BACTERIAL WARFARE: EXPLORING THE CONTACT DEPENDENT INHIBITION SYSTEM IN *MORAXELLA CATARRHALIS*

By

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(Under the Direction of Eric Lafontaine)

ABSTRACT

Moraxella catarrhalis is an important and emerging cause of infectious disease, including 15-20% of otitis media cases in children and ~10% of respiratory infections in adults with chronic obstructive pulmonary disease. Children have been reported to have a high carriage rate, there is not an efficacious vaccine, and there has been a rapid emergence of antibiotic resistance in clinical isolates. Additionally *M. catarrhalis* infections may become even more prevalent due to the effectiveness of conjugate vaccines at reducing the incidence of otitis media caused by *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae*. Hence, *M. catarrhalis* causes significant health problems and the development of a vaccine is highly desirable. Identifying potential vaccine candidates, testing their role in pathogenesis, and testing their ability to provide protection in an animal model are the first steps to developing a vaccine. To address this, we selected MhaB1 and MhaB2, which are part of a potential contact dependent inhibition system (CDI), as our candidates. To our knowledge, this is the first time a CDI system has been used in a vaccine study. The data demonstrate that MhaB1 and MhaB2 are CDI proteins and their cognitive immunity genes provide protection. Also, MhaB1 and MhaB2 play an important role in the ability of *M. catarrhalis* to colonize the chinchilla nasopharynx. Moreover, when vaccinated with portions of these proteins,

the chinchillas produce MhaB specific antibodies, which decrease the ability of wild-type *M. catarrhalis* to colonize the chinchilla nasopharynx. In summary, this study describes the establishment of the chinchilla model to perform vaccine efficacy studies for *M. catarrhalis*, demonstrates that *M. catarrhalis* has a CDI system, and shows that this CDI system plays an important role in pathogenesis. To our knowledge, our data are the first report of testing a CDI system using an animal model.

INDEX WORDS: *Moraxella catarrhalis*, Contact Dependent Inhibition System, Vaccine Development, Chinchilla

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CHAPTER 1

INTRODUCTION

The most common infections caused by *Moraxella catarrhalis* are otitis media and lower respiratory tract infections resulting in exacerbation of chronic obstructive pulmonary disease (COPD). *Moraxella catarrhalis* is the third leading cause of otitis media worldwide behind *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (1-8). Otitis media is the number one reason for children being prescribed antibiotics and more than 80% of infants experience at least one episode of this disease by the age of three, and *M. catarrhalis* is the causative agent in ~20% of these cases (9, 10). The annual costs associated with management of otitis media are upwards of $5 billion, and direct medical care expenditures alone account for $2-3 billion (1, 4, 7, 11-15). Recurring ear infections are prevalent and occur during the crucial period when a child is developing speech and language skills, which can delay the development of communication and learning (16, 17). Clearly, otitis media is a significant health and economic problem, and *M. catarrhalis* contributes substantially to this burden.

Moraxella catarrhalis is also the second most common cause of respiratory infections in adults with chronic obstructive pulmonary disease (COPD) (14, 18-20). Direct medical care costs are greater than $14 billion each year and there are ~10 million visits to physicians are related to COPD (21-24). Half of the exacerbations are caused by bacterial infections and *M. catarrhalis* is responsible for ~10% of cases. These infections contribute prominently to the progression of COPD by augmenting inflammation, oxidative stress, and tissue damage in the airways. Additionally, *M. catarrhalis* has been associated with diseases such as bronchitis, conjunctivitis, and sinusitis in recent years (5, 8, 14, 25-39). This increase, coupled with the emergence of

antibiotic resistance, suggests that *M. catarrhalis* infections may become more prevalent and difficult to treat. This emphasizes the need to improve our understanding of pathogenesis by this understudied bacterium in order to identify targets for intervention and prevention.

The purpose of this study is to validate the potential vaccine targets MhaB1 and MhaB2, and explore their role in pathogenesis. MhaB1 and MhaB2 are hypothesized to be contact dependent inhibition (CDI) proteins and previous studies indicated they are also involved in adherence (40-42). Contact dependent inhibition is a method for bacteria to target neighboring bacteria using a toxin/anti-toxin system. These systems have been proposed to be involved in virulence, but their role is unknown (43). The first hypothesis is to determine if MhaB1 and MhaB2 are part of a CDI system. The second hypothesis is to determine if MhaB1 and MhaB2 are involved in the colonization process.

The majority of knowledge about CDI systems has been generated studying *Escherichia coli* and *Burkholderia* species. This project is the first time that the potential CDI system in *M. catarrhalis* has been studied. Additionally, MhaB1 and MhaB2 are both potential inhibitor proteins that share a common transporter protein. This is the first system that has been identified to have two inhibitor proteins for one transporter for display on the bacterial surface. Other species specifying multiple inhibitor proteins typically encode one transporter protein for each inhibitor. Our study represents the first instance that the role of a CDI system is explored using an animal model, and also the first report that CDI inhibitor proteins are tested as vaccine antigens. Moreover, the work described in this dissertation is the first report of the use of the chinchilla model to perform vaccine efficacy studies for *M. catarrhalis*.

We anticipate that MhaB1 and MhaB2 are functional CDI inhibitor proteins. Additionally, we expect to identify the potential immunity proteins, which will protect against the inhibition activity. This system is expected to play a role in the virulence of

M. catarrhalis. It is predicted that these genes will play a major role in the colonization process and that vaccination with a portion of these proteins will reduce the ability of wild-type *M. catarrhalis* to colonize the chinchilla nasopharynx.

CHAPTER 2

LITERATURE REVIEW

Moraxella catarrhalis

Moraxella catarrhalis is a gram-negative staining diplococcus bacterium (44). It is considered to be strictly a human organism and was thought to be a commensal organism, but in the late 1980s it became apparent that *M. catarrhalis* was a primary cause of acute otitis media (2, 32, 44, 45). Additionally, *M. catarrhalis* is able to cause respiratory infections in patients with chronic obstructive pulmonary disease (COPD), bronchitis, conjunctivitis, and sinusitis (5, 8, 14, 25-39). These diseases and the impact they have on public health will be discussed in detail.

Moraxella catarrhalis has had a variety of names starting with *Micrococcus catarrhalis*. It was then moved into the *Neisseria* genus due to the similarity in phenotype and ecology. When DNA analysis was done, it became apparent that *M. catarrhalis* was not similar to *Neisseria* species and was then moved into *Branhamella.* Finally, it was moved into the genus *Moraxella* due to genetic and biochemical similarity even though the majority of *Moraxella* species are rod-shaped (46). Within the species there is a high degree of similarity between isolates when comparing their virulence factors, metabolic pathways, and gene content (47).

Studying *M. catarrhalis* has been limited due to lack of commonly used genetic tools such as genomes sequenced, cloning vectors, and reporter plasmids. In 2006, Wang and colleagues developed the first cloning vector for use with *M. catarrhalis* (48, 49). More recently the first annotated sequences of *M. catarrhalis* were published (BBH18 by de Vries *et al*.) (50). In total there are 12 published and annotated sequences now available (47, 50). Of interest, all of the strains that have been

sequenced were isolated from patients with otitis media, COPD exacerbation, bronchiolitis, or bronchitis. Finally, in 2013 Evans *et al.* developed a LacZ-based transcriptional reporter system for *M. catarrhalis* (51). The development of these tools will help to drive the research exploring *M. catarrhalis* virulence mechanisms.

Disease and Public Health Interest

What was once thought to be a commensal organism has now been identified as an organism that causes a variety of diseases including otitis media, exacerbations of patients with COPD, bronchitis, conjunctivitis, and sinusitis (5, 8, 14, 25-39). While many of these diseases are not life threating, they are very costly. *Moraxella catarrhalis* is the third leading cause of otitis media behind *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae*. It is estimated that 80% of children have a case of acute otitis media by age 3 and *M. catarrhalis* causes ~20% of these cases (1-10, 15, 52-55). The shear number of cases causes otitis media to be a very expensive disease. In the U.S. alone, there are approximately 25 million visits made to pediatricians each year. *Moraxella catarrhalis* causes 3-5 million of these cases (1-8, 15, 52-55). Every year the costs associated with the management of otitis media are approximately $5 billion, with $2-3 billion being due to direct medical care expenditures (1, 4, 7, 11-15). In addition to causing otitis media, *M. catarrhalis* is the second most common cause of respiratory infections with COPD (14, 18-20). COPD has staggering direct medical care costs (more than $14 billion per year) and is the fourth leading cause of death in the U.S. (21-24, 56). Due to these staggering medical costs, *M. catarrhalis* has a significant impact on public health.

As mentioned above, *M. catarrhalis* is the third leading cause of otitis media, but what is otitis media? Otitis media occurs when the middle ear becomes infected and there is a build up of fluid. Depending on how severe this fluid build up is determines if it is considered to be acute or not. When the fluid builds up there is an increase in

pressure, which is very painful. The symptoms are very general and varied, but typically include ear tugging, irritability, and/or fever. In order to have an accurate diagnosis the tympanic membrane must be examined. During acute otitis media, the tympanic membrane is cloudy and has moderate to severe bulging. These acute infections typically occur when the child is between 6 and 12 months old. Otitis media with effusion occurs when there is chronic inflammation and typically affects children between the ages of 3 and 7. In this case, the child lacks the typical signs associated with acute otitis media, but there is a glue-like fluid behind the tympanic membrane. There can be transient hearing loss associated with this type of otitis media. This is a disease that affects children during their developmental years. During each episode, the child has reduced hearing and repeated incidences can lead to loss of hearing and development as well as impairment in speech. While this is not life threatening, otitis media is the leading reason for doctor's visits, antibiotic prescriptions, and surgical procedures. All of this makes otitis media a very expensive disease. Additionally, there is an increase in antibiotic resistance among strains, which will be discussed later (57, 58).

Chronic obstructive pulmonary disease (COPD) is a progressive disease that typically has two forms. The first form is defined by the overproduction of mucus. This overproduction causes the airways to clog and breathing becomes difficult. The second form is damage to the lungs such as prolonged exposure to cigarette smoke or other lung irritants. While, neither of these are caused by bacteria, both create an environment in the lungs that is ideal for infection. These infections or exacerbations cause a worsening of the lung damage, which then can lead to death. Since this disease is progressive the medical costs are substantial. Direct medical care costs are estimated to be over $14 billion per year. *Moraxella catarrhalis* is estimated to cause 10% of exacerbation cases (21-24).

Although *M. catarrhalis* is best known for causing otitis media and exacerbations of COPD patients, it can also cause numerous other diseases and these diseases have been increasing in recent years. Bronchitis occurs when the bronchi become inflamed. Conjunctivitis is inflammation of the outermost layer of the eye and the inner surface of the eyelid. Finally sinusitis is inflammation of the sinuses. In recent years, there has been an increase in *M. catarrhalis* causing these diseases (2, 8, 27-32, 59-62).

The diseases associated with *M. catarrhalis* are treatable, except for the fact that *M. catarrhalis* has rapidly gained resistance to β-lactams. It is important to note that *S. pneumoniae* and nontypeable *H. influenzae* are susceptible to β-lactams and since they are the number one and two causes of otitis media β-lactams are the first antibiotic used to treat otitis media. Starting in the late 1980s and into the 1990s, *M. catarrhalis* went from very few isolates to over 90% of the isolates being resistant to β-lactams (29, 31, 32, 59-61, 63-69). Also, when *M. catarrhalis* and nontypeable *H. influenzae* cause a coinfection, there is an increase in the antibiotic resistance in the biofilm (70). It has been demonstrated that *M. catarrhalis* releases outer membrane vesicles that contain a various outer membrane proteins. These vesicles have the ability to promote the survival of *S. pneumoniae* and *H. influenzae* (71). *Moraxella catarrhalis* has demonstrated that it can rapidly acquire antibiotic resistance and this resistance can also protect other susceptible bacteria.

A vaccine is desirable for *M. catarrhalis* because of the increase in antibiotic resistance, the high rate of infection, and the high cost of treatment. While focus for developing a vaccine would be on otitis media, it is possible that this vaccine would also be beneficial to COPD patients. In order to develop a vaccine for *M. catarrhalis*, there are several steps necessary. First, potential targets need to be identified. Once these targets have been identified, they need to be tested. In order to test them, there needs to be an animal model.

Animal Models

Moraxella catarrhalis is an exclusively human organism and studying pathogenesis, as well as the stringent testing of vaccine candidates, has been hindered by the lack of an animal model that mimics human infection. To date, the most commonly used model has been a pulmonary clearance test in which bacteria are deposited in the lungs of mice (72-78). Lungs are aseptically removed, tissues are homogenized, and dilutions of the homogenates are spread onto agar plates in order to enumerate viable organisms. While this model has provided important data, it is limited to measuring the rate at which bacteria are cleared over a very short period of time because *M. catarrhalis* persists for <24hours in the murine lungs. Another drawback is that mice do not develop pneumonia. Hence, the rapid clearance and failure to cause disease limit the usefulness of this model.

Attempts to use various other models of infection in mice have been unsuccessful. In one of these attempts, Melhaus and Ryan directly inoculated *M. catarrhalis* into the middle ear of three strains of mice, but there was no evidence of *M. catarrhalis* causing a middle ear infection in any of the animals (79). This was done in parallel with *S. pneumoniae* and nontypeable *H. influenzae* and the model was successful with these organisms although it was dose and strain of mouse dependent (79). They concluded that this model of infection was not useful for studying *M. catarrhalis*.

The pulmonary clearance test does not provide a biologically relevant window in which to study the interactions between *M. catarrhalis* and the tissues. Because of the rapid clearance and the lack of disease it is not a good model for vaccine studies. Additionally, it is uncommon to do co-infection studies using the pulmonary clearance test because there are better models of infection for mice using *S. pneumoniae* and

nontypeable *H. influenzae*. This led to exploring other animal models for studying *M. catarrhalis*.

Recent studies have demonstrated the value of the chinchilla to examine *M. catarrhalis* host-pathogen interactions *in vivo* (70, 80-83). Following intranasal inoculation, *M. catarrhalis* causes symptoms of disease (inflammation of the tympanic membrane, middle ear effusions) and colonizes the nasopharynx for ~2 weeks (80-82). Therefore, chinchillas provide an advantage over the mouse pulmonary clearance test in that *M. catarrhalis* persists in their nasopharynx for several days. This imparts greater confidence in the data obtained by comparing the difference in colonization between two experimental conditions (vaccinated vs. sham-vaccinated animals, WT vs. mutant strains) as it provides a more physiologically relevant time frame to monitor bacterial clearance.

The chinchilla model has been an invaluable tool to study the pathogenesis of nontypeable *H. influenzae* and *S. pneumoniae* and to develop vaccines for these organisms (84-87). The course of disease (nasopharyngeal colonization, ascension of the Eustachian tubes, development of middle ear effusions, clearance of fluids, return to homeostasis) is similar to that in children with otitis media (88-93). Immunological parallels between chinchillas and humans have been demonstrated. For example, middle ear fluids collected from chinchillas and children infected with nontypeable *H. influenzae* contain Abs that bind to the same antigenic determinants of the adhesion OMP P5 (94). Chinchillas also produce homologs of human antimicrobial peptides, and at least two of them (cBD-1 and cCRAMP) have been shown to have bactericidal activity against *M. catarrhalis* (95-98). Kerschner and colleagues analyzed host cDNA libraries generated from the middle ear mucosa of chinchillas infected with nontypeable *H. influenzae*, and discovered that the cDNA sequences displayed greater phylogenetic similarities to human genes than to other rodent species (99-101). These investigators

also noted similarities with human infection in the pattern of host defense genes expressed in chinchilla tissues.

Another benefit of using the chinchilla model is this model is also used for other organisms that cause otitis media (e.g. *S. pneumoniae* and nontypeable *H. influenzae*). This allows for co-infection studies. These studies are useful, because multiple organisms often cause otitis media and by utilizing the same animal model for all the organisms can lead to new discoveries. One such discovery by Armbruster and colleagues demonstrated that co-infection by *H. influenzae* and *M. catarrhalis* increased the antibiotic resistance within the biofilm and reduced clearance by the host due to quorum signaling by *H. influenzae* (70). Having a biologically relevant animal model is necessary to promote the necessary vaccination studies.

While, *M. catarrhalis* is a human specific organism and it has been problematic developing an animal model of infection, the chinchilla model is the best that is available. It provides a biologically relevant window in which to observe interactions between the bacteria and the host tissues. Additionally, while *M. catarrhalis* is unable to cause otitis media when it is used alone, but with the help of a quorum sensing molecule from *H. influenzae*, *M. catarrhalis* is capable of migrating to the middle ear and cause disease (70). For these reasons, we believe the chinchilla animal model is currently the best animal model to use.

Virulence Factors

There have been a number of virulence factors that have been identified and characterized in *M. catarrhalis*. These factors are involved in adherence, iron and nutrient acquisition, serum resistance, and biofilm formation. In the Lafontaine laboratory the focus is on adherence proteins. The reasoning behind targeting adherence proteins is that adherence is the first step to infection and if this step can be blocked, then the bacteria will not get a foothold to cause disease. For this reason, I am

going to focus on the virulence factors that contribute to adherence. Many of these adherence factors have additional functions. Some of these adhesins that will be highlighted are the UspA family, Hag, McaP, and OMP CD. There is also the two-partner secretion and putative contact dependent inhibition proteins (MhaB1, MhaB2, and MhaC), which will be discussed in a later section because they are the focus of this project.

The UspA family consists of the proteins UspA1, UspA2, and UspA2H. These proteins are the most extensively characterized proteins in *M. catarrhalis*. This family of proteins was first studied due to the fact that antibodies are produced against it during infection. Monoclonal antibodies were produced and utilize to identify UspA (102). Later it was discovered that UspA was actually encoded by two different genes, i.e. UspA is two separate proteins, UspA1 and UspA2 (103). In 2000 Lafontaine *et al.* discovered the hybrid UspA2H (104). UspA2H is a hybrid of UspA1 and UspA2 where the C-terminus is virtually identical to the C-terminus of UspA2 and the N-terminus shares motifs, which are considered to be unique to UspA1 (104). While UspA1 and UspA2 are found in most clinical isolates, UspA2H is present in only 21% of isolates (105). UspA2H is the least well studied of these proteins, but it has been determined to be involved in adherence (104, 106). While these proteins share a common epitope for the monoclonal antibody that was first used to identify them, they have divergent functions.

UspA1 is a phase variable trimeric autotransporter that mediates adherence to human epithelial cells (104, 107). The phase variation is regulated at the transcriptional level due to the presence of a poly(G) tract located upstream of the *uspA1* gene. Changes in the length of the poly(G) tract changes the expression. Additionally these changes can directly correlate to adherence of *M. catarrhalis* to human epithelial cells (107). Additional studies have demonstrated that UspA1 has an affinity to members of the carcinoembryonic antigen related cell adhesion molecule (CEACAM) subfamily. In

particular, human CEACAM1, which is commonly found on epithelial cells that compose the respiratory tract (108-110). Overall, UspA1 is considered to be a major adhesin for *M. catarrhalis*.

UspA2 in addition to mediating adherence to human epithelial cells is involved in serum resistance (111, 112). The subpopulation of *M. catarrhalis* that does not demonstrate serum resistance lacks UspA2 or has very low transcription of it (113, 114). UspA2 is able to bind the complement factors C3, C4b-binding protein, and vitronectin to resist killing by normal human serum (112, 115-117). UspA1 can also bind C3 and C4b, but UspA2 is considered to be more important to serum resistance than UspA1.

The UspA proteins are potential vaccine candidates due to their involvement in adherence and evading the immune system. Also, antibodies are produced against these proteins during the course of infection, which demonstrates that these proteins are targets for the immune system. Interestingly when gene expression was analyzed using the chinchilla model, UspA2 was slightly down regulated after 48 hours (82). This does not mean UspA2 is not involved in the initial colonization process, as this down regulation was observed for many of the virulence factors involved in adherence. Overall, UspA1, UspA2, and UspAH are good vaccine targets and play a major role in the virulence of *M. catarrhalis*.

Another autotransporter that in involved in adherence is Hag (MID). Hag is a 200 kDa protein that is responsible for hemagglutination, autoagglutinations, and has an IgD binding domain (118). Additionally, children have a mucosal immune response to Hag when they have been exposed to *M. catarrhalis* (119). Hag does not bind to all cell types, but it has been demonstrated to adhere to A549 (human type II alveolar lung epithelium), primary cultures of human middle ear epithelial cells, and normal human bronchial epithelium (41, 120, 121). Interestingly, Hag has a preference for the cilia on the normal human bronchial epithelium. Not only do *M.* catarrhalis Hag mutants not

adhere to ciliated cells, but when Hag is expressed in a nonadherent *H. influenzae* there is a 27% increase in adherence to the ciliated cells (122). Hag is another phase variable protein regulated by a poly(G) tract like UspA1 (123). Biofilm formation by *M. catarrhalis* is repressed when Hag is expressed (124). This is an interesting phenotype that needs to be explored further. Due to the fact that Hag is involved in adherence to a variety of human epithelial cells and antibodies against it are produced in humans make it a good target for vaccine development.

The third autotransporter that is involved in adherence is McaP. Like the UspA proteins and Hag, McaP has multiple functions. In addition to adhering to conjunctival, type II alveolar lung epithelium, and polarized human bronchial cells, McaP has phospholipase B activity (125). Further studies demonstrated that the N-terminal passenger domain mediates adherence to human epithelial cells (126). McaP was the first lipolytic enzyme that was characterized in *M. catarrhalis*. Further studies need to be conducted to determine the potential of McaP as a vaccine antigen.

Using transposon mutagenesis and selecting mutants with a reduction in adherence to human epithelial cells resulted in the detection of OMPCD. This is a 55 kDa protein with structural similarity to bacterial porins (127). Not only is this protein an adhesin but also following infection and colonization by *M. catarrhalis* patients were found to have anti-OMPCD antibodies in both otitis media and COPD patients. Also, in children the level of anti-OMPCD antibodies in the serum inversely correlated with the severity of infection (128). This demonstrates that OMPCD is a good vaccine target and antibodies against it can help to provide protection against infection.

Other surface exposed proteins that are potential vaccine candidates are proteins, which are involved in iron and nutrient acquisition. These proteins include transferrin-binding protein, (TbpA and TbpB), lactoferrin-binding proteins (LbpA and LbpB) and CopB. Anti-TbpA, -TbpB, -LbpA, and -LbpB antibodies have been found in

the serum of patients, which make them a potential vaccine candidate, but there is not a lot of other knowledge about these proteins (119, 129). CopB has been characterized the most. There is an increase in expression of CopB under iron limiting conditions and it mediates the binding of iron from transferrin and lactoferrin sources (130-132). These proteins have potential to be vaccine candidates, and they play a role in virulence, but they are not as well studied as the adhesins.

Moraxella catarrhalis has a plethora of virulence factors, which can be targeted as vaccine antigens. The focus of this report has been on the virulence factors involved in adherence. The majority of these proteins also have other functions such as serum resistance in the case of UspA2. Most likely a single protein will not be enough, but it is possible that a combination of these proteins would provide protection. There are also other factors that have not been fully explored. These include MhaB1 and MhaB2, the target of this project.

Contact Dependent Inhibition

Bacterial contact dependent inhibition (CDI) was first reported by Aoki and colleagues in 2005 (133). CDI systems are a way for bacteria to compete with closely related bacteria using a large surface expressed protein that has inhibitory activity at the C-terminus. Aoki *et al.* discovered that CDI systems are composed of three parts. The large CdiA, which has the inhibitor activity at the c-terminus (CT), the CdiB, which is the transporter for CdiA, and CdiI, which is the immunity protein that blocks the inhibitory activity (Figure 2.1). What occurs in this process is, CdiA and CdiB are a two-partner secretion (TPS) system, which allows for the surface expression of CdiA. Once CdiA is expressed on the surface of the inhibitor cell, it is able to come into contact with another bacterial cell (target) and the CdiA-CT (C-terminus of the CdiA protein) can be cleaved and imported into the target cell. Once the CdiA-CT is inside the target cell there are two options. The first option is if the target cell has the CdiI, then CdiI will bind the CdiA-

CT and prevent inhibition activity and the target cell will continue to grow and divide. The second option is if the target cell lacks the CdiI, in this case the CdiA-CT is able to have activity due to the lack of the CdiI. The CdiA-CTs have a wide range of activities including nucleases, adenosine deaminases, ADP-ribosyl cyclases, metallopeptidases, and there are other that have been yet to determined (40, 43, 133-139). Additionally, each CdiA-CT and CdiI forms a unique pair, which causes this family to be very diverse.

This system has been studied extensively in *E. coli* and this system has been found in other bacteria including *Burkholderia* species, *Yersinia pestis*, *Dickeya dadantii, Neisseria meningitidis*, and others (40). While, the CDI system is involved in virulence, it is not known what role the system plays during infection (43, 139-143). It has been hypothesized that this system is used as a communication method to shape bacterial communities (135). Most of the work in this area has focused on determining how these systems work.

