at 94°C for 30 s, an annealing step at 50°C for 1 min, and an elongation step at 72°C for 5 min. The fusion product was subsequently amplified by *Pfu* in the third PCR with the forward primer P9 and the reverse primer P14 (Table 1). This amplicon consisted of 816 bp of upstream sequence of *oppA* and 930 bp of downstream sequence of *oppA* flanking the kanamycin cassette. The amplicon was purified from a 1% agarose gel with the Qiaquick gel extraction kit (Qiagen, Valencia, CA) and sent for sequencing to the RPCI DNA sequencing facility.

**Mutant construction.** The isogenic *oppA* mutant strain was constructed by transformation of *M. catarrhalis* strain 035E (28) with the overlap extension PCR

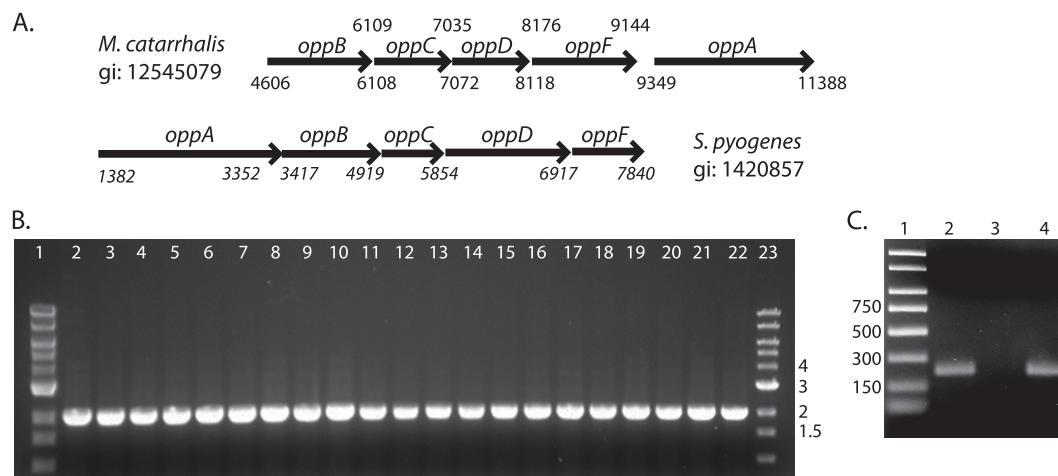


FIG. 1. Characterization of the *oppA* gene. (A) Schematic illustration of *oppA* with the other oligopeptide transporter genes in the genomes of *M. catarrhalis* ATCC 43617 and *S. pyogenes*. Numbers indicate the initial and terminal nucleotide positions of each gene within the GenBank sequence. (B) Ethidium bromide-stained agarose gel showing amplicons of the *oppA* gene amplified from 20 clinical isolates of *M. catarrhalis* and the ATCC 43617 strain. Lanes 1 and 23, DNA standards; lanes 2 to 11, amplicons from middle ear fluid isolates 135, 238, 555, 2901, 3584, 3614, 5488, 6952, 8184, and 9483, respectively; lane 12, amplicon from ATCC 43617; lanes 13 to 22, amplicons from sputum isolates 5P34B1, 10P58B2, 12P15B1, 14P25B1, 29P24B1, 33P25B2, 39P29B2, 55P18B3, 63P62B1, and 96P9B1, respectively. The sizes of the DNA standards (in kilobases) are indicated on the right. (C) Results of reverse transcriptase PCR (RT-PCR) with RNA extracted from *M. catarrhalis* 035E to detect *oppA* transcript. Lane 1, DNA standards; lane 2, RT-PCR product; lane 3, RT-PCR in the absence of reverse transcriptase; lane 4, PCR product from genomic DNA template. DNA standards are noted in base pairs on the left.

product. Briefly, strain 035E was grown to an optical density at 600 nm ( $OD_{600}$ ) of 0.2. A volume of 100  $\mu$ l of the bacterial culture was then spread on a BHI agar plate and air dried. Two circles (2-cm diameter) were marked on the plate. Either 30  $\mu$ l of distilled water or 30  $\mu$ l of water containing 100 ng of DNA was dropped onto these circles, followed by incubation at 37°C with 5%  $CO_2$  for 5 h. Bacteria within each circle were harvested with a cotton swab and spread on BHI plates containing 50  $\mu$ g/ml kanamycin. After 24 h of incubation at 37°C with 5%  $CO_2$ , numerous colonies were present on the selection plate following DNA transformation but no visible colony was present on the water control plate. The mutant colonies were examined by PCR and sequencing as described in Results.

**Cloning of the *oppA* gene.** The pCATCH plasmid is an expression vector engineered to express recombinant lipoprotein and has been used previously to express *M. catarrhalis* lipoproteins (3, 11). The 1,967-bp *oppA* gene encoding the mature OppA protein was amplified from *M. catarrhalis* strain ATCC 43617 with primers P7 and P8 (Table 1) and ligated into pCATCH between restriction sites NcoI and BamHI. The ligation mixture was transformed into chemically competent *E. coli* strain Top10 and grown on a kanamycin (50- $\mu$ g/ml) selection plate. The OppA expression plasmid was named pCATCH/OppA.

**Southern blot assay.** Southern blot assays were performed with genomic DNA that was restricted with PvuII and NdeI using a Hoefer TransVac vacuum blotting unit according to the manufacturer's instructions (Hoefer, San Francisco, CA). Probes were biotinylated with an NEBlot Phototope-Star detection kit (New England Biolabs) according to the manufacturer's instructions.

**Expression and purification of His-OppA protein.** pCATCH/OppA was transformed into *E. coli* strain BL21(DE3) to express OppA as a lipoprotein with a thrombin-cleavable C-terminal hexahistidine tag. A volume of 500 ml LB broth containing 50  $\mu$ g/ml kanamycin was inoculated with 20 ml overnight culture of bacteria harboring the expression vector. Following growth to an  $OD_{600}$  of 0.6, OppA expression was induced with 3 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) 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 (20 mM sodium phosphate, 500 mM NaCl, 1 mg/ml lysozyme, 100  $\mu$ g/ml Pefabloc [Roche, Indianapolis, IN], pH 7.4) and mixed with a nutator for 30 min at 4°C. The suspension was then sonicated with a Branson Sonifier 450 at setting 6, using an 80% pulsed cycle of 4 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 supernatant containing His-OppA protein was saved for protein purification.

His-OppA was purified with Talon metal affinity resin (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions. Two milliliters of a 50% suspension of the Talon resin was loaded onto a 20-ml chromatography column. The resin storage buffer was drained, and the remaining 1-ml beads were equil-

ibrated with 2  $\times$  10 ml binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4). After equilibration, the Talon beads were incubated with 10 ml of bacterial supernatant in the column with both ends capped for 20 min at room temperature with rocking. The unbound protein was drained, and the bound protein was washed two times with 20 ml wash buffer (20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, 10 mM beta-mercaptoethanol, pH 7.4) and eluted with 5 ml elution buffer (20 mM sodium phosphate, 500 mM NaCl, 150 mM imidazole, pH 7.4) by gravity flow. The eluates were collected every 500  $\mu$ l in each tube. Tubes containing concentrated protein fractions were pooled and subjected to buffer exchange into phosphate-buffered saline (PBS) by using a Centricon YM-10 filter device (Millipore Corporation, Danvers, MA). The concentration of the purified protein was determined by bicinchoninic acid assay (Pierce, Rockford, IL). The quality of the purified protein was examined by SDS-PAGE and Coomassie blue staining.

