IDENTIFICATION AND CHARACTERIZATION OF FACTORS CONTRIBUTING TO THE PATHOGENICITY OF *MORAXELLA CATARRHALIS*

by

SEAN WILLIAM BUSKIRK

(Under the Direction of Eric Lafontaine)

ABSTRACT

Moraxella catarrhalis is a human-specific pathogen and causative agent of otitis media in children and respiratory tract infections in adults. During the course of pathogenesis, *M. catarrhalis* is able to colonize and persist in the human host through processes such as adherence to the mucosal epithelium and resistance to complement-mediated killing.

The first part of this dissertation describes the identification and characterization of the protein responsible for cardiolipin synthesis in *M. catarrhalis* (MclS) and the contribution of cardiolipin to bacterial adherence to human epithelial cells. To study the role of MclS, we constructed an *mclS* insertion mutant strain that lacks expression of MclS and an *mclS* point mutant strain that expresses an enzymatically-inactive version of MclS. Both mutant strains are unable to synthesize cardiolipin and are defective in their adherence to human epithelial cells *in vitro*, thereby suggesting that MclS mediates adherence in an indirect manner. We hypothesize that cardiolipin contributes to adherence through its interaction with surface-displayed adhesins. Examination of outer membrane vesicles revealed that cardiolipin is present in the outer membrane of *M. catarrhalis*. However, five major proteinaceous adhesins (UspA1, Hag, McaP, OMP CD, MhaB1/MhaB) were all expressed and displayed on the surface of the cardiolipin-

deficient strains. Conversely, expression of lipooligosaccharide, which has previously been implicated in adherence of *M. catarrhalis* to epithelial cells, may depend on the presence of cardiolipin.

The second part of this dissertation describes a series of experiments designed to identify factors that contribute to complement resistance of *M. catarrhalis*. Several factors are currently known to contribute to complement resistance including UspA2 which mediates the majority of resistance exhibited by *M. catarrhalis*. Using experimental evolution, we demonstrate that an *M. catarrhalis* strain lacking expression of UspA2 is able to develop complement resistance through repeated exposure to human serum. We intend to identify the mutations that confer complement resistance in the evolved lineages using whole-genome sequencing. Ultimately, we hope to gain a better understanding of the factors that impact complement resistance in *M. catarrhalis*.

INDEX WORDS: *Moraxella catarrhalis*, Virulence, Adherence, Cardiolipin, Complement resistance, Experimental evolution

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DEDICATION

While I acknowledge that countless friends and family members have contributed to my accomplishments, I have decided to dedicate this dissertation to my late grandfathers, Arlington "Ty" Frutchey and Richard "Dick" Buskirk. I have always cherished the time that we spent together as I was growing up, and both of you have made a lasting impact in my life. From you I have learned so many life lessons. I know you would be proud of me today.

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CHAPTER 1

INTRODUCTION

Moraxella catarrhalis is a Gram negative diplococcus and human-specific pathogen of the mucosa. It is a causative agent of otitis media in children and exacerbations of chronic obstructive pulmonary disease in adults. Once considered simply a commensal, today *M. catarrhalis* is recognized as a significant public health concern. Treatment of *M. catarrhalis* infection has become troublesome due to the wide-spread resistance to β-lactam antibiotics. The introduction of vaccines against non-typeable *Haemophilus influenzae* and *Streptococcus pneumoniae* is predicted to shift the landscape of the human mucosa resulting in an increase in the incidence and prevalence of *M. catarrhalis* carriage. Disease caused by *M. catarrhalis* imparts a large financial burden on the United States, in part due to the lack of a licensed vaccine to combat *M. catarrhalis* infection. To promote the development of effective therapeutics, research is being conducted into how *M. catarrhalis* is able to cause disease in humans.

Infection of humans by *M. catarrhalis* requires colonization of the mucosal epithelium followed by persistence in the host. Numerous factors have been identified in *M. catarrhalis* that mediate processes essential for colonization and persistence. In particular, two traits, adherence to epithelial cells and resistance to the activity of the complement cascade, have a strong correlation with virulence of *M. catarrhalis*. Adherence is necessary for the initial colonization of the host, whereas complement resistance is crucial to persistence in the host. Ultimately, both of these traits are vital to *M. catarrhalis* pathogenesis, and the factors responsible for mediating adherence and complement resistance represent excellent targets for development of

therapeutics. In this dissertation, I describe my research on the role and synthesis of a factor, cardiolipin, that I demonstrate is associated with virulence of *M. catarrhalis*. In addition, I venture into the use of experimental evolution to identify factors involved in complement resistance.

CHAPTER 2

LITERATURE REVIEW

Taxonomy of *Moraxella catarrhalis*

Moraxella catarrhalis was first isolated in 1882 from a patient with bronchitis and was originally named *Micrococcus catarrhalis* [1]. However, nomenclature of the bacterium has changed on several occasions since its initial discovery [2]. Based on morphological similarities, *M. catarrhalis* was moved to the genus *Neisseria* in the mid-20th century [3]. In fact, due to the similarity in appearance and microbiological characterization, *M. catarrhalis* was often confused with *Neissiera cinerea* [3]. Eventually, this error was recognized, and based on DNA hybridization studies the bacterium was re-assigned a new genus, *Branhamella* [4]. Finally, 16S RNA sequencing data revealed that *M. catarrhalis* is most closely related to *Moraxella* species, thus prompting the change to its current nomenclature. Further genetic sequencing data confirms the appropriate classification of the bacterium [5-6]. Unlike the majority of the species in the *Moraxella* genus, *M. catarrhalis* is not rod-shaped and does infect humans [7]. Genomic comparisons show that *M. catarrhalis* is very similar to *Acinetobacter* and *Psychrobacter* species, both of which are members of the Moraxellaceae family (NCBI Blast).

Microbiology of *Moraxella catarrhalis*

M. catarrhalis is a Gram-negative, unencapsulated, non-motile, non-hemolytic, aerobic diplococcus. In a laboratory setting, *M. catarrhalis* is commonly propagated on complex media such as Todd Hewitt Media or Brain-Heart Infusion Media, though a defined media has been

described [8]. When grown on agar, *M. catarrhalis* forms round, opaque colonies that are distinguished by their ability to be pushed across the agar, much like a hockey puck [3]. In broth, bacteria aggregate and sink after a short period of time. Growth of *M. catarrhalis* is optimal at 37°C, but the bacteria can also grow at slower rates at temperatures as low as 22°C. Metabolically, *M. catarrhalis* does not utilize sucrose, glucose, maltose, and lactose; however, it does reduce nitrite and nitrate as well as produce oxidase and DNase [3].

The *Moraxella catarrhalis* Genome

In 2001, the first *M. catarrhalis* genome was sequenced and deposited into the GenBank database (accession numbers AX067426 through AX067466). The partial genome originated from isolate ATCC 43617 but was never completely assembled. Not until 2007 was the ATCC 43617 genome annotated [9].

The first completely assembled and annotated genome was completed in 2010. While the original publication stated that the genome was from isolate RH4, it was later corrected to that of isolate BBH18. The roughly 1.9 Mbp genome of BBH18 comprises approximately 1,900 genes. When compared to the genome of ATCC 43617, the gene content was highly conserved. According to bioinformatics analyses, the majority (~62%) of putative proteins were most similar to those encoded by *Psychrobacter* species, suggesting that these organisms are closely related [10].

In 2011, the genomes of 12 additional isolates were assembled and annotated. The isolates, which were geographically and clinically diverse, possessed genomes that ranged in size from 1.78 to 1.96 Mbp. Of the 2,383 gene clusters identified in the 12 genomes, 1,755 genes (74%) were present in each isolate (termed the core genome) while 628 genes (26%) were

distributed between the isolates. According to the finite supragenome model, 85% of genes encoded by the entire *M. catarrhalis* species are found between these 12 genomes. Genes encoding all known virulence factors were present in each of the strains, with only minor exceptions. Due to sequencing difficulties attributed to identical regions of the genes, a conclusion regarding the presence or absence of *uspA2*, *uspA2H*, *mhaB1*, and *mhaB2* could not be reached [11].

All *M. catarrhalis* isolates possess a single chromosome while some also contain a plasmid. Only two plasmids have been isolated from clinical *M. catarrhalis* isolates: pLQ510 from isolate E22 [12] and pEMCJH04 from isolate 6.12 [13]. Since its discovery, pLQ510 has been manipulated and several resulting plasmids (i.e. pWW102B, pWW115, pASE222) are now commonly used for cloning purposes [14-16]. Designed to be a shuttle vector, the plasmid pWW102B was generated by combining regions from pLQ510 with regions from pACYC184 and is able to replicate in *Escherichia coli*, *H. influenza*, and a small number of *M. catarrhalis* isolates [16]. The plasmid pWW115 is a spontaneous deletion mutant of pWW102B and is able to replicate in all *M. catarrhalis* isolates tested, though it no longer replicates in *E. coli* [14]. Generated from pWW115, the plasmid pASE222 is a *lacZ*-based reporter plasmid that is utilized to study regulation of individual genes in *M. catarrhalis* [15].

Moraxella catarrhalis Carriage and Disease

Up until the late 20th century, *M. catarrhalis* was viewed by many as solely a commensal. Today *M. catarrhalis* is also recognized as one of the leading bacterial causes of otitis media in children and exacerbations of chronic obstructive pulmonary disease (COPD) in adults. While *M. catarrhalis* is commonly found colonizing the nasopharynx of humans, disease results upon

migration of the bacterium to other regions including the middle ear and upper respiratory system [17]. Nearly 70% of infants are colonized by *M. catarrhalis* within their first 12 months [18], though carriage rates drop to 1-5% in adults [17].

Otitis Media

Otitis media, Latin for "inflammation of the middle ear," is common in children and causes more visits to the pediatrician's office (30 million annually) than any other disease in the United States [19]. In fact, approximately 75% of children develop otitis media before the age of three with the majority having three or more episodes [20-21]. Significant speech and language delays are displayed in children who develop otitis media, especially those with recurring episodes of the disease. *M. catarrhalis* is responsible for up to 20% of cases (3-4 million in the US), which makes it the third leading bacterial cause of otitis media in children, behind only non-typeable *Haemophilus influenzae* (*NTHi*) (~26%) and *Streptococcus pneumoniae* (~23%). An estimated $150 million are spent annually to treat children with infected by *M. catarrhalis* [22-25].

Exacerbations of COPD

COPD is the third leading cause of mortality in the United States, responsible for over 130,000 deaths annually [26]. An additional 25 million Americans are currently believed to be living with the disease [27]. COPD is a progressive disease that leads to the development of emphysema and chronic bronchitis. Complications often arise when a pathogen such as *M. catarrhalis* infects the respiratory tract of an individual with COPD, resulting in exacerbation of the disease. *M. catarrhalis* is one of the leading causes of COPD exacerbations, resulting in 2-4 million cases each year. Overall, 1-5% of adults are colonized by *M .catarrhalis* [17].

Other Diseases Caused by Moraxella catarrhalis

In addition to otitis media and COPD exacerbations, *M. catarrhalis* is also responsible for ~20% of all cases of bacterial sinusitis [25]. *M. catarrhalis* has also been shown to cause conjunctivitis, laryngitis, pneumonia, bacteraemia, endocarditis, meningitis, and arthritis, although these instances are rare and often occur in immunocompromised patients [21].

Public Health Concern

In addition to the financial burden described above, *M. catarrhalis* represents a significant public health concern due to the recent increase in prevalence of disease associated with this pathogen, the limited treatment options, and lack of a commercially available vaccine.

The incidence and prevalence of *M. catarrhalis* infection and colonization has increased recently in part due to the introduction of the pneumococcal conjugate vaccine that targets the capsule of *S. pneumoniae* isolates [28-29]. Like *M. catarrhalis*, *S. pneumoniae* colonizes the nasopharynx and causes both otitis media in children and infections in adults with COPD. Therefore, the two bacteria compete against each other for resources in the host. The pneumococcal vaccine, which targets *S. pneumoniae*, opens the niche for *M. catarrhalis*. According to the CDC, the pneumococcal vaccine is recommended for children under the age of 5 and adults over the age of 65, exactly the individuals most susceptible for infection by *M. catarrhalis*. In fact, in countries that the pneumococcal vaccine is widely used, otitis media due to *M. catarrhalis* and *H. influenzae* has increased [28-29]. Furthermore, the development and implementation of a vaccine against NTHi, which also occupies the same niche, is expected to accelerate this shift of the microbial landscape toward *M. catarrhalis*.