CdiA and CdiB form a two-partner secretion system. These systems are found in a variety of gram-negative bacteria. In these systems, the protein is transported though the outer membrane using a specific transporter protein. It is common for adhesins to be secreted in this manner including FHA from *Bordetella pertussis* and HMW1/2 from *H. influenzae*. It has been demonstrated that FhaC (transporter for FHA) forms a beta barrel in the outer membrane, while FHA forms a beta helix (144-148). Many of the CdiA proteins have similarities to FHA (43, 149), including the proteins of interest for this study, MhaB1 and MhaB2 (41). The CdiB proteins have been demonstrated to transport the CdiA to the cell surface and without the CdiB there can not be inhibition due to the lack of CdiA on the cell surface (133). The CdiI is a very small protein (8.9 kDa in *E. coli*) and is necessary to be able to express the CdiA (43).

This system was first discovered in uropathogenic strains of *Escherichia coli* due to the observation that these strains were able to inhibit growth of other *E. coli* strains.

In *E. coli* EC93, the system is clustered into *cdiBAI*. In this system, the CdiA is very large (~319 kDa) and has hemagglutinin-repeats similar that of FHA from *B. pertussis* in the large conserved portion of CdiA. The CdiA C-terminus (CdiA-CT) is very divergent (when compared to other CdiAs from *E. coli*) and it is this C-terminus portion that confers the inhibition activity. Additionally, it is this portion that the CdiI must bind in order to inhibit the activity (40, 133, 135, 150, 151).

Using *E. coli,* it has also been shown that there are other ways to block inhibitory activity besides the presence of the immunity protein (CdiI). It is necessary for the inhibitory cells to be in logarithmic growth, and expression of pili on the target cells can prevent growth inhibition (133). Additionally, the mechanism of importing the CdiA-CT into the target cell is not conserved. Initially it was thought that BamA (YaeT) was the receptor on the outer membrane and AcrB transferred the CdiA-CT through the inner membrane for all CDI systems, but this was found to be dependent on the highly variable BamA loops (150, 152, 153). The identification of the contact with the loops of BamA demonstrated that CDI is restricted to targeting the same species (153).

These systems are polymorphic and the CdiA-CT and cognate CdiI lack similarity to other pairs. There is a relatively well-conserved VENN motif before the start of the CdiA-CT (active portion). In some proteins, this motif has been degraded or is not present in certain species. In *Burkholderia* species there is a (Q/E)LYN motif instead of the VENN motif (134, 140). These motifs also represent the sharp change in the well-conserved portion of CdiA to the very polymorphic CT (40, 142). Another interesting aspect of these systems is downstream of the *cdiA/cdiI* are orphan pairs. These orphan pairs are composed of a small CT-like ORFs followed by a *cdiI*. These orphan pairs, while the CT-like ORF has similarity to the *cdiA*, the immunity genes may not share any similarity (142). Even more interesting is the fact that these orphan pairs appear to be mobile and can be exchanged between bacteria. Also, while there is not evidence that

the orphan CdiA-CTs are expressed there is evidence that the CdiIs can be expressed (142). Essentially, each system contains an arsenal of CdiA-CT/CdiI pairs that can be traded around and protect against other bacteria targeting the cell (142). It has been demonstrated that when these orphan pairs are fused with the conserved region of the *cdiA*, they can form a functional CDI system. This demonstrates the diversity of these systems and the extent at which these systems are used if the bacteria are collecting the CdiA-CT/CdiI of other bacteria in order to protect themselves from this killing.

It has been demonstrated that the CdiA-CT from unrelated bacteria can be placed on the conserved region of the CdiA from *E. coli* and form functional CDI systems. These chimeric CDI systems demonstrate that the conserved region is the portion that binds to the target cell and initiates the intake process of the CdiA-CT into the target cell. Additional studies to explore how this import process occurs showed that both the CdiA-NT (conserved) and CdiA-CT (active) regions are deposited on the target cell, but only the CdiA-CT is translocated into the target cells (136). In addition to these studies, Ruhe *et al.* did a detailed study on how the CDI system from *E. coli* EC93 is imported into the target cell. While other enteric gammaproteobacterial species (e.g. *Salmonella enterica* Typhimurium, *Enterobacter aerogenes*, and *Citrobacter freundii*) are naturally resistant to the CDI system from *E. coli* EC93, they can be made susceptible when BamA from *E. coli* is expressed in these cells. This is independent of what CdiA-CT is being expressed (153). Even more powerful is the fact that the BamA loops 6 and 7 from *E. coli* can be grafted onto the BamA from another species and this will cause that species to become susceptible to *E. coli* EC93. This demonstrates that the CDI system is limited by its ability to initiate the import process not the activity of the CdiA-CT (153).

These CDI systems are a rapidly changing family. While each system has it own unique group of CdiA/CdiI pairs, there are some similarities between systems. For

example an orphan pair from *E. coli* EC93 is similar to that of *E. coli* UPEC 536. The CdiA-CTs have 75% identity while the CdiIs have 35% identity. When these pairs are mix-matched the CdiIs have a lower affinity for the CdiA-CT, but the $CdiI^{UPEC536}$ is able to block the activity of $CdiA\text{-}CT^{EC93}$, but the $CdiI^{EC93}$ is unable to block the activity of $CdiA\text{-}CT^{UPEC536}$ (142). This helps to support the theory that these pairs are constantly mutating and changing in order to diversify.

There are many questions that have yet to be answered about CDI systems. Very little is known about how they are regulated. It has been shown that some are under very tight regulation. For example, the CDI system in *Dickeya dadantii* 3937 is expressed when on plants, but is not expressed when grown on laboratory media (rich or minimal media) (40). Also, it was demonstrated that the CDI system played a role in pathogenesis in *D. dadantii*. Unfortunately, this role is not known and more studies are needed to determine how the CDI system affects the ability of *D. dadantii* to cause disease in plants. Additionally, while many of these systems have been identified in various species of bacteria, in depth studies have not been done with the various systems. The CDI system for *E. coli* EC93 is the only system that has been studied in depth, which leaves many questions about the other systems that have been identified. One potential system that has been identified and has yet to be explored is the CDI system in *M. catarrhalis*.

MhaB1 and MhaB2

The potential vaccine targets this study focuses on are MhaB1 and MhaB2. These are part of a two-partner secretion system in *M. catarrhalis*, in which MhaC transports MhaB1 and MhaB2 through the outer membrane (41). These targets first came to our attention due to their similarity to FHA from *B. pertussis*. FHA is a component of all licensed whooping cough vaccines in the U.S. Initially, MhaB1 (MchA2) and MhaB2 (MchA1) were thought to be adherence proteins since lack of

expression in wild type *M. catarrhalis* resulted in a decrease in adherence to a wide variety of human cell lines (41, 42). Based on bioinformatics analysis MhaB1 was suggested to be a CdiA protein (40). This changed the direction of the research from adherence to studying the CDI system in *M. catarrhalis*. Later, MhaB2 was also identified as a CdiA protein.

MhaB1 and MhaB2 fit into the CDI family of proteins. Along with MhaC, they form a two-partner secretion system. Also, MhaB1 and MhaB2 are large proteins (184 kDa and 201 kDa respectively). The first 1,200 amino acids of MhaB1 and MhaB2 are identical within strains and overall they are 99% identical across strains. The C-termini are very divergent having only 16% identity (41). This also fits with them being part of a CDI system, because CdiA proteins typically have a well-conserved portion followed by a very divergent CT. Additionally, sequencing the Mha locus from wild type *M. catarrhalis* O35E demonstrated the presence of orphan pairs downstream of both *mhaB1* and *mhaB2* (data not shown).

The identification of MhaB1 and MhaB2 as potential CdiA proteins opened up many more questions. First and for most, do MhaB1 and MhaB2 function as a CDI system with MhaC? What are the immunity genes? What role could this system play in pathogenesis? There has not been a CDI system identified where there are two CdiA (MhaB1 and MhaB2) proteins for one CdiB (MhaC). Also, the role of a CDI system has never been studied in an animal model. These are some of the points I will address in the following chapters.

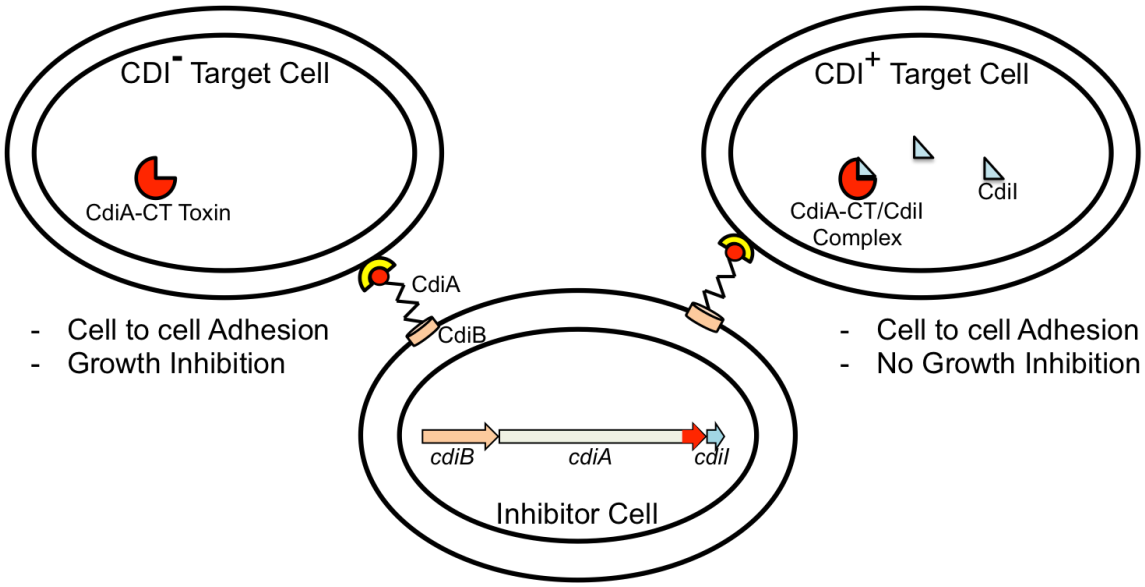

Figure 2.1: Contact-dependent growth inhibition (CDI) systems are composed of three genes, *cdiB*, *cdiA*, and *cdiI*. The cell that contains these genes is called the inhibitor cell, when these genes are expressed, CdiB transports CdiA through the outer membrane and CdiA comes in contact with a target cell. The CdiA C-terminus is then imported into the target cell. This is the portion of CdiA that has the inhibition activity. At this point there are two options for the cell. If the cell is lacks a CDI system the CdiA-CT toxin is able to inhibit growth through its activity. These activities are typically DNases or RNases. The second option is if the target cell has the same CDI system, then the CdiI will bind the CdiA-CT and prevent growth inhibition.

Adapted from Ruhe *et al.* (135).

CHAPTER 3

CHARACTERIZATION OF THE CONTACT DEPENDENT INHIBITION SYSTEM, MhaB1 AND MhaB2, IN *MORAXELLA CATARRHALIS*

Shaffer TL and Lafontaine ER. Characterization of the contact dependent inhibition system, MhaB1 and MhaB2, in *Moraxella catarrhalis*.

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Abstract

Moraxella catarrhalis is the third leading cause of otitis media causing ~20% of cases in children and causes ~10% of respiratory infections in adults with chronic obstructive pulmonary disease. The majority of the pathogenesis research on *M. catarrhalis* has been focused on adherence proteins. Two of these proteins, MhaB1 and MhaB2, have been suggested to be part of a contact dependent inhibition system. Initial studies on these proteins have demonstrated they are involved in the colonization process of the chinchilla nasopharynx and are potential vaccine targets. Contact dependent inhibition systems have been demonstrated to be involved in virulence in other bacteria. To determine if these proteins are part of a contact dependent inhibition system, we adapted the published contact dependent inhibition assays for *M. catarrhalis*. These data demonstrated that MhaB1 and MhaB2 are contact dependent inhibition molecules. In addition, the immunity protein has been identified for MhaB1 and there is a potential MhaB2 immunity identified. Finally, using the contact dependent inhibition system from wild type *M. catarrhalis* O35E, we demonstrated that there are susceptible strains among wild type clinical isolates. These data indicate that *M. catarrhalis* O35E has two functional contact dependent inhibition inhibitor proteins, which can be used to target other strains of *M. catarrhalis*.

Introduction

Bacterial contact dependent inhibition (CDI) is a method to target closely related bacteria using a toxin/anti-toxin system. In these systems, inhibitor proteins (CdiA) are expressed on the bacterial surface by an OMP like porin transporter protein (CdiB) in a two-partner secretion manner. When CdiA comes in contact with a target cell, a portion of its C-terminus (CdiA-CT) is imported into the target cell. If the target cell lacks a CDI system, it undergoes growth inhibition. If the target cell possesses a CDI system and expresses a cognate immunity protein (CdiI), the latter will bind the CdiA-CT and prevent

growth inhibition (40, 133, 135, 153-155). The C-termini of the CdiA proteins are polymorphic and vary drastically in their sequence and inhibitory function they specify; therefore each CdiA has its own unique CdiI, which confers protection. There can be limited cross protection between CdiI depending on how similar the cognitive CdiA-CTs are (153).

The CDI systems from *Escherichia coli* strains are the most well studied systems. In these systems, the CDI genes are clustered in the same genomic locus (*cdiBAI*), with CdiA being a large (~319 kDa) hemagglutinin-repeat protein (133). It also shares similarity to FHA from *Bordetella pertussis*. In contrast, the CdiI is a very small 8.9 kDa protein (133). In addition to having the main CdiA/CdiI pair, there are "orphan *cdiA-CT/cdiI*" genes downstream of the main CDI locus (142). These *cdiA-CT/cdiI* orphans are similar but not identical to the full-length *cdiA-CT*. It appears that these pairs each have a unique activity and a unique immunity gene, which suggests these pairs develop and change together. While there are data demonstrating the orphan immunity genes are expressed, there is no indication that the orphan *cdiA-CT* can be utilized unless attached to the main *cdiA* (142). These immunity genes can help protect a cell against other CDI systems, so it follows that cells would collect a variety of *cdiI* genes. However, it is not readily apparent why the *cdiA-CTs* are also being collected. One theory is that these regions can be duplicated, and the *cdiA-CT* can be "loaded" onto the full-length *cdiA* gene. Duplication would allow the extra *cdiA-CT/cdiI* pairs to diverge and overtime these small changes further diversify the region (135, 142, 153).

The mechanism of how these CDI systems operate has been determined, but the way they function remains unknown. *Burkholderia* species possess a variety of CDI systems, with many strains having multiple systems. It is common for one single strain to dominate an ecological niche, and it is speculated that CDI systems may be responsible for the domination of a species in an ecological niche (134, 140, 152).

Interestingly the CDI systems from *Erwinia chrysanthemi* and *Dickeya dadantii* appear to be necessary for virulence, but the roles these systems play in virulence are not understood (40, 135).

Moraxella catarrhalis is a human specific organism that causes otitis media in children and respiratory exacerbations in adults with chronic obstructive pulmonary disease (COPD) (2, 3, 9, 11, 12, 16, 17, 21-24, 27, 46, 52, 55, 57, 58, 86). There is an interest in developing a vaccine against *M. catarrhalis*, as it has rapidly developed resistance to β-lactams and it is the third leading cause of otitis media behind *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae*. The first antibiotics prescribed to children are typically β-lactams, because *S. pneumoniae* and nontypeable *H. influenzae* are susceptible to β-lactams (2, 3, 8, 9, 11, 12, 16, 17, 27, 32, 46, 52, 55, 57, 58, 86). To date, the majority of potential vaccine candidates from *M. catarrhalis* have been adherence proteins (1, 6, 44, 46). Included in this group are MhaB1 and MhaB2.

Initial studies on MhaB1 (MchA2) and MhaB2 (MchA1) characterized them as adherence proteins (41, 42). MhaB1 and MhaB2 share similarity to FHA, an adhesin, from *B. pertussis*, and *M. catarrhalis* mutants lacking expression of MhaB1 and/or MhaB2 have a decrease in adherence to human epithelial cells (41, 42). Due to their similarities to FHA, it is common for CDI proteins to be characterized as adhesins. However, MhaB1 possesses a VENN motif characteristic of CDI inhibitor proteins, suggesting it may be part of a CDI system (40).

MhaB1 and MhaB2 are part of a two-partner secretion system, in which MhaC is responsible for transporting MhaB1 and MhaB2 to the bacterial cell surface (41). While MhaB1 and MhaB2 are considered small for CdiA proteins, they are actually large proteins, 184 kDa and 201 kDa, respectively. MhaB1 and MhaB2 from *M. catarrhalis* O35E have 100% identity in the first 1,200 amino acids, while the remaining C-termini

share only a 16% identity. When MhaB1 and MhaB2 from *M. catarrhalis* O35E are compared to MhaB1 and MhaB2 proteins from other *M. catarrhalis* isolates, the first 1,200 amino acids share 99% identity while the C-termini are very divergent (41). Additionally, the region surrounding the *mha* locus has highly repetitive sequences reminiscent of "orphan *cdiA-CT/cdiI*" genes. The arrangement of the CDI gene locus, *mhaB1CB2* of *M. catarrhalis* is unique as MhaB1 and MhaB2 share a common transporter. Typically there is only one inhibitor gene per transporter gene in CDI systems. MhaB1 and MhaB2 are potential CDI inhibitor proteins from *M. catarrhalis* have that been shown to be necessary for colonization of the chinchilla nasopharynx, but this system has yet to be fully characterized (40, 156).

In this study, MhaB1 and MhaB2 were tested for their ability to inhibit the growth of target cells and potential immunity genes were identified and tested for their ability to protect target cells from growth inhibition. Additionally, wild type *M. catarrhalis* O35E was examined for its ability to target other wild type strains of *M. catarrhalis*. The goal of these experiments was to determine if MhaB1 and MhaB2 are a part of a CDI system, and if so to determine whether this CDI system could be used to target other *M. catarrhalis* strains.

Materials and Methods

Plasmids, Bacterial Strains, and Growth Conditions

The bacterial strains and plasmids used are listed in Table 3.1. *Moraxella catarrhalis* was cultured in Todd-Hewitt (TH) broth (BD Diagnostic Systems) or on TH agar plates at 37° C in the presence of 7.5% CO_2. When appropriate, *M. catarrhalis* mutants were supplemented with chloramphenicol (1 µg/mL), kanamycin (20 µg/mL), Zeocin™ (5 µg/mL), spectinomycin (15 µg/mL), and streptomycin (75 µg/mL). Cells were grown in broth for performing CDI assays and for making electrocompetent cells. In all other experiments, the *M. catarrhalis* cells were grown on TH agar plates.

Escherichia coli was cultured in Luria-Bertani (LB) broth (Fisher Bioreagents) or on LB agar plates at 37° C in the presence of 7.5% CO_2. Recombinant *E. coli* bacteria were supplemented with chloramphenicol (15 µg/mL), kanamycin (50 µg/mL), spectinomycin (200 µg/mL), and Zeocin™ (50 µg/mL) when appropriate. Cells were grown in LB broth for use with HiSpeed® Plasmid Midi Kit (QIAGEN) and on LB agar plates for all other conditions.

Recombinant *Haemophilus influenzae* DB117 was cultured in Brain Heart Infusion (BHI) broth (BD Diagnostic Systems) or on BHI agar supplemented with nicotinamide adenine dinucleotide (NAD) (10 µg/mL) and Heme (500 µg/mL). When necessary spectinomycin (50 µg/mL) or streptomycin (75 µg/mL) was added to the media. All strains were grown overnight at 37° C in the presence of 7.5% CO_2.

Plasmid DNA was isolated from *E. coli* using HiSpeed® Plasmid Midi Kit (QIAGEN) according to the manufacturer's specifications. Plasmids from *M. catarrhalis* or *H. influenzae* DB117 were isolated using the QIAprep Spin Miniprep Kit (QIAGEN) following the manufacturer's specifications.

Recombinant DNA Methods, PCR and Cloning

Standard molecular biology techniques were performed as described elsewhere (157). *Moraxella catarrhalis* DNA was isolated using the Easy-DNA™ Kit (Invitrogen™ Life Technologies™) according to the manufacturer's protocol. This DNA was then sheared and through various subcloning steps used to create plasmids pCC1.CB1i and pCC1.B2i. These subcloning steps were done using the CopyControl PCR cloning kit (Epicentre). These plasmids were then used to create a panel of mutants in *M. catarrhalis* O35E.

Restriction enzymes, *SphI* and *PacI* were used to digest pCC1.CB1i, removing a 1.4 kb fragment midway through *mhaB1*. A 1.2 kb spectinomycin resistance ($Spec^R$) cassette from pSPECR was ligated into pCC1.CB1i using End-It™ (Epicentre) to blunt

the ends, effectively generating in the plasmid pCC1.CB1KOi (158). Random transposon mutagenesis performed to insert a kanamycin (kan) resistance cassette into the potential *mhaB1i* of pCC1.CB1KOi. This insertion was achieved using the EZ-Tn5™<KAN-2> Insertion Kit (Epicentre) per manufacturer's instructions. Sanger sequencing confirmed the location of the insertion and the resulting plasmid with a deletion mutant in *mhaB1* and insertion in *mhaB1i* was named pCC1.CB1.iKO. Similar methods were used to create pCC1.B2KOi, a plasmid in which a deletion mutant was made in the *mhaB2* open reading frame (ORF). Using restriction enzymes *RsrII* and *NheI*, a 2.8 kb fragment was deleted from pCC1.B2KOi and replaced with the 0.4 kb Zeocin™ (Zeo) resistance cassette from pEM7Zeo (Invitrogen). Once Sanger sequencing confirmed this mutant the entire insert was digested out from pCC1, using the flanking *BamHI* sites. The insert was ligated into linearized pUC19 resulting in pUC19.B2KOi. The potential *mhaB2i* has a unique *EcoRI* site which was used to linearize pUC19.B2KOi. A chloramphenicol (cat) resistance cassette from pACYC184 was ligated to the linear pUC19.B2KOi, but no attempts resulted in a plasmid with *mhaB2i* interrupted with cat resistance (159).

Natural transformation was used to generate mutants in *M. catarrhalis* O35E. Briefly, plate grown overnight cultures of *M. catarrhalis* O35E were resuspended in TH broth and spotted onto TH agar plates, plasmids (20 µL) were added to the spotted bacteria, and plates were incubated at 37° C in the presence of 7.5% CO_2 for 4 hours. The bacteria were then resuspended in TH broth serially diluted and plated on TH agar plates supplemented with the appropriate antibiotics. Mutants were confirmed by PCR. Using this method O35E.B2KO (*mhaB2* deletion mutant), O35E.B1B2KO (*mhaB1* and *mhaB2* deletion mutants), and O35E.B1B2.B1iKO (*mhaB1*, *mhaB1i*, and *mhaB2* mutants) were made (Figure 3.1). It is important to note that each mutant was made separately and at different times.

The standard pWW115 plasmid could not be used to complement the mutant *M. catarrhalis* strains with the immunity genes, because it only carries a $Spec^R$ marker, which was used to create a mutation in the *mhaB1* gene. To address this, a $Spec^R$ from pLS88 was inserted in pWW115, and the resulting plasmid was designated pStrep (158). The potential *mhaB1i* and *mhaB2i* genes were PCR amplified using Platinum *Pfx* DNA polymerase (Invitrogen). The primers used are described in Table 3.2, and these PCR products were ligated into pStrep using T4 Ligase (New England Biolabs® Inc.).

Nucleotide Sequence Analysis

All plasmids were sequenced at the University of Michigan sequencing core (http://seqcore.brcf.med.umich.edu/ Accessed 2014 Jun 8). The resulting chromatograms were analyzed and assembled using the Sequencher software (Gene Codes Corporation). Sequence analysis was performed using Vector NTI (Invitrogen™ Life Technologies™).

CDI Assays

The published CDI assays were adapted to fit *M. catarrhalis* (40, 133, 134, 153). *Moraxella catarrhalis* strains were grown on TH agar plates overnight. The bacteria was resuspended and diluted to an optical density at 600 nm (OD_{600}) of 0.2. The target and inhibitor strains were then combined at the specified ratios. Time points were taken at 0 and 4 hours. During each of these time points, the OD_{600} of cultures was recorded and samples were serially diluted and plated. The inhibitor and target cells were distinguished using antibiotic resistance markers unique to each strain.

Results

Testing MhaB1 and MhaB1i as a CDI System

To determine if *mhaB1* and *mhaB1i* form a *cdiA/cdiI* pair, we developed a CDI assay from the literature to test inhibition based on the presence of MhaB1. All of the MhaB1 assays were done in an MhaB2 negative background to ensure that any

inhibition activity could be directly linked to MhaB1. In Figure 3.2, the ability of *M. catarrhalis* O35E.B2KO (inhibitor) to inhibit O35E.B1B2.B1iKO (target) is tested. In this experiment there were 1,000 inhibitor cells to 1 target cell. Additionally a control target strain was included, O35E.B1B2KO to insure that the inhibition is due to the lack of MhaB1i. When *mhaB1i* is knocked out the MhaB1$^+$ inhibitor strain is able to inhibit the growth of the target strain (MhaB1$^-$ MhaB1i$^-$). This indicates that MhaB1 is a CdiA inhibitor protein.

