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Sulfate-binding protein, CysP, is a candidate vaccine antigen of *Moraxella catarrhalis*

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ABSTRACT

Moraxella catarrhalis causes otitis media in children and respiratory tract infections in adults with chronic obstructive pulmonary disease (COPD). A vaccine to prevent *M. catarrhalis* infections would have an enormous impact globally in preventing morbidity caused by *M. catarrhalis* in these populations. Using a genome mining approach we have identified a sulfate binding protein, CysP, of an ATP binding cassette (ABC) transporter system as a novel candidate vaccine antigen. CysP expresses epitopes on the bacterial surface and is highly conserved among strains. Immunization with CysP induces potentially protective immune responses in a murine pulmonary clearance model. In view of these features that indicate CysP is a promising vaccine antigen, we conducted further studies to elucidate its function. These studies demonstrated that CysP binds sulfate and thiosulfate ions, plays a nutritional role for the organism and functions in intracellular survival of *M. catarrhalis* in human respiratory epithelial cells. The observations that CysP has features of a vaccine antigen and also plays an important role in growth and survival of the organism indicate that CysP is an excellent candidate vaccine antigen to prevent *M. catarrhalis* otitis media and infections in adults with COPD.

1. Introduction

Moraxella catarrhalis is an exclusively human pathogen that is an important cause of otitis media in children [1–3]. Approximately 80% of children experience an episode of otitis media by age 3, and 10–30% of children experience recurrent otitis media [4–6]. Recurrent otitis media causes hearing loss during a critical period of development and is associated with delays in speech and language development [7,8]. A recent worldwide systematic review estimated 709 million new cases of otitis media and 31 million cases of chronic suppurative otitis media occur annually [9]. Otitis media is a leading cause of healthcare visits worldwide, and its complications are important causes of preventable hearing loss [6]. As the most common reason for children to receive antibiotics, otitis media is important in driving the global crisis in antibiotic resistance [10,11]. Thus, an effective vaccine to prevent otitis media would be of immense benefit to prevent global morbidity, reduce healthcare costs, and ameliorate global antibiotic resistance by reducing antibiotic administration in children. Some of these benefits are being seen with national vaccine programs with the pneumococcal conjugate vaccines [12–19].

The three primary causes of otitis media are *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* and *M. catarrhalis*. Pneumococcal conjugate vaccines are changing the etiology of otitis media by altering nasopharyngeal colonization patterns. The vaccines have reduced otitis media and nasopharyngeal colonization by vaccine serotypes, resulting in "replacement" by non-vaccine serotypes, nontypeable *H. influenzae* and *M. catarrhalis* [19–22]. These gram-negative pathogens are increasing in importance in otitis media and are likely to increase further.

The gold standard for determining the etiology of otitis media has been culture of middle ear fluid obtained by tympanocentesis. However, biofilms play a central role in the pathogenesis of otitis media; middle ear fluid is often sterile by culture but may contain abundant pathogens in the form of a biofilm [23]. PCR detects pathogens in middle ear fluids at higher rates compared to culture alone, particularly in the case of *M. catarrhalis* [2,24–26]. Therefore,

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studies that rely on culture of middle ear fluid alone assess only a subset of cases of otitis media and underestimate the role of *M. catarrhalis* as an otopathogen. *M. catarrhalis* is isolated from 5% to 20% of middle ear fluids in otitis media whereas PCR identifies *M. catarrhalis* in 30–50% of middle ear fluids in children with otitis media [25,26]. A recent systematic review found that, on average, PCR analyses of middle ear fluid 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 [2]. Thus, a successful vaccine for otitis media should protect against *S. pneumoniae*, nontypeable *H. influenzae* and *M. catarrhalis*.

We have used genome mining to identify *M. catarrhalis* vaccine antigens [27], including substrate binding proteins of ATP binding cassette (ABC) transporter systems, such as OppA, a peptide transporter, and SBP2, an arginine transporter [28–30]. Guided by this approach, the present study identified CysP, a substrate binding protein of an ABC transporter system, as a candidate vaccine antigen. Here we show that CysP is highly conserved among strains, expresses epitopes on the bacterial surface and induces potentially protective responses in a mouse model. The work further shows that CysP binds sulfate and thiosulfate, functions in uptake of sulfate for growth, and plays a role in intracellular survival of *M. catarrhalis* in respiratory epithelial cells.