For cases of otitis media, *M. catarrhalis* infections are often self-limiting so doctors often employ a 'watch and wait' approach [30]. When treatment is necessary, antibiotics are prescribed. Beta-lactam antibiotics, such as penicillin, ampicillin, and amoxicillin, were once the first line of treatment; however, *M. catarrhalis* quickly developed resistance, and beta-lactams are no longer effective at treating *M. catarrhalis* infections [31]. Beta-lactams, which include penicillin derivatives and cephalosporins, are bactericidal agents that inhibit peptidoglycan synthesis and subsequently compromise the integrity of the cell wall. The first beta-lactamase-producing *M. catarrhalis* isolate was discovered in the US in 1976 [32]. The spread of beta-lactam resistance was quite remarkable; by the early 1990's, approximately 90% of isolates had developed resistance [33-34]. Now, virtually all clinical isolates (upwards of 95%) are resistant due to the expression of beta-lactamase enzymes, namely BRO-1 and BRO-2 [31]. The BRO lactamases appears to have been acquired through horizontal transfer since the G+C content of the BRO genes (31%) differs significantly from the rest of the *M. catarrhalis* genome (41%). Furthermore, the genes likely originated from a Gram positive bacterium since the BRO enzymes are localized to the membrane via a lipid anchor, a characteristic common for beta-lactamases of Gram positive organisms but rare in Gram negative bacteria [35]. In addition to protecting *M. catarrhalis* from treatment, these secreted beta-lactamases also protect other respiratory pathogens such as *NTHi* and *S. pneumoniae* from antibiotics [36] through the release of outer membrane vesicles [37].

Because of the limited treatment options and the recent increase in prevalence, a vaccine against *M. catarrhalis* is highly desirable. Researchers have predicted that a vaccine that prevents otitis media due to *S. pneumoniae*, NTHi, and *M. catarrhalis* would save $1.3 billion annually in medical and parental-time costs and would substantially improve the healthcare of

infants in the United States [38]. While patients often recover from *M. catarrhalis* infection, their immunity is strain-specific [17]. Therefore, patients are susceptible to other strains of *M. catarrhalis*, often resulting in future re-infection. A vaccine targeting a conserved component of the bacterium would alleviate this issue.

Virulence Traits of *Moraxella catarrhalis*

In order to colonize and persist in the human host, *M. catarrhalis* has evolved a multitude of virulence traits that promote replication, immune evasion, and pathogenicity. Specifically, *M. catarrhalis* is able to adhere to and invade human epithelial cells, bind to the extracellular matrix, resist the activity of complement, form biofilms, and acquire nutrients from the host. The following sections will detail each of these processes.

Adherence to Epithelial Cells

The ability of *M. catarrhalis* to attach to epithelial cells is required for colonization and persistence in humans. Various studies have demonstrated that *M. catarrhalis* adheres to a wide range of human epithelial cell types that correspond to sites of colonization or infection. Numerous human cell lines have been utilized to characterize adherence factors *in vitro* including A549 (type II pneumocytes), HEp-2 (laryngeal), Chang (conjuctival), Detroit 562 (pharyngeal), NCIH 292 (lung epidermoid), and 16HBE14o- (bronchial). Adherence is a multi-factorial process for which several factors, called adhesins, have been identified.

UspA1: The Ubiquitous surface protein A1 (UspA1) is an outer membrane protein and one of the major adhesins of *M. catarrhalis*, present in practically all (99%) isolates examined [39]. Like many *M. catarrhalis* adhesins, UspA1 is an autotransporter, a protein that mediates its own translocation into the outer bacterial membrane [40]. More specifically, UspA1 is

characterized as a trimeric autotransporter, sometimes referred to as an oligomeric coiled-coil adhesin, with significant similarity to the YadA protein of *Yersinia* spp. and Hia protein of *Haemophilus* spp. [41-42]. Electron micrographs show that UspA1 possesses a lollipop-like structure with a membrane-anchored domain, stalk region, and head group [43]. UspA1 contains an N-terminal signal sequence, an N-terminal passenger domain containing the protein's activity (stalk and head regions), and a C-terminal translocator domain formed by beta-barrels for pore formation in the membrane [44].

UspA1 directly binds numerous epithelial cell lines including HEp-2, A549, and Chang [45-46]. UspA1 is the only *M. catarrhalis* adhesin for which the host receptor is known. The stalk region of UspA1 binds the Carcinoembryonic Antigen-related Cellular Adhesion Molecule 1 (CEACAM-1), a signaling molecule found at the surface of various cell types including those in the oropharynx and lower respiratory tract [47]. Of note, CEACAM-1 is also a receptor for several other mucosal pathogens in addition to *M. catarrhalis* [47-48].

The regulation of UspA1 has been well-studied. Transcription of UspA1 is regulated by phase-variation: a poly-G tract located directly upstream of the ORF is vulnerable to addition or subtraction of G residues, thereby leading to changes in UspA1 expression [49]. UspA1 has also been shown to be up-regulated at 26˚, the temperature that can be reached in the human nasopharynx in colder climates [50].

In addition to adherence, UspA1 has also been demonstrated to contribute to binding of extracellular matrix proteins fibronectin and lamanin [51-52], serum resistance [53], and biofilm formation [54].

Hag: Hemagglutinin (Hag), also called the *M. catarrhalis* immunoglobulin D binding protein (MID), is another major adhesin of *M. catarrhalis*. The protein is expressed in approximately 84% of isolates while the gene is present in 100% [55]. Hag, like UspA1, is a trimeric autotransporter protein composed of a C-terminal translocator domain and an N-terminal globular head/passenger domain containing its activity [56]. While Hag has been shown to directly mediate binding to a number of epithelial cell lines [57], it displays specificity to ciliated cells [58]. Hag also contributes to the pathogenicity of *M. catarrhalis* through erythrocyte agglutination, binding of soluble IgD in a non-immune fashion, and mitogenically stimulating B-cells [59].

Like UspA1, Hag is subject to phase variation due to a poly G tract [55]. However, the tract is located within the 5' portion of the ORF of Hag suggesting that its regulation is at the translational level instead of the transcriptional level as in UspA1 [55]. In contrast to UspA1, Hag is a negative effector of biofilm formation and has been shown to be down-regulated at 26˚ [50].

McaP: The *M. catarrhalis* adherence protein (McaP) is yet another highly conserved autotransporter. Unlike UspA1 and Hag, McaP is a conventional autotransporter [60]. Conventional autotransporters differ from trimeric autotransporters in that the former functions as a monomer and therefore possesses a much longer translocator domain containing mulitple pore-forming beta-barrels. McaP is composed of an N-terminal signal sequence and passenger domain, a short linker sequence, and a C-terminal transporter domain [60]. The passenger domain directly mediates binding to a wide range of cell lines including Chang, A549, HEp-2, NCIH 292, and 16HBE14o$^-$ [61].

In addition to being an adhesin, McaP also exhibits esterase and phospholipase B activities, evidenced by the ability of McaP to cleave short-chain fatty acids as well as phosphatidylcholine (PC) and lysophosphatidylcholine [61]. However, the biological relevance of these lipolytic activities has not yet been determined.

MhaB1, MhaB2, & MhaC: The *M. catarrhalis* FHA-like proteins B1, B2, and C (MhaB1, MhaB2, MhaC) are components of a two-partner secretion system (TPS) that together contribute to the adherence to various human epithelial cell types. The Mha proteins possess significant similarity to the well-characterized FHA proteins of *Bordetella pertussis*. MhaC forms a pore in the outer membrane to facilitate the transport of MhaB1 and/or MhaB2. Once transported to the outer membrane, the MhaB1 proteins directly mediate adherence to epithelial cells, though the exact mechanism is not yet known. The Mha proteins are expressed in 63% of isolates tested. The N-terminus of MhaB1 and MhaB2 are practically identical while the C-terminus contains only 16% identity at the amino acid level [62]. Recent findings suggest that the Mha proteins are also involved in contact-dependent growth inhibition, a process that contributes to competition between bacteria (unpublished).

OMP CD: The Outer Membrane Protein CD (OMP CD) is a highly-conserved, heat-modifiable porin protein expressed by *M. catarrhalis* [63-64]. OMP CD mediates adherence to A549 and HEp-2 cells [65-66], and adherence is dependent on two individual regions of OMP CD, one being a motif resembling a thrombospondin-type 3 repeat (TT3R) [67]. In addition, OMP CD contributes to other virulence-associated traits including serum resistance [65] and binding of middle ear mucin [68].

LOS: Lipooligosaccharide (LOS) is the principle surface glycolipid of *M. catarrhalis*. Unlike lipopolysaccharide, LOS lacks repeating O-antigen polysaccharide side chains [69]. LOS consists of an oligosaccharide core and lipid A, linked together by 3-deoxy-d-manno-octulosonic acid (KDO) residues. There are three serotypes of *M. catarrhalis* based upon the structure of their LOS: A (72% of isolates), B (21%), and C (2%) [70]. The structure of LOS within a single isolate of *M. catarrhalis* varies significantly, mostly in the lipid A region [71].

Biosynthesis of the oligosaccharide core, lipid A, and KDO regions of LOS requires numerous enzymes, many of which have been characterized in *M. catarrhalis* [72-80]. Mutagenesis of these enzymes demonstrated that the LOS structure is required for and contributes to adherence and invasion of epithelial cells [81-82] as well as serum resistance [83-84]. However, LOS may not mediate adherence or serum resistance directly since the alteration of LOS compromises membrane integrity and likely affects the surface display of other molecules in the membrane [79].

Type IV Pili: *M. catarrhalis* expresses a Type IV Pilus (TFP) which is required for natural genetic transformation and contributes to adherence to A549 epithelial cells and biofilm formation [85-87]. The TFP exists as a filamentous surface appendage, first observed in electron microscopic images [86]. Three genes are required for expression and function of the TFP: *pilA* which is the major pilin subunit, *pilQ* which is the outer membrane secretin, and p*ilT* which mediates the disassembly and retraction of the pili [86]. TFP has been shown to be expressed by all isolates tested and exhibits high sequence similarity between isolates [85]. While TFP is expressed under most conditions, high levels of expression are induced under iron limitation [86] and cold shock at 26°C [88].

Other Factors Contributing to Adherence to Epithelial Cells: The ubiquitous surface protein A2 hybrid (UspA2H) is an adhesin expressed by a minority of isolates (20%) that exhibits sequence similarity to UspA1 [89]. UspA2H also displays significant similarity to the ubiquitous surface protein A2 (UspA2), both of which will be detailed in the following section describing complement resistance. The *M. catarrhalis* metallopeptidase-like adhesin (McmA) directly mediates adherence to several epithelial cell lines (NCIH292, A549, Chang, Hep-2) and biofilm formation, though little else is currently know about the protein [90]. A recent study identified 15 genes involved in adherence to A549 and Detroit 562 cell lines, but the mechanisms have not yet been investigated [91].

Binding to Proteins of the Extracellular Matrix: In addition to binding to epithelial cells, *M. catarrhalis* also expresses factors which mediate binding to extracellular matrix (ECM) proteins such as fibronectin, laminin, and vitronectin [51-52, 59]. This binding may enhance the bacterial colonization and/or biofilm formation in the host. In the case of vitronectin, UspA2-mediated binding also contributes to the bacteria's resistance of complement, further described below [59].

Complement Resistance

Pathogens have developed several unique mechanisms to avoid recognition and clearance by the host immune system. For *M. catarrhalis*, one such mechanism of immune evasion is to resist the activity of the complement cascade. The complement system is composed of proteases that cleave additional complement proteases resulting activation and amplification of innate immune mechanisms. The ultimate goal of the cascade is bacterial clearance, and this can be attained through complement's direct role in bacterial lysis, opsonization, and chemotaxis.

The complement cascade can be activated through three pathways: classical, alternative, and lectin. The classical pathway begins when an antibody binds an epitope on the bacterial surface. Complement proteins (i.e. C1 complex) recognize the bound antibody and trigger the remainder of the cascade. Complement is activated via the alternative pathway when a protein (i.e. C3b) directly binds the microbial surface. The lectin pathway begins when a mannose-binding lectin recognizes and binds a sugar residue on the bacterial surface. Each of the three pathways results in the formation of the Membrane Attack Complex (MAC), which is the assembly of proteins responsible for bacterial lysis. In addition to direct killing, the cleaved complement proteins also recruit lymphocytes (chemotaxis) and enhance phagocytosis (opsonization).

Resistance to both the classical and alternative pathways has been demonstrated through exposure *of M. catarrhalis* to normal human serum. In addition to being found in blood, complement is also part of mucosal secretions and is therefore crucial in the recognition and clearance of *M. catarrhalis*. The UspA proteins (1, 2, and 2H) directly inhibit the activity of complement, while several other factors (CopB, OMP CD, OMP E, LOS) have been implicated as well.

UspA2/UspA2H: The major factors mediating serum resistance in *M. catarrhalis* are UspA2/UspA2H. Both proteins are closely related genetically and structurally to the previously described autotransporter UspA1; however, unlike UspA1, *M. catarrhalis* isolates appear to encode for either UspA2 (80% of isolates) or UspA2H (20%) but not both [89]. While UspA2 has only 43% identity to UspA1, each contain an internal regions consisting of 135 amino acids with 93% identity [44]. UspA2H, meanwhile, is a hybrid protein, possessing an N-terminus with high identity to UspA1 and a C-terminus with high identity to UspA2 [89]. UspA2 plays a

significant role in serum resistance while UspA2H impacts both serum resistance and adherence to epithelial cells [89, 92]. The N-terminal head region of UspA2 and UspA2H contains a domain responsible for binding the ECM glycoprotein vitronectin. Binding of vitronectin prevents the formation of the MAC, the final step in the complement cascade leading to bacterial lysis [59]. In addition, UspA2/UspA2H binds and inactivates C3 and the C4 binding protein (C4bp) thereby interrupting the alternative and classical complement pathways, respectfully [53].