Next, we wanted to test if complementing the target strain (MhaB1$^-$ MhaB1i$^-$) with pStrep.B1i (MhaB1i$^+$) could restore target strain growth. Figure 3.3 indicates that having *mhaB1i* present on the plasmid is sufficient for preventing the growth inhibition phenotype of MhaB1. The empty vector control had no effect on the growth inhibition. Together, these experiments show that MhaB1 and MhaB1i form a functional CDI system.

Testing MhaB2 and MhaB2i as a CDI System

The limitations of this experimental model system prevented testing MhaB2 and MhaB2i in the same manner. This complication is addressed in the discussion of this paper, but briefly an *mhaB2i* mutant could not be made and without this mutant the previous experiments could not be repeated using MhaB2. Since the *mhaB2i* mutant could not be constructed, a new target strain was needed. In an alternate strategy, wild type strains that were previously identified to lack expression of MhaC, MhaB1, and MhaB2 (41) were utilized as target strains. The plan was to identify wild type *M. catarrhalis* strains were used as target strains in order to identify a strain sensitive to both MhaB1 and MhaB2 and then complement sensitive isolates with pStrep.B2i (MhaB2i$^+$). It was determined that MhaB1 and MhaB2 both inhibit the growth of *M. catarrhalis* TTA24 and this strain was used to test the ability of MhaB2 as a CDI protein. The inhibitor strains were *M. catarrhalis* O35E lacking expression of MhaB1 or MhaB1

and MhaB2 (Figure 3.4). The MhaB1 and MhaB2 double knockout was utilized as a control to ensure any inhibition activity was due to the presence of MhaB2. After confirming the inhibitory properties of MhaB2 using *M. catarrhalis* TTA24 as the target strain, attempts were made to complement TTA24 with pStrep. Unfortunately, this strain was not amenable to uptaking and/or replicating pStrep via both electroporation and natural transformation.

Targeting Wild Type Strains of *M. catarrhalis* with MhaB1 or MhaB2

Since it is hypothesized that CDI systems are a mechanism for bacterial competition, we investigated whether MhaB1 and MhaB2 could inhibit other wild type strains, and if so, to determine if there were varying levels of susceptibility among *M. catarrhalis* isolates. To do this, CDI assays were performed using O35E.B1KO, O35E.B2KO, and O35E.B1B2KO as the inhibitor strains and various wild type clinical isolates of *M. catarrhalis* as the target strains. These strains were combined at a ratio of 1 inhibitor cell to 1,000 target cells. The wild type strains were selected because they lacked expression of MhaC and MhaB1 or MhaB2 had been previously tested (41).

MhaB1 and MhaB2 have the ability to target McGHS2, 11P29B1 and TTA24 strains, while 7P94B1, P44, and TTA37 are resistant to both MhaB1 and MhaB2 (Figure 3.5 and Figure 3.6). It was surprising that target strains 7P94B1, P44, and TTA37 were not inhibited despite lacking reactivity to MhaC or MhaB antibodies (41). All of these strains were tested with O35E.B1B2KO as the inhibitor to ensure that the observed inhibition was due to the presence of MhaB1 or MhaB2 (data not shown).

Discussion

Contact dependent inhibition (CDI) is used for shaping a bacterial community by providing a simultaneous mechanism for both competition (lack CDI) and cooperation (posses CDI) amongst related bacteria. *Moraxella catarrhalis* is known to interact with other bacteria, particularly *H. influenzae* and together these bacteria cooperate to resist

clearance by the immune system (70). However it is not known if and how different *M. catarrhalis* strains interact with one another. In this study we identified and characterized the CDI system from *M. catarrhalis* O35E and determined that this CDI system can indeed target other strains of *M. catarrhalis*.

The *M. catarrhalis* O35E CDI system is unique in that there are two inhibitor proteins (MhaB1 and MhaB2) for one transporter (MhaC). Previous work determined that MhaB2 is expressed under normal growth conditions, but MhaB1 cannot be detected by western blot unless MhaB2 is knocked out (41). Interestingly enough, MhaB2 is downregulated and MhaB1 is upregulated after 24 hours in the chinchilla nasopharynx (82). This could indicate that MhaB2 is used to compete/cooperate with bacteria under normal laboratory conditions, and MhaB1 is used to compete/cooperate with bacteria under pathogenic conditions within a host.

MhaB1 has the VENN motif that is present in many of the CDI inhibitor proteins, which led to the initial hypothesis that MhaB1 is part of a CDI system (40). Using *M. catarrhalis* O35E, mutants were created to test MhaB1 as a CDI inhibitor protein. Our data indicate that MhaB1 is an inhibitor protein, as it can cause growth inhibition of O35E lacking expression of MhaB1 and MhaB1i (MhaB1 immunity protein) but cannot inhibit growth when MhaB1i is intact. Additionally, the immunity gene (*mhaB1i*) was identified and plasmid complementation of MhaB1i into the MhaB1/MhaB1i deficient O35E rescues the strain from growth inhibition. From these data we conclude that MhaB1 and MhaB1i form a functional CDI system. MhaB1 was expected to be more efficient at inhibiting growth, but a downstream orphan immunity gene is able to provide partial protection against the MhaB1-CT, a phenomenon has been demonstrated with other CDI systems (142). When MhaB1i (186 amino acids) is aligned with the translated orphan immunity gene (179 amino acids), they are over 70% identical with the last 110

amino acids having 100% identity. Further studies are required to determine if this orphan immunity gene truly provides partial protection against MhaB1 growth inhibition.

Attempts were made to characterize MhaB2 and its potential immunity protein in the same manner as MhaB1. Multiple attempts were made to interrupt *mhaB2i*, but none of these were successful. It appears that the *mhaB2-CT* is able to form a small open reading frame on its own. In order to remove this portion of *mhaB2* it would be necessary to PCR amplify the immunity region and downstream sequence. Unfortunately, this region is highly repetitive and we were unable to design primers that would bind in one location and provide enough flanking region for recombining a mutant construct into the *M. catarrhalis* genome. In order to create this mutation, more sequence downstream of *mhaB2i* would be needed.

Previous work had tested a variety of *M. catarrhalis* clinical isolates for the presence of MhaC and MhaB1/MhaB2 (41). Strains that did not react with MhaC or MhaB antibodies were selected and used to determine if any of these strains were susceptible to inhibition by MhaB1 and MhaB2. Both MhaB1 and MhaB2 consistently inhibited McGHS2, 11P29B1, and TTA24. In order to determine if *mhaB2i* is the immunity gene for MhaB2, attempts were made to add pStrep and pStrep.B2i to these strains. However, these strains had not been previously tested to see if pWW115 could replicate in them (48). The attempts to complement with pStrep and pStrep.B2i were unsuccessful. While this was not the desired result, some interesting findings were gleamed from these data.

First, while all of these strains (McGHS2, 7P94B1, 11P29B1, P44, TTA24, and TTA37) appear to lack a CDI system, they display varying levels of susceptibility to MhaB1 and MhaB2. It was expected that all of these strains would be susceptible to contact dependent inhibition, but these data indicate that even though these bacteria lack expression of the transporter and the large portion of the inhibitor protein they have

immunity proteins. We hypothesize that these strains actually possess immunity genes in their genomes, but further studies with additional sequencing are required to determine if these clinical isolates truly possess immunity genes or if this resistance mechanism occurs through other methods.

This is the first study to demonstrate that *Moraxella catarrhalis* has a fully functional CDI system with MhaB1 and MhaB2 acting as CDI inhibitor proteins. Our data also indicate that MhaB1i is indeed the immunity protein for MhaB1, but further experiments are required to conclusively demonstrate the potential MhaB2i is the immunity protein for MhaB2. Interestingly, these two inhibitor proteins (MhaB1 and MhaB2) share a common transporter protein (MhaC), indicating a unique relationship that has not been demonstrated in other CDI systems. Finally, we demonstrate the susceptibility of various *M. catarrhalis* clinical isolates to growth inhibition by MhaB1 and MhaB2 in wild type *M. catarrhalis* O35E. Additional studies are needed to fully understand how the CDI system in *M. catarrhalis* operates and to determine the exact impact of this system on bacterial-bacterial interactions both under normal laboratory conditions and within a host.

Strain	Description	Source
M. catarrhalis		
O35E	Wild type isolate from middle ear effusion (Dallas, TX)	E. Hansen (78)
O35E.B1KO	*mhaB1* isogenic mutant of strain O35E, spec	This Study
O35E.B2KO	*mhaB2* isogenic mutant of strain O35E, zeo	This Study
O35E.B1B2KO	*mhaB1mhaB2* double isogenic mutant of strain O35E, spec and zeo	This Study
O35E.B1B2.B1iKO	*mhaB1mhaB2mhaB1i* triple isogenic mutant of strain O35E, spec, zeo, and kan	This Study
McGHS2	Wild type disease isolate (Toledo, OH)	G. Hageage (41)
7P94B1	Wild type isolate from the sputum of an adult with COPD (Buffalo, NY)	T. Murphy (160)
P44LN2	Wild type disease isolate (Johnson City, TN)	S. Berk (102)
P44-80	Wild type disease isolate (Johnson City, TN)	S. Berk (102)
TTA24	Wild type isolate from tracheal aspirates of patients with confirmed *M. catarrhalis* pneumonia (Johnson City, TN)	S. Berk (78)
TTA37	Wild type isolate from transtracheal aspirate (Johnson City, TN)	S. Berk (102, 103)
E. coli		
Epi300	Cloning strain	Epicentre
H. influenzae		
DB117	Cloning strain	(161)
Plasmid		
pCC1	Cloning vector, cat	Epicentre
pCC1.CB1i	pCC1 containing and expressing m*haC mhaB1* and *mhaB1i*, cat	This Study
pCC1.CB1KOi	pCC1.CB1i with a deletion mutation in *mhaB1,* cat and spec	This Study
pCC1.CB1iKO	pCC1.CB1KOi with an insertion mutation in *mhaB1i,* cat, spec, and kan	This Study
pCC1.B2i	pCC1 containing 2 kb upstream *mhaB2* and *mhaB2i,* cat	This Study
pCC1.B2KOi	pCC1.B2i with a deletion mutation in *mhaB2*, cat and zeo	This Study
pUC19	Cloning vector, ampicillin (amp)	(162)
pUC19.B2KOi	pUC19 with 2 kb upstream *mhaB2* and *mhB2i* sequence, amp and zeo	This Study
pWW115	Cloning vector, spec	(48)
pStrep	pWW115 with a streptomycin resistance cassette, spec and strep	This Study
pStrep.B1i	pStrep containing *mhaB1i*, spec and strep	This Study
pStrep.B2i	pStrep containing *mhaB2i*, spec and strep	This Study
pSPECR	Source of spectinomycin cassette	(158)
pLS88	Source of streptomycin cassette	(163)
pEM7Zeo	Source of Zeocin™ cassette	Invitrogen
pACYC184	Source of chloramphenicol cassette	(159)

Table 3.1: Strains and Plasmids Used in this Study.

Primer	Sequence
mhaB1i F	5'-TGG GTG AAA TCA TTA GAC ATA TGA A-3'
mhaB1i R	5'-TGA TGT CTT GTA AAT CAC CAT TGC-3'
mhaB2i F	5'-GCC ATG AAT GAT AAA AAC AAA CAC-3'
mhaB2i R	5'-GGG TCT TTT GGT TCC TAA AAA TAA A-3'

Table 3.2: Primers Used in this Study.

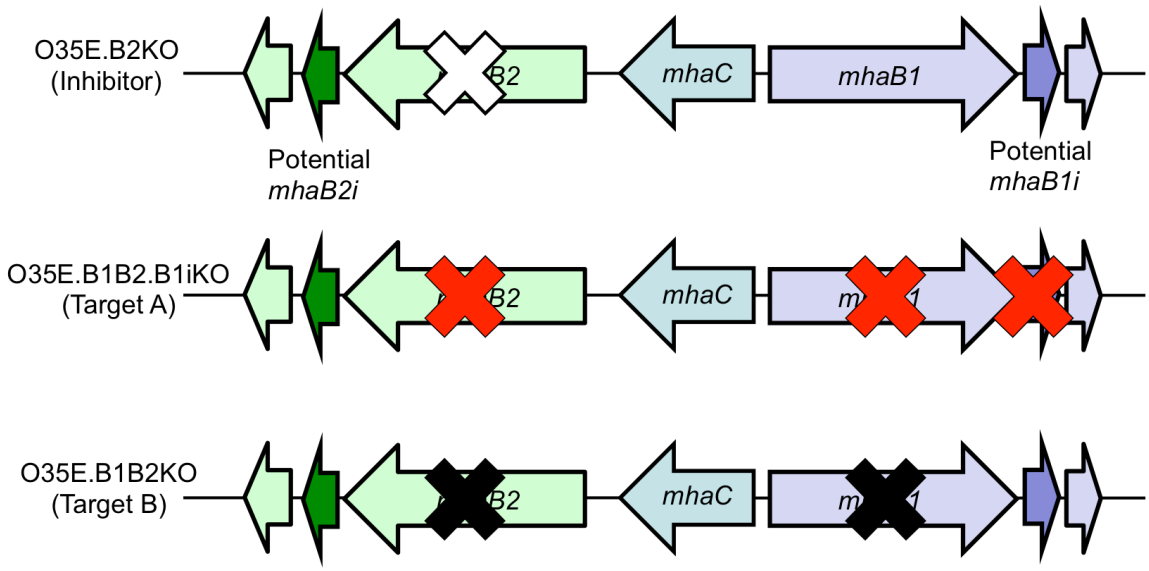

Figure 3.1: *Moraxella catarrhalis* O35E mutants used to test MhaB1 and MhaB1i as a CDI system. Isogenic *M. catarrhalis* O35E mutants used to test if MhaB1 is a CDI inhibitor protein. The inhibitor strain (O35E.B2KO) is expressing MhaB1, MhaB1i, and MhaB2i. The lack of MhaB2 expression ensures that any inhibition activity that occurs is due to MhaB1 being expressing. Target A (O35E.B1B2.B1iKO) lacks expression of MhaB1, MhaB2, and MhaB1i but expresses MhaB2i. It is expected that Target A will be inhibited, because the MhaB1 immunity protein (MhaB1i) is not expressed. Target B (O35E.B1B2) lacks expression of MhaB1 and MhaB2, but has both immunity genes (*mhaB1i* and *mhaB2i*) intact. It is expected that growth will not be inhibited in this strain.

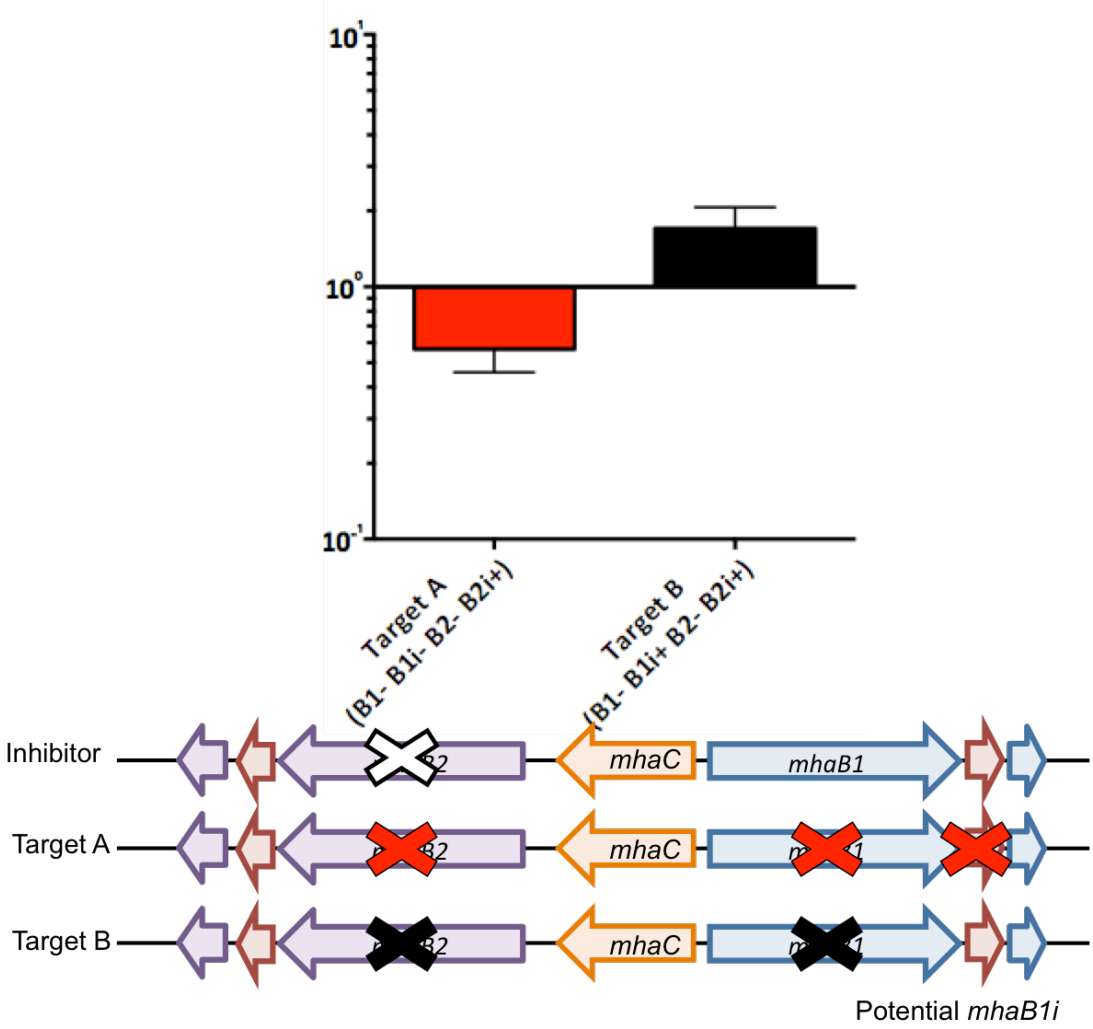

Figure 3.2: MhaB1 as a CDI inhibitor protein. The inhibitor strain O35E.B2KO expresses MhaB1 and MhaB1i. The inhibitor was combined with the target strains at a ratio of 1,000:1 (inhibitor:target). As expected, O35E.B1B2.B1iKO (Target A) underwent growth inhibition due to the MhaB1 immunity protein (MhaB1i) not being expressed (Figure 3.1). The control, O35E.B1B2KO (Target B) (Figure 3.1) continued to grow due to the presence of the intact *mhaB1i* gene.

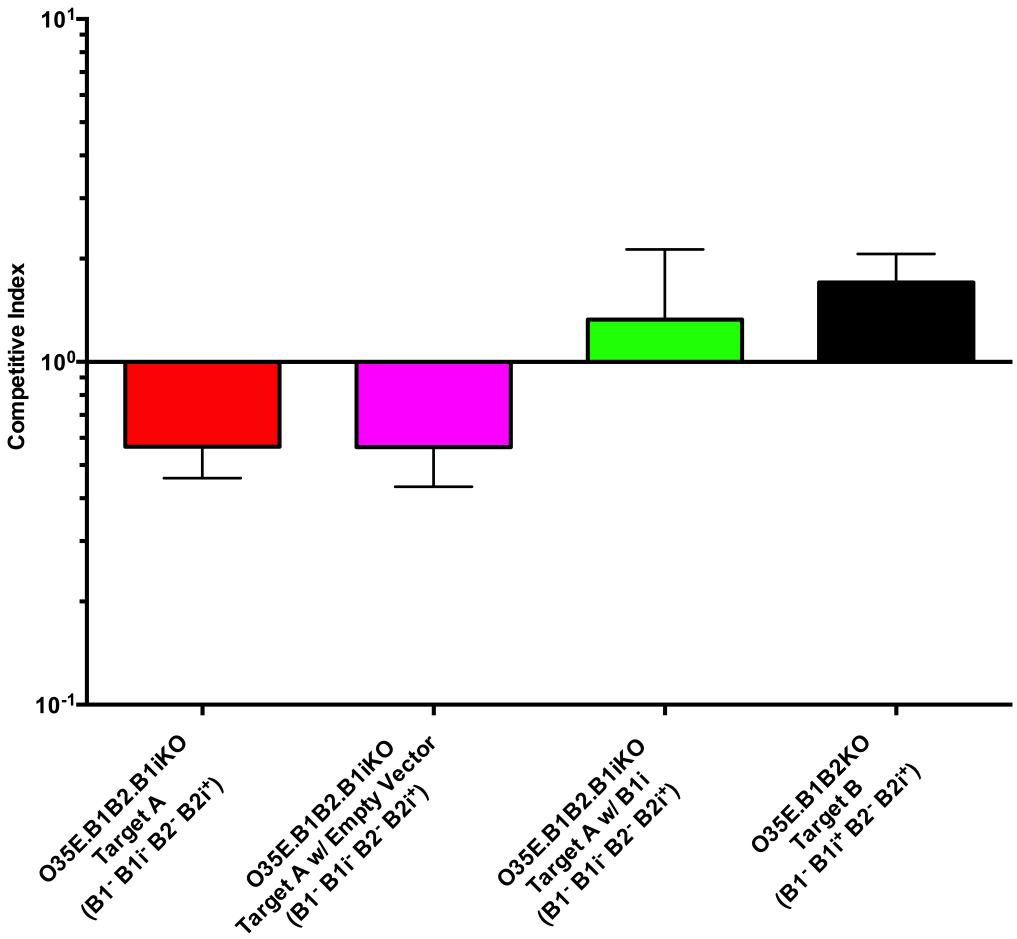

Figure 3.3: MhaB1i as a CDI immunity protein. The inhibitor strain, O35E.B2KO, expresses MhaB1 and MhaB1i. This strain was combined with the various target strains at a ratio of 1,000 inhibitor cells to 1 target cell. The first column represents the target strain O35E.B1B2.B1iKO (Target A from Figure 3.1), which lacks expression of MhaB1 and MhaB1i and as expected growth is inhibited. The second column is also Target A (Figure 3.1) except it also contains the empty vector pStrep and its growth is inhibited also. The third column is the same strain, but instead of the pStrep it contains pStrep.B1i and in this growth is not inhibited due to the presence of *mhaB1i* on the plasmid. In the final column, the target strain is O35E.B1B2KO (Target B Figure 3.1), which has *mhaB1i* intact and as expected growth is not inhibited.

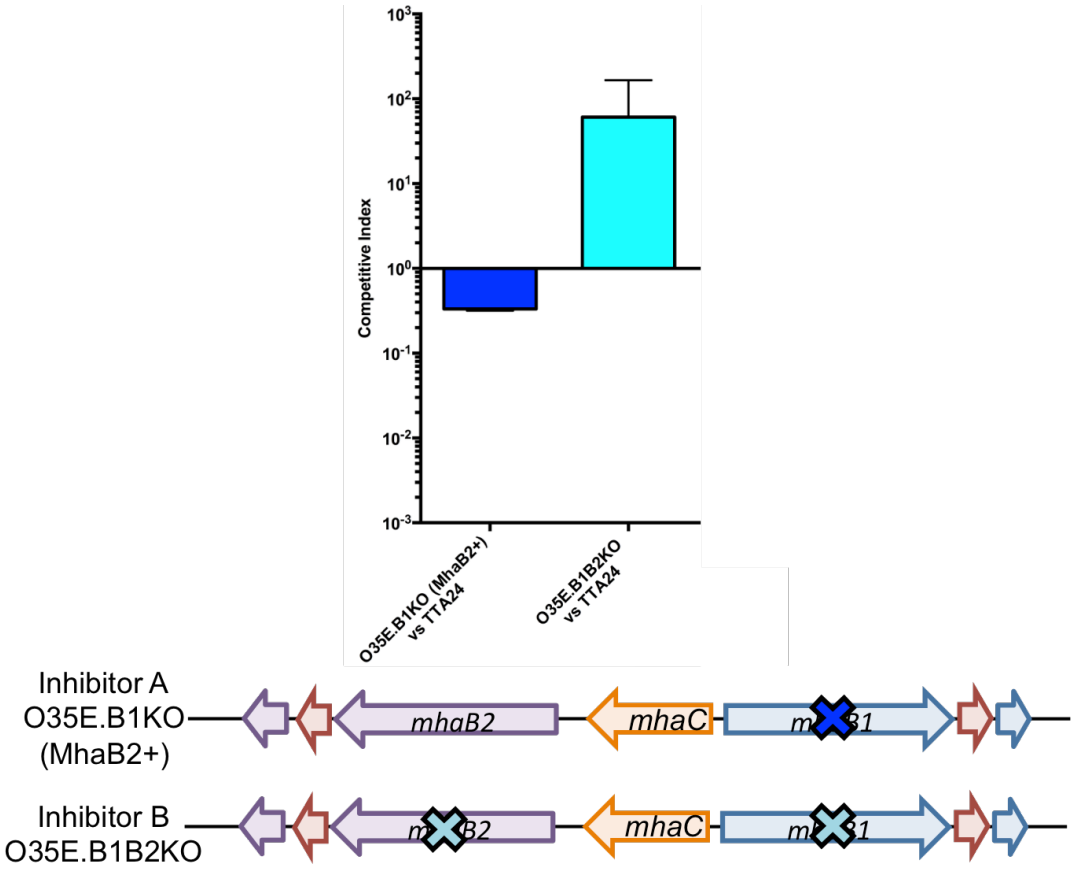

Figure 3.4: MhaB2 targets *M. catarrhalis* strain TTA24. MhaB2 is able to inhibit the growth of *M. catarrhalis* strain TTA24. The first column is a combination of the inhibitor strain O35E.B1KO (MhaB2$^+$) and the target TTA24 at a ratio of 1 inhibitor cell to 1,000 target cells. The second column is a combination of the inhibitor strain O35E.B1B2KO and the target strain TTA24 at a ratio of 1 inhibitor cell to 1,000 target cells. O35E.B1KO is able to inhibit the growth of the target TTA24, while the mutant lacking MhaB2 expression is unable to inhibit TTA24. TTA24 lacked expression of MhaC and MhaB1/B2 by western blot, so presumably they lack the CDI system in *M. catarrhalis*.