2. Materials and methods

2.1. Bacterial strains

M. catarrhalis strain O35E was provided by Eric Hansen. *M. catarrhalis* strains 135, 555, 2015, 5193, 6955, R40, R42, R62, R64, and R76 were middle ear fluid isolates obtained via tympanocentesis provided by Howard Faden in Buffalo NY and Janet Casey in Rochester NY. *M. catarrhalis* was grown on brain heart infusion (BHI) plates at 35 °C with 5% CO_2 or in BHI broth with shaking at 37 °C. Chemically defined medium (CDM) was used for growth curve experiments [31]. All methods are described in detail in the Supplement.

2.2. Construction of cysP knockout mutants and cysP revertant

Mutant construction was accomplished using overlap extension PCR and homologous recombination as described previously [28,30,32].

An effort was made to complement the mutation using previously described methods [33]. We were unable to accomplish complementation but generated a revertant using a modification of this method described in the Supplement.

cysP knockout mutants were constructed in 10 middle ear fluid strains by transforming with the fragment used to engineer the mutant in strain O35E.

2.3. Cloning, expression and purification of recombinant CysP

The *cysP* gene from *M. catarrhalis* strain O35E was cloned into the plasmid pCATCH, which expresses recombinant lipoproteins in *Escherichia coli*, using previously described methods modified as described in the Supplement [28,34].

2.4. Development of antiserum to recombinant CysP

Purified recombinant CysP was sent to Covance (Denver, PA) for antibody production in New Zealand White rabbits using a 59-day protocol as previously described [35].

2.5. Whole-cell ELISA

To assess binding of antibodies to epitopes on the bacterial surface, whole-cell enzyme-linked immunosorbent assay (ELISA) was performed as described previously [30]. In previous work, we have used both flow cytometry and whole cell ELISA to assess the extent to which the putative *M. catarrhalis* vaccine antigens OppA and SBP2 express epitopes on the bacterial surface; in each case the two methods have yielded identical results [28,30]. In evaluating CysP in the present study, we performed both whole cell ELISA and flow cytometry using the wild type strain and its *cysP* mutant. Because both assays showed identical results for CysP in the wild type strain, and because our experience with two other vaccine antigens (OppA and SBP2) showed identical results for a series of strains, we performed whole cell ELISAs in triplicate, with mutant controls for each of the 10 strains in the present study.

2.6. Immunization of mice

All animal studies were approved by the University at Buffalo Institutional Animal Care and Use Committee. Balb/c mice were immunized subcutaneously as previously described [28,30].

2.7. Mouse pulmonary clearance model

To determine if immunization with CysP induces potentially protective responses *in vivo*, the mouse pulmonary clearance model was performed as described previously [28,30].

2.8. Thermal shift assays

To assess the binding of sulfate and thiosulfate to purified CysP, thermal shift assays were performed using the Stratagene Mx3005P real-time PCR instrument (Stratagene, LaJolla CA) as previously described [35–37].

2.9. Assessment of bacterial growth

Growth curves were performed using the Bioscreen C Automated Growth Curve Analysis System (Oy Growth Curves AB, Helsinki, Finland). *M. catarrhalis* strains were grown in broth overnight with shaking. Overnight cultures were diluted (1:1000 for BHI and 1:250 for CDM) and used to inoculate cultures to perform growth curves in 300 μl volumes with 5 replicate wells for each growth condition in each experiment. Optical density measurements were taken at 600 nm at 30 min intervals.

2.10. Adherence and invasion assays with A549 human respiratory epithelial cells

Quantitative adherence and invasion assays were performed with A549 cells as previously described [35].

3. Results

3.1. Identification of the cysP gene

As part of a genome mining approach to identify vaccine antigens of *M. catarrhalis*, we previously analyzed the genome of strain ATCC 43617 (accession numbers AX067426–AX067466) to identify open reading frames (ORFs) that were predicted to be potentially surface exposed [27]. Of 348 ORFs that were predicted to be surface exposed, 14 had homology to substrate binding proteins of ABC transporter systems. This approach led to the identification of Oligopeptide Binding Protein A (OppA) and Substrate Binding