UspA2 is present on outer membrane vesicles secreted from *M. catarrhalis*, possibly conferring complement resistance to neighboring bacteria [93]. UspA2 is regulated at the transcriptional level due to the presence of heteropolymeric repeats (AGAT) located upstream of the ORF [59]. It has also been shown to be up-regulated upon cold shock at 26° in the same manner as UspA1 [94].

Other Factors Contributing to Complement Resistance: While UspA2/UspA2H is the major complement resistance factor expressed by *M. catarrhalis*, several other factors have been implicated. Like UspA2, UspA1 confers resistance directly by binding C3 and C4bp, albeit at lower affinities [53, 95]. The mechanism by which the remaining factors (CopB, OMP CD, OMP E, LOS) mediate resistance is unknown, though *M. catarrhalis* strains lacking each individual factor are more susceptible to serum than their *wild-type* counterparts [65, 80, 96-97]. Regarding LOS, its absence would likely weaken and increase the permeability of the outer membrane, leading to bacterial lysis [70]. Recently, a study revealed that the disulfide bond formation system also plays a role in complement resistant, apparently affecting evasion of the classical pathway through the alteration of LOS [91].

Biofilm Formation

Biofilm formation is another mechanism that aids in *M. catarrhalis* colonization and persistence in the host. Biofilm formation by *M. catarrhalis* has been observed both *in vitro* and *in vivo* in children with otitis media [54, 98]. According to the International Union of Pure and Applied Chemistry (IUPAC), a biofilm is defined as an "aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance adhere to each other and/or a surface" [99]. The process of biofilm formation requires initial attachment to a surface, followed by biofilm maturation, and ultimately dispersion to surrounding locations. Microbes found within a biofilm are often more resistant to medical treatments, including antibiotics, leading to increased persistence. The physical arrangement of the biofilm provides the inner-most bacteria with a protective barrier that prevents penetration of drugs and immune factors. This inner core of bacteria also replicate at a lower rate, leading to antibiotic tolerance [100-101]. Finally, competition between bacteria is limited within a biofilm, leading to a diverse population with higher fitness than the original infecting strain [102].

A study in 2006 found that *M. catarrhalis*, along with *S. pneumoniae* and *H. influenzae*, produce biofilms in the middle ear of children with otitis media, indicating a role for biofilm formation in pathogenesis [98]. Polymicrobial infections have the potential to produce biofilms with increased virulence potential. For *M. catarrhalis* in particular, recent studies have shown that co-infection with *NTHIi* or *S. pneumoniae* yields increased levels of biofilm formation, antibiotic resistance, and bacterial persistence [103-104].

A number of factors mediating biofilm formation in *M. catarrhalis* have been identified using either static culture and/or continuous flow chambers as *in vitro* models. Positive

effectors of biofilm formation include UspA1 [54, 105], UspA2 [106], UspA2H [105], McmA [90], and the TFP [86]. In contrast, Hag/MID and host factor 1 (Hfq), a global regulator protein, are negative effectors of biofilm formation [54]. Interestingly, each of these factors has also been linked to other virulence-associated traits, specifically adherence to epithelial cells and serum resistance.

Nutrient Acquisition

In order to persist in the host for extended periods of time, all pathogens must acquire specific nutrients from the local environment, and *M. catarrhalis* is no exception. While *M. catarrhalis* cannot metabolize exogenous carbohydrates, it can utilize acetate, lactate, and fatty acids as carbon and/or energy sources. Other molecules, such as the amino acids argenine and proline, are required for *M. catarrhalis* growth. The outer membrane protein M35 is a porin that is required for growth in nutrient-limiting conditions; although it has been implicated in the uptake glutamic acid, its complete role has yet to be determined, [107]. Recently, a zinc uptake ABC transporter (ZnuA) was shown to be required for zinc acquisition in conditions of low zinc concentrations [108].

Iron is quite possibly the single most important nutrient/cofactor for pathogenic bacteria to acquire. *M. catarrhalis* has developed special mechanism to scavenge for iron within the host. In the host, free iron is rare; instead, iron is usually found in a bound to a carrier such as lactoferrin, transferrin, heme, and hemoglobin. Researchers have identified two *M. catarrhalis* lactoferrin-binding proteins, LbpA and LbpB [109] and two transferrin-binding proteins, TbpA and TbpB [110]. LbpA and TbpA are TonB-dependent integral membrane proteins which form a channel through which transferrin and lactoferrin, respectively, are transported [111].

Meanwhile, LbpB and TbpB appear to facilitate the process [112-113]. Spaniol et al. found that expression of LbpA, LbpB, TbpA, and TbpB are all increased during cold shock [94]. CopB contributes to the uptake of both lactoferrin and transferrin, though direct binding has not been demonstrated [114]. The OMP HumA directly binds heme and allows the bacterium to utilize heme as its sole source of iron [115]. Likewise, MhuA directly binds hemoglobin, which *M. catarrhalis* can also use as its only iron source [116].

Invasion of Epithelial Cells

Adherence to epithelial cells and ECM proteins is a critical step in the colonization of the host. To aid in persistence at the site of colonization, several researchers have suggested that *M. catarrhalis* invades epithelial cells to evade immune clearance. The first paper examining this phenomenon revealed through electron microscopy that epithelial cells form lamellapodia and filopodia enveloping the bacterium [117]. The bacteria actively promote uptake by the non-professional phagocytes via macropinocytosis [117]. The exact mechanism of uptake is yet to be elucidated; however clathrin, microfilaments, Rho-type GTPases, and phosphoinositide 3-kinase (PI3K)-dependent contractile mechanisms may play a role [81, 117]. The biological relevance of epithelial cell invasion was corroborated by the detection of *M. catarrhalis* within the tonsillar epithelium of infected patients [118].

UspA1 appears to play a direct role in part since competitive inhibition for its binding site on CEACAM-1 reduces invasion [48, 81]. LOS has also been implicated, although its role may be indirect since alteration of LOS is known to affect the display of outer membrane proteins [81]. Likewise, ZnuA is required for invasion *in vitro* and persistence in the mouse model, though the mechanism is yet to be determined [108].

Animal Models for *Moraxella catarrhalis*

Since *M. catarrhalis* is a human-specific pathogen, there is a shortage of appropriate animal models to study infection. The mouse pulmonary clearance model and chinchilla model of otitis media have been the most commonly used animal models to study *M. catarrhalis* pathogenesis, though models of acute otitis media have been described for the mouse [119-121], rat [122], and guinea pig [123]. The mouse pulmonary clearance model supports the study of lower respiratory tract infections. However the mice are able to clear the bacteria within 24 hours of inoculation and do not develop disease [124]. To alleviate these issues, the chinchilla model of otitis media has been utilized. This model is more appropriate to study processes such as colonization since bacteria are recovered from the nasopharynx up to two weeks post-inoculation [85]. The model is not perfect, however, since the chinchillas do not develop otitis media following *M. catarrhalis* colonization [125].

Mouse Model of Pulmonary Clearance

The development of a murine model to study *M. catarrhalis* infection was first described in 1981 when researchers inoculated mice via aerosol with various bacterial species collected from normal human flora. Infection by *M. catarrhalis* resulted in a large influx of granulocytes to the lungs [124, 126-127]. Further characterization of the model revealed the rapid clearance of *M. catarrhalis* from the lung within 48 hours following endotracheal inoculation [124]. Unhanand et al. noted that the rate of clearance varied depending on the isolate of *M. catarrhalis* [127]. While most studies utilize aerosol challenge [128], several employ an endobronchial route of inoculation [129-130].

Since its characterization, the mouse pulmonary clearance model has been used to test numerous potential vaccine candidates. Immunization of mice with outer membrane vesicles isolated from *M. catarrhalis* yielded systemic antibodies that recognize outer membrane proteins and LOS. Furthermore, immunization resulted in enhanced pulmonary clearance following challenge. Passive immunization using sera from these immunized mice also enhanced clearance following homologous and heterologous challenge [129]. This study confirmed the potential of *M. catarrhalis* surface antigens as vaccine candidates.

Over the next two decades, many *M. catarrhalis* factors have been investigated as potential vaccine candidates using the mouse model of pulmonary clearance. First, passive immunization of mice with monoclonal antibodies against CopB [130], UspA [131], and LOS [132] resulted in enhanced clearance of *M. catarrhalis* in murine lungs. Active immunization of mice with purified protein including UspA [133], OMP CD [134-136], Hag/MID [137], msp22 [138-139], msp75 [138], Znu [108], M35 [140], and OppA [141] have also increased the rate of clearance in the *in vivo* system. Conjugate vaccines where LOS is attached to either an endogenous *M. catarrhalis* protein (UspA, OMP CD [142]) or an exogenous protein (tetanus toxoid, high-molecular-weight proteins from NTHi [143], diphtheria toxin [144]) also promote clearance in the *in vivo* model. These studies utilized various routes of immunization including mucosal/intra-Peyer's patch [134, 145], intranasal [139, 141, 144, 146], intramuscular [134], intratracheal [145], and subcutaneous [144], and all have been effective to some extent. Many of these vaccination approaches resulted in the production of bactericidal antibodies (anti-LOS [147], anti-UspA [133]) isolated from the mucosa and/or serum.

The mouse model has also been used to examine the clearance rate of mutant strains compared to their WT counterparts. This approach has implicated Hag/MID [137], LOS [148],

and OMP J2 [149] in bacterial persistence in the murine model. In a related experiment, Kyd et al. tested a lab-evolved variant in the mouse model and found that neutrophil recruitment to the lung was reduced and bacterial clearance was delayed. The variant was produced through serial passages, selecting only cells that failed to aggregrate in broth. Attenuation of the bacterial variant was attributed to altered OMP (i.e. UspA1, OMP CD, and an unknown protein) and LOS expression [150].

Chinchilla Model of Otitis Media and Nasopharyngeal Colonization

The chinchilla model of otitis media was originally developed in 1976 to study otitis media caused by *Streptococcus pneumoniae* [151]. The chinchilla was selected as an appropriate model in part because the animal's middle ear structure is similar to that of humans. The chinchilla model of otitis media has since been adapted to study other causative agents of otitis media such as *H. influenzae* [152] and *M. catarrhalis* [153]. A preliminary study found that, given intranasally, *M. catarrhalis* successfully colonizes the chinchilla nasopharynx. Despite an absence of otitis media following nasopharyngeal colonization, researchers concluded that the chinchilla model is excellent for studying the early stages of *M. catarrhalis* pathogenesis [125]. Follow-up studies revealed that passive inhalation of *M. catarrhalis* lead to nasopharyngeal colonization for at least two weeks. However, at no point during the experiment was *M. catarrhalis* detected in the fluid of the middle ear. Histology revealed local inflammation indicating that *M. catarrhalis* is exposed to various aspects of the host immune system [85]. Recently, it has been shown that following inoculation with *M. catarrhalis*, chinchillas produce antibodies that recognize major bacterial antigens and, notably, many of the same antigens that are recognized by antibodies produced in humans who have recovered from *M. catarrhalis* infection [154].

Researchers have reported conflicting results when attempting to induce otitis media in chinchillas via direct inoculation of the middle ear. One study reports that chinchillas that receive transbullar inoculation of *M. catarrhalis* develop otitis media accompanied by middle ear effusion and severe inflammatory changes in the tubotympanum. Bacteria were recovered from these chinchillas up to five days post-inoculation [153]. Meanwhile, a second group found that inoculation of the middle ear cavity produced a self-limited inflammation, although no viable bacteria were recovered from the middle ear 24 hours post inoculation suggesting that no infection occurred [155].

Otitis media is gaining appreciation as a polymicrobial infection, and the chinchilla is an excellent model to study infection by multiple pathogens. Armbruster et al. demonstrated that *M. catarrhalis* persistence in the chinchilla is greatly increased when co-infected with *H. influenzae*, a result that was dependent on LuxR-related quorum sensing between the organisms [103]. Brockson et al. took co-infection one step further and introduced another human pathogen, respiratory syncytial virus (RSV), which is commonly isolated in patients with bacterial otitis media [156]. A challenge regimen consisting of intranasal introduction of *H. influenzae* followed by *M. catarrhalis* followed by RSV resulted in symptoms of otitis media that are absent from infection solely due to *H. influenzae* and RSV. The observations, bullous myringitis and the presence of blood at the bullar orifice of the Eustachian tube, were thereby attributed to *M. catarrhalis*-induced otitis media [157]. These studies suggest that co-infection aids in the initial colonization of the chinchilla and assists the migration of *M. catarrhalis* from the nasopharynx to the middle ear.