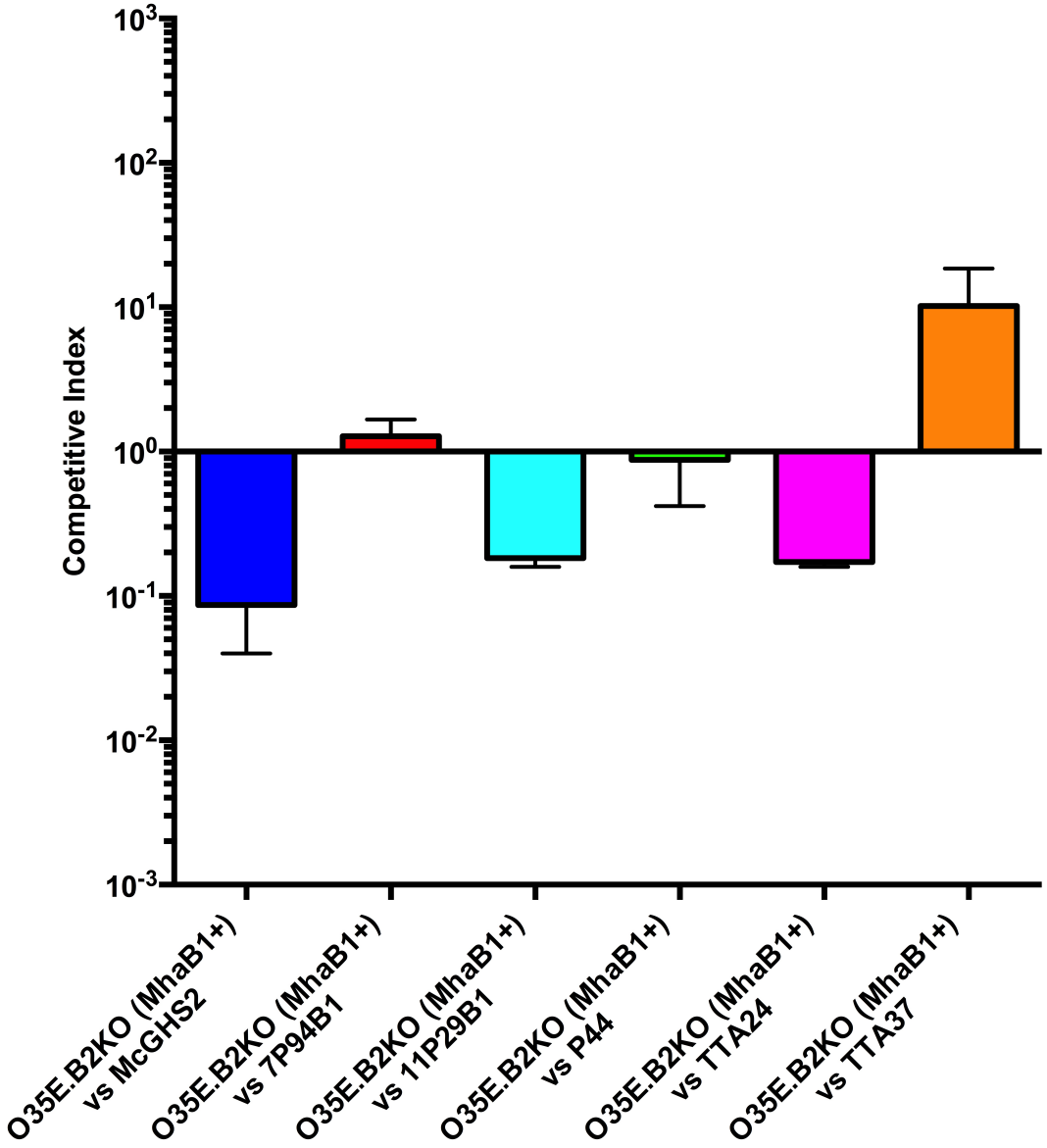

Figure 3.5: MhaB1 from O35E is able to target various wild type *M. catarrhalis* strains. Various wild type isolates were selected from the isolates that had been previously tested by western blot to determine if they had MhaC and/or MhaB1/MhaB2. All of these target strains were presumed to lack the CDI system from *M. catarrhalis*. The inhibitor strain for this assay is O35E.B2KO, which is expressing MhaB1 as the inhibitor protein. The various *M. catarrhalis* strains are the target cells in this assay. The inhibitor to target ratio was 1:1,000.

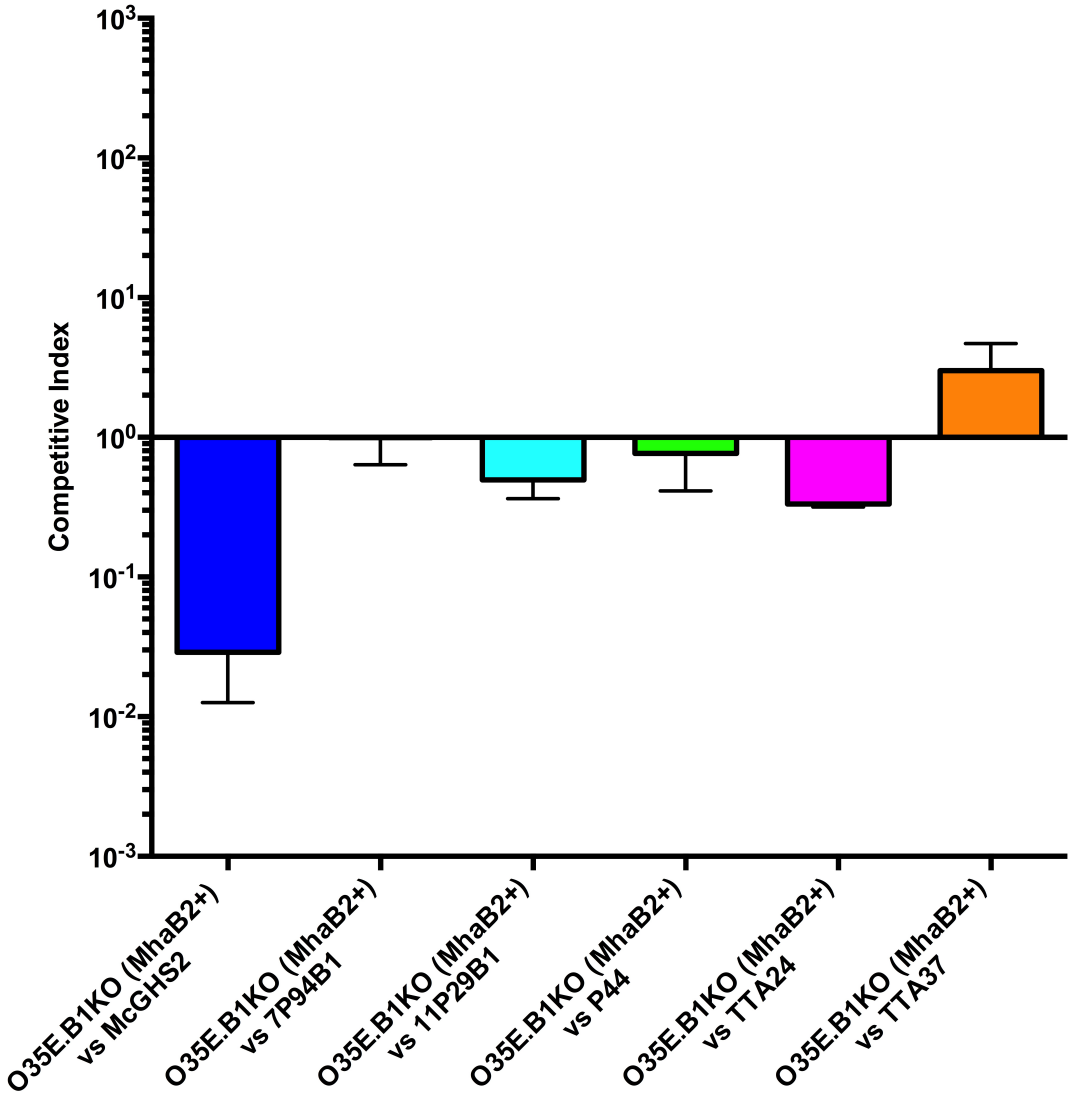

Figure 3.6: MhaB2 from O35E is able to target various wild type *M. catarrhalis* strains. Various wild type isolates were selected from the isolates that had been previously tested by western blot to determine if they had MhaC and/or MhaB1/MhaB2. Therefore, they presumably lacked the CDI system from *M. catarrhalis*. The inhibitor strain for this assay is O35E.B1KO, which is expressing MhaB2 as the inhibitor protein and the various *M. catarrhalis* strains are the target cells in this assay. The inhibitor to target ratio was 1:1,000.

CHAPTER 4

USE OF THE CHINCHILLA MODEL TO EVALUATE THE VACCINOGENIC POTENTIAL OF THE *MORAXELLA CATARRHALIS* FILAMENTOUS HEMAGGLUTININ-LIKE PROTEINS MhaB1 AND MhaB2

Shaffer TL, Balder R, Buskirk SW, Hogan RL, Lafontaine ER (2013) Use of the Chinchilla Model to Evaluate the Vaccinogenic Potential of the *Moraxella catarrhalis* Filamentous Hemagglutinin-like Proteins MhaB1 and MhaB2. PLoS ONE 8(7): e67881. doi:10.1371/journal.pone.0067881

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Abstract

Moraxella catarrhalis causes significant health problems, including 15-20% of otitis media cases in children and ~10% of respiratory infections in adults with chronic obstructive pulmonary disease. The lack of an efficacious vaccine, the rapid emergence of antibiotic resistance in clinical isolates, and high carriage rates reported in children are cause for concern. In addition, the effectiveness of conjugate vaccines at reducing the incidence of otitis media caused by *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* suggests that *M. catarrhalis* infections may become even more prevalent. Hence, *M. catarrhalis* is an important and emerging cause of infectious disease for which the development of a vaccine is highly desirable. Studying the pathogenesis of *M. catarrhalis* and the testing of vaccine candidates have both been hindered by the lack of an animal model that mimics human colonization and infection. To address this, we intranasally infected chinchillas with *M. catarrhalis* to investigate colonization and examine the efficacy of a protein-based vaccine. The data reveal that infected chinchillas produce antibodies against antigens known to be major targets of the immune response in humans, thus establishing immune parallels between chinchillas and humans during *M. catarrhalis* infection. Our data also demonstrate that a mutant lacking expression of the adherence proteins MhaB1 and MhaB2 is impaired in its ability to colonize the chinchilla nasopharynx, and that immunization with a polypeptide shared by MhaB1 and MhaB2 elicits antibodies interfering with colonization. These findings underscore the importance of adherence proteins in colonization and emphasize the relevance of the chinchilla model to study *M. catarrhalis*-host interactions.

Introduction

Moraxella catarrhalis is a leading cause of otitis media worldwide along with *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae* (NTHi) (1-8). More than 80% of infants experience at least one episode of this disease by the age of

three, and *M. catarrhalis* is the causative agent in ~20% of these cases. Likewise, otitis media is the number one reason for which children are prescribed antibiotics (9, 10). In the U.S., ~25 million visits are made annually to pediatrician offices for treatment of this painful infection and of these, 3-5 million are precipitated by *M. catarrhalis* (1-8, 15, 52-55). The annual costs associated with management of otitis media are upwards of $5 billion, and direct medical care expenditures alone account for $2-3 billion (1, 4, 7, 11-15). The disease is a significant source of distress, as it produces a rapidly expanding middle ear abscess that exerts pressure on the tympanic membrane and causes acute stabbing pain. After the onset of otitis media, fluid persists in the middle ear for weeks to months and interferes with hearing. Recurring ear infections are prevalent and occur during the crucial period when a child is developing speech and language skills. Hence, many children spend most of the first 2-3 years of life with some hearing impairment because of multiple episodes of otitis media, which can delay the development of communication and learning. The WHO has estimated that chronic/recurrent otitis media occurs in 65-330 million people and is the major cause of hearing loss in developing countries (16, 17). Clearly, otitis media is a significant health and economic problem, and *M. catarrhalis* contributes substantially to this burden.

Moraxella catarrhalis is also the second most common cause of respiratory infections in adults with chronic obstructive pulmonary disease (COPD) (14, 18-20). This disease is the fourth leading cause of death in the U.S., surpassed only by heart attack, cancer, and stroke (56). Each year ~10 million visits to physicians are related to COPD, and the costs associated with treatment are enormous—direct medical care costs alone are greater than $14 billion (21-24). Worldwide, COPD ranks as the fourth leading cause of death, killing more people than TB or HIV/AIDS, and is predicted to be third by 2030 (164, 165). The course of this debilitating disease is characterized by intermittent exacerbations, half of which are caused by bacterial infections. These

infections, of which *M. catarrhalis* causes ~10% of cases, contribute prominently to the progression of COPD by augmenting inflammation, oxidative stress, and tissue damage in the airways. In recent years, *M. catarrhalis* has also been increasingly associated with diseases such as bronchitis, conjunctivitis, and sinusitis (5, 8, 14, 25-39). Long considered to be a harmless commensal of the respiratory tract, *M. catarrhalis* is now recognized as an important cause of infectious disease and a significant source of morbidity.

Moraxella catarrhalis infections are a matter of concern due to the rapid emergence of antibiotic resistance in clinical isolates, high carriage rates in children, and the current lack of a vaccine. More than 90% of *M. catarrhalis* strains are now resistant to β-lactams (61, 65-69, 166, 167), which are generally the first antibiotics prescribed to treat otitis media. The genes specifying this resistance appear to be of Gram-positive origin (63, 64) suggesting that *M. catarrhalis* can readily acquire genes conferring resistance to additional antibiotics via horizontal gene transfer. Carriage rates as high as 81% have been reported in children (8, 168). In one study, Faden and colleagues analyzed the nasopharynx of 120 children over a two-year period and showed that 77% of patients became colonized with *M. catarrhalis* (45). These investigators also observed a direct relationship between colonization with *M. catarrhalis* and development of otitis media. This high carriage rate, coupled with the emergence of antibiotic resistance, suggests that *M. catarrhalis* infections may become more prevalent and difficult to treat, emphasizing the need to improve our understanding of pathogenesis by this understudied bacterium in order to identify targets for intervention and prevention.

To cause disease, *M. catarrhalis* must first colonize the nasopharynx and then spread to distal sites such as the middle ear and the lower respiratory tract. Hence, one key event that occurs early in pathogenesis by the organism is adherence to the mucosal surface of the nasopharynx because it leads to colonization. Crucial to this

process are afimbrial adherence proteins (adhesins), which mediate binding of bacteria to host cells (169-175). *Moraxella catarrhalis* expresses many afimbrial adhesins including UspA1 (104), UspA2H (104), MhaB1 and MhaB2 (41), MchA1 and MchA2 (42), Hag/MID (120, 122), OMPCD (127, 176) and McaP (125, 126). These molecules were characterized by demonstrating a decrease in the adherence of mutant strains to human airway cells *in vitro*, but their contribution to nasopharyngeal colonization, or utility as vaccine antigens has not been evaluated *in vivo*. In the present study, we utilized a chinchilla model to demonstrate that wild-type *M. catarrhalis* colonizes the nasopharynx for seven days, a mutant lacking expression of the adherence proteins MhaB1 and MhaB2 is impaired in its ability to colonize the nasopharynx, and immunization with a polypeptide shared by MhaB1 and MhaB2 elicits antibodies impeding nasopharyngeal colonization and promoting clearance.

Materials and Methods

Plasmids, Bacterial Strains, Growth Conditions, and Culture of Human Epithelial Cells *in vitro*

Strains and Plasmids are described in Table 4.1. Wild-type (WT) *M. catarrhalis* isolates were routinely cultured using Todd-Hewitt agar plates (THA, BD Diagnostic Systems). The *M. catarrhalis* isogenic mutant strain O35E.B1B2 was propagated on THA supplemented with 15 µg/mL spectinomycin and 5 µg/mL zeocin. The *hag* transposon mutant O35E.TN2, the *ompCD* mutant strain O35E.CD1, and the *uspA2* serum-sensitive mutant O35E.2 were cultured using THA containing 20 µg/mL kanamycin. For colonization experiments, tissues and nasopharyngeal lavages collected from infected animals were plated onto THA supplemented with 5 µg/mL vancomycin and 2.5 µg/mL trimethoprim to suppress the growth of the chinchilla flora. *Escherichia coli* was grown using Luria-Bertani (LB) medium (Fisher BioReagents) containing 15 µg/mL chloramphenicol or 100 µg/mL ampicillin. All stains were cultured

at 37°C in the presence of 7.5% CO_2. The human cell line HEp-2 (laryngeal epithelium; ATCC CCL-23) was cultured as previously reported (125).

Recombinant DNA Methods, PCR and Cloning

Standard molecular biology techniques were performed as described elsewhere (120, 125, 127, 157). Genomic DNA was obtained using the Easy-DNA™ kit (Invitrogen™Life Technologies™). A 1-kb amplicon encompassing amino acids (aa) 72-399 of the *M. catarrhalis* O35E MhaB1 protein was generated with primers P1 (5'-CGG GAT CCG TTA TTT CTG ACA GTC AAG CA-3'; *BamHI* site underlined) and P2 (5'-CGC TCG AGT ATT ACC TTG CAA GTT GGC AGT-3'; *XhoI* site underlined). This DNA fragment was excised from an agarose gel, purified with the High Pure PCR Product Purification Kit (Roche Applied Science), restricted with the endonucleases *BamHI* and *XhoI* (New England Biolabs® Inc.), and ligated into the *BamHI* and *XhoI* sites of the vector pGEX4T-2 (GE Healthcare Life Sciences) yielding plasmid pGEX-MhaB. This plasmid was sequenced to verify that no mutations were introduced during PCR and to confirm that the protein expressed from pGEX-MhaB corresponds to residue 72-399 of *M. catarrhalis* O35E MhaB1 fused to an N-terminal Glutathione-*S*-transferase (GST) tag. Plasmid DNA used as template in sequencing reactions was obtained with the QiAprep Spin Miniprep Kit (Qiagen). A similar approach was used to obtain the plasmid pGEX-McaP, which expresses residues 51-333 of *M. catarrhalis* O35E McaP joined to GST. The PCR product cloned into pGEX-McaP was amplified with primers P3 (5'-CGG GAT CCC AAG AAT TTA GCC AAA CCG TA-3'; *BamHI* site underlined) and P4 (5'-CGG TCG AGT CCC TGA AGG GTG AAT TTT ATC AGC-3'; *XhoI* site underlined). *Moraxella catarrhalis* O35E genomic DNA was used as the template in all PCR-based cloning experiments.

Nucleotide Sequence Analysis

Plasmids were sequenced at the University of Michigan sequencing core (http://seqcore.brcf.med.umich.edu/ Accessed 2013 Jun 4). Chromatograms were analyzed and assembled with the Sequencher software (Gene Codes Corporation). Sequence analysis was performed using Vector NTI (Invitrogen™ Life Technologies™).

Protein Preparation

Outer membrane proteins were obtained from *M. catarrhalis* strains using the EDTA procedure of Murphy and Loeb (177). The method used to prepare whole-cell lysates is described elsewhere (178, 179). The His-tagged recombinant protein His-MhaB was obtained as previously outlined by Balder *et al.* (41). The plasmids pGEX-MhaB and pGEX-McaP were introduced in the *E. coli* strain TUNER™ (EMD Millipore) for the purpose of overexpressing and purifying the recombinant proteins GST-MhaB and GST-McaP, respectively. Expression was induced by adding isopropyl-β-$_D$-thiogalactopyranoside (IPTG, final concentration of 1mM) to broth cultures and incubating for 5 hours at 37°C with agitation (200-rpm). Bacteria were pelleted, followed by treatment with the BugBuster® HT protein extraction reagent (EMD Millipore) supplemented with rLysozyme™ (EMD Millipore) under the recommended conditions. Recombinant proteins were then purified using a GST Spin Purification Kit (Thermo Scientific Pierce) per the manufacturer's instructions. Protein concentrations were determined with a bicichoninic acid (BCA) Protein assay kit (Thermo Scientific Pierce).

Analysis of Selected Antigens

Equivalent protein amounts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were visualized by staining gels with the ProtoBlue™ Safe reagent (National Diagnostics). Alternatively, the resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore) for western blot analysis. After transfer, the PVDF membranes were submersed in StartingBlock™ T20. After this incubation, the membranes were washed

with Phosphate-Buffered Saline (PBS) supplemented with 0.05% (vol/vol) Tween 20, followed by 1 hour incubation at room temperature with secondary Abs conjugated to Horse Radish Peroxidase (HRP) diluted in StartingBlock™ T20. After washing off the excess secondary Abs with PBS+0.05% Tween 20, protein bands were visualized by chemiluminescence using the Luminata™ Crescendo Western HRP substrate (EMD Millipore) and Foto/Analyst Luminary/FX imaging system (Fotodyne Inc.).

For ELISA, duplicate wells of Immulon™ 2HB plates (Thermo Scientific Nunc) were coated overnight at 4°C with ~1 µg of purified GST-MhaB protein. Excess unbound protein was removed by washing the wells with PBS+0.05% Tween 20, and the wells were then filled with PBS+0.05% Tween 20 containing 3% (wt/vol) dry milk and incubated for 1 hour at room temperature. After washing with PBS+0.05% Tween 20, the wells were probed overnight at 4°C with primary Abs diluted in PBS+0.05% Tween 20+3% dry milk. After this incubation, the wells were washed with PBS+0.05% Tween 20, followed by overnight incubation at 4°C with secondary Abs conjugated to HRP and diluted in PBS+0.05% Tween 20+3% dry milk. After washing off the excess secondary Abs with PBS+0.05% Tween 20, 100 µL of SureBlue™ TMB Microwell Peroxidase Substrate (KPL) was added to wells. Color development indicative of antibody binding was measured by determining the absorbance of well contents at a wavelength of 650 nm using a µQuant™ Microplate Spectrophotometer (BioTek®). End-point titers were determined as described by Song *et al.* (180) and correspond to the highest fold dilution giving an optical density at 650 nm greater than the mean value plus 3 standard deviations of pre-immune samples.

Antibodies

The murine monoclonal Abs 10F3 [specific for the *M. catarrhalis* iron transport protein CopB (132)], 5D2 [specific for the *M. catarrhalis* adhesin Hag (118)], 17H4 [specific for the *M. catarrhalis* serum resistance protein UspA2 (111)], and 1D3 [specific

for the *M. catarrhalis* adhesin OMPCD (62)], His-tag® (EMD Millipore) and GST-Tag™ were used as primary Abs in western blot experiments in combination with goat anti-mouse HRP (IgG+IgA+IgM) secondary Abs (SouthernBiotech). For experiments using chinchilla samples as primary Abs (ELISA, Western Blot), goat anti-rat abs conjugated to HRP were utilized for detection. Goat anti-rat HRP (IgG) and HRP (IgG+IgA+IgM) were purchased from SouthernBiotech. Goat anti-rat HRP (IgA) Abs were obtained from Bethyl Laboratories, Inc.

Adherence Assays

The WT *M. catarrhalis* strains O35E, O12E, and McGHS1 were preincubated for 30 min at 37°C with samples (serum, nasopharyngeal lavage fluids) collected from naïve and vaccinated chinchillas. These bacteria were then used to perform adherence assays as previously described by Lipski and colleagues (126). Briefly, bacteria were incubated for 30 min with HEp-2 human laryngeal cells seeded in 24-well tissue culture plates at a multiplicity of infection of 100 bacteria to 1 epithelial cell. The infected cells were then washed to remove unbound bacteria and treated with a solution containing saponin. Well contents were mixed, serially diluted, and spread onto agar plates to count colony-forming units (CFU). This value was used to calculate the number of inoculated bacteria that bound to HEp-2 cells. The adherence of *M. catarrhalis* preincubated with samples from control chinchillas (*i.e.* immunized with PBS) was set at 100%. The adherence of *M. catarrhalis* preincubated with samples from chinchillas vaccinated with the His-MhaB protein is presented as the percentage (± standard error) of that of *M. catarrhalis* preincubated with samples from control chinchillas. These assays were performed in triplicate in three or more separate experiments.