**Development of antisera to OppA and whole bacteria.** Purified recombinant His-OppA protein was sent to Covance (Denver, PA) for antibody production in New Zealand White rabbits using a 118-day protocol. Briefly, 250  $\mu$ g purified OppA was emulsified 1:1 in complete Freund's adjuvant for initial subcutaneous injection. Subsequent immunizations followed a 3-week cycle of boosts with 125  $\mu$ g OppA emulsified 1:1 in incomplete Freund's adjuvant. Test bleeds were taken approximately 10 days after the boosts. Final bleeds were taken 2 weeks after the 5th boosts.

Rabbit anti-035E antisera were raised by Covance according to a protocol described previously (18). New Zealand White rabbits were injected subcutaneously and intramuscularly twice with a 4-week interval with  $10^9$  CFU of *M. catarrhalis* 035E emulsified with incomplete Freund's adjuvant (1:1). Blood samples were collected 2 weeks after the final injection.

Rabbit antiserum to OMP CD was produced in our laboratory according to a protocol described by Adlowitz et al. (2).

**SDS-PAGE and immunoblot assay.** Whole-cell extracts of *M. catarrhalis* clinical strains, 035E, and the 035E/*oppA* mutant were prepared by suspending a loop of bacterial colonies grown on the BHI agar plates in 100  $\mu$ l PBS. The bacterial suspension was then mixed with 100  $\mu$ l 2 $\times$  SDS-PAGE sample buffer (Laemmli buffer) and boiled for 10 min. Twenty microliters of each whole-cell extract was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane.

For the immunoblot assay with the rabbit anti-035E antiserum, the membrane was incubated with a 1:5,000 dilution of the primary antibody in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) followed by a 1:2,000 dilution of the peroxidase-labeled goat anti-rabbit IgG antibody (KPL, Gaithersburg, MD) in TBST as secondary antibody. Bands were detected by using the Super-



Signal West Pico chemiluminescence kit (Thermo Fisher Scientific Inc., Rockford, IL). The image was acquired with an AlphaImager (Alpha Innotech Corporation, San Leandro, CA).

For immunoblotting with the rabbit anti-OppA or anti-CD antibody, the primary antibodies used were a 1:2,000 dilution of test bleed anti-OppA antiserum, a 1:15,000 dilution of final-bleed anti-OppA antiserum, and a 1:2,000 dilution of anti-CD antiserum. A 1:2,000 dilution of the peroxidase-labeled goat anti-rabbit IgG antibody was the secondary antibody followed by colorimetric detection.

For immunoblotting with the mouse antisera pooled following mucosal immunization with recombinant OppA protein, *M. catarrhalis* 035E, or PBS control, the membranes were incubated with a 1:10,000 dilution of the antisera, probed with a 1:25,000 dilution of peroxidase-conjugated anti-mouse IgG, and developed with chemiluminescence.

**Serum from adults with COPD.** Serum samples were obtained from adults enrolled in our COPD Study Clinic at the Buffalo Veterans Affairs Medical Center (33, 48). Patients were seen monthly and at times when an exacerbation was suspected. At each visit, serum and expectorated sputum samples were collected and bacteria present in the sputum were identified. Serum samples collected ~1 month before (preacquisition) and ~1 month after (postclearance) acquisition and clearance of *M. catarrhalis* were subjected to whole-cell enzyme-linked immunosorbent assays (ELISAs) to analyze the human systemic immune response to *M. catarrhalis* infection (33). Nineteen pairs of serum samples with a positive response to homologous infecting strains were used in ELISAs with purified recombinant OppA protein in this study to examine the OppA-specific antibody response. Eight pairs of serum samples collected 2 months apart from patients who never had positive sputum culture for *M. catarrhalis* during the study were used as negative controls.

**ELISA.** ELISA was carried out by coating the wells of a 96-well microtiter Immunolon 4 plate (Thermo Labsystems, Franklin, MA) with 500 ng of purified recombinant His-OppA protein overnight in coating buffer (0.1 M Na<sub>2</sub>CO<sub>3</sub> and 0.1 M NaHCO<sub>3</sub>, pH 9.6). Equal volumes of coating buffer were added to control wells for each well that received OppA. The plate was washed once with PBST (0.5% Tween 20 in PBS buffer) and blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature, after which the plate was washed once again with PBST. Paired COPD patient sera (preacquisition and postclearance) described above were diluted 1:2,000 in diluent buffer (1% BSA in PBST) and added to the sham-coated control wells and His-OppA-coated sample wells in parallel. After incubation for 1 h at room temperature, the plate was washed 4 times with PBST and a 1:6,000 dilution of peroxidase-labeled secondary antibody, goat anti-human IgG (KPL, Gaithersburg, MD), was added. After another 1 h of incubation, the plate was washed 5 times with PBST and developing reagent was added to the wells. The reaction was allowed to proceed in the dark for 10 min and stopped with 2 M sulfuric acid. The absorbance at 450 nm was determined using a Bio-Rad model 3550-UV microplate reader.

The OD value of the sham-coated control wells was subtracted from the OD value of each corresponding sample well to give a normalized OD of each sample. The percent change in the normalized OD between paired samples was calculated with the following formula: [(OD of postclearance sample - OD of preacquisition sample)/OD of preacquisition sample] × 100. ELISAs were repeated in four independent experiments. The percent change in OD of each paired sample was determined as the average of the values derived from the four experiments. The mean and standard deviation (SD) of the percent change of control sera were calculated. The cutoff for a significant percent change of the sample sera was set as (mean + 2 SD) of percent change of controls as previously described (3, 5).

**Whole-cell ELISA.** *M. catarrhalis* strain 035E and the 035E/*oppA* mutant strain were grown in BHI broth to an OD<sub>600</sub> of 0.2, harvested by centrifugation, and resuspended in PBS. A volume of 100 μl of the suspension was added to each well of the 96-well microtiter Immunolon 4 plate (Thermo Labsystems, Franklin, MA). Wells with PBS alone were included as controls. The rest of the experiment was performed according to the protocol described above for ELISA with OppA. Primary antibodies, either rabbit anti-OppA antibody, rabbit anti-CD antibody (positive control), or rabbit anti-protein 140 (protein 140 is a non-surface-exposed protein) (negative control), diluted in 2-fold serial dilutions from 1:10,000 to 1:40,000, were added to the wells. The secondary antibody was a 1:4,000 dilution of peroxidase-labeled goat anti-rabbit IgG (KPL, Gaithersburg, MD).