The development of the chinchilla model has provided researchers the opportunity to characterize individual virulence factors in an *in vivo* system. The TFP was found to promote

persistence in the chinchilla by mediating association with the airway mucosa [85]. The MhaB proteins, which have been implicated in adherence to epithelial cells, have been shown to be crucial for nasopharyngeal colonization of the chinchilla [154]. Furthermore, Hoopman et al. examined *M. catarrhalis* gene expression during colonization of the chinchilla nasopharynx using a microarray and identified more than 100 *M. catarrhalis* genes that are up-regulated and over 200 gene that are down-regulated compared to growth *in vitro* [158]. The model has also been utilized to examine the vaccinogenic potential of particular antigens. Shaffer et al. demonstrate that immunization with MhaB1 and MhaB2 elicited antibodies that obstructed colonization of the nasopharynx [154].

Phospholipids in Bacteria

Phospholipids (PL) are amphipathic molecules that contain a hydrophobic tail and a hydrophilic, phosphorylated head. Due to their polarity, PL arrange into bilayers, such as those composing biological membranes. PL often diffuse laterally within the membrane and directly interact with other macromolecules such as proteins, lipids, and carbohydrates. Bacterial membranes are chiefly composed of three major phospholipids: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Each has its own unique set of characteristics which contributes to its cellular function. PG and CL are anionic PLs whereas PE is a zwitterionic PL with a net neutral charge.

The synthesis of bacterial PLs has been best-studied in *E. coli*. Figure 2.1 summarizes the biosynthetic pathway of the major prokaryotic PLs. Cytidine diphosphate (CDP-DAG) is the precursor to each of the major PLs. CDP-DAG is converted to PG by phosphatidylglycerophosphate (PGP) synthase (PgsA) and PGP phosphatase (PgpA, PgpB,

PgpC), with PGP serving as the intermediate. CDP-DAG is converted to PE by phosphatidylserine (PS) synthase (PssA) and PS decarboxylase (Psd), with PS serving as the intermediate. CL is either synthesized from two molecules of PG (ClsA) or one molecule of PG and one molecule of PE (ClsC) [159].

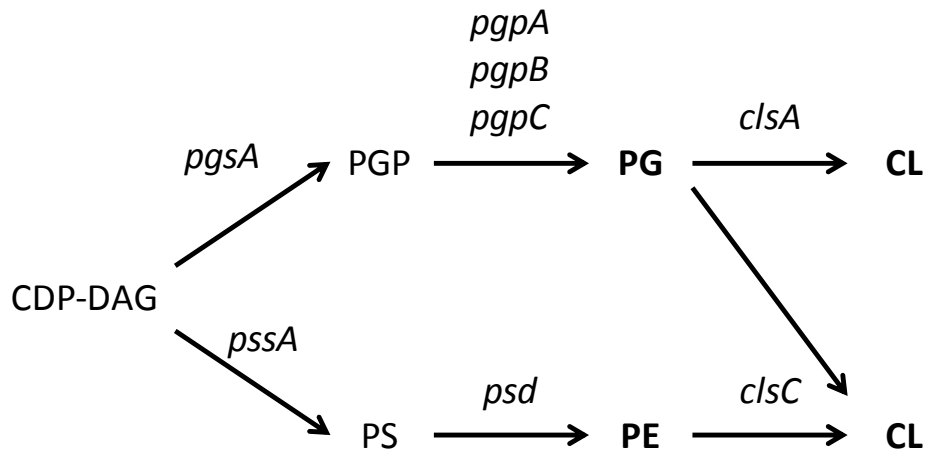

Figure 2.1: Biosynthetic pathway of major bacterial PLs. The genes responsible for the synthesis of PE, PG, and CL are indicated. The major PLs of bacteria are bolded.

Phospholipids of Moraxella catarrhalis

Phospholipids have been well-studied in several bacteria such as *E. coli*; however, only a single report exists that examines the PLs of *M. catarrhalis*. Through the use of isotopic labeling (C^{14} of glucose, P^{32} of phosphoric acid), Beebe and Wlodkowski, found that PE and PG were the

major phospholipid constituents of *M. catarrhalis* membranes, each representing 25-50% of the total lipid composition. Meanwhile, CL was the third-most prevalent PL at 5-30%. A small amount (3%) of phosphatidylcholine (PC) was also detected, which is interesting as PC is only found in approximately 10% of bacterial species [160]. Compared to exponential growth, *M. catarrhalis* during stationary phase had increased levels of PG and reduced levels of PE, while the amount of CL was not significantly affected [161]. This finding is unique since the level of CL (and PE) of most bacteria increase during this transition [162].

Role Phospholipids in Virulence

While CL has not been previously implicated in virulence, several other PLs have been. For example, PE has been shown to contribute to virulence-associated traits in *Brucella abortis* and *E. coli*. In *B. abortis*, PE is required for intracellular survival in macrophages and HEp-2 cells, the maturation of the replicative *Brucella*-containing vacuole, and colonization in a murine model. PE-deficient *B. abortis* membranes display altered membrane properties and increased sensitivity to various detergents. It appears that the lipid composition influences the structure or function of certain surface protein complexes such as the type IV secretion system (VirB), the major virulence factor of *B. abortis* [163]. In *E. coli*, the enzymes responsible for the synthesis of PE, PssA and Psd, are mandatory for motility and chemotaxis. Strains of *E. coli* with mutated *pssA* and *psd* genes completely lack flagella, an observation which is in part explained by data demonstrating the down-regulation of flagellar gene transcription in these strains. Researchers hypothesize that alteration of the membrane from the absence of PE may affect the assembly of the flagella, which subsequently down-regulates the expression of flagellar genes [164].

Both the human pathogen *Legionella pneumophila* and the plant pathogen *Agrobacterium tumefaciens* require PC for virulence. In *L. pneumophila*, PC promotes intracellular survival in macrophages and high multiplicity cytotoxicity. The absence of PC in *L. pneumophila* results in low levels of flagellar proteins, which have been associated with reduced cytotoxicity and cellular adherence. Additionally, the observed phenotypes in PC-deficient *L. pneumophila* may be due to diminished function of the Dot/Icm IVb secretion system that injects bacterial effectors into the host cells [165]. In *A. tumefaciens*, PC is required for tumor formation and infection of *Kalanchoë* plants. This observation is attributed to the absence of type IV secretion machinery in PC-deficient bacteria. However, PC did not affect the global expression of membrane-associated proteins [166].

Staphylococcus aureus requires expression of a PL-altering enzyme for resistance to innate immune response. The *mprF* gene encodes for an amino acid-PG synthase, an enzyme that catalyzes the transfer of lysine to a molecule of PG via an esterification reaction. The resulting PL, lysyl-PG, contributes to the resistance of *S. aureus* to several host defensins and protegrins. Bacteria devoid of lysyl-PG are readily killed by human neutrophils and are attenuated in mice [167]. The attachment of lysine neutralizes the anionic nature of PG, providing the PL with novel properties. This may explain why *Sinorhizobium meliloti* lacking lysyl-PG display increased susceptibility to polymyxin B, a cationic antibiotic [168].

Cardiolipin

CL, also called diphosphatidylglycerol, was originally discovered in animal hearts, hence its nomenclature [169]. It is a rather unique PL given its dimeric structure of four acyl chains and a dianionic head group. CL is found almost exclusively in the plasma membrane of bacteria

and mitochondrial membranes of eukaryotes. Due to its relatively small head and large tail, CL is considered a high curvature lipid, localizing to regions of the membrane with high negative curvature such as cell poles and septa [170].

In general, prokaryotes and eukaryotes utilize different substrates and enzymes to synthesize CL. Eukaryotes synthesize CL from a molecule of PG and a molecule of CDP-DAG via a CL synthase (CLS) with phosphatidyl transferase activity. The reaction, which releases cytidine monophosphate (CMP) as a by-product, requires divalent cations such as Mg^{+2}, Mn^{+2}, or Co^{+2} and high pH. In eukaryotes, CL is synthesized in the mitochondria and is thereby the only PL synthesized outside of the endoplasmic reticulum. In the mitochondria, 25% of the inner membrane is composed of CL while only 4% of the outer membrane consists of CL. Although mainly found in eukaryotes, this type of CLS is also found in certain bacteria including *Streptomyces coelicolor* and most actinomycetes [171]. Likewise, a prokaryotic-type CLS has been in the parasite *Typanosoma brucei* [172].

Only recently was it discovered that some bacteria may possess two related, yet different types of CLS. In *E. coli*, there are three paralogous genes predicted to encode CLS. The first gene, now termed *clsA*, was the first to be discovered and is now the best-studied [173-174]. It converts two molecules of PG to one molecule of CL, with a molecule of glycerol as a by-product [174]. The mechanism by which the second *E. coli* gene, *clsB*, synthesizes CL has not been thoroughly investigated. However, Tan et al. revealed that the third gene product, ClsC, synthesizes CL from one molecule of PG and one molecule of PE [159]. Since the two types of CLS share sequence similarity, it is likely that proteins that were originally identified by bioinformatics as ClsA orthologs may in fact utilize a ClsC-type mechanism for CL synthesis [159]. As previously stated, CL is found predominantly in the plasma membrane of bacteria.

However, small amounts of CL have been detected in the outer membrane of *E. coli* [175] and *Salmonella enterica* serovar *typhimurium* [176].

The three CLS of *E. coli* are each members of the Phospholipase D (PLD) Superfamily, along with CLS from most other bacteria [177]. In addition to CLS, this superfamily contains PLDs, PS synthases, a poxviridae envelope protein, the *Yersinia* murine toxin (Ymt), and the Nuc endonuclease [178]. Members of this superfamily are distinguished by the presence of a $HXKX_4D$ (HKD) motif [179]. Most PLD Superfamily proteins, including CLS, contain two HKD motifs, both of which must be intact to maintain activity [180]. In addition to being required for proper folding of the protein [181], the histidine residue is directly involved in catalyzing the transphosphatidylation reaction [178, 182-183]. Most CLSs, including ClsA of *E. coli*, are predicted to contain transmembrane helices that localize the enzyme to the inner membrane [159, 184], and it appears that the catalytic site is located on the periplasmic side of inner membrane [185].

The function of CL has been studied in both eukaryotes and prokaryotes and is directly related to the intrinsic properties of the PL. Yeast has been the model organism to study CL in eukaryotic system. Researchers have found that CL binds tightly to and is required by the cytochrome complexes of the respiratory chain [186] and serves as a proton trap for oxidative phosphorylation [187]. Additionally, CL appears to play a major role in the regulation of apoptosis [188].

In prokaryotes, researchers have shown that CL spontaneously assembles into micro-domains. Since CL is known to bind and regulate a large number of membrane-associated proteins, the importance of compartmentalization is apparent for processes such as cell division,

energy metabolism, and membrane transport, all of which CL has been shown to impact. For example, the activity of DnaA and MinD, proteins directly involved in the cell cycle and cell division, is controlled by CL micro-domain formation [189]. CL is also required for the conformation, activity, and localization of the SecYEG protein translocation complex that secretes proteins through the plasma membrane [190].

Furthermore, CL form micro-domains in regions of high membrane curvature such as the polar and septal region [170]. Microscopy revealed the presence of CL in these regions in numerous bacterial species including *E. coli* [191], *Bacillus subtilis* [192] *Pseudomonas putida* [193] and *Streptococcus pyogenes* [194]. Many proteins are found at the poles and septa [195], and some proteins localize to these regions because of recruitment by CL. For example, the proteins that synthesize PLs (such as CLS) are localized to septal regions along with CL in *B. subtilis* [196], and the *Vibrio chlolera* type II secretion apparatus (Eps) co-localizes with CL at the bacterial poles [197].

CL has long been known to play a role in osmoregulation. When exposed to conditions of increasing osmolality, the level of CL rises in several bacteria including *E. coli* [198] *B. subtilis* [199], *S. aureus* [200], and *Rhodobacter sphaeroides* [201]. This observation was determined to be a result of induction of CLS transcription in *E. coli* [191] and *B. subtilis* [202]. Furthermore, *E. coli* and *B. subtilis* strains that possess low levels of CL displayed a growth defect when placed under osmotic stress [191, 202]. The role of CL in the osmotic stress response is in part due to its functional relationship with the osmoregulator ProP. ProP is a osmolyte-H^+ symporter that results in the accumulation of proline, glycine betaine and ectoine in the cytoplasm [203]. Romantsov et al. found that ProP co-localizes with CL micro-domains at

the polar and septal regions of *E. coli*. Furthermore, ProP activity is dependent on the presence of CL [191].

Conveniently, the molecule 10-*N*-nonyl acridine orange (NAO) naturally associates with CL and can be used to detect and visualize CL in both eukaryotes and prokaryotes. Originally used for detection of CL in mitochondria [204-205], the approach has been adapted for use in bacteria [206]. NAO has a high affinity for anionic PLs (i.e. CL and PG). However, following excitation, NAO emits red fluorescence (640 nm) when associated with CL while green fluorescence (525 nm) results from NAO association with PG. This difference is attributed to dimer formation and pi-pi bond stacking that occurs when NAO is associated with CL [207]. The viability of bacteria is unaffected by NAO at concentrations of 100-200 nM, thereby avoiding artifacts that may result from cell death [191, 206]. While the study of CL has progressed significantly due to the detection and visualization of CL by NAO, the method is not effective in all organisms [208].