Intranasal Inoculation of Chinchillas with *M. catarrhalis*

The method used to inoculate the nasopharynx of chinchillas was adapted from that described by Luke *et al.* (83), Bakaletz and colleagues (80), and more recently by

Hoopman *et al.* (82). Healthy adult chinchillas (*Chinchilla lanigera*) were purchased from Rauscher's Chinchilla Ranch (LaRue, Ohio). Prior to inoculation the animals were anesthetized by injecting ketamine (10 mg/kg, Fort Dodge®) and xylazine (2 mg/kg, Lloyd Laboratories) intramuscularly (i.m.). Once anesthetized, the animals were placed on their stomach. Using a 26 ½ gauge needle attached to 1 cc syringe, 0.2 mL of a *M. catarrhalis* suspension containing ~$1x10^9$ CFU was delivered intranasally (i.n.) by administering 5-10 µL droplets to alternating nasal openings and allowing droplets to be brought into the nasopharynx by the animal's breathing. A total volume of 0.1 mL was administered per naris. *Moraxella catarrhalis* strains used to inoculate chinchillas were cultured on THA for 16-20 hr at 37°C. These plate-grown bacteria were suspended to a concentration of ~$5x10^9$ CFU/mL in PBS supplemented with 0.15% gelatin (PBSG) to maintain the viability of the organism. The *M. catarrhalis* suspension was also diluted and 100 µL aliquots were immediately spread onto THA supplemented with vancomycin and trimethoprim to determine the number of CFU inoculated into the nasal passages of the chinchillas. Back titration of inoculum was performed for all challenge experiments.

Viable *M. catarrhalis* was recovered from the nasopharynx of infected animals by performing nasopharyngeal lavages or by collecting and homogenizing nasopharyngeal tissues. Lavages were performed under anesthesia. Using a 1 cc syringe and a 26 ½ gage needle, 0.5 mL of PBSG was delivered at the entrance of one naris (in form of 5-10 µL droplets) by passive inhalation and collected from the other naris (as it is exhaled) utilizing a needle-free 1 cc syringe. Portions of these lavages were serially diluted and plated onto THA supplemented with vancomycin and trimethoprim. After 24 hr incubation at 37°C, CFU were counted to determine the number of viable *M. catarrhalis* bacteria present in the fluids.

To harvest nasopharyngeal tissues, chinchillas were first anesthetized as described above. While under anesthesia, the animals were euthanized by delivering 1

mL Beuthanasia®-D solution (Schering-Ploug Animal Health) via cardiac injection. This was accomplished by inserting 21 gauge, 1 ½ inch needle into the chest cavity beneath the xyphoid process and injecting the euthanasia solution into the heart. After assurance of death, decapitation was performed. Standard dissection techniques were used to remove the eyes, mandibles and soft tissues covering the skulls. Following this, the heads were bisected along the nasal septum to expose the interior structure of the nasopharynx. The mucosa of the nasopharynx and of the ethmoid and nasal turbinates were collected, weighed, and placed in 2 mL of PBSG. The nasopharyngeal tissues were then shredded, homogenized using a sterile glass dounce and pestle (Kimble Chase Life Science and Research Products), serially diluted and plated onto selective media to determine the number of viable *M. catarrhalis* organisms.

Immunization of Chinchillas

Serum and nasopharyngeal lavage fluids were collected from anesthetized chinchillas prior to immunization. Nasal fluids were collected as described above and stored at -80°C for later use. Blood was drawn by cardiac puncture. This was accomplished by inserting 21 gauge, 1 ½ inch needle into the chest cavity beneath the xyphoid process and removing blood directly from the heart. The samples were allowed to clot, centrifuged to remove red blood cells, and the sera were stored at -80°C. Blood samples and nasopharyngeal lavage fluids were also collected on days 19 and 44 post-immunization.

Vaccination was performed under anesthesia. Groups of chinchillas were immunized with PBS (control animals) or 80 µg of the His-MhaB protein. PBS and protein preparation were mixed with Complete Freund's Adjuvant (CFA) in a 1:1 ratio (vol/vol) and administered subcutaneously (s.c.). Booster vaccinations were performed on days 23 and 72. Animals were boosted with PBS or 80 µg of His-tagged protein mixed with Incomplete Freund's Adjuvant (IFA).

Animal Research Ethic Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Georgia. All efforts were made to minimize suffering.

Statistical Analysis

The paired *t* test was used to analyze data from adherence assays. *P* values <0.05 were found to be statistically significant. The results of nasopharyngeal colonization experiments were examined with the Wilcoxon signed rank test. All statistical analyses were performed using the Graph Pad Prism software.

Results

Use of the Chinchilla Model to Examine Colonization of the Nasopharynx by *M. catarrhalis*

To study *M. catarrhalis* colonization and persistence *in vivo*, we developed the ability to utilize the chinchilla model of nasopharyngeal colonization. Figure 4.1 shows the results of calibration experiments in which chinchillas were inoculated intranasally (i.n.) with 10^9 colony-forming units (CFU) of the wild-type (WT) isolate O35E. At the indicated times post-infection, animals were anesthetized and nasopharyngeal lavage fluids were collected, diluted and spread onto selective agar plates to suppress the growth of the chinchilla flora and accurately count viable *M. catarrhalis* CFU. Following this, chinchillas were euthanized and nasopharyngeal tissues were harvested, weighed, homogenized, diluted, and plated. After overnight incubation at 37°C, CFU were counted to determine the number of viable *M. catarrhalis* bacteria present in lavage fluids and tissues. The results shown in Figure 4.1 demonstrate that we obtain reproducible and consistent numbers, comparable to those reported by Luke *et al.* for the WT isolate 7169 (83) and Hoopman and colleagues for strain O35E (82).

After establishing the model, we tested the hypothesis that mutants lacking expression of adherence proteins will not colonize as effectively as WT *M. catarrhalis*. To accomplish this, cohorts of chinchillas were challenged with WT *M. catarrhalis* O35E and the mutant strain O35E.B1B2, which is unable to express the filamentous hemagglutinin-like proteins MhaB1 and MhaB2 (41). These molecules are associated with the outer membrane of *M. catarrhalis* and are secreted in a Two-Partner Secretion manner via the outer membrane protein MhaC. MhaB1 and MhaB2 are involved in adherence to several human epithelial cell types that are relevant to the pathogenesis of *M. catarrhalis* (lung, laryngeal, conjunctival). The adhesins also resemble the filamentous hemagglutinin FHA, which is the major colonization factor of *Bordetella pertussis* and a component of all vaccines that are currently licensed for use in children to protect against whooping cough (CDC website. Available: http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/pert.pdf. Accessed 2013 Jun 4). Figure 4.2 shows that lack of expression of MhaB1 and MhaB2 causes an 18.5-fold reduction in the number of viable *M. catarrhalis* bacteria recovered from nasopharyngeal tissues 72 hr post-infection. These results indicate that the filamentous hemagglutinin-like proteins are involved in *M. catarrhalis* ability to colonize and persist in the chinchilla nasopharynx. Lavages (prior to collecting tissues) were not performed in these experiments in order to generate a single value representing the total number of bacteria present in the nasal passageways at the experimental end-point.

Use of the Chinchilla Model to Perform Vaccine Studies

To test the hypothesis that a vaccine containing *M. catarrhalis* adherence proteins protects against colonization *in vivo*, chinchillas were immunized subcutaneously (s.c.) with a recombinant form of MhaB1 and MhaB2. Three independent vaccination trials were performed and the experimental timeline is depicted in Figure 4.3. The recombinant protein used to immunize chinchillas corresponds to aa

72-399 of MhaB1 fused to six N-terminal histidine residues. This portion of MhaB1 is 99% identical to aa 72-99 of MhaB2 in all *M. catarrhalis* isolates characterized to date, and murine Abs against this polypeptide were previously shown to react with both MhaB1 and MhaB2 (41). This shared region of MhaB1 and MhaB2 also displays sequence similarity to the portion of *B. pertussis* FHA that is a component of all licensed vaccines for whooping cough (data not shown).

Serum and nasopharyngeal lavage fluids were collected from chinchillas and analyzed by western blot and ELISA. The results are shown in Figure 4.4 and demonstrate that the animals produced serum Abs reacting with the adhesins in the outer membrane of *M. catarrhalis* (Figure 4.4A) and with a GST-tagged version of MhaB1/MhaB2 (Figure 4.4B and 4.4D). The data also indicate that chinchillas developed mucosal Abs binding to the shared region of MhaB1 and MhaB2 (Figure 4.4C). Serum and lavage fluids from the control animals vaccinated with PBS did not contain Abs against the adhesins (data not shown). Following this, we performed *in vitro* adherence assays in which *M. catarrhalis* was incubated with serum or lavage fluids from immunized chinchillas prior to infecting HEp-2 laryngeal cells. These experiments revealed that chinchilla Abs against MhaB1 and MhaB2 significantly decrease the adherence of multiple WT *M. catarrhalis* isolates to epithelial cells (Figure 4.5A and 4.5B). The data also indicate that this inhibitory effect is dependent on the concentration of Abs.

After confirming that chinchillas produced Abs against MhaB1 and MhaB2, and demonstrating that these Abs interfere with adherence to airway cells, we challenged the animals with ~10^9 CFU of the WT O35E and determined bacterial loads in nasopharyngeal tissues three days post-infection. Figure 4.6 shows that vaccination with the His-tagged MhaB protein causes a 9.3-fold reduction in the number of viable *M. catarrhalis* bacteria recovered from the nasopharynx of chinchillas compared to sham-

immunized animals. These results substantiate the data obtained when comparing the ability of the mutant O35E.B1B2 to colonize the nasopharynx to that of it progenitor strain O35E (Figure 4.2). The results also support the hypothesis that a vaccine containing *M. catarrhalis* adherence proteins will elicit the production of Abs blocking colonization and promoting clearance.

Moraxella catarrhalis Proteins Targeted by the Chinchilla Immune Response During Colonization

To gain more insight into the immune response of the chinchilla to *M. catarrhalis*, we inoculated four animals i.n. with 10^9 CFU of the WT strain O35E on three consecutive occasions (21 days apart). Seven days after the last challenge, serum samples were collected and analyzed by western blot. Figure 4.7 shows that chinchillas produced Abs against several *M. catarrhalis* antigens during colonization including the iron acquisition protein CopB, the serum-resistance factor UspA2, and the adhesins OMPCD and Hag. Of significance, these four molecules have been shown to be major targets of systemic and mucosal antibody responses in humans (62, 119, 160, 181-186). Infected chinchillas did not produce detectable levels of Abs against the shared region of MhaB1 and MhaB2 (data not shown).

Discussion

The success of the immunization program against *S. pneumoniae* and has placed more emphasis on *M. catarrhalis* as a frequent cause of ear infections. Vaccination of children with Prevnar®, which contains capsular polysaccharides from seven different *S. pneumoniae* serotype strains, affords protection against otitis media caused by the organism (57% efficacy) (187). Likewise, an investigational vaccine containing the capsule of 11 distinct *S. pneumoniae* serotype strains conjugated to protein D of *H. influenzae* was shown to reduce the incidence of ear infection caused by *S. pneumoniae* (57% efficacy) and NTHi (35% efficacy) (188). Significantly, Synflorix™,

a capsule-protein D conjugate vaccine comprising capsular polysaccharides from 10 different *S. pneumoniae* serotype strains, was licensed in Europe in 2009. While these studies demonstrate that prevention of otitis media can be achieved, the widespread administration of capsule-protein D conjugate vaccines protecting against both *S. pneumoniae* and NTHi, along with the continued expansion of the *S. pneumoniae* vaccination program (a version of Prevnar® covering 13 capsule serotypes was licensed in 2010), will result in *M. catarrhalis* becoming an even more prevalent cause of infectious disease. Evidence of such a shift has been observed in children with otitis media as well as in children and adults with sinusitis (59, 60, 189). Therefore, the prevention of *M. catarrhalis* infections would make a significant contribution to improving children's health. Though otitis media would be the primary target, a vaccine against the organism would also be of value to adults at high risk of infection, especially those with COPD.

Moraxella catarrhalis is an exclusively human associated organism and studying pathogenesis, as well as the stringent testing of vaccine candidates, has been hindered by the lack of an animal model that mimics human infection. To date, the most commonly used model has been a pulmonary clearance test in which bacteria are deposited in the lungs of mice (72-78). Viable organisms are enumerated by aseptically removing the lungs, homogenizing the tissues, and spreading dilutions of the homogenates onto agar plates. While this model has provided important data, it is limited to measuring the rate at which bacteria are cleared over a very short period of time because *M. catarrhalis* persists for <24-hr in the murine lungs. Another drawback is that mice do not develop pneumonia. Hence, the rapid clearance and failure to cause disease limit the usefulness of this model.

Recent studies have demonstrated the value of the chinchilla to examine *M. catarrhalis* host-pathogen interactions *in vivo* (70, 80-83). Following intranasal

inoculation, *M. catarrhalis* causes symptoms of disease (inflammation of the tympanic membrane, middle ear effusions) and colonizes the nasopharynx for ~2 weeks (80-82). Therefore, chinchillas provide an advantage over the mouse pulmonary clearance test in that *M. catarrhalis* persists in their nasopharynx for several days. This imparts greater confidence in the data obtained by comparing the difference in colonization between two experimental conditions (vaccinated vs. sham-vaccinated animals, WT vs. mutant strains) as it provides a more physiologically relevant time frame to monitor bacterial clearance. The chinchilla model has been an invaluable tool to study the pathogenesis of NTHi and *S. pneumoniae* and to develop vaccines for these organisms (84-87). The course of disease (nasopharyngeal colonization, ascension of the Eustachian tubes, development of middle ear effusions, clearance of fluids, return to homeostasis) is similar to that in children with otitis media (88-93). Immunological parallels between chinchillas and humans have been demonstrated. For example, middle ear fluids collected from chinchillas and children infected with NTHi contain Abs that bind to the same antigenic determinants of the adhesion OMP P5 (94). Chinchillas also produce homologs of human antimicrobial peptides, and at least two of them (cBD-1 and cCRAMP) have been shown to have bactericidal activity against *M. catarrhalis* (95-98). Kerschner and colleagues analyzed host cDNA libraries generated from the middle ear mucosa of chinchillas infected with NTHi, and discovered that the cDNA sequences displayed greater phylogenetic similarities to human genes than to other rodent species (99-101). These investigators also noted similarities with human infection in the pattern of host defense genes expressed in chinchilla tissues. Our data showing that chinchillas infected with *M. catarrhalis* produce Abs against antigens known to be major targets of the immune response in humans further underscore the usefulness of the model (Figure 4.7). To our knowledge, this is the first demonstration of immunological parallels between chinchillas and humans during *M. catarrhalis* infection.

We discovered that lack of expression of the filamentous hemagglutinin-like proteins MhaB1 and MhaB2 decreases recovery of viable *M. catarrhalis* cells from the chinchilla nasopharynx approximately 20-fold (Figure 4.2). This reduction is most likely caused by a defect in adherence to the airway mucosa. MhaB1 and MhaB2 mediate adherence to respiratory cells *in vitro* and resemble FHA, the major adhesin and colonization factor of *B. pertussis* (41). Moreover, Abs against MhaB1 and MhaB2 reduce *in vitro* adherence of *M. catarrhalis* (Figure 4.5) and decrease the number of viable organisms recovered from the nasopharynx of chinchillas infected with the WT strain O35E (Figure 4.6). Taken together our data suggest that MhaB1 and MhaB2 are critical factors for colonization. Hoopman and colleagues recently used the chinchilla and DNA microarray technology to determine global transcriptome expression by *M. catarrhalis in vivo* (82). More than 100 ORFs of strain O35E, including *mhaB1*, were found to be upregulated 24-hr after introducing the organism in the nasopharynx. Another 200 genes were shown to be downregulated, and the ORF specifying MhaB2 (MchA1) exhibited some of the highest levels of repression. Therefore, it is tempting to speculate that lack of MhaB1 is responsible for the reduced number of viable O35E.B1B2 cells recovered from the chinchilla nasopharynx during our colonization experiments (Figure 4.2). However, the contribution of MhaB2 cannot be ruled out. The transcriptome analysis showing decreased *mhaB2* expression levels were performed with samples collected 24-hr post-inoculation, whereas we calculated bacterial loads in the nasopharynx 3 days after infection. It is possible that expression of *mhaB2* (and *mhaB1*) changes during this 48-hr period. Interestingly, microarray data also indicate that expression of the *uspA2* and *hag* genes is downregulated (82). The western blot results of Figure 4.7 show that infected chinchillas produce Abs against UspA2 and Hag, demonstrating their expression *in vivo*. Clearly, understanding the individual contribution of MhaB1 and MhaB2 to colonization and persistence is a key area for future study.

Although lack of MhaB1 and MhaB2 reduces the recovery of viable O35E.B1B2 cells from the chinchilla nasopharynx, the mutant retained colonization capabilities (Figure 4.2), which implies that additional factors contribute to this process. Luke *et al.* used the chinchilla model to show that a type IV pilus mutant of *M. catarrhalis* strain 7169 does not colonize as effectively as the WT parent isolate (83). The pilus-negative mutant exhibited 7.67-, 2.56-, and 9.6-fold reductions in recovery of viable organisms from nasopharyngeal, nasoturbinate, and ethmoid turbinate tissues, respectively. The mutant also showed lower adherence to epithelial cells *in vitro* (83). Strain O35E expresses a type IV pilus (190), which presumably contributed to colonization in our experiments. Our laboratory demonstrated that *M. catarrhalis* has strict tropism for ciliated cells of the human respiratory tract and that the autotransporter adhesin Hag is responsible for this phenotypic trait (122). Brockson and colleagues recently reported that *M. catarrhalis* exhibits similar ciliotropism in the chinchilla nasal passageways (81). Hag may therefore play a role in colonization and persistence. Other potential colonization factors include UspA1 (binds to human CEACAM-1 receptor (106, 110, 191), chinchillas express a homologue of human CEACAM-1 shown to be necessary for colonization by NTHi (192)) and genes that are a part of the truncated denitrification regulon, specifically MC ORF1550 (encodes a protein of unknown function, highly upregulated in the chinchilla nasopharynx 24-hr post inoculation, mutation in the gene causes a decrease in the ability of strain O35E to survive in the chinchilla nasopharynx over a 3-day period (82).

The results of vaccination experiments validate the role of MhaB1 and MhaB2 as critical factors for colonization. Subcutaneous immunization with a polypeptide common to both molecules elicits the production of serum Abs reacting with the proteins in the outer membrane of *M. catarrhalis* (Figure 4.5A). Vaccinated animals also develop mucosal Abs binding to the shared region of MhaB1 and MhaB2 (Figure 4.5C). These

Abs not only block *M. catarrhalis* adherence *in vitro*, but also reduce nasopharyngeal colonization of the WT strain O35E by one order of magnitude (Figure 4.6). The MhaB proteins function as adhesins and mediate a key step in pathogenesis by *M. catarrhalis*. To cause disease, the organism must first colonize the nasopharynx and then spread to distal sites such as the middle ear and the lower respiratory tract. Hence, adherence to the mucosal surface of the nasopharynx is critical. MhaB1 and MhaB2 are surface-located and thus are readily accessible to Abs and the host immune response. In addition, the proteins are well conserved among clinical isolates of diverse clinical and geographical origins (41, 42). Therefore, MhaB1 and MhaB2 possess many attributes of excellent vaccine candidates. Our results showing that Abs against the shared region of MhaB1 and MhaB2 blocks adherence of multiple WT *M. catarrhalis* isolates suggests that immunization with the proteins will have broad-spectrum activity. Of note, this shared region of MhaB1 and MhaB2 displays sequence similarity to the portion of *B. pertussis* FHA that is a component of all vaccines that are currently licensed for use in children to protect against whooping cough. Future studies will be aimed at exploring the vaccinogenic potential of MhaB1 and MhaB2 with adjuvants that readily translate to human studies, immunization routes that promote robust mucosal immunity, measuring colonization at multiple intervals post-inoculation, and testing additional *M. catarrhalis* isolates.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: ERL TLS RB RJH. Performed the experiments: ERL TLS RB SWB. Analyzed the data: ERL TLS RB RJH. Contributed reagents/materials, analysis tools: ERL RJH. Wrote the paper: ERL TLS.

Table 4.1: Strains and Plasmids Used in this Study.

Strain	Description	Source
M. catarrhalis		
O35E	WT isolate from middle ear effusion (Dallas, TX)	(111)
O35E.B1B2	*mhaB1mhab2* double isogenic mutant of strain O35E, spectinomycin and zeocin resistant	(41)
O35E.TN2	*hag* transposon mutant of strain O35E, kanamycin resistant	(121)
O35E.2	*uspA2* isogenic mutant of strain O35E, kanamycin resistant	(111)
O35E.CD1	*ompCD* isogenic mutant of strain O35E, kanamycin resistant	(127)
O12E	WT isolate from middle ear effusion (Dallas, TX)	(104)
McGHS1	WT isolate from patient with respiratory infection (Toledo, OH)	(120)
E. coli		
EPI300™	Cloning strain for recombinant DNA methods	Epicentre® (Illumina®)
TUNER™	Expression strain for protein purification purposes	EMD Millipore
Plasmids		
pGEX4T-2	Protein expression vector, ampicillin resistant	GE Healthcare Life Sciences
pGEX-MhaB	pGEX4T-2 expressing O35E MhaB1 aa 72-399 joined to a GST N-terminal tag (GST-MhaB), ampicillin resistant	This Study
pGEX-McaP	pGEX4T-2 expressing O35E McaP aa 51-333 joined to a GST N-terminal tag (GST-McaP), ampicillin resistant	This Study
PRBHis.MhaB.72.399	pETcoco-1 expressing O12E MhaB1 aa 72-399 joined to 6 N-terminal histidine residues (His-MhaB), chloramphenicol resistant	(41)

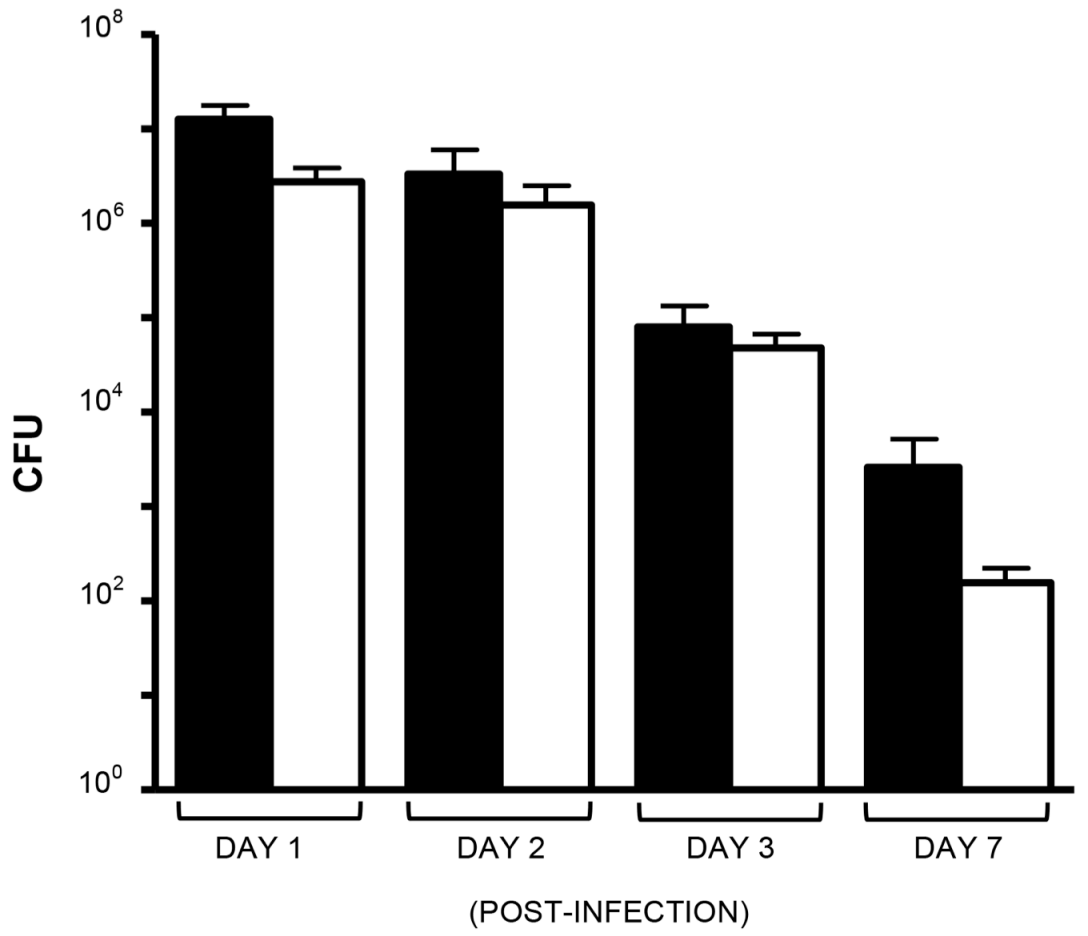

Figure 4.1: Recovery of WT *M. catarrhalis* O35E from the nasopharynx of chinchillas. Animals were inoculated with ~1x10^9 CFU. Results are expressed as the mean (± standard error) CFU/mL (lavage fluids, black bars) or CFU/g (nasopharyngeal tissues, open bars). Each column represents at least 4 animals, and each experimental condition was tested on at least two separate occasions.