**Flow cytometry.** *M. catarrhalis* was grown to mid-logarithmic phase, and 100 μl of culture was centrifuged at 4,000 × g for 5 min. The bacterial pellet was resuspended in an appropriate dilution of heat-inactivated (56°C for 30 min) rabbit serum in PBS. Appropriate dilutions were determined by performing initial experiments at multiple 2-fold dilutions to identify the dilution of serum that yielded the greatest signal-to-noise ratio. Two negative controls were in-

cluded: (i) bacteria incubated in PBS alone and (ii) bacteria incubated with rabbit antiserum directed at a nonsurface protein, protein 140. Bacteria incubated with polyclonal antiserum from rabbits immunized with whole bacterial cells were used as a positive control. Bacteria were incubated with antisera for 1 h at 37°C. The bacteria were washed with PBS and resuspended in a 1:10 dilution of antibody conjugated to fluorescein isothiocyanate (KPL). After 30 min of incubation at 37°C, the sample was diluted with 900 μl PBS and fluorescence was detected with a FACSCalibur flow cytometer using Cell Quest 3.1 software (BD Biosciences, San Jose, CA).

**Mucosal immunization of mice.** Groups of six BALB/c mice were immunized intranasally with either 50 μg purified recombinant OppA plus 1 μg cholera toxin, formalin-killed *M. catarrhalis* 035E plus 1 μg cholera toxin, or 1 μg cholera toxin alone in PBS buffer as a sham immunization control. Intranasal immunization was performed on days 0, 10, and 20 by placing a pipette tip with 5 μl immunogen at each nostril of a conscious mouse for it to sniff. Immunized mice were challenged on day 28 as described below.

**Mouse pulmonary clearance model.** The mouse pulmonary clearance model was used as described previously (43). An overnight culture of *M. catarrhalis* 035E was inoculated into 100 ml BHI broth with an OD<sub>600</sub> of 0.05 and grown to an OD<sub>600</sub> of 0.3. Bacteria were collected by centrifugation and resuspended in 10 ml PCGM buffer (4.3 mM NaHPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, 5 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.1% gelatin, pH 7.3). An aliquot of suspension was diluted and plated to determine the starting concentration of bacteria, which was approximately 1 × 10<sup>9</sup> CFU per ml in each experiment. Ten milliliters of the bacterial suspension was placed in the nebulizer of a Glas-Col inhalational exposure system, model 099C A4212 (Glas-Col, Terre Haute, IN). Immunized mice were challenged using this inhalation system with the following settings: 10 min of preheating, 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.

Three hours postchallenge, the mice were anesthetized by inhalation of isoflurane and bled by retro-orbital puncture. Serum was heat inactivated at 56°C for 30 min and stored at -20°C for later use. After serum was collected, the anesthetized mouse was euthanized by administration of additional isoflurane. Lungs were then harvested and homogenized on ice in 5 ml PCGM buffer using a tissue homogenizer. Aliquots (20 μl) of undiluted and 1:10 diluted lung homogenate were plated in duplicate and incubated at 35°C with 5% CO<sub>2</sub> overnight. Colonies were counted the following day. The statistical significance of colony counts between groups of immunized and sham-immunized mice was determined by two-tailed *t* tests. A *P* value of ≤0.05 was considered significant.

## RESULTS

**Identification and characterization of the *oppA* gene.** The genomic sequence of *M. catarrhalis* strain ATCC 43617 was available as 41 contigs in GenBank (accession numbers AX067426 to AX067466). Computer programs predicted approximately 1,800 ORFs in the genome (44, 57). These ORFs were analyzed previously in our laboratory to identify putative surface proteins as vaccine candidates (44), leading to the identification of the *oppA* gene on contig 34 (AX067459, gi12545079), and it was predicted to encode a lipoprotein. Homolog analysis by NCBI BLASTn search using the full-length sequence of the *oppA* gene revealed significant homology of this gene to the *oppA* genes in *Streptococcus pyogenes* strains with approximately 69% identity and 83% similarity.

The *oppA* gene is the last ORF in a cluster of five being translated in the same frame. The four ORFs preceding the *oppA* gene exhibited significant homologies to the *oppB*, *oppC*, *oppD*, and *oppF* genes of *S. pyogenes*, respectively. Specifically, the identities of these genes between *M. catarrhalis* ATCC43617 (AX067459, gi12545079) and an *S. pyogenes* strain (X89237, gi1420857) are 68.5% for *oppB*, 75% for *oppC*, 70.8% for *oppD*, and 69.2% for *oppF*. The organization of these five genes in both strains is depicted in Fig. 1A with nucleotide positions referring to their locations in the GenBank sequence. Generally, the five genes encoding the five proteins of the

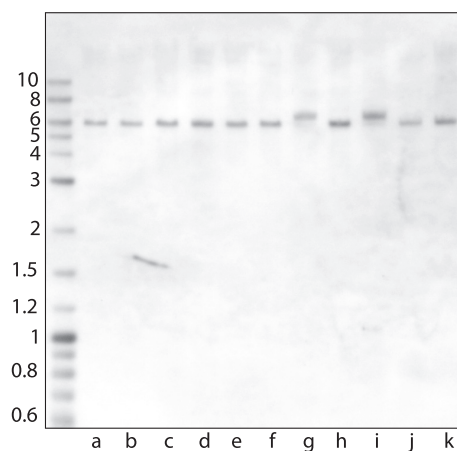


FIG. 2. Southern blot assay. Purified genomic DNA of *M. catarrhalis* strains was restricted with PvuII and NdeI and hybridized with a 200-bp probe corresponding to *oppA*. Lane a, 035E; lanes b through f, otitis media strains 135, 555, 3584, 5488, and 6952, respectively; lanes g through k, COPD strains 6P29B1, 29P24B1, 33P25B2, 55P18B3, and 96P9B1, respectively. Molecular size markers are noted on the left in kilobases.

oligopeptide transport system are organized in an operon except for additional copies of the *oppA* gene, if present (30). Consistent with this observation, the *oppA* gene of *M. catarrhalis* is present in the immediate vicinity of the other oligopeptide transporter genes, presumably constituting a single operon. Interestingly, although all five genes from *M. catarrhalis* showed significant homologies to corresponding genes from *S. pyogenes*, the organization of these five genes is different in these two bacterial species with the *oppA* genes being located in opposite extremities of the operon. This may be of significance in terms of mRNA stability in different intracellular environments of the organisms where multiple mechanisms of mRNA inactivation are present (40).

Bacteria carry from 2 to 5 copies of *oppA* genes in their genome (30). However, a BLAST search of a database containing genome sequences of 5 *M. catarrhalis* strains including ATCC 43617, 7169, 46P47B1, 103P14B1, and 12P80B1 identified only a single *oppA* gene. This database is accessible at the NCBI website ([http://camp.mic.med.buffalo.edu/blast/blast\\_cs.html](http://camp.mic.med.buffalo.edu/blast/blast_cs.html)). Similarly, only a single *oppA* gene is present in the two strains of *M. catarrhalis* whose genomes are published (ATCC 43617 and RH4) (13, 57).

To further evaluate whether strains of *M. catarrhalis* have one or more copies of the *oppA* gene, a Southern blot assay was performed with 10 clinical isolates including 5 middle ear fluid isolates that caused otitis media and 5 isolates from the sputum of adults with COPD. A single band is observed in each strain (Fig. 2). Eight strains have a band of identical size whereas two strains have bands that are slightly larger. Thus, we conclude that strains of *M. catarrhalis* have a single *oppA* gene based on (i) analysis of genome sequences of 7 strains and (ii) results of PCR with 20 strains (Fig. 1B and below) and Southern blot assay (Fig. 2).