CHAPTER 3

MORAXELLA CATARRHALIS EXPRESSES A CARDIOLIPIN SYNTHASE THAT IMPACTS ADHERENCE TO HUMAN EPITHELIAL CELLS[1]

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[1] Buskirk S.W. and E.R. Lafontaine. 2014. *J. Bacteriol*. 196(1):107-120.

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Abstract

The major phospholipid constituents of *Moraxella catarrhalis* membranes are phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin (CL). However, very little is known regarding the synthesis and function of these phospholipids in *M. catarrhalis*. In this study, we discovered that *M. catarrhalis* expresses a cardiolipin synthase (CLS), termed MclS, that is responsible for the synthesis of CL within the bacterium. The nucleotide sequence of *mclS* is highly conserved throughout *M. catarrhalis* isolates and is predicted to encode a protein with significant amino acid similarity to the recently characterized YmdC/ClsC protein of *Escherichia coli*. Isogenic *mclS* mutant strains were generated in *M. catarrhalis* isolates O35E, O12E, and McGHS1 and contained no observable levels of CL. Site-directed mutagenesis of a highly conserved HKD motif of MclS also resulted in a CL-deficient strain. *Moraxella catarrhalis*, which depends on adherence to epithelial cells for colonization of the human host, displays significantly reduced levels of adherence to HEp-2 and A549 cell lines in the *mclS* mutant strains compared to WT bacteria. The reduction in adherence appears to be attributed to the absence of CL. These findings mark the first instance in which a CLS has been related to a virulence-associated trait.

Introduction

Moraxella catarrhalis is a human-specific pathogen of the mucosa and causative agent of otitis media in children and respiratory infections in adults. After only non-typeable *Haemophilus influenzae* (*NTHi*) (~26%) and *Streptococcus pneumoniae* (~23%), *M. catarrhalis* is a leading bacterial cause of otitis media in children, responsible for up to 20% of cases [22-25]. Nearly 70% of infants are colonized by *M. catarrhalis* within their first 12 months [18]. *Moraxella catarrhalis* is also the second leading cause of bacterial exacerbations of chronic obstructive pulmonary disease (COPD), responsible for approximately 10% of cases [17]. In the United States, COPD currently stands as the fourth leading cause of death. Exacerbations of COPD due to *M. catarrhalis* are responsible for an estimated $2 billion each year in medical costs and healthcare [209].

Currently, there is no licensed vaccine available to prevent *M. catarrhalis* infection. A vaccine is desirable, however, due to the high prevalence, antibiotic resistance, and financial burden associated with *M. catarrhalis* infection. The vast majority of clinical isolates of *M. catarrhalis* (>95%) are now resistant to the beta-lactamase family of antibiotics that was once considered a front-line treatment for the disease [210]. This antibiotic resistance was acquired rapidly over a 10-15 year period in which the resistance spread from few isolates to the majority [211-213]. In addition, the incidence and prevalence of disease due to *M. catarrhalis* infection is expected to increase in the United States following the introduction of vaccines against the upper respiratory tract pathogens *S. pneumoniae* and *NTHi* [214]. Finally, a vaccine to protect against the top three causes of otitis media would save upwards of $1.3 billion annually and substantially improve the overall health status of infants [38].

Moraxella catarrhalis is a gram-negative, unencapsulated, aerobic diplococcus. Several virulence factors of *M. catarrhalis* have been identified and characterized, many of which are transported through the plasma membrane and are either localized to the outer membrane (i.e. outer membrane proteins, OMPs) or secreted outside of the cell. These molecules then mediate processes such as adherence to epithelial cells, complement resistance, biofilm formation, and nutrient acquisition in order to colonize and cause disease in the human host. Many of these traits are multifactorial. For example, *M. catarrhalis* expresses several adhesins including UspA1[89], Hag/MID [57], McaP [61], OMP CD [65], and the FHA-like proteins MhaB1 and MhaB2 [62] that mediate adherence to human epithelial cells.

The phospholipase D (PLD) superfamily is composed of a group of proteins with various functions, yet all members contain a signature $HXKX_4DX_6G(G/S)$ motif (HKD) [180, 215]. This superfamily includes prokaryotic and eukaryotic PLD, bacterial cardiolipin synthases (CLS) and phosphatidylserine synthases (PSS), Poxviridae envelope proteins, and bacterial endonucleases. The PLD, CLS, and PSS all contain two HKD motifs that associate to form the active site [181-182] and catalyze reactions that synthesize or modify phospholipids (PL). The histidine and lysine residues within the HKD motif are required for the enzymatic activity of the proteins [159, 180]. Members of the PLD superfamily have been studied in many bacterial species and several have been shown to exhibit virulence-associated characteristics. These include PLD superfamily members of *Neisseria gonorrhoeae* [216], *Acinetobacter baumannii* [217], *Chlamydophila pneumoniae* [218], *Rickettsia prowazekii* [219], *Yersinia pestis* [178], *Arcanobacterium haemolyticum* [220] and *Corynebacterium pseudotuberculosis* [221]. Furthermore, *N. gonorrhoeae* and *A. haemolyticum* express a PLD that have specifically been

shown to impact adherence to human epithelial cells [216, 220]. To our knowledge, a role for CLS in microbial pathogenesis has not been reported.

Three types of CLS have been characterized and identified; all three catalyze the formation of the PL cardiolipin (CL), though the substrates differ. Eukaryotic-type CLS synthesize CL from one molecule of cytidine diphosphate (CDP)-diacylglycerol and one molecule of phosphatidylglycerol (PG). Though expressed predominantly in eukaryotes, eukaryotic-type CLS are also expressed by *Streptomyces coelicolor* and most actinobacteria [171]. The eukaryotic-type CLS is not a member of the PLD superfamily, unlike the other two types of CLS. The first type, classically referred to as the prokaryotic-type CLS, has been characterized in several bacteria including *Escherichia coli* [222], *Bacillus firmus* [223], and *Pseudomonas putida* [224]. This type of CLS catalyzes the condensation of two molecules of PG to form CL [174]. Recently, a third type of CLS, termed YmdC/ClsC, has been identified and characterized in *E. coli* [159]. Like the prokaryotic-type CLS, YmdC/ClsC contains two HKD motifs and is a member of the PLD superfamily; however, it generates CL from one molecule of PG and one molecule of phosphatidylethanolamine (PE) [159]. Prior to this study, the presence of *ymdC/clsC*-like CLS had not been confirmed in any bacterial species outside of *E. coli*, though bioinformatic tools have suggested that YmdC/ClsC-type CLS are prevalent [159]. Some bacteria, including *E. coli*, express multiple types of CLS. In these organisms, the regulation of CLS depends on growth conditions [159, 222, 225].

Cardiolipin is an anionic PL present in energy transducing membranes, primarily the inner mitochondrial membrane of eukaryotes and the plasma membrane of prokaryotes. Along with PG and PE, CL is a major PL constituent of *M. catarrhalis* membranes [161]. Composed of four acyl chains and a head group with two negative charges, CL has high intrinsic curvature and

is found predominantly in membrane regions with negative curvature such as poles or septa. In fact, studies in *E. coli* and *Bacillus subtilis* have shown that CL localizes to the polar and septal regions of bacteria forming microdomains [192, 226]. Furthermore, CL is required for the polar localization of the *E. coli* osmoregulator ProP, which may explain why CLS-deficient bacteria are more susceptible to osmotic stress [191, 202]. The presence of CL in bacterial membranes is required for efficient protein translocation across the plasma membrane in both Sec-dependent and Sec-independent mechanisms [190, 227]. In *E. coli*, the CL content of bacterial membranes varies depending on growth conditions. Cardiolipin levels are highest during stationary growth due to the up-regulation of both the expression and activity of CLS [225, 228].

Here we demonstrate that *M. catarrhalis* expresses a CLS that impacts adherence to human epithelial cells. Nucleotide sequence analysis revealed that *M. catarrhalis* contains a gene predicted to encode a member of the PLD superfamily displaying significant similarity to the YmdC/ClsC protein of *E. coli*. This gene, termed *mclS*, is highly conserved throughout *M. catarrhalis* isolates and is located immediately upstream of a gene predicted to encode a protein with similarity to ProP of *E. coli*. An *M. catarrhalis* isogenic mutant strain lacking expression of *mclS* was found to contain no observable levels of CL, thereby confirming that *mclS* encodes a CLS. Likewise, a strain of *M. catarrhalis* with a mutated HKD motif lacks CL, demonstrating that the intact motif is required for CLS activity. Both mutant strains exhibit reduced levels of adherence to human epithelial cells lines, suggesting that the CLS activity, and subsequently CL, is required for *wild-type* (WT) levels of adherence. This work documents the first instance in which a CLS contributes to a virulence-associated trait.

Materials and Methods

Strains, plasmids, tissue culture cell lines, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3.1. *Moraxella catarrhalis* was grown at a temperature of 37˚C using Todd-Hewitt (TH) medium (Difco™). Where indicated, TH medium was supplemented with antibiotics at the following concentrations: spectinomycin (spec, 15 µg/mL), kanamycin (kan, 20 µg/mL), Zeocin™ (zeo, 5 µg/mL), chloramphenicol (cat, 1 µg/mL), and streptomycin (stm, 75 µg/mL).

Escherichia coli was grown at a temperature of 37˚C using Luria Bertani (LB) medium (Difco™). Where indicated, LB medium was supplemented with cat and/or spec at concentrations of 15 µg/mL and 200 µg/mL, respectively. To isolate plasmid DNA from TransforMax™ EPI300™ *E. coli* harboring a pCC1™-based plasmid, strains were grown on LB agar (approximately 20 mL per plate) supplemented with 30 µL of CopyControl™ Induction Solution to induce the transition of plasmid maintenance from single-copy to multi-copy.

The human cell lines A549 (ATCC CCL-185, type II pneumocytes) and HEp-2 (ATCC CCL-23, laryngeal epithelium) were cultured in Ham's F-12 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (gibco® Life Technologies™), 0.15% (wt/vol) sodium bicarbonate (Cellgro®), and GlutaMAX-I at the manufacturer's recommendation (gibco® Life Technologies™) at a temperature of 37˚C with atmosphere of 92.5% air-7.5% CO_2. In addition, the medium for HEp-2 also contained 1 mM sodium pyruvate (Cellgro®) and MEM nonessential amino acids at the manufacturer's recommendation (Cellgro®).

Recombinant DNA techniques

Standard molecular biology techniques were performed as previously described [229]. Genomic DNA was extracted from *M. catarrhalis* using the Easy-DNA™ kit (Invitrogen™ Life Technologies™) according to the manufacturer's recommendations. Amplicons used for cloning, constructing isogenic mutants, and sequencing were generated with Platinum® *Pfx* DNA polymerase (Invitrogen™ Life Technologies™) using genomic DNA extracted from *M. catarrhalis*. All other DNA fragments were amplified using *Taq* DNA polymerase (Invitrogen™ Life Technologies™). Plasmids were isolated from bacteria using QIAprep® Spin Miniprep Kit (Qiagen®) according to the manufacturer's recommendations. When necessary, DNA was purified using DNA Precipitation Solution (Epicentre® Illumina®) according to the manufacturer's recommendations. For DNA electrophoresis, 1-Kb Plus DNA Ladder (Invitrogen™ Life Technologies™) was loaded as the DNA size marker.

Cloning of M. catarrhalis O35E mclS gene in E. coli

Based on the available sequence data from *M. catarrhalis* strain ATCC 43617, oligonucleotide primers P1 and P2 (see Table 3.2) were designed to amplify the *mclS* gene from O35E including the upstream region likely containing regulatory sequence. An amplicon of 1542 base pairs (bp) was generated by PCR using the aforementioned primers. To generate blunt ends, the *mclS* amplicon was treated with the End-It™ DNA End Repair Kit (Epicentre® Illumina®). The resulting *mclS* amplicon was ligated into pCC1™ using the CopyControl™ PCR Cloning Kit (Epicentre® Illumina®), per the manufacturer's instructions, yielding the plasmid pCC1.mclS. The construct was sequenced to verify that no mutations were introduced during PCR and to determine the orientation of the *mclS* gene in vector pCC1™.

Construction of M. catarrhalis mutants

The plasmid pSPEC^{R} was restricted with the endonuclease *PstI* and a 1.2-kilobase (kb) DNA fragment corresponding to the spectinomycin resistance (spec^{R}) cassette was purified from agarose gel slices using the High Pure™ PCR Product Purification Kit (Roche Applied Science). After treatment with the End-It™ DNA End Repair Kit (Epicentre® Illumina®), the spec^{R} cassette was then ligated into a unique *NsiI* site located near the middle of the *mclS* ORF in the plasmid pCC1.mclS. The resulting construct, pCC1.mclS.spec, was sequenced to verify insertion of the spectinomycin marker at the intended location.