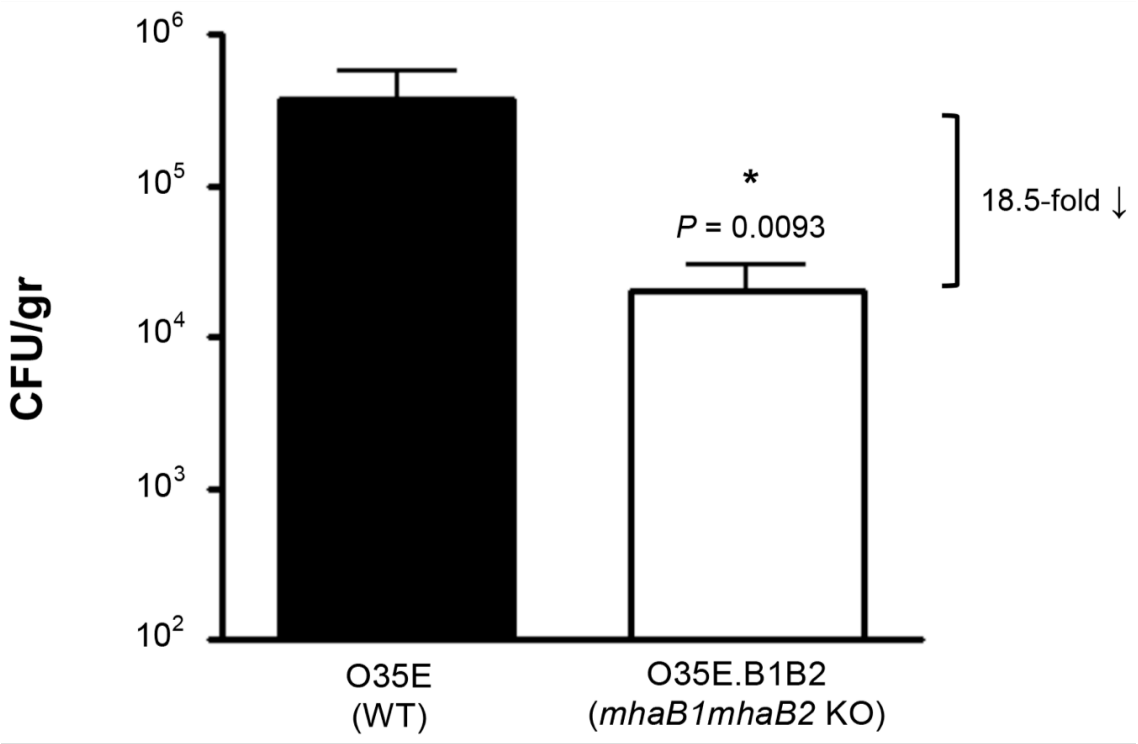

Figure 4.2: Recovery of *M. catarrhalis* from the nasopharynx of chinchillas three days post-infection. Animals were inoculated with ~1x10^9 CFU. Results are expressed as the mean (± standard error) CFU/g of nasopharyngeal tissues. Strains were tested in parallel on two separate occasions. Each column represents 12 animals. The asterisk indicates that the reduction in the number of bacteria is statistically significant (Wilcoxon signed rank test).

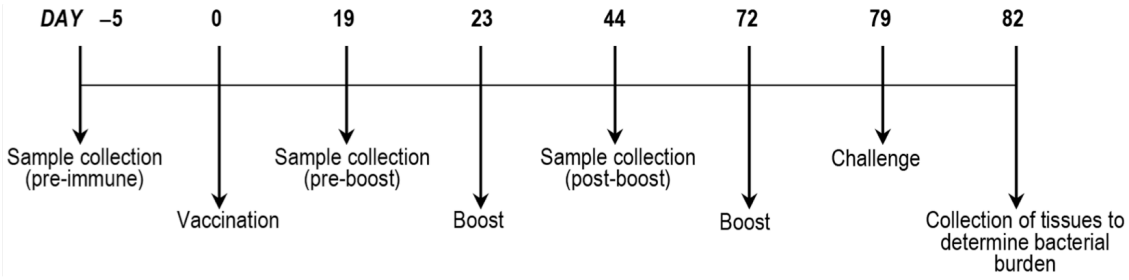

Figure 4.3: Timeline of vaccination experiments.

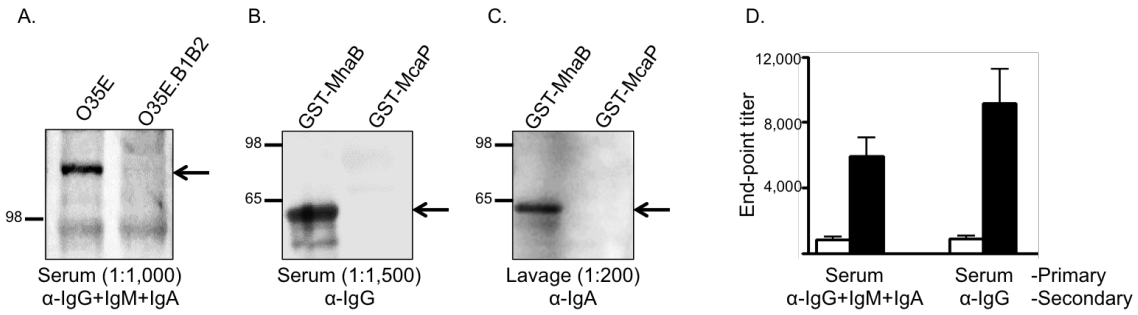

Figure 4.4: Western blot and ELISA analysis of samples from chinchillas immunized with the His-tagged MhaB protein. Western blot (panels A, B, C): Equivalent protein amounts were resolved by SDS-PAGE, transferred to PVDF and probed with the indicated primary and secondary Abs. Post-boost serum and lavage samples taken on Day 44 of the vaccination experiments (see Figure 4.3) were pooled and used as primary Abs at the dilution shown in parentheses. Goat α-rat Abs conjugated to HRP were used as secondary Abs. Panel A: western blot of outer membrane protein preparations from the WT *M. catarrhalis* strain O35E and the *mhaB1mhaB2* mutant O35E.B1B2. Panels B and C: western blot of the purified recombinant proteins GST-tagged MhaB and GST-tagged McaP (used as a negative control). Arrows indicate proteins specifically reacting with chinchilla Abs α-MhaB1/MhaB2. MW markers are shown to the left in kDa. ELISA (panel D): Individual serum samples were serially diluted and place in duplicate wells of plates coated with GST-tagged MhaB. Goat α-rat Abs conjugated to HRP were used as secondary Abs. The results are expressed as the mean (± standard deviation) end-point titer of samples from n=12 animals. Individual titers were determined using pre-immune samples as background. Open bars correspond to pre-boost samples taken on Day 19 of the vaccination experiments while black bars represent post-boost samples collected of Day 44 (see figure 4.3).

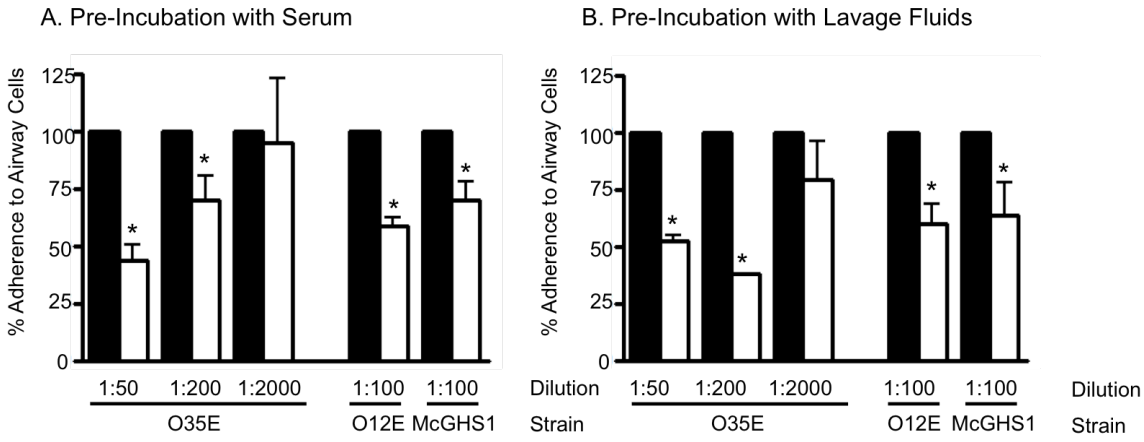

Figure 4.5: Inhibition of adherence with samples from chinchillas immunized with His-tagged MhaB protein. The WT *M. catarrhalis* strains O35E, O12E, and McGHS1 were preincubated for 30 min at 37°C with pooled samples from chinchillas sham-vaccinated with PBS (black bars) or with pooled samples from chinchillas immunized with His-tagged MhaB at dilutions of 1:50, 1:100, 1:200, and/or 1:2000. These bacteria were then used to perform adherence assays. The adherence of *M. catarrhalis* preincubated with samples from chinchillas vaccinated with PBS was set at 100%. The adherence of *M. catarrhalis* preincubated with samples from chinchillas immunized with His-tagged MhaB is expressed as the percentage (± standard error) of that of *M. catarrhalis* preincubated with samples from chinchillas vaccinated with PBS. Assays were performed in triplicate on three separate occasions. The asterisks indicate that the reduction in adherence is statistically significant (*P* values <0.05, paired *t* test). Post-boost samples taken on Day 44 of vaccination experiments (see Figure 4.3) were pooled and used in these assays.

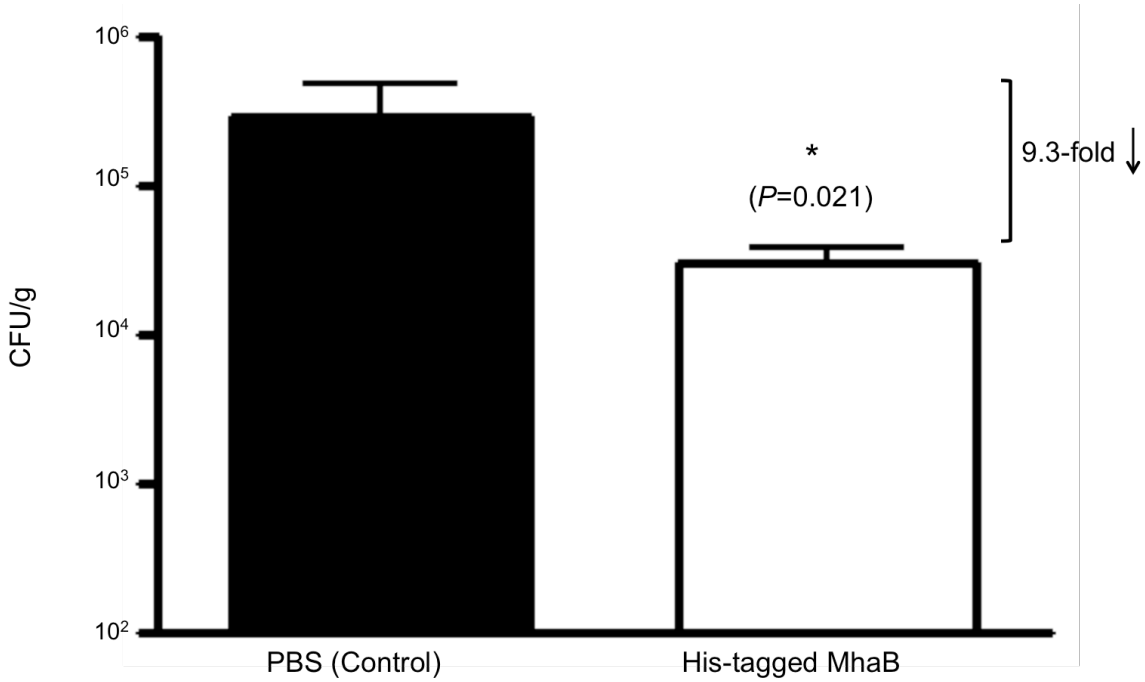

Figure 4.6: Recovery of WT *M. catarrhalis* O35E from the nasopharynx of immunized chinchillas three days post infection. Results are expressed as the mean (± standard error) CFU/g of nasopharyngeal tissues (note the log scale). The asterisk indicates that the reduction in the number of bacteria is statistically significant (Wilcoxon signed rank test, *P* value is shown in parentheses). Control and His-tagged MhaB groups were tested in parallel on three separate occasions. Each column represents 12 animals (groups of n=4 animals/experiment).

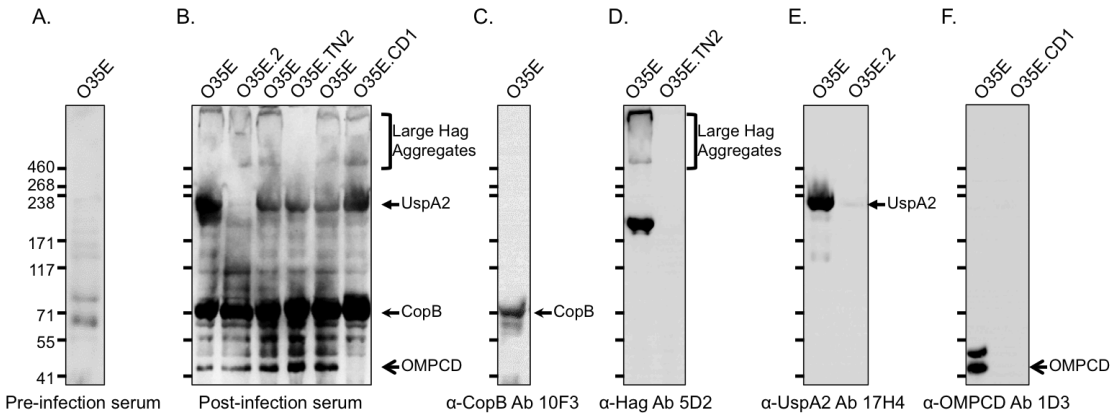

Figure 4.7: Western blot analysis of serum from chinchillas inoculated with the WT *M. catarrhalis* strain O35E. Equivalent amounts of whole cell lysates (WT *M. catarrhalis* O35E, *uspA2* KO strain O35E.2, *hag* transposon mutant strain O35E.TN2, and *ompCD* KO strain O35E.CD1) were resolved by SDS-PAGE, transferred to PVDF and probed ith the indicated primary Abs. Panels A and B: Pre- and post-infection serum samples were pooled and used as primary Abs at a dilution of 1:250. Goat α-rat IgG conjugated to HRP were used as secondary Abs. Controls: The murine monoclonal Abs 10F3 (Panel C, α-CopB), 5D2 (Panel D, α-Hag), 17H4 (Panel E, α-UspA2), and 1D3 (Panel F, α-OMPCD) were used as primary Abs in combination with goat α-mouse HRP-(IgG+IgA+IgM) secondary Abs. These controls were included to verify the identity of proteins recognized by post-infection chinchilla serum in panel B. MW markers are shown to the left in kDa.

CHAPTER 5

CONCLUSION

There is a need for a vaccine against *M. catarrhalis* due to the antibiotic resistance of most strains and the increase in infections caused by *M. catarrhalis*. While the immunization program against *S. pneumoniae* has been successful, it has also resulted in an increase in otitis media cases caused by *M. catarrhalis*. When children are vaccinated with Prevnar®, which contains capsular polysaccharides from seven different *S. pneumoniae* serotype strains, they gain protection against otitis media caused by the organism (57% efficacy) (187). Similarly, an investigational vaccine against *S. pneumoniae* and nontypeable *H. influenzae* was shown to reduce the incidence of ear infections caused by *S. pneumoniae* (57% efficacy) and nontypeable *H. influenzae* (35% efficacy). This vaccine contains the capsule of 11 distinct *S. pneumoniae* serotype strains conjugated to protein D of *H. influenzae* (188). Finally, Synflorix™ was licensed in Europe in 2009. This is a capsule-protein D conjugate vaccine comprising capsular polysaccharides from 10 different *S. pneumoniae* serotype strains. These studies demonstrate that prevention of otitis media can be achieved. Studies indicate the widespread administration of protecting against both *S. pneumoniae* and nontypeable *H. influenzae* and the continued expansion of the *S. pneumoniae* vaccination program (a version of Prevnar® covering 13 capsule serotypes was licensed in 2010) will result in *M. catarrhalis* infections being even more prevalent. In addition to this shift being observed in otitis media cases the shift has also been observed in both children and adults with sinusitis (59, 60, 189). Therefore, the prevention of *M. catarrhalis* infections would make a significant contribution to improving children's health.

Though otitis media would be the primary target, a vaccine against the organism would also be of value to adults at high risk of infection, especially those with COPD.

To our knowledge contact dependent inhibition (CDI) systems have not been used as targets for vaccine development, but in this study we test the potential of MhaB1 and MhaB2, CDI inhibitor proteins, as vaccine candidates. Initially, MhaB1 and MhaB2 were identified as adhesins for *M. catarrhalis* due to the decrease in adherence of mutants lacking MhaB1 and/or MhaB2 expression (41, 42). Upon further study, MhaB1 and MhaB2 were identified as potential inhibitor proteins for a CDI system. It was common for CDI inhibitor proteins to be characterized as adhesins before CDI systems were identified (40, 133). In the CDI field, it is common belief that any adherence properties CDI proteins have are a due to them being "sticky." Since these proteins adhere to other bacterial cells during CDI, it is necessary for them to have adherence properties. Regardless of whether MhaB1 and MhaB2 are truly involved in adherence, they are CDI proteins and play an important role in colonization and are good vaccine targets as demonstrated in Chapter 4 (156).

While CDI systems have not been used as vaccine targets, the inhibitor proteins have many of the desired characteristics of a successful vaccine candidate. The inhibitor proteins are surface-exposed, have been demonstrated to be involved in pathogenesis, and the majority of the protein is well conserved within species (40, 43, 133, 134, 142). For these reasons, we characterized and tested the vaccine potential of the dual CDI system in *M. catarrhalis*.

One interesting aspect of the CDI system from *M. catarrhalis* is there are two inhibitor proteins, MhaB1 and MhaB2, and while each has their own immunity protein, but they share the same transporter, MhaC. Thus, *M. catarrhalis* is capable of deploying two distinct inhibitors proteins using the same CDI system machinery. Using wild type *M. catarrhalis* O35E, MhaB1 was determined to be an inhibitor protein and its cognitive

immunity protein (MhaB1i) was identified (Figure 3.2). Expression of MhaB1i from a plasmid rescued the mutant lacking expression of MhaB1i from growth inhibition (Figure 3.3). We anticipated that there would be more inhibition by MhaB1; however, there is an orphan immunity gene downstream of *mhaB1/mhaB1i*, which has a high degree of similarity to *mhaB1i*. MhaB1i is 186 amino acids, and the orphan immunity protein is 179 amino acids. When these sequences are aligned the last 110 amino acids have 100% identity and overall the two proteins have over 70% identity. Previous work by Poole *et al.* demonstrated that these orphans could provide partial protection in other systems (142). We hypothesize that this partial protection is also occurring in the system we used to test MhaB1. To further evaluate whether this orphan immunity gene provides protection; two potential experiments could be performed. One method would be to generate a new mutant using our current mutant (lacks expression of MhaB2, MhaB1, and MhaB1i) by interrupting the ORF of the Orphan immunity gene with another unique antibiotic cassette. The other option would be to remove the entire *mha* locus and the downstream orphan pairs, then use pWW115 to express the desired immunity genes and use these as the target strain.

Unfortunately, determining if MhaB2 is an inhibitor protein was more difficult. A mutant lacking expression of its cognitive immunity protein (MhaB2i) could not be generated. We were successful in using wild type isolates of *M. catarrhalis* as target strains for growth inhibition by MhaB2. These strains were selected because they had previously been shown to lack expression of MhaC and MhaB1/B2 (41). Since these strains lacked expression of the conserved parts of the CDI system, we hypothesized that they would be sensitive to inhibition by MhaB2. Interestingly enough, not all strains were sensitive (Figure 3.2) to inhibition by MhaB2. The strains that were sensitive to inhibition by both MhaB1 and MhaB2 (Figure 3.4) were selected for further study. These two strains, McGHS2 and TTA24, have not been well studied and it was not known if the

plasmid pStrep could replicate in these strains. Attempts to transform pStrep into these strains using both electroporation and natural transformation were unsuccessful. While MhaB2 was shown to be a CDI protein, MhaB2i was not conclusively demonstrated to be its cognate immunity protein. Based on the fact that *mhaB2* could be cloned in *E. coli* with the potential *mhaB2i* (Table 3.1, plasmid pCC1.B2i), we are confident that *mhaB2i* has been identified and future work will conclusively demonstrate this. Currently I am collaborating with Dr. Hayes' (leader in the CDI field) laboratory to identify the activities of MhaB1 and MhaB2. Though the process of determining the activity of MhaB2, the identification MhaB2i will be confirmed.

While testing wild type *M. catarrhalis* strains, we discovered that the strains had different levels of susceptibility. All of the strains tested were used as target strains because it was believed that they lacked the CDI system. We anticipated that all strains would be sensitive to inhibition by MhaB1 and MhaB2. This was not the case, because P44LN2 and TTA37 were resistant to inhibition by MhaB1 (Figure 3.5) and 7P94B1 was resistant to inhibition by MhaB2 (Figure 3.6). In the future, we would like to expand the strains tested to include strains that have expression of MhaC and MhaB1/B2 in order to see if these also have varying levels of susceptibility. Also, using primers that correspond to *mhaC* and the identical portion of *mhaB1* and *mhaB2*, we would like to see if there are any remnants of the conserved portions of the CDI system in the strains that lack expression of MhaC and MhaB1/B2. In addition to characterizing the CDI system in *M. catarrhalis*, we also wanted to test the vaccine potential of these proteins.

Next, the vaccine potential of MhaB1 and MhaB2 were examined, but this required an animal model. Due to *M. catarrhalis* being an exclusively human associated organism studying pathogenesis and the stringent testing of vaccine candidates has been hindered by the lack of an animal model that mimics human infection. To date, the most commonly used model has been a pulmonary clearance test in which bacteria are

deposited in the lungs of mice (72-78). Following inoculation of the lungs, they are removed aseptically, the tissues are homogenized, and dilutions of the homogenates are spread onto agar plates to enumerate viable organisms. While this model has provided important data, it is limited to measuring the rate at which bacteria are cleared over a very short period of time because *M. catarrhalis* persists for <24-hr in the murine lungs. Another drawback is that mice do not develop pneumonia. Hence, the rapid clearance and failure to cause disease limit the usefulness of this model.

Following models developed for *S. pneumoniae* and nontypeable *H. influenzae*, recent studies examining *M. catarrhalis* host-pathogen interaction *in vivo* have demonstrated the value of the chinchilla model (70, 80-83). *Moraxella catarrhalis* is able to cause symptoms of disease following intranasal inoculation and colonize the nasopharynx for ~2 weeks (80-82). Therefore, chinchillas provide an advantage over the mouse pulmonary clearance test in that *M. catarrhalis* persists in their nasopharynx for several days providing a biologically relevant window to study infection. In addition, this imparts greater confidence in the data obtained by comparing the difference in colonization between two experimental conditions (vaccinated vs. mock-vaccinated animals, WT vs. mutant strains). The chinchilla model has been an invaluable tool to study the pathogenesis of nontypeable *H. influenzae* and *S. pneumoniae* and to develop vaccines for these organisms (84-87). The course of disease is similar to children suffering from otitis media. Nasopharyngeal colonization is established, followed by ascension into the Eustachian tubes, development of middle ear effusions, clearance of fluids, and finally the return to homeostasis when the bacteria are cleared (88-93). Immunological parallels between chinchillas and humans have been demonstrated. For example, middle ear fluids collected from chinchillas and children infected with nontypeable *H. influenzae* contain antibodies that bind to the same antigenic determinants of the adhesion OMP P5 (94). Kerschner and colleagues analyzed host

cDNA libraries generated from the middle ear mucosa of chinchillas infected with nontypeable *H. influenzae*, and discovered that the cDNA sequences displayed greater phylogenetic similarities to human genes than to other rodent species (99-101). These investigators also noted similarities with human infection in the pattern of host defense genes expressed in chinchilla tissues. Our data showing that chinchillas infected with *M. catarrhalis* produce antibodies against antigens known to be major targets of the immune response in humans further underscore the usefulness of the model (Figure 4.7). To our knowledge, this is the first demonstration of immunological parallels between chinchillas and humans during *M. catarrhalis* infection.