In bacteria carrying multiple copies of *oppA* genes, the *oppA* copies are generally not identical and the OppA proteins play both redundant and unique physiological roles (20). This

would intuitively increase the complexity of OppA-based vaccine development for that species, if applicable. A single copy of the *oppA* gene in *M. catarrhalis* was therefore considered an appealing feature in our study on vaccine investigation.

**Sequence conservation of *oppA* among strains.** The *oppA* gene was evaluated for sequence conservation among *M. catarrhalis* strains in 10 sputum isolates from adults with COPD and 10 middle ear fluid isolates from children with otitis media. The 2,039-bp full-length *oppA* genes were amplified by PCR with primers P1 and P2 (Table 1) from these 20 strains and the ATCC 43617 strain as a positive control. A single band of the expected size was present in each strain (Fig. 1B), suggesting that the *oppA* gene is present and similar in gene length among *M. catarrhalis* strains.

In order to examine the sequence conservation of the *oppA* gene, PCR products from the 20 clinical isolates and the ATCC 43617 strain were purified and sequenced. Nucleotide sequences and translated amino acid sequences were aligned with the MacVector program by which the *oppA* homology among these strains was calculated. The results showed that the DNA sequence of the PCR product from ATCC 43617 was identical to the *oppA* gene sequence in GenBank, which validated the sequence information derived from our PCR products. Overall, a small number of nucleotide variations were present in each strain. The nucleotide variations collected from all strains were distributed at 18 discrete and consecutive nucleotide positions scattered over the 2,039 bases of the full-length *oppA* gene. Nucleotide variations at 12 of these positions were silent, and those at the other 6 positions gave rise to 3 amino acid changes. The gene identity scores among these strains range from 98.7% to 100%. These data indicate that the *oppA* gene is highly conserved among *M. catarrhalis* strains.

Of interest, sequence analysis revealed that nucleotide variations occurring at 10 of 18 positions were found exclusively in sputum isolates, whereas nucleotide variations occurring at another 5 sites were found exclusively in middle ear fluid isolates. The nucleotide variations at the remaining 3 positions were present in strains of both clinical sources. This observation suggests that while *oppA* varies slightly in adaptation to a different environmental niche, the gene is even more conserved within a specific ecological group of strains.

**Transcription of the *oppA* gene.** Once sequence conservation of the *oppA* gene was confirmed, we examined if the *oppA* gene is transcribed in *M. catarrhalis*. The *M. catarrhalis* 035E strain was originally isolated from the middle ear fluid of a patient with otitis media in Dallas, TX, and was subsequently widely used in *M. catarrhalis* studies. RT-PCR for *oppA* gene expression was performed with total RNA extracted from this strain. A 223-bp fragment within the *oppA* gene was amplified by PCR with P3 and P4 primers (Table 1), using genomic DNA extracted from 035E as template (Fig. 1C, lane 4). A DNA product of the same length was produced by RT-PCR (lane 2), but it is, however, absent in the negative control lacking the reverse transcriptase (lane 3). This result indicated that the *oppA* gene is transcribed in *M. catarrhalis* strain 035E.

**Recombinant His-OppA protein.** The *M. catarrhalis oppA* gene encodes a predicted lipoprotein with a 23-amino-acid signal peptide at the amino terminus, which contains a consensus lipoprotein signal peptidase (LSP) recognition site, LAAC. The mature OppA protein after LSP cleavage consists

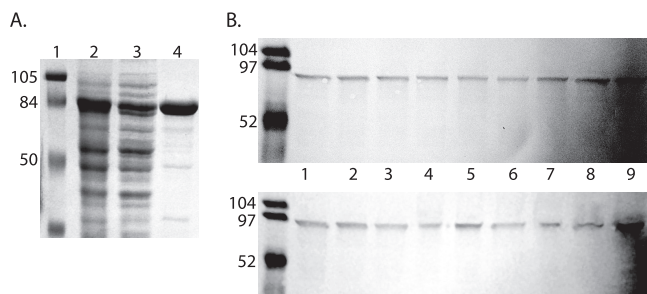


FIG. 3. (A) Coomassie blue-stained SDS gel of His-OppA purification products. *E. coli* BL21(DE3) harboring the His-OppA expression plasmid, pCATCH-OppA, was induced by 3 mM IPTG for 4 h, and the whole-bacterial-cell lysate was extracted and subjected to sonication. After centrifugation, the clarified supernatant was incubated with Talon metal affinity resin and His-OppA was eluted with imidazole. Lane 1, protein standards (kilodaltons); lane 2, whole-bacterial-cell lysate following IPTG induction; lane 3, supernatant containing His-OppA; lane 4, purified His-OppA. (B) Immunoblot assays with rabbit anti-OppA antiserum. Whole-cell lysates of clinical isolates of *M. catarrhalis* assayed with rabbit antiserum raised to purified recombinant (1:15,000 dilution) OppA. Blots were probed with peroxidase-conjugated goat anti-rabbit IgG (1:2,000 dilution) and developed with horseradish peroxidase color developer. Lane 1, molecular mass markers (kilodaltons); lanes 2 to 9, upper panel, whole-cell lysates of middle ear fluid isolates 155, 238, 555, 2910, 3584, 3614, 5488, and 6952, respectively; lanes 2 through 9, lower panel, whole-cell lysates of COPD sputum isolates 5P34B1, 10P58B2, 12P15B2, 29P24B1, 33P25B2, 39P29B2, 55P18B3, and 63P62B1, respectively.

of 656 amino acids. The *oppA* gene region encoding the mature OppA protein was inserted into pCATCH vector to express recombinant OppA as a lipoprotein with a C-terminal hexahistidine tag in *E. coli* BL21(DE3) as described in Materials and Methods. A Coomassie blue-stained SDS-PAGE gel (Fig. 3A) shows that the recombinant His-OppA protein (~85 kDa) is present in the bacterial lysate following induction with IPTG (lane 2) and that it was retained in the supernatant of the lysate as a soluble protein after sonication (lane 3). Following affinity purification with the Talon metal affinity resin, the recombinant protein was purified to over 95% purity (lane 4) and stored in PBS for other applications.

**Characterization of *oppA* mutant.** The isogenic *oppA* mutant 035E strain was constructed by replacing the *oppA* gene with a nonpolar kanamycin resistance cassette via homologous recombination. The region of mutagenesis is illustrated in Fig. 4A. A 97-bp sequence at the 5' end and a 137-bp sequence at the 3' end of the *oppA* gene were retained in the mutant strain as a result of optimal primer designs for the overlap extension PCR. After transformation of the 035E strain with the purified PCR product, the resulting mutagenesis was confirmed by PCR of the wild-type (WT) and the mutant strains. An 816-bp F1 fragment and a 930-bp F3 fragment were present in both strains while the 835-bp F2 was present only in the mutant strain and a 739-bp *oppA* gene fragment was present only in the WT strain (Fig. 4B, left panel). PCR with the forward primer of F1 and the reverse primer of F3 gave rise to a 3,526-bp amplicon from the WT strain and a 2,541-bp amplicon from the mutant strain, as expected (Fig. 4B, right panel). Sequences of these two amplicons confirmed that the targeted *oppA* gene was knocked out in the mutant strain while the upstream and downstream genes flanking *oppA* were completely preserved.