The plasmid pCC1.mclS.spec was introduced into *M. catarrhalis* strains O35E, O12E, and McGHS1 by natural transformation to generate *mclS* isogenic mutants via allelic exchange. This pCC1™-based construct does not replicate in *M. catarrhalis* due to the absence of a suitable origin of replication. We identified isolates that incorporated the inactivated copy of *mclS* in their genome via homologous recombination by selecting for transformants resistant to spectinomycin. Proper allelic exchange was confirmed by PCR with the oligonucleotide primers P3 and P4 (Table 3.2), which yielded a DNA fragment of 2.3-kb in the mutant strains O35E.mclS, O12E.mclS, McGHS1.mclS. Of note, the same primers, P3 and P4, yielded a DNA fragment of 1.1-kb in the WT isolates O35E, O12E, and McGHS1. This 1.2-kb shift in the size of the PCR products is consistent with disruption of the *mclS* gene with the spec^{R} cassette in the genome of *M. catarrhalis*.

The *mclS* mutant strains were complemented by re-introducing the WT copy of the *mclS* gene in its original locus, yielding strains O35E.mclS repaired, O12E.mclS repaired, and McGHS1.mclS repaired. This was accomplished per the congression procedure described by

Balder et al [62]. Briefly, primers P1 and P2 were used to amplify the 1.5-kb WT copy of *mclS* from strain O35E. The purified *mclS* amplicon was then combined with an amplicon (generated using primers P13 and P14) containing the *rpsL* gene of *M. catarrhalis* strain O35E.SM100, which specifies resistance to streptomycin. This mixture was introduced in the mutant strains O35E.mclS, O12E.mclS, and McGHS1.mclS by natural transformation. Streptomycin resistant and spectinomycin sensitive transformants were screened by PCR to verify that the WT copy of the *mclS* gene had been re-introduced in its original location in the *M. catarrhalis* genome. This re-introduction was confirmed by sequencing the gene of the complemented strains.

Site-directed mutagenesis of the mclS gene

The QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) was utilized to introduce a point mutation in the second HKD motif of the *mclS* gene cloned in the plasmid pCC1.mclS. To accomplish this, we designed the 30-mer mutagenesis primers P15 and P16 (Table 3.2) to introduce guanine in place of adenine at nt position 1313 of the *mclS* open reading frame (ORF), which changes the codon AAA (specifies lysine 438) to AGA (codes for arginine). Mutagenesis, which was performed as recommended by the manufacturer, produced the plasmid pCC1.mclS.K438R. The construct was sequenced to verify that only the intended mutation (i.e. lysine at position 438 to arginine, $K^{438}\rightarrow R$) was introduced in the *mclS* gene. The point mutation was introduced into the O35E.mclS mutant strain by congression as described previously. Streptomycin resistant and spectinomycin sensitive transformants were screened by PCR and confirmed by sequencing the *mclS* gene.

Sequence analysis

Plasmids and PCR products were sequenced by the University of Michigan Sequencing Core. Chromatograms were assembled using Sequencher 5.0 (Gene Codes Corp). Sequence analysis was performed using Vector NTI 10.1 (Invitrogen™ Life Technologies™).

BlastP (NCBI) was used to compare the predicted amino acid sequence of MclS and the gene products of MCORF 819 and MCORF 821 with proteins from the online database compiled by GenBank. Protein domains and structural features of the gene products of *mclS*, MCORF 819 and MCORF 821 were predicted using a variety of algorithms/programs. The presence of transmembrane helices (TMH) was predicted by TM Pred (ExPASy) or TMHMM 2.0 (Center for Biological Sequence Analysis). The presence of signal peptides was predicted by SignalP 4.1 (Center for Biological Sequence Analysis) and TatP 1.0 (Center for Biological Sequence Analysis). The Phobius algorithm (Stockholm Bioinformatics Centre) specifically differentiates between transmembrane topology and signal peptides. ProSite (ExPASy) was utilized to identify a variety of protein domains, families, and functional sites.

Protein preparation and western blot analysis

Whole-cell lysates were prepared and western blots were performed as previously described [230]. Outer membrane proteins were obtained using the EDTA method outlined by Murphy et al [231]. Equivalent proteins loads were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (EMD Millipore) for western blot analysis. SeeBlue® Plus2 Pre-Stained Standard (Invitrogen™ Life Technologies™) was loaded as the protein size marker. The membranes were probed with the primary antibodies as described below, followed by goat anti-

mouse Ig(H+L)-horseradish peroxidase (HRP) secondary antibody (SouthernBiotech) at 1:10,000. Signals were detected using Luminata™ Crescendo Western HRP Substrate (EMD Millipore) and the Foto/Analyst Luminary/FX imaging system (Fotodyne Inc.).

Flow cytometry

Flow cytometry was performed as described previously [60], with slight modification. Briefly, a 50 µL aliquot of a 250 Klett suspension of *M. catarrhalis* was incubated with primary antibody for 30 minutes at 37°C with shaking. After a series of washes and resuspension in phosphate buffered saline with 0.15% gelatin (PBSG, wt/vol), the bacteria were incubated with goat anti-mouse Ig(H+L) conjugated with Alexa Fluor 488 (Invitrogen™ Life Technologies™) at 1:100 in darkness for 30 minutes at 37°C with shaking. After a series of washes and resuspension in PBSG, an equal volume of PBSG supplemented with 4% paraformaldehyde (wt/vol) was added. Samples were analyzed by the BD™ LSR II Flow Cytometer (BD Biosciences) and BD FACSDiva™ (BD Biosciences). The Alexa Fluor 488 fluorescence from the labeled bacteria was measured through a 525-nm bandpass filter in which 50,000 events were measured. The experiments were repeated on two separate occasions.

Antibodies

The western blot and flow cytometry procedures detected the *M. catarrhalis* adhesins UspA1 and OMP CD using the murine monoclonal antibodies (mAbs) 24B5 [44-45] and 5E8 [232], respectively, as the primary antibodies. The procedures also detected the following proteins using the indicated polyclonal antibodies (pAbs) as the primary antibodies: Hag [mouse serum recognizing the C-terminus of Hag [233]], McaP [mouse serum recognizing the region of McaP consisting of amino acids 51-650 [60]], and MhaB1/2 [mouse serum recognizing a portion

of the identical region of MhaB1 and MhaB2, amino acids 72-399 [62]]. For western blots, membranes were probed with mAbs at 1:100 and pAbs at 1:5,000. For flow cytometry, bacteria were incubated with mAbs at 1:200 and pAbs at 1:5,000.

RNA isolation

Plate-grown *M. catarrhalis* was used to generate a 230 Klett suspension in TH broth. RNA from *M. catarrhalis* was extracted by mechanical disruption (Mini Beadbeater-1, Biospec Products) with the RNAprotect® Bacteria Reagent (Qiagen®) and the RNeasy® Mini Kit (Qiagen®). The procedure was then continued per the manufacturer's recommendation. To ensure degradation of genomic DNA, a second DNase I treatment was performed using RiboPure™ - Bacteria (Ambion®) according to the manufacturer's protocols. The quality and quantity of extracted RNA was determined by visualization of 16S and 23S rRNA bands during gel electrophoresis and spectrophotometric analysis of the absorbance of the samples at 260 nm (A_{260}) and 280 nm (A_{280}) (SmartSpec™ Plus, Bio-Rad).

Reverse transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR)

For both RT-PCR and qRT-PCR, RNA was isolated and cDNA was synthesized using the same procedure. cDNA was synthesized from extracted RNA using the ThermoScript™ RT-PCR System (Invitrogen™ Life Technologies™) as recommended by the manufacturer. The cDNA was synthesized using the provided random hexamer primer to generate a cDNA library from *M. catarrhalis*. Equal amounts of RNA from each strain were used for cDNA synthesis according to the A_{260} spectrophotometric readings. In separate parallel reactions, the ThermoScript™ reverse transcriptase enzyme was withheld to control for DNA contamination and the isolated RNA was withheld to control for contamination of reagents.

For RT-PCR experiments, the PCR reaction was performed using *Taq* DNA Polymerase (Invitrogen™ Life Technologies™). Genomic DNA obtained from *M. catarrhalis* served as the template for the positive control for the PCR. Extension times varied depending on the expected fragment size (one minute per kb). Oligonucleotide primers P5 and P10 (Table 3.2) were designed to determine transcription linkage between *mclS* and MCORF 819 while primers P8 and P9 were designed to determine transcription linkage between *mclS* and MCORF 821. Oligonucleotide primers P1 and P4 were used to monitor transcription of *mclS*. Likewise, primers P5 and P6 were used to monitor transcription of MCORF 819, and primers P7 and P8 were to monitor transcription of MCORF 821. As a control, transcription of *mcaP*, which is constitutively expressed in *M. catarrhalis*, was monitored using primers P11 and P12. Each RT-PCR experiment was conducted on two separate occasions.

For qRT-PCR experiments, the iQ™ SYBR® Green Supermix (BioRad) was utilized for the amplification of the cDNA template as recommended by the manufacturer. Gene expression was measured and analyzed by the BioRad iQ™5 Optical System and Software. Oligonucleotide primers P17 and P18 (Table 3.2) were designed to monitor gene expression of MCORF 821. The reference control gene *mcaP*, which is constitutively expressed in *M. catarrhalis* and shown to be unaffected in the *mclS* mutants (Figure 3.S4 and 3.S5G-3.S5I), was monitored using primers P19 and P20 (Table 3.2). The qPCR reactions were performed in triplicate, and the entire qRT-PCR process was conducted on three individual occasions. All primers used for qPCR were validated to ensure minimal dimerization and non-specific binding.

Extraction of bacterial PL

Todd Hewitt broth (30 mL) was seeded with *M. catarrhalis* and grown overnight in a shaker (200 rpm) at 37˚C. Bacterial cultures were then centrifuged for ten minutes at 3,500 x g. Supernatant was discarded and the pellet was resuspended in 5 mL PBSG. Bacterial suspensions were transferred to polypropylene copolymer (PPCO) centrifuge tubes (Oak Ridge™). Phospholipids were then extracted by the method of Bligh and Dyer [234]. Briefly, the bacterial suspension was mixed with 18.75 mL of a 1:2 (v/v) chloroform:methanol (Acros Organics®; Fisher Scientific®) and vortexed. Next, 6.25 mL of chloroform was added to the mixture and vortexed. Finally, 6.25 mL of distilled water was added and vortexed. After a 5 minute centrifugation at 2,500 x g, the bottom phase was transferred to a new tube using a Pasteur pipette. The solvent was evaporated over a stream of nitrogen gas (AirGas®). The dried PL were resuspended in 200 µL of 1:2 methanol:chloroform and transferred to glass vials (Supelco® Analytical). Phospholipids preps were stored at -20˚C.

Thin layer chromatography (TLC)

Phospholipid preps (10-40 µL) were spotted on silica gel high performance TLC (HP-TLC) plates with inorganic binder (Analtech). Plates were developed in one dimension using a mobile phase consisting of 65:25:10 chloroform:methanol:acetic acid (Fisher Scientific®). Spots were visualized by spraying plates with Molybdenum Blue spray reagent (Sigma Life Science™). Phospholipids spots were identified by comparison with the following standards: PE, PG, and CL (Sigma Life Science™).

Adherence assays

Quantitative adherence assays were performed as previously described [57]. Briefly, a 24-well plate was seeded with epithelial cells (HEp-2 or A549) the day before the assay to generate a monolayer. On the day of the assay, freshly grown bacteria were resuspended in PBSG to an optical density (OD) of 230 Klett (~10^9 CFU/mL). Portions of the suspension were used to inoculate wells containing epithelial cells at a multiplicity of infection (MOI) of 100:1. Meanwhile, an aliquot of the bacterial suspension was diluted and plated to determine the inoculum. The 24-well plate was centrifuged to facilitate contact between the bacteria and epithelial cell monolayer. The plate was then incubated for 30 minutes at 37°C. The wells were washed five times with PBSG to remove non-adherent bacteria. Cells were treated with a saponin solution [5% saponin (wt/vol, Acros Organics®) and 0.85% EDTA (wt/vol, Fisher Scientific®) in PBSG] for five minutes to release the bacteria from the epithelial cells. The bacteria were then diluted and plated on TH agar to enumerate following incubation at 37°C. Percent adherence was calculated by dividing the number of adherent bacteria by the inoculum. Experiments were performed in duplicate or triplicate on at least three occasions.

Survival in PBSG, tissue culture (TC) media, and saponin solution

Moraxella catarrhalis strains were first cultured onto TH agar plates supplemented with appropriate antibiotics. Bacterial cultures were diluted and portions were distributed into tubes containing PBSG, TC media (HEp-2 cell culture media), or saponin solution. Tubes were incubated at 37°C for 15 minutes (saponin) or 30 minutes (PBSG, TC media), with the incubation time corresponding to the period in which the bacteria were exposed to the each reagent in the adherence assay procedure. Aliquots were removed and plated on TH agar to

determine survival. Percent survival was calculated by dividing the number of CFU post-incubation by the number of CFU pre-incubation. Survival experiments were performed in duplicate on at least three separate occasions.