We discovered that lack of expression of the filamentous hemagglutinin-like proteins MhaB1 and MhaB2 decreases recovery of viable *M. catarrhalis* cells from the chinchilla nasopharynx approximately 20-fold (Figure 4.2). This reduction is most likely caused by a defect in adherence to the airway mucosa or the loss of the CDI system, which may play a role in bacterial cell-to-cell communication. Our data supports MhaB1 and MhaB2 being involved in adherence. MhaB1 and MhaB2 mediate adherence to respiratory cells *in vitro* and resemble FHA, the major adhesin and colonization factor of *B. pertussis* (41, 42). Moreover, antibodies against MhaB1 and MhaB2 reduce *in vitro* adherence of *M. catarrhalis* (Figure 4.5) and decrease the number of viable organisms recovered from the nasopharynx of chinchillas infected with the WT strain O35E (Figure 4.6). Taken together our data suggest that MhaB1 and MhaB2 are critical factors for colonization.

Recently the chinchilla model and DNA microarray technology was used to determine global transcriptome expression by *M. catarrhalis in vivo* by Hoopman and colleagues (82). Among the upregulated genes was *mhaB1*. There were more than 100 ORFs of strain O35E upregulated 24-hr after introducing the organism in the nasopharynx. In addition, another 200 genes were shown to be downregulated. In this

group *mhaB2* (*mchA1*) exhibited some of the highest levels of repression. Due to these findings, it is tempting to speculate that lack of MhaB1 is responsible for the reduction of viable *M. catarrhalis* O35E.B1B2 cells recovered from the chinchilla nasopharynx during our colonization experiments (Figure 4.2). However, the contribution of MhaB2 cannot be ruled out, because the transcriptome analysis showing decreased *mhaB2* expression levels were performed with samples collected 24-hr post-inoculation, whereas we calculated bacterial loads in the nasopharynx 3 days after infection. It is possible that expression of *mhaB2* (and *mhaB1*) changes during this 48-hr period. Interestingly, microarray data also indicate that expression of the *uspA2* and *hag* genes is downregulated (82). The western blot results of Figure 4.7 show that infected chinchillas produce antibodies against UspA2 and Hag, demonstrating their expression *in vivo*. Clearly, understanding how MhaB1 and MhaB2 contribute to colonization and persistence is a key area for future study and this knowledge will help improve the understanding of *M. catarrhalis* and CDI systems.

The results of vaccination experiments validate the role of MhaB1 and MhaB2 as critical factors for colonization. Subcutaneous immunization with a polypeptide common to both molecules elicits the production of serum antibodies reacting with the proteins in the outer membrane of *M. catarrhalis* (Figure 4.5A). Vaccinated animals also develop mucosal antibodies binding to the shared region of MhaB1 and MhaB2 (Figure 4.5C). These antibodies not only block *M. catarrhalis* adherence *in vitro*, but also reduce nasopharyngeal colonization of the WT strain O35E by one order of magnitude (Figure 4.6). The MhaB1 and MhaB2 proteins are involved in colonization and mediate a key step in pathogenesis by *M. catarrhalis*. To cause disease, the organism must first colonize the nasopharynx and then spread to distal sites such as the middle ear and the lower respiratory tract. Hence, the ability to colonize the mucosal surface of the nasopharynx is critical. MhaB1 and MhaB2 are surface-located and thus are readily

accessible to antibodies and the host immune response. In addition, the proteins are well conserved among clinical isolates of diverse clinical and geographical origins (41, 42). Therefore, MhaB1 and MhaB2 possess many attributes of excellent vaccine candidates. Our results showing that antibodies against the shared region of MhaB1 and MhaB2 blocks adherence of multiple WT *M. catarrhalis* isolates suggests that immunization with the proteins will have broad-spectrum activity. Of note, this shared region of MhaB1 and MhaB2 displays sequence similarity to the portion of *B. pertussis* FHA that is a component of all vaccines that are currently licensed for use in children to protect against whooping cough.

Future studies will be aimed at exploring the vaccinogenic potential of MhaB1 and MhaB2 with adjuvants that readily translate to human studies, immunization routes that promote robust mucosal immunity, measuring colonization at multiple intervals post-inoculation, and testing additional *M. catarrhalis* isolates. In addition to these studies, we would like to test the colonization ability of mutants that express full length MhaB1 and MhaB2, but have point mutations so these proteins lack CDI activity. This would help to answer the question of their role in the colonization process. If they are involved in adherence, the colonization process should not be affected. If it is the CDI activity that is promoting the colonization there should be a decrease in colonization.

This project was the first time to our knowledge that a CDI system was tested for its vaccinogenic potential, the first time a CDI system was tested in an animal model, and the first time chinchillas were used in a vaccine study for *M. catarrhalis*. In addition, we were able to demonstrate that *M. catarrhalis* has a CDI system with two inhibitor proteins, MhaB1 and MhaB2. This is the first time a CDI system has been characterized where there are two inhibitor proteins that share the same transporter. MhaB1 and MhaB2 from *M. catarrhalis* O35E are able to inhibit the growth of other strains of *M. catarrhalis* to varying degrees. Moreover, MhaB1 and MhaB2 play an important role in

the colonization process of the chinchilla nasopharynx. When the chinchillas were vaccinated with MhaB, they produced antibodies against MhaB. These antibodies not only caused a decrease in colonization *in vivo*, but also could block adherence by *M. catarrhalis* to human epithelial cells *in vitro*. Together all these data demonstrates MhaB1 and MhaB2 are very promising vaccine candidates.

REFERENCES

1. **Cripps AW, Otczyk DC, Kyd JM.** 2005. Bacterial otitis media: a vaccine preventable disease? Vaccine **23:**2304-2310.

2. **Enright MC, McKenzie H.** 1997. *Moraxella* (*Branhamella*) *catarrhalis*--clinical and molecular aspects of a rediscovered pathogen. Journal of medical microbiology **46:**360-371.

3. **Faden H.** 2001. The microbiologic and immunologic basis for recurrent otitis media in children. European journal of pediatrics **160:**407-413.

4. **Giebink GS, Kurono Y, Bakaletz LO, Kyd JM, Barenkamp SJ, Murphy TF, Green B, Ogra PL, Gu XX, Patel JA, Heikkinen T, Pelton SI, Hotomi M, Karma P.** 2005. Recent advances in otitis media. 6. Vaccine. The Annals of otology, rhinology & laryngology. Supplement **194:**86-103.

5. **Karalus R, Campagnari A.** 2000. *Moraxella catarrhalis*: a review of an important human mucosal pathogen. Microbes and infection / Institut Pasteur **2:**547-559.

6. **Murphy TF.** 2005. Vaccine development for non-typeable H*aemophilus influenzae* and *Moraxella catarrhalis*: progress and challenges. Expert review of vaccines **4:**843-853.

7. **Pichichero ME, Casey JR.** 2002. Otitis media. Expert opinion on pharmacotherapy **3:**1073-1090.

8. **Verduin CM, Hol C, Fleer A, van Dijk H, van Belkum A.** 2002. *Moraxella catarrhalis*: from emerging to established pathogen. Clinical microbiology reviews **15:**125-144.

9. **American Academy of Pediatrics Subcommittee on Management of Acute Otitis M.** 2004. Diagnosis and management of acute otitis media. Pediatrics **113:**1451-1465.

10. **Arguedas A, Kvaerner K, Liese J, Schilder AG, Pelton SI.** 2010. Otitis media across nine countries: disease burden and management. International journal of pediatric otorhinolaryngology **74:**1419-1424.

11. **Klein JO.** 1994. Otitis media. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **19:**823-833.

12. **Klein JO.** 2000. The burden of otitis media. Vaccine **19 Suppl 1:**S2-8.

13. **Klein JO, Teele DW, Pelton SI.** 1992. New concepts in otitis media: results of investigations of the Greater Boston Otitis Media Study Group. Advances in pediatrics **39:**127-156.

14. **Murphy TF.** 1996. *Branhamella catarrhalis*: epidemiology, surface antigenic structure, and immune response. Microbiological reviews **60:**267-279.

15. **Stool SE, Field MJ.** 1989. The impact of otitis media. The Pediatric infectious disease journal **8:**S11-14.

16. **Acuin J.** 2004. Chronic suppurative otitis media. Clinical evidence**:**710-729.

17. **Berman S.** 1995. Otitis media in developing countries. Pediatrics **96:**126-131.

18. **Murphy TF, Brauer AL, Grant BJ, Sethi S.** 2005. *Moraxella catarrhalis* in chronic obstructive pulmonary disease: burden of disease and immune response. American journal of respiratory and critical care medicine **172:**195-199.

19. **Sethi S, Evans N, Grant BJ, Murphy TF.** 2002. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. The New England journal of medicine **347:**465-471.

20. **Sethi S, Murphy TF.** 2001. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. Clinical microbiology reviews **14:**336-363.

21. **Hunter MH, King DE.** 2001. COPD: management of acute exacerbations and chronic stable disease. American family physician **64:**603-612.

22. **Hurd S.** 2000. The impact of COPD on lung health worldwide: epidemiology and incidence. Chest **117:**1S-4S.

23. **Strassels SA, Smith DH, Sullivan SD, Mahajan PS.** 2001. The costs of treating COPD in the United States. Chest **119:**344-352.

24. **Sullivan SD, Ramsey SD, Lee TA.** 2000. The economic burden of COPD. Chest **117:**5S-9S.

25. **Ahmed A, Broides A, Givon-Lavi N, Peled N, Dagan R, Greenberg D.** 2008. Clinical and laboratory aspects of *Moraxella catarrhalis* bacteremia in children. The Pediatric infectious disease journal **27:**459-461.

26. **Berner R, Schumacher RF, Brandis M, Forster J.** 1996. Colonization and infection with *Moraxella catarrhalis* in childhood. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology **15:**506-509.

27. **Bingen E, Cohen R, Jourenkova N, Gehanno P.** 2005. Epidemiologic study of conjunctivitis-otitis syndrome. The Pediatric infectious disease journal **24:**731-732.

28. **Brook I.** 2005. The role of bacteria in chronic rhinosinusitis. Otolaryngologic clinics of North America **38:**1171-1192.

29. **Brook I.** 2005. Microbiology and antimicrobial management of sinusitis. The Journal of laryngology and otology **119:**251-258.

30. **Brook I, Foote PA, Frazier EH.** 2005. Microbiology of acute exacerbation of chronic sinusitis. The Annals of otology, rhinology, and laryngology **114:**573-576.

31. **Buznach N, Dagan R, Greenberg D.** 2005. Clinical and bacterial characteristics of acute bacterial conjunctivitis in children in the antibiotic resistance era. The Pediatric infectious disease journal **24:**823-828.

32. **Catlin BW.** 1990. *Branhamella catarrhalis*: an organism gaining respect as a pathogen. Clinical microbiology reviews **3:**293-320.

33. **Christensen JJ.** 1999. *Moraxella* (*Branhamella*) *catarrhalis*: clinical, microbiological and immunological features in lower respiratory tract infections. APMIS. Supplementum **88:**1-36.

34. **Neumayer U, Schmidt HK, Mellwig KP, Kleikamp G.** 1999. *Moraxella catarrhalis* endocarditis: report of a case and literature review. The Journal of heart valve disease **8:**114-117.

35. **Nigrovic LE, Kuppermann N, Malley R, Bacterial Meningitis Study Group of the Pediatric Emergency Medicine Collaborative Research Committee of the American Academy of P.** 2008. Children with bacterial meningitis presenting to the emergency department during the pneumococcal conjugate vaccine era. Academic emergency medicine : official journal of the Society for Academic Emergency Medicine **15:**522-528.

36. **Stefanou J, Agelopoulou AV, Sipsas NV, Smilakou N, Avlami A.** 2000. *Moraxella catarrhalis* endocarditis: case report and review of the literature. Scandinavian journal of infectious diseases **32:**217-218.

37. **Thorsson B, Haraldsdottir V, Kristjansson M.** 1998. *Moraxella catarrhalis* bacteraemia. A report on 3 cases and a review of the literature. Scandinavian journal of infectious diseases **30:**105-109.

38. **Turner HR, Taylor MR, Lockwood WR.** 1985. *Branhamella catarrhalis* endocarditis in a patient receiving hemodialysis. Southern medical journal **78:**1021-1022.

39. **Utsunomiya T, Nakahara K, Kuramochi M, Hashiba K, Uzuka Y, Matsumoto K.** 1984. *Branhamella* (*Neisseria*) *catarrhalis* endocarditis after insertion of a mitral prosthesis: a case report. Nihon Naika Gakkai zasshi. The Journal of the Japanese Society of Internal Medicine **73:**1506-1511.

40. **Aoki SK, Diner EJ, de Roodenbeke CT, Burgess BR, Poole SJ, Braaten BA, Jones AM, Webb JS, Hayes CS, Cotter PA, Low DA.** 2010. A widespread

family of polymorphic contact-dependent toxin delivery systems in bacteria. Nature **468:**439-442.

41. **Balder R, Hassel J, Lipski S, Lafontaine ER.** 2007. *Moraxella catarrhalis* strain O35E expresses two filamentous hemagglutinin-like proteins that mediate adherence to human epithelial cells. Infection and immunity **75:**2765-2775.

42. **Plamondon P, Luke NR, Campagnari AA.** 2007. Identification of a novel two-partner secretion locus in *Moraxella catarrhalis*. Infection and immunity **75:**2929-2936.

43. **Aoki SK, Poole SJ, Hayes CS, Low DA.** 2011. Toxin on a stick: modular CDI toxin delivery systems play roles in bacterial competition. Virulence **2:**356-359.

44. **Mawas F, Ho MM, Corbel MJ.** 2009. Current progress with *Moraxella catarrhalis* antigens as vaccine candidates. Expert review of vaccines **8:**77-90.

45. **Faden H, Harabuchi Y, Hong JJ.** 1994. Epidemiology of *Moraxella catarrhalis* in children during the first 2 years of life: relationship to otitis media. The Journal of infectious diseases **169:**1312-1317.

46. **Murphy TF, Parameswaran GI.** 2009. *Moraxella catarrhalis*, a human respiratory tract pathogen. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **49:**124-131.

47. **Davie JJ, Earl J, de Vries SP, Ahmed A, Hu FZ, Bootsma HJ, Stol K, Hermans PW, Wadowsky RM, Ehrlich GD, Hays JP, Campagnari AA.** 2011. Comparative analysis and supragenome modeling of twelve *Moraxella catarrhalis* clinical isolates. BMC genomics **12:**70.

48. **Wang W, Hansen EJ.** 2006. Plasmid pWW115, a cloning vector for use with *Moraxella catarrhalis*. Plasmid **56:**133-137.

49. **Wang W, Attia AS, Liu L, Rosche T, Wagner NJ, Hansen EJ.** 2006. Development of a shuttle vector for *Moraxella catarrhalis*. Plasmid **55:**50-57.

50. **de Vries SP, van Hijum SA, Schueler W, Riesbeck K, Hays JP, Hermans PW, Bootsma HJ.** 2010. Genome analysis of *Moraxella catarrhalis* strain BBH18, [corrected] a human respiratory tract pathogen. Journal of bacteriology **192:**3574-3583.

51. **Evans AS, Pybus C, Hansen EJ.** 2013. Development of a LacZ-based transcriptional reporter system for use with *Moraxella catarrhalis*. Plasmid **69:**180-185.

52. **Del Beccaro MA, Mendelman PM, Inglis AF, Richardson MA, Duncan NO, Clausen CR, Stull TL.** 1992. Bacteriology of acute otitis media: a new perspective. The Journal of pediatrics **120:**81-84.

53. **Faden H, Duffy L, Wasielewski R, Wolf J, Krystofik D, Tung Y.** 1997. Relationship between nasopharyngeal colonization and the development of otitis

media in children. Tonawanda/Williamsville Pediatrics. The Journal of infectious diseases **175:**1440-1445.

54. **Faden H, Stanievich J, Brodsky L, Bernstein J, Ogra PL.** 1990. Changes in nasopharyngeal flora during otitis media of childhood. The Pediatric infectious disease journal **9:**623-626.

55. **Ruuskanen O, Heikkinen T.** 1994. Otitis media: etiology and diagnosis. The Pediatric infectious disease journal **13:**S23-26; discussion S50-24.

56. **Website N-N** 2009, posting date. Chart Book on Cardiovascular, Lung, and Blood Diseases. Morbidity and Mortality. [Online.]

57. **Coticchia JM, Chen M, Sachdeva L, Mutchnick S.** 2013. New Paradigms in the Pathogenesis of Otitis Media in Children. Frontiers in pediatrics **1:**52.

58. **Qureishi A, Lee Y, Belfield K, Birchall JP, Daniel M.** 2014. Update on otitis media - prevention and treatment. Infection and drug resistance **7:**15-24.

59. **Brook I, Foote PA, Hausfeld JN.** 2006. Frequency of recovery of pathogens causing acute maxillary sinusitis in adults before and after introduction of vaccination of children with the 7-valent pneumococcal vaccine. Journal of medical microbiology **55:**943-946.

60. **Brook I, Gober AE.** 2007. Frequency of recovery of pathogens from the nasopharynx of children with acute maxillary sinusitis before and after the introduction of vaccination with the 7-valent pneumococcal vaccine. International journal of pediatric otorhinolaryngology **71:**575-579.

61. **Klugman KP.** 1996. The clinical relevance of in-vitro resistance to penicillin, ampicillin, amoxycillin and alternative agents, for the treatment of community-acquired pneumonia caused by *Streptococcus pneumoniae*, *Haemophilus influenzae,* and *Moraxella catarrhalis*. The Journal of antimicrobial chemotherapy **38 Suppl A:**133-140.

62. **Murphy TF, Kirkham C, DeNardin E, Sethi S.** 1999. Analysis of antigenic structure and human immune response to outer membrane protein CD of *Moraxella catarrhalis*. Infection and immunity **67:**4578-4585.

63. **Bootsma HJ, Aerts PC, Posthuma G, Harmsen T, Verhoef J, van Dijk H, Mooi FR.** 1999. *Moraxella* (*Branhamella*) *catarrhalis* BRO beta-lactamase: a lipoprotein of gram-positive origin? Journal of bacteriology **181:**5090-5093.

64. **Bootsma HJ, van Dijk H, Vauterin P, Verhoef J, Mooi FR.** 2000. Genesis of BRO beta-lactamase-producing *Moraxella catarrhalis*: evidence for transformation-mediated horizontal transfer. Molecular microbiology **36:**93-104.

65. **Esel D, Ay-Altintop Y, Yagmur G, Gokahmetoglu S, Sumerkan B.** 2007. Evaluation of susceptibility patterns and BRO beta-lactamase types among clinical isolates of *Moraxella catarrhalis*. Clinical microbiology and infection : the

official publication of the European Society of Clinical Microbiology and Infectious Diseases **13:**1023-1025.

66. **Kadry AA, Fouda SI, Elkhizzi NA, Shibl AM.** 2003. Correlation between susceptibility and BRO type enzyme of *Moraxella catarrhalis* strains. International journal of antimicrobial agents **22:**532-536.

67. **Manninen R, Huovinen P, Nissinen A.** 1997. Increasing antimicrobial resistance in *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in Finland. The Journal of antimicrobial chemotherapy **40:**387-392.

68. **Richter SS, Winokur PL, Brueggemann AB, Huynh HK, Rhomberg PR, Wingert EM, Doern GV.** 2000. Molecular characterization of the beta-lactamases from clinical isolates of *Moraxella* (*Branhamella*) *catarrhalis* obtained from 24 U.S. medical centers during 1994-1995 and 1997-1998. Antimicrobial agents and chemotherapy **44:**444-446.

69. **Schmitz FJ, Beeck A, Perdikouli M, Boos M, Mayer S, Scheuring S, Kohrer K, Verhoef J, Fluit AC.** 2002. Production of BRO beta-lactamases and resistance to complement in European *Moraxella catarrhalis* isolates. Journal of clinical microbiology **40:**1546-1548.

70. **Armbruster CE, Hong W, Pang B, Weimer KE, Juneau RA, Turner J, Swords WE.** 2010. Indirect pathogenicity of *Haemophilus influenzae* and *Moraxella catarrhalis* in polymicrobial otitis media occurs via interspecies quorum signaling. mBio **1**.

71. **Schaar V, Nordstrom T, Morgelin M, Riesbeck K.** 2011. *Moraxella catarrhalis* outer membrane vesicles carry beta-lactamase and promote survival of Streptococcus pneumoniae and Haemophilus influenzae by inactivating amoxicillin. Antimicrobial agents and chemotherapy **55:**3845-3853.

72. **Becker PD, Bertot GM, Souss D, Ebensen T, Guzman CA, Grinstein S.** 2007. Intranasal vaccination with recombinant outer membrane protein CD and adamantylamide dipeptide as the mucosal adjuvant enhances pulmonary clearance of *Moraxella catarrhalis* in an experimental murine model. Infection and immunity **75:**1778-1784.

73. **Kyd JM, Cripps AW, Murphy TF.** 1998. Outer-membrane antigen expression by *Moraxella* (*Branhamella*) *catarrhalis* influences pulmonary clearance. Journal of medical microbiology **47:**159-168.

74. **Liu DF, McMichael JC, Baker SM.** 2007. *Moraxella catarrhalis* outer membrane protein CD elicits antibodies that inhibit CD binding to human mucin and enhance pulmonary clearance of *M. catarrhalis* in a mouse model. Infection and immunity **75:**2818-2825.

75. **Maciver I, Unhanand M, McCracken GH, Jr., Hansen EJ.** 1993. Effect of immunization of pulmonary clearance of *Moraxella catarrhalis* in an animal model. The Journal of infectious diseases **168:**469-472.

76. **Murphy TF, Kyd JM, John A, Kirkham C, Cripps AW.** 1998. Enhancement of pulmonary clearance of *Moraxella* (*Branhamella*) *catarrhalis* following immunization with outer membrane protein CD in a mouse model. The Journal of infectious diseases **178:**1667-1675.

77. **Peng D, Choudhury BP, Petralia RS, Carlson RW, Gu XX.** 2005. Roles of 3-deoxy-D-manno-2-octulosonic acid transferase from *Moraxella catarrhalis* in lipooligosaccharide biosynthesis and virulence. Infection and immunity **73:**4222-4230.

78. **Unhanand M, Maciver I, Ramilo O, Arencibia-Mireles O, Argyle JC, McCracken GH, Jr., Hansen EJ.** 1992. Pulmonary clearance of *Moraxella catarrhalis* in an animal model. The Journal of infectious diseases **165:**644-650.

79. **Melhus A, Ryan AF.** 2003. A mouse model for acute otitis media. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica **111:**989-994.

80. **Bakaletz LO.** 1995. Viral potentiation of bacterial superinfection of the respiratory tract. Trends in microbiology **3:**110-114.

81. **Brockson ME, Novotny LA, Jurcisek JA, McGillivary G, Bowers MR, Bakaletz LO.** 2012. Respiratory syncytial virus promotes *Moraxella catarrhalis*-induced ascending experimental otitis media. PloS one **7:**e40088.

82. **Hoopman TC, Liu W, Joslin SN, Pybus C, Sedillo JL, Labandeira-Rey M, Laurence CA, Wang W, Richardson JA, Bakaletz LO, Hansen EJ.** 2012. Use of the chinchilla model for nasopharyngeal colonization to study gene expression by *Moraxella catarrhalis*. Infection and immunity **80:**982-995.

83. **Luke NR, Jurcisek JA, Bakaletz LO, Campagnari AA.** 2007. Contribution of *Moraxella catarrhalis* type IV pili to nasopharyngeal colonization and biofilm formation. Infection and immunity **75:**5559-5564.

84. **Bakaletz LO.** 2009. Chinchilla as a robust, reproducible and polymicrobial model of otitis media and its prevention. Expert review of vaccines **8:**1063-1082.

85. **Forsgren A, Riesbeck K, Janson H.** 2008. Protein D of *Haemophilus influenzae*: a protective nontypeable *H. influenzae* antigen and a carrier for pneumococcal conjugate vaccines. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **46:**726-731.

86. **Giebink GS.** 1997. Vaccination against middle-ear bacterial and viral pathogens. Annals of the New York Academy of Sciences **830:**330-352.

87. **Giebink GS.** 1999. Otitis media: the chinchilla model. Microbial drug resistance **5:**57-72.

88. **Bakaletz LO, Daniels RL, Lim DJ.** 1993. Modeling adenovirus type 1-induced otitis media in the chinchilla: effect on ciliary activity and fluid transport function of

eustachian tube mucosal epithelium. The Journal of infectious diseases **168:**865-872.

89. **Bakaletz LO, Kennedy BJ, Novotny LA, Duquesne G, Cohen J, Lobet Y.** 1999. Protection against development of otitis media induced by nontypeable *Haemophilus influenzae* by both active and passive immunization in a chinchilla model of virus-bacterium superinfection. Infection and immunity **67:**2746-2762.

90. **Bakaletz LO, Murwin DM, Billy JM.** 1995. Adenovirus serotype 1 does not act synergistically with *Moraxella* (*Branhamella*) *catarrhalis* to induce otitis media in the chinchilla. Infection and immunity **63:**4188-4190.

91. **Kennedy BJ, Novotny LA, Jurcisek JA, Lobet Y, Bakaletz LO.** 2000. Passive transfer of antiserum specific for immunogens derived from a nontypeable *Haemophilus influenzae* adhesin and lipoprotein D prevents otitis media after heterologous challenge. Infection and immunity **68:**2756-2765.