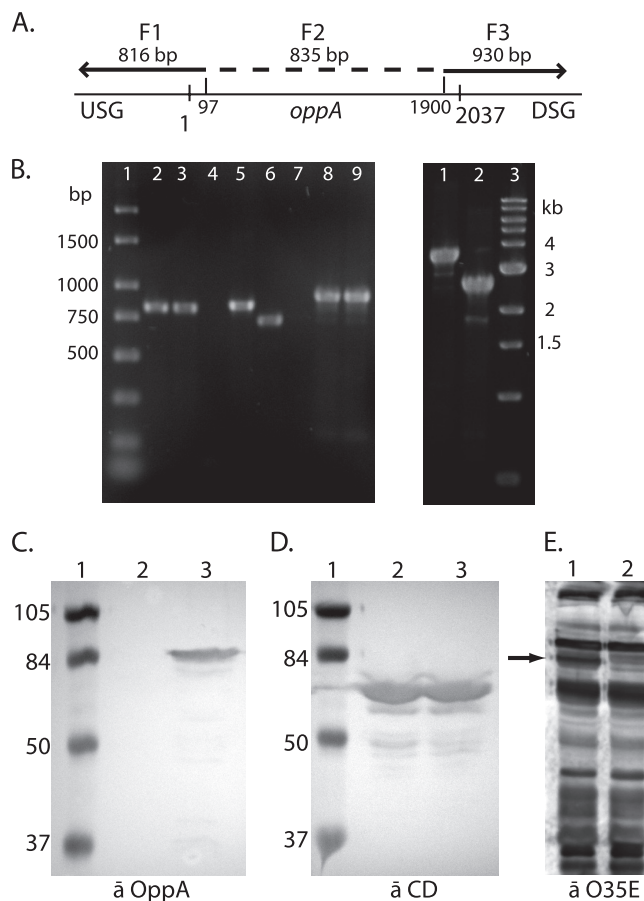


FIG. 4. Construction and characterization of the isogenic *oppA* mutant *M. catarrhalis* 035E strain. The *oppA* gene (from nt 97 to 1900) was replaced by the kanamycin resistance gene via homologous recombination in the *oppA* mutant. (A) Schematic depiction of the transforming DNA fragment in relation to the *oppA* gene in the genome. Numbers under the line indicate the nucleotide position within the *oppA* gene. USG, upstream gene; DSG, downstream gene. (B) Paired PCRs to examine the targeted mutagenesis in the *oppA* mutant compared to the wild type. (Left panel) Lane 1, DNA standards; lanes 2 and 3, PCR of the F1 fragment; lanes 4 and 5, PCR of the F2 fragment; lanes 6 and 7, PCR of a region (nt 1073 to 1811) of the *oppA* gene; lanes 8 and 9, PCR of the F3 fragment; lanes 2, 4, 6, and 8, PCR with the wild-type DNA as template; lanes 3, 5, 7, and 9, PCR with the *oppA* mutant DNA as template. (Right panel) PCR of the whole region of genes involved in the mutagenesis with the forward primer of F1 and the reverse primer of F3; lane 1, PCR with the wild-type DNA as template; lane 2, PCR with the mutant DNA as template; lane 3, DNA standards noted in kilobases. (C and D) Immunoblot assays of whole-cell lysates of the wild-type *M. catarrhalis* 035E and the *oppA* mutant. (C) Immunoblot with rabbit anti-OppA antibody. (D) Immunoblot with rabbit anti-CD antibody. Lanes 1, protein standards (kilodaltons); lanes 2, whole-cell lysate of the *oppA* mutant; lanes 3, whole-cell lysate of wild-type 035E. (E) Immunoblot assay with rabbit antiserum raised to whole cells of *M. catarrhalis* 035E. Lane 1, whole-cell lysate of the wild type; lane 2, whole-cell lysate of the *oppA* mutant. The arrow denotes OppA protein in the wild-type strain.

**OppA expression in *M. catarrhalis* strain 035E.** Rabbit anti-OppA antibody was raised against purified recombinant OppA as described in Materials and Methods. This antibody was used in immunoblot analysis to examine the expression of OppA in *M. catarrhalis*. The immunoblot assay was performed with



whole-cell extracts of both WT and the *oppA* mutant 035E strain. OMP CD is a constitutively expressed *M. catarrhalis* protein (47); antiserum to OMP CD was used as a protein expression control in the assay. Antibodies raised to recombinant OppA specifically recognized the OppA protein from the whole-cell extracts of the WT strain; the band is absent in the whole-cell extracts of the mutant strain (Fig. 4C). The anti-CD antibody recognized OMP CD from the whole-cell extracts of both strains (Fig. 4D). We conclude that the native OppA protein is recognized by the rabbit anti-OppA antibody raised to purified recombinant OppA. The results also indicate that OppA is expressed in *M. catarrhalis* 035E during growth *in vitro* while the protein is absent in the *oppA* mutant strain.

**Immunogenicity of the OppA protein.** Immunodominant antigens in bacteria have been considered to have great potential as targets for immunotherapy and have been frequently selected as vaccine antigens (9, 46, 60). In order to further evaluate OppA as a vaccine antigen, we characterized the immunogenicity of the OppA protein in *M. catarrhalis*. First, we assessed if the native OppA protein is immunogenic in a mammalian host presented with the whole organism. To answer this question, rabbit antiserum was raised against the whole organism of *M. catarrhalis* 035E and the presence of antibodies to OppA in the rabbit antiserum was examined by immunoblot assay.

Whole-cell extracts of the 035E WT and *oppA* mutant strains were separated by SDS-PAGE and subjected to immunoblot assay with the rabbit anti-035E antiserum. A band of ~84 kDa was present in the WT strain but absent in the mutant strain (Fig. 4E). These data suggest that rabbit antiserum raised to whole bacterial cells of strain 035E contains antibodies that bind OppA. Therefore, we conclude that native OppA protein is an immunogenic protein in *M. catarrhalis*.

To determine whether antibodies raised by immunization with recombinant OppA recognized epitopes on OppA of multiple strains of *M. catarrhalis*, immunoblot assays were performed with rabbit antiserum to recombinant OppA. Figure 3B shows that rabbit antiserum raised to recombinant OppA recognized a single band in whole-bacterial-cell lysates corresponding to the size of OppA. The band was present in 8 of 8 middle ear fluid isolates from children with otitis media and 8 of 8 sputum isolates from adults with COPD. We conclude that immunization with recombinant OppA induces antibodies that bind native OppA in multiple strains of *M. catarrhalis*.

**Human systemic antibody response to OppA.** We next assessed if OppA is an antigenic protein of *M. catarrhalis* during infection of the human respiratory tract. Immunoassays with convalescent patient serum can not only identify antigenic proteins expressed during infection to a level sufficient to induce immune responses but also indicate possible bacterial targets of the host immune system. To examine whether or not OppA is expressed by *M. catarrhalis* during infection in adults with COPD and induces an immune response, ELISAs were performed to measure the serum IgG response to OppA following *M. catarrhalis* infection.