Growth rate experiments

Moraxella catarrhalis strains were first cultured onto TH agar plates supplemented with appropriate antibiotics. These plate-grown bacteria were used to inoculate sidearm flasks containing 20 mL of TH broth to an OD of 50 Klett units. The cultures were then incubated with shaking (200 rpm) at a temperature of 37°C for up to 7 hours. The OD of each culture was determined every 60 minutes using a Klett™ Colorimeter (Scienceware®). To examine growth of *M. catarrhalis* strains under osmotic stress, TH broth was supplemented with either sodium chloride (Fisher Scientific®) or D-sorbitol (Sigma Life Science™). Growth curves were repeated on at least three separate occasions.

Statistical methods

All statistical analyses were performed using the Mann-Whitney test and GraphPad Prism® 4 software (GraphPad Software). P-values of <0.05 were considered statistically significant.

Accession numbers of newly determined sequences

The nucleotide sequences of *mclS* from *M. catarrhalis* isolates O12E and McGHS1 were deposited into the GenBank database in February 2013 under the accession number KC692996 and KC692997, respectively.

Results

Identification of an ORF in M. catarrhalis that is predicted to encode a member of the PLD superfamily of proteins

Analysis of the published genomic sequence of *M. catarrhalis* isolate ATCC 43617 (GenBank accession numbers AX067426 to AX067466 [9]) using the NCBI BlastP algorithm identified a gene, MCORF 820, predicted to encode a phospholipase D/transphosphatidylase. Further investigation revealed that the predicted protein contains two HXKXDXG(G/S) motifs, or HKD motifs, which suggests that MCORF 820 belongs to the of the PLD superfamily of molecules [180]. The amino acid residues comprising the HKD motif are required for activity [159, 180, 183]. Most members of the PLD superfamily contain two HKD motifs, which associate to form the active site [181-182]. Specifically, the histidine residues within the active site are crucial as one serves as the nucleophile and forms the phosphoenzyme intermediate while the other acts as a general acid to cleave the phosphodiester bond [181-182].

MCORF 820 also displayed significant similarity to the recently characterized YmdC/ClsC protein of *E. coli* strain K12 substrain W3110 [159] (E value of 9e-113) and CLS of various bacterial species. The greatest identity was to a predicted CLS of *Psychrobacter* species (ZP 10790049.1) with greater than 50% identity at both the nucleotide and amino acid levels (E value of 0.0). Due to the sequence similarity of MCORF 820 to bacterial CLS, the gene was named the *Moraxella catarrhalis* cardiolipin synthase (*mclS*).

The 1,629 bp ORF of *mclS* is predicted to encode a 542 amino acid (aa) protein with a molecular weight of 62 kDa. According to the ProSite algorithm (ExPASy), the N-terminus of MclS is predicted to contain a 23 aa signal peptide followed immediately by a cysteine residue,

which serves as a lipid attachment site for either a palmitoyl or diacylglycerol group (score = 5.000). This is in contrast to the results from TM Pred service (ExPASy) that indicated a possible N-terminal transmembrane domain of 18-20 aa in length (scores of 1711 and 1380). Additionally, there were no Sec or Tat signal sequences detected within *mclS* by SignalP 4.1 and TatP1.0, respectively [235-236]. Figure 3.1 depicts the structural features identified in MclS.

MclS is conserved throughout M. catarrhalis isolates.

In order to evaluate conservation of the *mclS* gene product, we sequenced the ORF from three *M. catarrhalis* strains commonly used in our laboratory: O35E, O12E, and McGHS1. Additionally, we searched the genomic sequences of ten *M. catarrhalis* isolates available through the NCBI genomic BLAST service [11] for the *mclS* gene. All strains specified a highly conserved *mclS* gene product. Eight single nucleotide polymorphisms (SNPs) were identified among the 14 sequences analyzed, and three were found to specify aa substitutions: C-A at nt 508 (arginine → serine substitution), G-A at nt 1081 (valine → isoleucine substitution), and C-T at nt 1205 (alanine → valine substitution). No missense SNPs were observed within the HKD motifs, signal sequence, or lipid attachment site. There were no obvious trends between SNPs and the geographical, anatomical, and disease sources of these *M. catarrhalis* isolates.

Genetic organization of the mclS locus

Examination of the *mclS* genetic locus of strain ATCC 43617 revealed that the ORF is flanked upstream by MCORF 819, which is predicted to encode a GTP-cyclohydrolase I (GCYH-I, NCBI BlastP, E-value 4d-99), and downstream by MCORF 821, which is predicted to encode a protein of the Major Facilitator Superfamily (MFS) (NCBI BlastP, E value 1.73e-5), as

diagramed in Figure 3.2A. The genes corresponding to MCORF 819 and MCORF 821 were also found flanking *mclS* in all 12 isolates of *M. catarrhalis* with sequenced genomes.

In prokaryotes, GCYH-I catalyzes the hydrolysis of GTP in the first committed step in the biosynthesis of tetrahydrofolate [237]. The 201 aa MCORF 819 gene product is predicted to have a molecular weight of 22.5 kDa. No signal peptides or TMH were predicted (SignalP, TatP, Phobius, TMHMM 2.0, ProSite). In *E. coli*, GCYH-I exists as a decamer (a pentamer of dimers) and requires zinc for activity [238-239].

The MFS is a diverse group of secondary transporters that facilitate transport across cytoplasmic or internal membranes. Substrates can vary but may include ions, sugar phosphates, neurotransmitters, nucleosides, amino acids, and peptides. The 509 aa MCORF 821 gene product is predicted to have a molecular weight of 56.6 kDa. According to the TMHMM algorithm, the MCORF 821 gene product possesses six TMH at the N-terminus, six TMH at the C-terminus, and a cytoplasmic region located between the two transmembrane domains, thereby indicating it is likely an integral membrane protein. Significant similarity was observed to ProP of *E. coli* (BlastP, 3e-22), an H+ osmoprotectant symporter with the capability of transporting proline and glycine betaine. Additionally, ProP has been shown to localize at the cell poles, a process that is dependent on CL [191, 240]. Due to the relationship between CL and ProP, we examined whether or not *mclS* is transcriptionally linked to MCORF 821. Though no data have been published that propose a functional relationship between CL and GCYH-I, we also investigated if a transcriptional-linkage exists between *mclS* and upstream gene, MCORF 819.

RNA was extracted from *M. catarrhalis* isolate O35E and analyzed by RT-PCR to determine if the flanking genes were transcriptionally linked to *mclS*. Primers were specifically

designed to span the intergenic regions between ORFs. The presence of amplicons of 475 bp and 487 bp in size are consistent with both MCORF 819 and MCORF 821 being transcriptionally linked to *mclS*, respectively (Figure 3.2B). The results demonstrate that *mclS* is co-transcribed with both MCORF 819 and MCORF 821.

Construction of M. catarrhalis strains that lack expression of a WT mclS gene product

To investigate the biological role of MclS, a mutation was engineered in the *mclS* gene of *M. catarrhalis* WT isolates O35E, O12E, and McGHS1. This was accomplished by inserting a spectinomycin resistance cassette near the middle of the ORF and introducing the disrupted *mclS* gene into the *M. catarrhalis* genome via homologous recombination. The approach yielded the isogenic mutant strains O35E.mclS, O12E.mclS, and McGHS1.mclS (Table 3.1). To verify that the mutant strains lack expression of the *mclS* gene, RT-PCR was utilized, and the results of these experiments are shown in Figure 3.3. Using oligonucleotide primers internal to the *mclS* transcript (P1 and P4, see Figure 3.3A), a DNA fragment of the expected size was amplified in the WT isolate O35E (Figure 3.3B, lane 5), but was absent in the isogenic mutant strain O35E.mclS (Figure 3.3B, lane 7). Using control primers specific for the *mcaP* gene, RT-PCR analysis demonstrated that equivalent amounts of RNA from the WT and mutant strains were analyzed (lanes 14-17 in Figure 3.3B). Similar results were obtained when analyzing expression of the *mclS* gene in the *M. catarrhalis* strains O12E, O12E.mclS, McGHS1, and McGHS1.mclS (data not shown). Taken together, the data indicate that our mutagenesis approach successfully abrogated expression of the *mclS* gene product in the mutant strains O35E.mclS, O12E.mclS, and McGHS1.mclS. Each of these mutants were also complemented by re-introducing the WT copy of the *mclS* gene into its original genomic locus, yielding strains O35E.mcls repaired, O12E.mclS repaired, and McGHS1.mclS repaired (Table 3.1). Proper allelic expression was

verified by PCR (data not shown) and sequencing of the *mclS* gene in the repaired strains. In addition, RT-PCR experiments using internal primers specific for the *mclS* transcript confirmed that the repaired strains express the *mclS* gene (lane 9 in Figure 3.3B for O35E.mclS repaired, data not shown for O12E.mclS repaired and McGHS1.mclS repaired).

MclS is responsible for the synthesis of CL in M. catarrhalis

Beebe et al. previously reported that CL is a major component of the *M. catarrhalis* cell envelope, constituting 5-30% of the total membrane PL [161]. Since *mclS* is the only gene in the genome predicted to encode a CLS, we hypothesized that *mclS* is solely responsible for production of CL in *M. catarrhalis*. To address this, PL were extracted from our panel of *M. catarrhalis* WT and mutant strains, separated by high performance thin layer chromatography (HP-TLC), and visualized by staining with a Molybdenum Blue spray reagent. Purified CL, PG, and PE were used as standards in these experiments in order to identify the various PL present in the cell envelope of the *M. catarrhalis* strains. Figure 3.4A clearly demonstrates that CL is produced by the WT (lane 1) and repaired (lane 3) strains while missing in the mutant O35E.mclS (lane 2). Similar results were obtained when analyzing PL produced by the O12E (Figure 3.4B) and McGHS1 (Figure 3.4C) strain sets.

It is possible that the *mclS* mutation in strains O35E.mclS, O12E.mclS, and McGHS1.mclS results in polar effects on expression of the co-transcribed genes MCORF 819 and MCORF 821 (see Figure 3.2), which in turn abrogate accumulation of CL in the cell envelope of *M. catarrhalis*. To rule out this possibility, we examined expression of MCORF 819 and MCORF 821 in the mutant strain O35E.mclS by use of RT-PCR. As shown in Figure 3.5B, amplicons of 212 bp (lane 3) and 210 bp (lane 12) indicating transcription of MCORF 819 and

MCORF 821, respectively, were generated in the *mclS* mutant strain. Furthermore, qRT-PCR analysis confirmed that the *mclS* insertion mutant strain expressed MCORF 821 at levels equal to, or even slightly higher than, the WT and repaired strains of O35E (Figure 3.S1). These results indicate that transcription of the flanking genes was not markedly affected by the mutation in *mclS*.

Sung et al. previously demonstrated that proteins of the PLD superfamily require the lysine residue in the C-terminal HKD motif for enzymatic activity [180]. It was reported that even the most conservative amino acid change, lysine to arginine, abolished its activity of human PLD. Expression, localization, and protein conformation of PLD were not affected by the mutation. Therefore, a point mutation (lysine at aa position 438 to arginine, $K^{438}\rightarrow R$) was engineered in the C-terminal HKD motif of the *mclS* gene product (see Figure 3.1) of isolate O35E, and the mutated ORF was reintroduced in its original genomic locus, yielding the strain O35E.mclS.K438R (Table 3.1). Confirmation that the mutated *mclS* gene was transcribed at levels equivalent to that of the WT and repaired strains was attained by RT-PCR (compare lane 11 to lanes 5 and 9 in Figure 3.3B) and qRT-PCR (Figure 3.S1). The PL profile of strain O35E.mclS.K438R was examined by HP-TLC, and these experiments revealed that expression of the mutated *mclS* gene product abolishes accumulation of CL (see lane 4 of Figure 3.4A). Taken together, our data conclusively demonstrates that MclS is a CLS responsible for the production of CL in the cell envelope of *M. catarrhalis* and that this function is conserved among isolates of various clinical and geographical origins. Moreover, site-directed mutagenesis of the *mclS* gene product demonstrates that the lysine at position 438 is necessary for CLS activity. This is the first study where the enzymatic activity of a bacterial CLS is abolished due to the generation of a point mutation. This result furthers validates the claim by Sung et al. that

mutagenesis of the lysine residue of the second HKD motif will abolish enzymatic activity of members of the PLD superfamily [180].

Cardiolipin-deficient M. catarrhalis exhibits WT growth in complete media and under osmotic stress

It has previously been shown that CLS is required for viability/optimal growth *in vitro* for some bacterial species including *E. coli* [222, 225]. Therefore, we tested whether or not the *mclS* mutant strains exhibit growth defects by culturing the bacteria in TH broth and monitoring the OD of the cultures over a period of six hours. As shown in Figure 3.6, the mutant strains O35E.mclS (panel A), O12E.mclS (panel B), and McGHS1.mclS (panel C) did not display growth defects when compared to the WT and repaired strains. Additionally, the *M. catarrhalis* strain expressing the lipolytically inactive form of *mclS* (O35E.mclS.K438R) grew at WT levels (Figure 3.6A). These results demonstrate that *mclS* gene product is not required for optimal growth of *M. catarrhalis in vitro*.