92. **Miyamoto N, Bakaletz LO.** 1997. Kinetics of the ascension of NTHi from the nasopharynx to the middle ear coincident with adenovirus-induced compromise in the chinchilla. Microbial pathogenesis **23:**119-126.

93. **Suzuki K, Bakaletz LO.** 1994. Synergistic effect of adenovirus type 1 and nontypeable *Haemophilus influenzae* in a chinchilla model of experimental otitis media. Infection and immunity **62:**1710-1718.

94. **Novotny LA, Jurcisek JA, Pichichero ME, Bakaletz LO.** 2000. Epitope mapping of the outer membrane protein P5-homologous fimbrin adhesin of nontypeable *Haemophilus influenzae*. Infection and immunity **68:**2119-2128.

95. **Harris RH, Wilk D, Bevins CL, Munson RS, Jr., Bakaletz LO.** 2004. Identification and characterization of a mucosal antimicrobial peptide expressed by the chinchilla (*Chinchilla lanigera*) airway. The Journal of biological chemistry **279:**20250-20256.

96. **McGillivary G, Bakaletz LO.** 2010. The multifunctional host defense peptide SPLUNC1 is critical for homeostasis of the mammalian upper airway. PloS one **5:**e13224.

97. **McGillivary G, Mason KM, Jurcisek JA, Peeples ME, Bakaletz LO.** 2009. Respiratory syncytial virus-induced dysregulation of expression of a mucosal beta-defensin augments colonization of the upper airway by non-typeable *Haemophilus influenzae*. Cellular microbiology **11:**1399-1408.

98. **McGillivary G, Ray WC, Bevins CL, Munson RS, Jr., Bakaletz LO.** 2007. A member of the cathelicidin family of antimicrobial peptides is produced in the upper airway of the chinchilla and its mRNA expression is altered by common viral and bacterial co-pathogens of otitis media. Molecular immunology **44:**2446-2458.

99. **Kerschner JE, Erdos G, Hu FZ, Burrows A, Cioffi J, Khampang P, Dahlgren M, Hayes J, Keefe R, Janto B, Post JC, Ehrlich GD.** 2010. Partial

characterization of normal and *Haemophilus influenzae*-infected mucosal complementary DNA libraries in chinchilla middle ear mucosa. The Annals of otology, rhinology, and laryngology **119:**270-278.

100. **Kerschner JE, Horsey E, Ahmed A, Erbe C, Khampang P, Cioffi J, Hu FZ, Post JC, Ehrlich GD.** 2009. Gene expression differences in infected and noninfected middle ear complementary DNA libraries. Archives of otolaryngology--head & neck surgery **135:**33-39.

101. **Kerschner JE, Khampang P, Samuels T.** 2010. Extending the chinchilla middle ear epithelial model for mucin gene investigation. International journal of pediatric otorhinolaryngology **74:**980-985.

102. **Helminen ME, Maciver I, Latimer JL, Klesney-Tait J, Cope LD, Paris M, McCracken GH, Jr., Hansen EJ.** 1994. A large, antigenically conserved protein on the surface of *Moraxella catarrhalis* is a target for protective antibodies. The Journal of infectious diseases **170:**867-872.

103. **Aebi C, Maciver I, Latimer JL, Cope LD, Stevens MK, Thomas SE, McCracken GH, Jr., Hansen EJ.** 1997. A protective epitope of *Moraxella catarrhalis* is encoded by two different genes. Infection and immunity **65:**4367-4377.

104. **Lafontaine ER, Cope LD, Aebi C, Latimer JL, McCracken GH, Jr., Hansen EJ.** 2000. The UspA1 protein and a second type of UspA2 protein mediate adherence of *Moraxella catarrhalis* to human epithelial cells in vitro. Journal of bacteriology **182:**1364-1373.

105. **Meier PS, Troller R, Grivea IN, Syrogiannopoulos GA, Aebi C.** 2002. The outer membrane proteins UspA1 and UspA2 of Moraxella catarrhalis are highly conserved in nasopharyngeal isolates from young children. Vaccine **20:**1754-1760.

106. **Brooks MJ, Sedillo JL, Wagner N, Wang W, Attia AS, Wong H, Laurence CA, Hansen EJ, Gray-Owen SD.** 2008. *Moraxella catarrhalis* binding to host cellular receptors is mediated by sequence-specific determinants not conserved among all UspA1 protein variants. Infection and immunity **76:**5322-5329.

107. **Lafontaine ER, Wagner NJ, Hansen EJ.** 2001. Expression of the *Moraxella catarrhalis* UspA1 protein undergoes phase variation and is regulated at the transcriptional level. Journal of bacteriology **183:**1540-1551.

108. **Hammarstrom S.** 1999. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. Seminars in cancer biology **9:**67-81.

109. **Hill DJ, Edwards AM, Rowe HA, Virji M.** 2005. Carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-binding recombinant polypeptide confers protection against infection by respiratory and urogenital pathogens. Molecular microbiology **55:**1515-1527.

110. **Hill DJ, Virji M.** 2003. A novel cell-binding mechanism of *Moraxella catarrhalis* ubiquitous surface protein UspA: specific targeting of the N-domain of carcinoembryonic antigen-related cell adhesion molecules by UspA1. Molecular microbiology **48:**117-129.

111. **Aebi C, Lafontaine ER, Cope LD, Latimer JL, Lumbley SL, McCracken GH, Jr., Hansen EJ.** 1998. Phenotypic effect of isogenic uspA1 and uspA2 mutations on *Moraxella catarrhalis* 035E. Infection and immunity **66:**3113-3119.

112. **Attia AS, Lafontaine ER, Latimer JL, Aebi C, Syrogiannopoulos GA, Hansen EJ.** 2005. The UspA2 protein of *Moraxella catarrhalis* is directly involved in the expression of serum resistance. Infection and immunity **73:**2400-2410.

113. **Bootsma HJ, van der Heide HG, van de Pas S, Schouls LM, Mooi FR.** 2000. Analysis of *Moraxella catarrhalis* by DNA typing: evidence for a distinct subpopulation associated with virulence traits. The Journal of infectious diseases **181:**1376-1387.

114. **Meier PS, Troller R, Heiniger N, Grivea IN, Syrogiannopoulos GA, Aebi C.** 2005. *Moraxella catarrhalis* strains with reduced expression of the UspA outer membrane proteins belong to a distinct subpopulation. Vaccine **23:**2000-2008.

115. **Attia AS, Ram S, Rice PA, Hansen EJ.** 2006. Binding of vitronectin by the *Moraxella catarrhalis* UspA2 protein interferes with late stages of the complement cascade. Infection and immunity **74:**1597-1611.

116. **Nordstrom T, Blom AM, Forsgren A, Riesbeck K.** 2004. The emerging pathogen *Moraxella catarrhalis* interacts with complement inhibitor C4b binding protein through ubiquitous surface proteins A1 and A2. Journal of immunology **173:**4598-4606.

117. **Nordstrom T, Blom AM, Tan TT, Forsgren A, Riesbeck K.** 2005. Ionic binding of C3 to the human pathogen *Moraxella catarrhalis* is a unique mechanism for combating innate immunity. Journal of immunology **175:**3628-3636.

118. **Pearson MM, Lafontaine ER, Wagner NJ, St Geme JW, 3rd, Hansen EJ.** 2002. A hag mutant of *Moraxella catarrhalis* strain O35E is deficient in hemagglutination, autoagglutination, and immunoglobulin D-binding activities. Infection and immunity **70:**4523-4533.

119. **Meier PS, Freiburghaus S, Martin A, Heiniger N, Troller R, Aebi C.** 2003. Mucosal immune response to specific outer membrane proteins of *Moraxella catarrhalis* in young children. The Pediatric infectious disease journal **22:**256-262.

120. **Bullard B, Lipski SL, Lafontaine ER.** 2005. Hag directly mediates the adherence of *Moraxella catarrhalis* to human middle ear cells. Infection and immunity **73:**5127-5136.

121. **Holm MM, Vanlerberg SL, Sledjeski DD, Lafontaine ER.** 2003. The Hag protein of *Moraxella catarrhalis* strain O35E is associated with adherence to human lung and middle ear cells. Infection and immunity **71:**4977-4984.

122. **Balder R, Krunkosky TM, Nguyen CQ, Feezel L, Lafontaine ER.** 2009. Hag mediates adherence of *Moraxella catarrhalis* to ciliated human airway cells. Infection and immunity **77:**4597-4608.

123. **Mollenkvist A, Nordstrom T, Hallden C, Christensen JJ, Forsgren A, Riesbeck K.** 2003. The *Moraxella catarrhalis* immunoglobulin D-binding protein MID has conserved sequences and is regulated by a mechanism corresponding to phase variation. Journal of bacteriology **185:**2285-2295.

124. **Pearson MM, Laurence CA, Guinn SE, Hansen EJ.** 2006. Biofilm formation by *Moraxella catarrhalis* in vitro: roles of the UspA1 adhesin and the Hag hemagglutinin. Infection and immunity **74:**1588-1596.

125. **Timpe JM, Holm MM, Vanlerberg SL, Basrur V, Lafontaine ER.** 2003. Identification of a *Moraxella catarrhalis* outer membrane protein exhibiting both adhesin and lipolytic activities. Infection and immunity **71:**4341-4350.

126. **Lipski SL, Akimana C, Timpe JM, Wooten RM, Lafontaine ER.** 2007. The *Moraxella catarrhalis* autotransporter McaP is a conserved surface protein that mediates adherence to human epithelial cells through its N-terminal passenger domain. Infection and immunity **75:**314-324.

127. **Holm MM, Vanlerberg SL, Foley IM, Sledjeski DD, Lafontaine ER.** 2004. The *Moraxella catarrhalis* porin-like outer membrane protein CD is an adhesin for human lung cells. Infection and immunity **72:**1906-1913.

128. **Harabuchi Y, Murakata H, Goh M, Kodama H, Kataura A, Faden H, Murphy TF.** 1998. Serum antibodies specific to CD outer membrane protein of *Moraxella catarrhalis*, P6 outer membrane protein of non-typeable *Haemophilus influenzae* and capsular polysaccharides of *Streptococcus pneumoniae* in children with otitis media with effusion. Acta oto-laryngologica **118:**826-832.

129. **Yu RH, Bonnah RA, Ainsworth S, Schryvers AB.** 1999. Analysis of the immunological responses to transferrin and lactoferrin receptor proteins from *Moraxella catarrhalis*. Infection and immunity **67:**3793-3799.

130. **Aebi C, Stone B, Beucher M, Cope LD, Maciver I, Thomas SE, McCracken GH, Jr., Sparling PF, Hansen EJ.** 1996. Expression of the CopB outer membrane protein by *Moraxella catarrhalis* is regulated by iron and affects iron acquisition from transferrin and lactoferrin. Infection and immunity **64:**2024-2030.

131. **Campagnari AA, Shanks KL, Dyer DW.** 1994. Growth of *Moraxella catarrhalis* with human transferrin and lactoferrin: expression of iron-repressible proteins without siderophore production. Infection and immunity **62:**4909-4914.

132. **Helminen ME, Maciver I, Latimer JL, Cope LD, McCracken GH, Jr., Hansen EJ.** 1993. A major outer membrane protein of *Moraxella catarrhalis* is a target for

antibodies that enhance pulmonary clearance of the pathogen in an animal model. Infection and immunity **61:**2003-2010.

133. **Aoki SK, Pamma R, Hernday AD, Bickham JE, Braaten BA, Low DA.** 2005. Contact-dependent inhibition of growth in *Escherichia coli*. Science **309:**1245-1248.

134. **Nikolakakis K, Amber S, Wilbur JS, Diner EJ, Aoki SK, Poole SJ, Tuanyok A, Keim PS, Peacock S, Hayes CS, Low DA.** 2012. The toxin/immunity network of *Burkholderia pseudomallei* contact-dependent growth inhibition (CDI) systems. Molecular microbiology **84:**516-529.

135. **Ruhe ZC, Low DA, Hayes CS.** 2013. Bacterial contact-dependent growth inhibition. Trends in microbiology **21:**230-237.

136. **Webb JS, Nikolakakis KC, Willett JLE, Aoki SK, Hayes CS, Low DA.** 2013. Delivery of CdiA Nuclease Toxins into Target Cells during Contact-Dependent Growth Inhibition. PloS one **8**.

137. **Iyer LM, Zhang D, Rogozin IB, Aravind L.** 2011. Evolution of the deaminase fold and multiple origins of eukaryotic editing and mutagenic nucleic acid deaminases from bacterial toxin systems. Nucleic Acids Res **39:**9473-9497.

138. **Zhang D, de Souza RF, Anantharaman V, Iyer LM, Aravind L.** 2012. Polymorphic toxin systems: Comprehensive characterization of trafficking modes, processing, mechanisms of action, immunity and ecology using comparative genomics. Biology direct **7:**18.

139. **Zhang DP, Iyer LM, Aravind L.** 2011. A novel immunity system for bacterial nucleic acid degrading toxins and its recruitment in various eukaryotic and DNA viral systems. Nucleic Acids Res **39:**4532-4552.

140. **Anderson MS, Garcia EC, Cotter PA.** 2012. The *Burkholderia* bcpAIOB genes define unique classes of two-partner secretion and contact dependent growth inhibition systems. PLoS genetics **8:**e1002877.

141. **Holberger LE, Garza-Sanchez F, Lamoureux J, Low DA, Hayes CS.** 2012. A novel family of toxin/antitoxin proteins in *Bacillus* species. FEBS letters **586:**132-136.

142. **Poole SJ, Diner EJ, Aoki SK, Braaten BA, t'Kint de Roodenbeke C, Low DA, Hayes CS.** 2011. Identification of functional toxin/immunity genes linked to contact-dependent growth inhibition (CDI) and rearrangement hotspot (Rhs) systems. PLoS genetics **7:**e1002217.

143. **Rojas CM, Ham JH, Deng WL, Doyle JJ, Collmer A.** 2002. HecA, a member of a class of adhesins produced by diverse pathogenic bacteria, contributes to the attachment, aggregation, epidermal cell killing, and virulence phenotypes of *Erwinia chrysanthemi* EC16 on *Nicotiana clevelandii* seedlings. Proc Natl Acad Sci U S A **99:**13142-13147.

144. **Clantin B, Delattre AS, Rucktooa P, Saint N, Meli AC, Locht C, Jacob-Dubuisson F, Villeret V.** 2007. Structure of the membrane protein FhaC: a member of the Omp85-TpsB transporter superfamily. Science **317:**957-961.

145. **Clantin B, Hodak H, Willery E, Locht C, Jacob-Dubuisson F, Villeret V.** 2004. The crystal structure of filamentous hemagglutinin secretion domain and its implications for the two-partner secretion pathway. Proc Natl Acad Sci U S A **101:**6194-6199.

146. **Jacob-Dubuisson F, Fernandez R, Coutte L.** 2004. Protein secretion through autotransporter and two-partner pathways. Biochimica et biophysica acta **1694:**235-257.

147. **Tommassen J.** 2007. Biochemistry. Getting into and through the outer membrane. Science **317:**903-904.

148. **Willems RJ, Geuijen C, van der Heide HG, Renauld G, Bertin P, van den Akker WM, Locht C, Mooi FR.** 1994. Mutational analysis of the *Bordetella pertussis* fim/fha gene cluster: identification of a gene with sequence similarities to haemolysin accessory genes involved in export of FHA. Molecular microbiology **11:**337-347.

149. **Mazar J, Cotter PA.** 2007. New insight into the molecular mechanisms of two-partner secretion. Trends in microbiology **15:**508-515.

150. **Aoki SK, Malinverni JC, Jacoby K, Thomas B, Pamma R, Trinh BN, Remers S, Webb J, Braaten BA, Silhavy TJ, Low DA.** 2008. Contact-dependent growth inhibition requires the essential outer membrane protein BamA (YaeT) as the receptor and the inner membrane transport protein AcrB. Molecular microbiology **70:**323-340.

151. **Aoki SK, Webb JS, Braaten BA, Low DA.** 2009. Contact-Dependent Growth Inhibition Causes Reversible Metabolic Downregulation in *Escherichia coli*. Journal of bacteriology **191:**1777-1786.

152. **Morse RP, Nikolakakis KC, Willett JLE, Gerrick E, Low DA, Hayes CS, Goulding CW.** 2012. Structural basis of toxicity and immunity in contact-dependent growth inhibition (CDI) systems. P Natl Acad Sci USA **109:**21480-21485.

153. **Ruhe ZC, Wallace AB, Low DA, Hayes CS.** 2013. Receptor Polymorphism Restricts Contact-Dependent Growth Inhibition to Members of the Same Species. mBio **4**.

154. **Hayes CS, Aoki SK, Low DA.** 2010. Bacterial Contact-Dependent Delivery Systems. Annu Rev Genet **44:**71-90.

155. **Koskiniemi S, Lamoureux JG, Nikolakakis KC, de Roodenbeke CT, Kaplan MD, Low DA, Hayes CS.** 2013. Rhs proteins from diverse bacteria mediate intercellular competition. P Natl Acad Sci USA **110:**7032-7037.

156. **Shaffer TL, Balder R, Buskirk SW, Hogan RJ, Lafontaine ER.** 2013. Use of the Chinchilla model to evaluate the vaccinogenic potential of the *Moraxella catarrhalis* filamentous hemagglutinin-like proteins MhaB1 and MhaB2. PloS one **8:**e67881.

157. **Sambrook J, Russell D.** 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press.

158. **Whitby PW, Morton DJ, Stull TL.** 1998. Construction of antibiotic resistance cassettes with multiple paired restriction sites for insertional mutagenesis of *Haemophilus influenzae*. FEMS microbiology letters **158:**57-60.

159. **Ray A, Skurray R.** 1984. Stabilization of the cloning vector pACYC184 by insertion of F plasmid leading region sequences. Plasmid **11:**272-275.

160. **Murphy TF, Brauer AL, Aebi C, Sethi S.** 2005. Antigenic specificity of the mucosal antibody response to *Moraxella catarrhalis* in chronic obstructive pulmonary disease. Infection and immunity **73:**8161-8166.

161. **St Geme JW, 3rd, de la Morena ML, Falkow S.** 1994. A *Haemophilus influenzae* IgA protease-like protein promotes intimate interaction with human epithelial cells. Molecular microbiology **14:**217-233.

162. **Yanisch-Perron C, Vieira J, Messing J.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

163. **Willson PJ, Albritton WL, Slaney L, Setlow JK.** 1989. Characterization of a multiple antibiotic resistance plasmid from *Haemophilus ducreyi*. Antimicrobial agents and chemotherapy **33:**1627-1630.

164. **Website WHO** 2008, posting date. World Heath Statistics. [Online.]

165. **Website WHO** 2008, posting date. Fact Sheet: The Top Ten Causes of Death. [Online.]

166. **Jacobs MR, Bajaksouzian S, Windau A, Good CE, Lin G, Pankuch GA, Appelbaum PC.** 2004. Susceptibility of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* to 17 oral antimicrobial agents based on pharmacodynamic parameters: 1998-2001 U S Surveillance Study. Clinics in laboratory medicine **24:**503-530.

167. **Johnson DM, Sader HS, Fritsche TR, Biedenbach DJ, Jones RN.** 2003. Susceptibility trends of *Haemophilus influenzae* and *Moraxella catarrhalis* against orally administered antimicrobial agents: five-year report from the SENTRY Antimicrobial Surveillance Program. Diagnostic microbiology and infectious disease **47:**373-376.

168. **Garcia-Rodriguez JA, Fresnadillo Martinez MJ.** 2002. Dynamics of nasopharyngeal colonization by potential respiratory pathogens. The Journal of antimicrobial chemotherapy **50 Suppl S2:**59-73.

169. **Beachey EH.** 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surface. The Journal of infectious diseases **143:**325-345.

170. **Boyle EC, Finlay BB.** 2003. Bacterial pathogenesis: exploiting cellular adherence. Current opinion in cell biology **15:**633-639.

171. **Gerlach RG, Hensel M.** 2007. Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. International journal of medical microbiology : IJMM **297:**401-415.

172. **Hauck CR.** 2002. Cell adhesion receptors - signaling capacity and exploitation by bacterial pathogens. Medical microbiology and immunology **191:**55-62.

173. **Jacob-Dubuisson F, Locht C, Antoine R.** 2001. Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. Molecular microbiology **40:**306-313.

174. **Niemann HH, Schubert WD, Heinz DW.** 2004. Adhesins and invasins of pathogenic bacteria: a structural view. Microbes and infection / Institut Pasteur **6:**101-112.

175. **St Geme JW, 3rd.** 1997. Bacterial adhesins: determinants of microbial colonization and pathogenicity. Advances in pediatrics **44:**43-72.

176. **Akimana C, Lafontaine ER.** 2007. The *Moraxella catarrhalis* outer membrane protein CD contains two distinct domains specifying adherence to human lung cells. FEMS microbiology letters **271:**12-19.

177. **Murphy TF, Loeb MR.** 1989. Isolation of the outer membrane of *Branhamella catarrhalis*. Microbial pathogenesis **6:**159-174.

178. **Cope LD, Lafontaine ER, Slaughter CA, Hasemann CA, Jr., Aebi C, Henderson FW, McCracken GH, Jr., Hansen EJ.** 1999. Characterization of the *Moraxella catarrhalis* uspA1 and uspA2 genes and their encoded products. Journal of bacteriology **181:**4026-4034.

179. **Patrick CC, Kimura A, Jackson MA, Hermanstorfer L, Hood A, McCracken GH, Jr., Hansen EJ.** 1987. Antigenic characterization of the oligosaccharide portion of the lipooligosaccharide of nontypable *Haemophilus influenzae*. Infection and immunity **55:**2902-2911.

180. **Song JM, Hossain J, Yoo DG, Lipatov AS, Davis CT, Quan FS, Chen LM, Hogan RJ, Donis RO, Compans RW, Kang SM.** 2010. Protective immunity against H5N1 influenza virus by a single dose vaccination with virus-like particles. Virology **405:**165-175.

181. **Chen D, Barniak V, VanDerMeid KR, McMichael JC.** 1999. The levels and bactericidal capacity of antibodies directed against the UspA1 and UspA2 outer

membrane proteins of *Moraxella* (*Branhamella*) *catarrhalis* in adults and children. Infection and immunity **67:**1310-1316.

182. **LaFontaine ER, Snipes LE, Bullard B, Brauer AL, Sethi S, Murphy TF.** 2009. Identification of domains of the Hag/MID surface protein recognized by systemic and mucosal antibodies in adults with chronic obstructive pulmonary disease following clearance of *Moraxella catarrhalis*. Clinical and vaccine immunology : CVI **16:**653-659.

183. **Mathers K, Leinonen M, Goldblatt D.** 1999. Antibody response to outer membrane proteins of *Moraxella catarrhalis* in children with otitis media. The Pediatric infectious disease journal **18:**982-988.

184. **Murphy TF, Brauer AL, Aebi C, Sethi S.** 2005. Identification of surface antigens of *Moraxella catarrhalis* as targets of human serum antibody responses in chronic obstructive pulmonary disease. Infection and immunity **73:**3471-3478.

185. **Murphy TF, Kirkham C, Liu DF, Sethi S.** 2003. Human immune response to outer membrane protein CD of *Moraxella catarrhalis* in adults with chronic obstructive pulmonary disease. Infection and immunity **71:**1288-1294.

186. **Stutzmann Meier P, Heiniger N, Troller R, Aebi C.** 2003. Salivary antibodies directed against outer membrane proteins of *Moraxella catarrhalis* in healthy adults. Infection and immunity **71:**6793-6798.

187. **Eskola J, Kilpi T, Palmu A, Jokinen J, Haapakoski J, Herva E, Takala A, Kayhty H, Karma P, Kohberger R, Siber G, Makela PH, Finnish Otitis Media Study G.** 2001. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. The New England journal of medicine **344:**403-409.

188. **Prymula R, Peeters P, Chrobok V, Kriz P, Novakova E, Kaliskova E, Kohl I, Lommel P, Poolman J, Prieels JP, Schuerman L.** 2006. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both *Streptococcus pneumoniae* and non-typable *Haemophilus influenzae*: a randomised double-blind efficacy study. Lancet **367:**740-748.

189. **Revai K, McCormick DP, Patel J, Grady JJ, Saeed K, Chonmaitree T.** 2006. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. Pediatrics **117:**1823-1829.

190. **Meier PS, Troller R, Heiniger N, Hays JP, van Belkum A, Aebi C.** 2006. Unveiling electrotransformation of *Moraxella catarrhalis* as a process of natural transformation. FEMS microbiology letters **262:**72-76.

191. **Hill DJ, Whittles C, Virji M.** 2012. A novel group of *Moraxella catarrhalis* UspA proteins mediates cellular adhesion via CEACAMs and vitronectin. PloS one **7:**e45452.

192. **Bookwalter JE, Jurcisek JA, Gray-Owen SD, Fernandez S, McGillivary G, Bakaletz LO.** 2008. A carcinoembryonic antigen-related cell adhesion molecule 1 homologue plays a pivotal role in nontypeable *Haemophilus influenzae*

colonization of the chinchilla nasopharynx via the outer membrane protein P5-homologous adhesin. Infection and immunity **76:**48-55.