ELISA was performed to measure antibodies to OppA in 19 pairs of patient serum samples (preacquisition and postclearance) and 8 pairs of negative-control samples as described in Materials and Methods. The percent change in OD between paired samples was calculated. The mean and SD values for

negative controls were -3.44% and 15.98%, respectively, representing a nonspecific percent change distribution with an upper limit of 28.52% (mean + 2 SDs) with 99% confidence. Therefore, any percent change in paired preacquisition and postclearance serum samples greater than 28.52% can be regarded as significant. According to this criterion, none of the 19 pairs of patient serum samples demonstrated significant percent changes. However, all serum samples, including our negative controls, have high levels of anti-OppA activity when tested with the purified recombinant OppA protein in ELISAs. Two pairs of serum samples were arbitrarily selected to titrate the anti-OppA level. Both gave titers over 1:10,000, which was defined by the highest dilution of the serum in OppA-coated wells giving an OD value 3-fold greater than that derived from the corresponding sham-coated control wells. Based on the results of these assays, we speculate that the preexisting high level of antibody to OppA most likely masked any possible anti-OppA immune response, if present, following an episode of *M. catarrhalis* infection.

**Surface exposure of OppA epitopes. (i) Whole-cell ELISA.** Surface exposure is an important property of vaccine antigens. Whole-cell ELISA was performed to examine the surface exposure of OppA epitopes on *M. catarrhalis*. OMP CD is a surface-exposed *M. catarrhalis* protein used as a control (47). Microtiter wells coated with both WT and *oppA* mutant 035E strains were incubated with the anti-OppA, anti-CD, and anti-protein 140 (nonsurface protein) antisera in ELISA. Anti-OppA antibody was captured by the WT strain but not by the *oppA* mutant strain (Fig. 5), while anti-CD antibody (positive control) was captured by both strains, and anti-protein 140 antibodies (negative control) were captured by neither strain. This result indicates that cells of the *oppA* mutant strain lack affinity for the anti-OppA antibody and that the binding of anti-OppA antibody to cells of the WT strain was specifically mediated by OppA protein. We conclude that the OppA protein has epitopes on the bacterial surface and that these epitopes are accessible to antibody binding. This characteristic suggests that OppA has potential as a protective immunogen.

**(ii) Flow cytometry.** As a second independent method to assess the exposure of OppA epitopes on the bacterial surface, antiserum raised to recombinant purified OppA was subjected to flow cytometry with wild-type 035E and OppA mutant bacteria. Rabbit antiserum raised to OppA demonstrates an increase in median fluorescence intensity from preimmune serum to immune serum with strain 035E as indicated by a shift of the curve to the right (Fig. 6A). An assay of the same antisera to the OppA mutant (Fig. 6B) shows a distinctly less prominent shift to the right from the preimmune to the immune serum, indicating that the OppA immune serum contains OppA-specific antibodies to epitopes on the bacterial surface. Figures 6C and D show absent binding of surface epitopes to the wild-type and OppA mutant strains with antiserum to protein 140, a non-surface-exposed protein. The experiment depicted in Fig. 6 was performed three times and yielded similar results each time.

Based on the results of whole-cell ELISAs and flow cytometry, we conclude that OppA expresses epitopes on the surface of the bacterial cell. This was a somewhat surprising result given the prediction of OppA as a periplasmic protein. This result is considered in the Discussion.

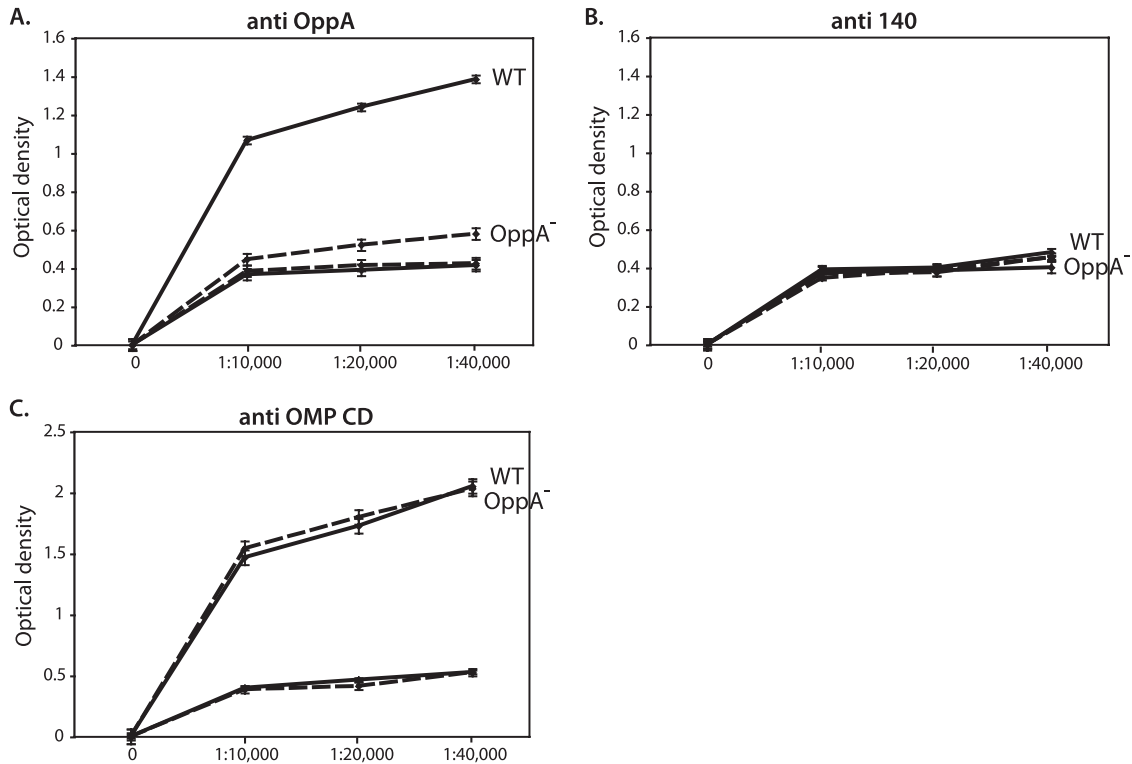


FIG. 5. Results of whole-cell ELISA with *M. catarrhalis* 035E wild type (WT; solid lines) and the *oppA* mutant (OppA<sup>-</sup>; dashed lines). *x* axes are dilutions of serum, and *y* axes are optical densities at 450 nm. Wells were incubated with rabbit anti-OppA (A), rabbit anti-protein 140 (protein 140 is a nonsurface protein) (B), and rabbit anti-OMP CD (CD is a known surface protein) (C). Corresponding prebleed sera from rabbits were tested simultaneously as controls. Error bars indicate standard deviations of triplicate values. Curves with solid lines indicate results with the wild type, and curves with dashed lines indicate results with the OppA<sup>-</sup> mutant.

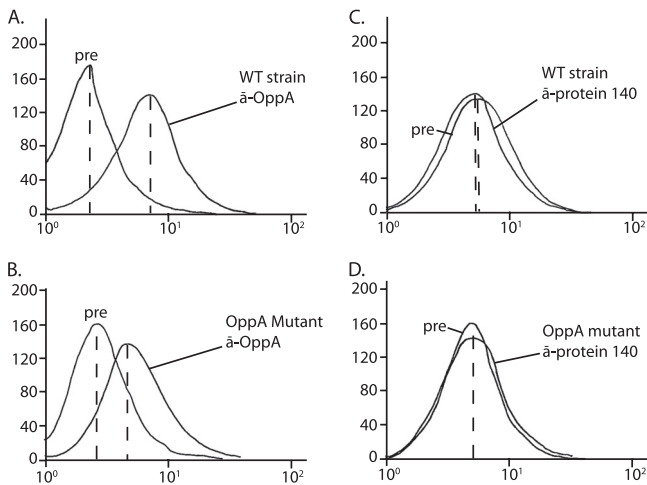


FIG. 6. Results of flow cytometry with *M. catarrhalis* wild-type 035E and OppA mutant. *x* axes are fluorescence, and *y* axes are cell counts. (A) Wild-type strain 035E assayed with rabbit antiserum to recombinant purified OppA (1:400) and preimmune serum (1:400). (B) OppA mutant assayed with rabbit antiserum to recombinant purified OppA (1:400) and preimmune serum (1:400). (C) Wild-type strain 035E assayed with rabbit antiserum to recombinant purified protein 140, a non-surface-exposed *M. catarrhalis* protein (1:200), and preimmune serum (1:200). (D) OppA mutant assayed with rabbit antiserum to recombinant purified protein 140 (1:200) and preimmune serum (1:200).

**Protective immune response.** To determine if mucosal immunization with OppA induces protective immunity against *M. catarrhalis* infection *in vivo*, the mouse pulmonary clearance model, in which groups of mice were immunized intranasally with recombinant purified OppA, was used. Parallel groups of mice were immunized simultaneously with either formalin-killed *M. catarrhalis* 035E as a positive control or PBS as a negative control. Mice were challenged with live *M. catarrhalis* 035E by an inhalation system on day 28 postimmunization. Three hours postchallenge, the lungs of mice were harvested and bacteria recovered from the lungs were quantified as colony counts. Protective immunity was evaluated as reduced number of colony counts in the OppA immunization group compared to that in the negative-control group. Statistical significance was analyzed by two-tailed *t* tests.

Mice immunized intranasally with OppA exhibited (i) production of anti-OppA antibodies in serum that recognized both native and recombinant OppA in immunoblot assays (Fig. 7A), (ii) production of IgA antibodies to OppA in bronchoalveolar lavage fluid (Fig. 7B), and (iii) significantly greater clearance of bacteria from lungs in the pulmonary clearance model (Fig. 7C, left panel). Intranasal immunization with OppA resulted in reduction of colony counts by approximately 1/2 log compared to the sham-immunized control, an effect comparable to that induced by immunization with killed whole organisms (positive control). The experiment depicted in Fig. 7C was repeated and yielded an identical result of enhanced clearance by approxi-



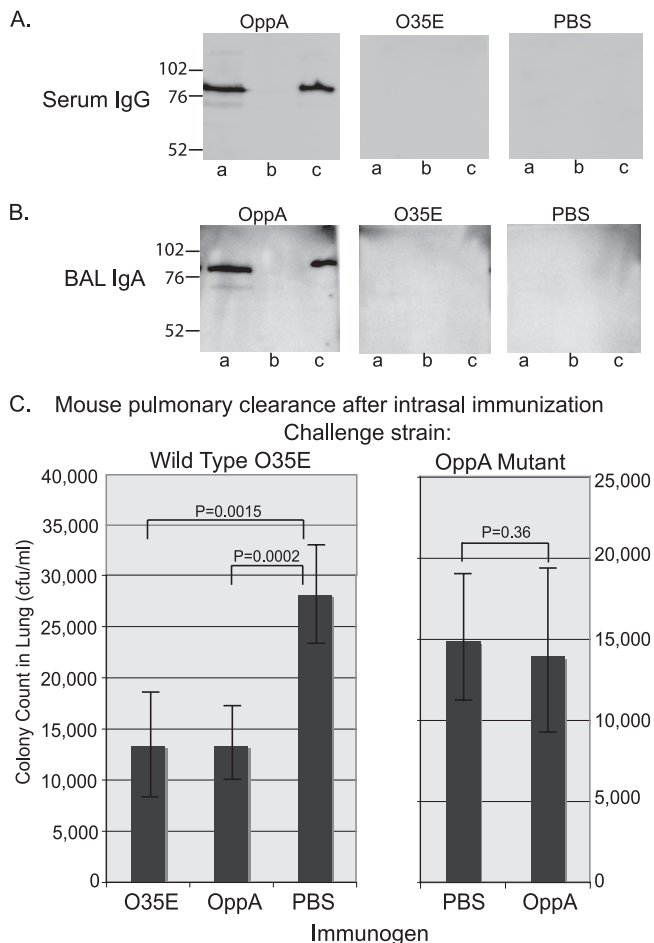


FIG. 7. Mucosal immunization of mice. (A) Immunoblot assays with sera (1:10,000) pooled from mice immunized intranasally with purified recombinant OppA (left), *M. catarrhalis* O35E (middle), and PBS (right). Blots were probed with peroxidase-conjugated anti-mouse IgG and developed with chemiluminescence. (B) Immunoblot assays with bronchoalveolar lavage (BAL) (1:50) fluid pooled from mice immunized intranasally with purified recombinant OppA (left), *M. catarrhalis* O35E (middle), and PBS (right). Blots were probed with peroxidase-conjugated anti-mouse IgA and developed with chemiluminescence. (A and B) Lanes a, whole-cell lysate of *M. catarrhalis* O35E; lanes b, whole-cell lysate of OppA mutant; lanes c, recombinant OppA. Molecular mass markers are noted on the left in kilodaltons. (C) (Left panel) Results of pulmonary clearance after aerosol challenge with *M. catarrhalis* O35E following intranasal immunization of groups of mice with *M. catarrhalis* O35E, recombinant OppA, and PBS. (Right panel) Results of pulmonary clearance after aerosol challenge with OppA mutant following intranasal immunization of groups of mice with recombinant OppA and PBS as noted. For both panels in panel C, at 3 h postchallenge, bacteria present in mouse lung homogenate were plated and incubated overnight. Colony counts were plotted. Error bars represent the standard errors of the means ( $n = 6$ ). Statistical significance of difference between groups was analyzed by two-tailed  $t$  test, and  $P$  values are noted.

mately 1/2 log of bacteria. To further assess the specificity of the enhanced pulmonary clearance, groups of mice were immunized intranasally with OppA and with PBS (negative control) and were challenged with the OppA mutant. The level of clearance of the OppA mutant in the OppA-immunized group was no different from the level of clearance observed in the

PBS group (Fig. 7C, right panel). These results indicate that intranasal immunization with OppA induces enhanced clearance of *M. catarrhalis* in the mouse pulmonary clearance model.

The level of enhanced clearance observed here is consistent with studies of other vaccine antigens in this model performed by other research groups (6, 15, 24). We conclude that mucosal immunization with purified recombinant OppA induces potentially protective immune responses against *M. catarrhalis*.

## DISCUSSION

*M. catarrhalis* is a significant cause of otitis media and exacerbations of COPD. Developing an effective vaccine against *M. catarrhalis* is therefore beneficial to both children and adults. Current vaccine studies have been focused on OMPs of *M. catarrhalis*, an unencapsulated Gram-negative bacterium. This endeavor is supported by a clinical trial with protein D (an OMP) from *H. influenzae*, also an unencapsulated Gram-negative bacterium, as a carrier for the pneumococcal conjugate vaccine. This conjugated vaccine induced protection in children against otitis media caused by pneumococcal vaccine serotypes and nontypeable *H. influenzae* (42).

The *oppA* gene was initially discovered in our search for surface proteins of *M. catarrhalis*. Homology analysis suggests that *oppA* encodes a soluble periplasmic protein of the oligopeptide transport system. We did not exclude OppA from potential vaccine antigens based on its predicted periplasmic localization because a number of predicted periplasmic components of the ABC transporters of Gram-negative bacteria have been shown to be immunogenic and to induce potentially protective immune responses (8, 16, 38, 39). In particular, OppA is an immunodominant protein of *Borrelia burgdorferi* (35) and *Yersinia pestis* OppA induces immune protection in mice (52).

Because oligopeptide permeases like OppA are predicted to be located in the periplasm, the observation that OppA of *M. catarrhalis* expresses epitopes on the bacterial surface is somewhat surprising. The observation is based on two independent assay systems (whole-cell ELISA and flow cytometry) utilizing an OppA mutant and rigorous controls (Fig. 5 and 6) and is consistent with the induction of a potentially protective response (Fig. 7). So how might a predicted periplasmic bacterial protein expose epitopes on the bacterial surface? One possibility is that epitopes are intermittently exposed when they bind oligopeptides from the environment for transport across the periplasm. Alternatively, the protein may be intermittently exposed during bacterial cell wall turnover and cell division. Oligopeptide permeases participate in numerous other cell functions in addition to peptide transport, and these include competence and sporulation (4, 22, 45), biofilm formation (23), adherence to host cells (12, 20), growth and intracellular survival (7), modulation of the expression of virulence factors (56), and antibiotic resistance (1). These observations and the results of the present investigation suggest that a broader interpretation of the structure and function of oligopeptide permeases as exclusively periplasmic transporters is in order.

OppA is highly conserved among strains of *M. catarrhalis*. Sequence conservation can be a result of selection pressure to preserve the essential physiological function of the protein.

Oligopeptides provide important nitrogen sources for bacterial amino acid synthesis and cell growth. In many bacteria, in order to achieve efficient oligopeptide binding, an OppA protein is produced with a higher stoichiometry than that of other components of the oligopeptide transporter by having multiple copies of the *oppA* gene in the genome (30). However, there is only a single copy of the *oppA* gene in the genome of *M. catarrhalis*. Having a single copy of the *oppA* gene does not necessarily mean that oligopeptide transport is less prominent in *M. catarrhalis*. In fact, enzymes for synthesis of all amino acids except proline and arginine are present in *M. catarrhalis* (57), which suggests that *M. catarrhalis* may demand highly active oligopeptide transport for potentially substantial *de novo* amino acid synthesis during infection. Although an *oppA* mutant 035E strain grows in enriched laboratory media in our study, an intact OppA protein could be essential for *M. catarrhalis* growth in the various microenvironments of the human respiratory tract, where nutrients may be relatively deficient.

Another factor that could contribute to the sequence conservation of the *oppA* gene is its presumed intermittent periplasmic localization. Genes that encode surface-exposed proteins in *H. influenzae* undergo point mutations driven by immune selective pressure during colonization; these changes allow the bacterium to evade host immune responses (17, 55). In contrast, OppA is presumably not continuously displayed to the host immune system. Furthermore, colonization by *M. catarrhalis* in the respiratory tract of adults with COPD is relatively brief, the mean duration of carriage being approximately 1 month (33). Therefore, the apparent shorter duration of colonization by *M. catarrhalis* and the surface exclusion of the OppA protein during at least parts of its growth cycle may preclude OppA-specific antibody from exerting immune selective pressure on the protein.

ELISAs with serum samples from adults with COPD revealed the presence of high levels of antibodies to OppA (>1:10,000) in serum. This result suggests that OppA is expressed by *M. catarrhalis* during infection and can induce a systemic immune response. We speculate that the antibody to OppA in preacquisition serum samples may be the result of a previous episode of *M. catarrhalis* infection or colonization. However, paired samples of preacquisition and postclearance serum did not demonstrate a significant increase of the anti-OppA antibody level following a new episode of *M. catarrhalis* infection. Before we conclude that OppA is not an immunodominant antigen of the *M. catarrhalis* strains causing the infection, other possibilities need to be considered as well. One possibility is that the purified recombinant OppA protein might cross-react with serum antibodies induced by oligopeptide binding proteins of other bacterial species in ELISAs. The cross-reactivity between recombinant OppA and these preexisting antibodies might mask the detection of an immune response to *M. catarrhalis*-specific epitopes on OppA following an episode of *M. catarrhalis* infection. A second possibility is that the native OppA epitopes presented on the cell surface of *M. catarrhalis* during infection might not be presented by the recombinant OppA protein coating the microtiter plate in ELISAs. Therefore, it is possible that ELISAs with recombinant OppA did not detect specific antibodies to native epitopes of OppA following infection. A third possibility is that OppA specifically induces a

mucosal immune response as opposed to a systemic immune response during *M. catarrhalis* infection and thus would be missed by immunoassays with serum. In order to clarify these possibilities, immunodominant B and T cell epitopes of OppA protein need to be identified and epitope-specific humoral, mucosal, and cellular immune responses should be examined further in future studies.

The observation that high levels of anti-OppA antibodies are present in patient sera, including the preacquisition serum samples, raises the question of how antibodies to OppA can play a protective role against *M. catarrhalis* infection when preexisting antibodies are present in patients who acquire infection. The lack of protection by the naturally occurring antibodies does not exclude potential protection by vaccine-induced immunity against bacterial infection. The naturally occurring anti-OppA antibody and the vaccine-induced antibody might exhibit different epitope profiles, different IgG subclass distributions, or different affinity/avidity indices, all of which are important determinants of protection. Furthermore, patients may show good antibody responses to an antigen following infection but fail to show a T cell response, while active immunization may induce T cell responses as well as a B cell response to the immunizing antigen (58). Finally, mucosal immunity may be critical in protection from *M. catarrhalis* infection. Ultimately, direct evaluation of vaccine-induced immunity in clinical trials will address these questions.

Currently the mouse pulmonary clearance model is the most widely used model to assess potential vaccine antigens of *M. catarrhalis*. A limitation of the model is that mice do not develop infections that simulate human infection. Rather, the endpoint is rate of clearance of bacteria from the lungs. The model is simple and reproducible, is performed in multiple centers, and measures a functional response. The observation that intranasal immunization with OppA induced enhanced pulmonary clearance raises the question of whether a mucosal route of immunization may be an effective approach for *M. catarrhalis* infections which are predominantly mucosal infections.

In this work, we evaluated the potential of *M. catarrhalis* OppA protein as a vaccine antigen. We showed that OppA (i) is highly conserved among strains of *M. catarrhalis* that cause otitis media and exacerbations of COPD, (ii) is an efficient immunogen, (iii) expresses epitopes that are exposed on the bacterial surface, and (iv) induces potentially protective immune responses following mucosal immunization in the mouse pulmonary clearance model. It will be interesting to study OppA further as a paradigm for ABC transporters as vaccine antigens in general. Future investigations are warranted to characterize immunodominant T or B cell epitopes of OppA; to characterize human systemic, mucosal, and cell-mediated immune responses to OppA epitopes; to further clarify the mechanisms of OppA-induced immune protection; and to investigate possible synergy between OppA and other vaccine antigens in multicomponent vaccine formulations.

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