Previous studies have demonstrated that CL-deficient *E. coli* displays reduced viability when grown under conditions of osmotic stress [191, 202]. To see if CL-deficient *M. catarrhalis* is also sensitive to osmotic pressure, we examined the growth rates of the WT and *mclS* mutant strains of O35E in broth containing high concentrations of either sodium chloride or sorbitol. There was no significant difference between WT and CL-deficient *M. catarrhalis* when grown in media with a high concentration of sodium chloride (Figure 3.S2A). Likewise, there was no significant difference between growth rates when grown in media with high concentrations of sorbitol (Figure 3.S2B). Therefore, we conclude that the viability of *M. catarrhalis*, unlike *E. coli*, does not depend on the presence of CL in conditions of high osmotic stress.

M. catarrhalis lacking CL exhibits reduced levels of adherence to human epithelial cells

Some members of the PLD superfamily have been shown to affect bacterial adherence to epithelial cells [216, 220]. Alteration in PL levels, specifically PE, has been shown to result in reduction in virulence-associated traits [163-164]. To date, no CLS has been associated with adherence or virulence in general. For these reasons, we tested our panel of *M. catarrhalis* strains for adherence to HEp-2 (laryngeal) and A549 (type II pneumocytes) human epithelial cell lines. In the isolate O35E, adherence to HEp-2 cell was reduced by 76% in the *mclS* insertion mutant strain compared to the WT strain (Figure 3.7A). The level of adherence in the *mclS* insertion mutant was nearly reduced to that of the O35E.ZCSM strain that lacks expression of three known *M. catarrhalis* adhesins (UspA1, Hag, and McaP). Adherence to A549 cells was also significantly reduced (62%) in the *mclS* insertion mutant strain (Figure 3.7B). Furthermore, the *mclS* mutant strains of isolates O12E and McGHS1 displayed a comparable reduction in adherence (Figure 3.7C-3.7F), except McGHS1.mclS which adhered to HEp-2 cells at a WT level. For each isolate, adherence of the repaired strains was restored to WT levels (Figure 3.7A-3.7F). To ensure that the adherence defect of the *mclS* mutant strains is authentic and not simply an artifact of the adherence assay procedure, we monitored the survival of the O35E strains in the reagents used in the procedure: PBSG, tissue culture (TC) media, and saponin solution. There is no difference in survival between the WT and *mclS* mutant strains of O35E, therefore demonstrating that the adherence defect is genuine (Figure 3.S3).

Taken together, our data indicate that expression of the *mclS* gene product impacts the adherence of *M. catarrhalis* to human epithelial cells. The results, however, do not specify if MclS is an adhesin that directly mediates binding to the surface of epithelial cells. It is possible that the effect of *mclS* expression on *M. catarrhalis* adherence is indirect. Absence of CL in the

M. catarrhalis membrane may perturb the proper surface display of adhesins, which in turn reduces binding to epithelial cells. To address this, we examined the adherence of strain O35E.mclS.K438R, which expresses a mutated *mclS* gene product that is lipolytically inactive and does not support the accumulation of CL in the *M. catarrhalis* membrane (see lane 4 in Figure 3.4A). These experiments revealed that the binding of O35E.mclS.K438R to HEp-2 and A549 cell lines was equivalent to that of the mutant strain O35E.mclS that lacks expression of the CLS (Figure 3.7A). Hence, the data supports the hypothesis that the contribution of *mclS* to adherence is indirect, possibly by modulating the proper surface display of *M. catarrhalis* adhesins on the bacterial surface through its CLS activity.

To determine if the expression and/or localization of a known adhesin is affected by the absence of CL, we examined whole-cell lysates and outer membrane preparations of the CL-deficient strains of O35E by western blot. The following adhesins are expressed at WT levels in the CL-deficient strains of O35E and detected in the whole-cell lysates: Hag, UspA1, McaP, OMP CD, and MhaB1/MhaB2 (Figure 3.S4A). Furthermore, the adhesins are localized to the outer membrane (Figure 3.S4B) as shown by detection of the proteins in the outer membrane preparations. Analysis of the strains by flow cytometry further confirmed that the adhesins UspA1, Hag, and McaP are not only expressed at WT levels in the *mclS* mutant strains, but that they are also localized and displayed on the outer membrane (Figure 3.S5). By using the monoclonal antibody (mAb) 24B5, we were able to detect a single epitope of UspA1 in both O35E and O35E.mclS, suggesting that UspA1 is folded and displayed correctly (Figure 3.S5A-3.S5C). Flow cytometry also detected Hag and McaP using polyclonal antibodies (pAbs) in both O35E and O35E.mclS (Figure 3.S5D-3.S5I). The negative control, O35E.ZCSM, which lacks Hag, UspA1, and McaP, displayed considerably reduced fluorescence for each of the antibodies

(Figure 3.S5C, 3.S5F, 3.S5I). Combined, the results demonstrate that the CL-deficient strains express and display five major *M. catarrhalis* adhesins (Hag, UspA1, McaP, OMP CD, and MhaB1/MhaB2) on the outer membrane at WT levels.

Discussion

In this study, we have shown that *M. catarrhalis* expresses a CLS, termed MclS, that contributes to the ability of the bacterium to adhere to human epithelial cells. The *mclS* gene is predicted to encode a member of the PLD superfamily and displays significant similarity to *ymdC/clsC* of *E. coli*. Sequence analysis of numerous *M. catarrhalis* isolates revealed that *mclS* is highly conserved throughout the species at both the nucleotide and amino acid level. Mutant strains that lack expression of the *mclS* gene product were constructed in *M. catarrhalis* isolates O35E, O12E, and McGHS1. In addition, a mutant strain was generated in isolate O35E that contained a point mutation in one of the two signature HKD motifs of *mclS*. The *mclS* insertion and point mutant strains were unable to synthesis CL, thus verifying MclS as a CLS. The *mclS* mutant strains also displayed significantly reduced levels of adherence to human epithelial cells when compared to their WT counterparts. As expected, the *mclS* repaired strains restored adherence to WT levels. The reduction in adherence was attributed to the absence of endogenous CL, as both the *mclS* insertion mutant strain and *mclS* point mutant strain adhered at similar levels. We hypothesize that the absence of CL from membranes of *M. catarrhalis* results in altered expression and/or display of adhesins on the outer membrane, thereby contributing to the reduction in adherence to epithelial cells.

The sequence and role of *mclS* appears to be conserved throughout *M. catarrhalis* isolates. The *mclS* gene was present in all fourteen *M. catarrhalis* isolates examined. Within the

1.6-kb ORF of *mclS*, only eight SNPs were identified. Three of these SNPs resulted in amino acid changes, none of which occurred in the signal peptide, lipid attachment site, or HKD motifs (Figure 3.1). In addition, mutation of *mclS* in three isolates of *M. catarrhalis* (O35E, O12E, and McGHS1) yielded strains that were unable to produce CL and displayed reduced levels of adherence to human epithelial cell lines compared to WT strains (Figures 3.4, 3.7). This data leads us to conclude that the sequence and role of *mclS* is conserved throughout the *M. catarrhalis* species.

MclS appears to be unique among CLS in that it is predicted to be a lipoprotein, and this property may aid in determining the subcellular localization of the protein within *M. catarrhalis*. The ProSite algorithm predicts the *mclS* gene product to be a lipid-anchored protein consisting of an N-terminal signal peptide followed by a cysteine residue at the amino acid position 24 serving as the lipid attachment site (Figure 3.1). The YmdC/ClsC protein, which has significant similarity with MclS, is not predicted to be a lipoprotein. Instead, YmdC/ClsC contains a N-terminal signal peptide and requires expression of a second protein, YmdB, in order to exhibit phospholipolytic activity [159]. Yamaguchi et al. has shown that the amino acid residue immediately following the lipid attachment site is a strong indicator of the localization of lipoproteins in *E. coli*. An aspartic acid at this position targets the lipoprotein for the plasma membrane, while the presence of any other amino acid results in localization at the outer membrane [241]. The BRO β-lactamases of *M. catarrhalis* (BRO-1 and BRO-2) are expressed as lipoproteins and are localized to the periplasmic leaflet of the outer membrane. Like BRO-1 and BRO-2, MclS possesses a lysine residue following the lipidated cysteine residue, suggesting outer membrane localization [35]. However, a prior study demonstrated that ClsA of *E. coli* is localized to the periplasmic leaflet of the plasma membrane [185]. Cardiolipin, the PL product

of CLS, is predominantly found in the plasma membrane of bacteria, though it has been detected in smaller amounts in the outer membrane of *E. coli* [242]. *Moraxella catarrhalis* does contain higher levels of CL than most bacteria, so it is possible that CL is present at higher levels in the outer membrane, comparatively [161, 242]. Further experiments must be conducted to determine the subcellular localization of both CL and MclS within *M. catarrhalis*.

Examination of the genetic organization of the *mclS* locus by RT-PCR revealed that *mclS* is transcriptionally linked to both MCORF 819 upstream and MCORF 821 downstream (Figure 3.2). Interestingly, the downstream gene MCORF 821 is predicted to encode a protein of the MFS with similarity to ProP, an osmosensory transporter that requires CL for polar localization in *E. coli* [191, 240]. In the *E. coli* genome, however, *proP* is not located near any of the three CLS genes. In conditions of osmotic stress, CL-deficient *B. subtilis* suffer reduced viability, suggesting a role for CL (direct or indirect) in the regulation of osmotic pressure [202]. We found that CL-deficient *M. catarrhalis* do not display reduced viability when grown in broth with high concentrations of sodium chloride or sorbitol (Figure 3.S2). This may be due to the possibility that MCORF 821 does not encode for a protein that is a functional homolog of ProP. It is also likely that *M. catarrhalis* expresses multiple osmoregulators whose functions may be unaffected by CL, thereby allowing the bacteria to display WT viability under osmotic pressure in the CL-deficient strains. While no functional relationship between the gene products of *mclS* and MCORF 819 is apparent, we are currently investigating how the transcriptional-linkage between two genes may impact their roles in *M. catarrhalis*.

The impact of CL on viability is one example of how the characteristics of CL-deficient bacteria differ between *M. catarrhalis* and *E. coli*. As demonstrated by the growth curves of isolates O35E, O12E, and McGHS1 (Figure 3.6), there were no significant differences in the

viability between the WT and CL-deficient strains of *M. catarrhalis*. The growth curves were extended for several days to include stationary phase, and still no significant difference were observed between the WT and *mclS* mutant strain (data not shown). In contrast, *clsA* is required by *E. coli* for WT growth rates during long-term incubation in stationary phase [225]. However, the role of *clsB* and *ymdC/clsC* under such conditions has not been investigated to our knowledge.

Our data indicates that MclS is the only CLS expressed by *M. catarrhalis*. As shown in Figure 3.4, the *mclS* mutant strains of *M. catarrhalis* did not contain detectable levels of CL. Moreover, a BlastP search yielded no additional CLS homologs in the *M. catarrhalis* genome. Interestingly, previous reports have shown that other bacteria including *E. coli* and *B. subtilis* express up to three CLS, which differentially contribute to CL synthesis depending on the growth phase of the bacteria [159, 192, 225]. It has been documented that the CL content of several bacteria including *E. coli* is highest during stationary phase [162, 225]. We did not observe any significant changes in CL content despite varying conditions including growth phase (data not shown). Our data agrees with the initial findings by Beebe et al. that CL levels in *M. catarrhalis* remain constant throughout logarithmic and stationary growth [161]. However, it is possible that our detection system (HP-TLC with Molybdenum Blue Spray Reagent to visualize) was not sensitive enough to perceive relatively small changes in CL content. In contrast to *E. coli*, CL levels in *M. catarrhalis* remain constant throughout exponential and stationary growth and are only dependent on a single CLS enzyme.

Recently, Tan et al. found that not all prokaryotic-type CLS utilize PG as the sole substrate for CL synthesis, prompting us to examine the substrates of MclS [159]. Based on the HP-TLC experiments (Figure 3.4), it is not apparent whether or not PG and PE levels are altered

in the *mclS* mutant strains compared to WT isolates in each instance. Again, it is possible that the detection methods are not sensitive enough to detect relatively small changes in PL levels. It does appear that the intensity of the spot corresponding to PG is increased in the *mclS* insertion mutant strain compared to the WT and repaired strains of O12E (Figure 3.4B, lanes 1-3). However, this observation alone cannot allow us to conclude which substrates MclS utilizes for CL synthesis. Based on the similarity of MclS to YmdC/ClsC, we sought to determine if PE is a substrate for MclS by generating PE-deficient strains of *M. catarrhalis,* which lack enzymes specific to the PE synthetic pathway. A BlastP search of the genome of *M. catarrhalis* isolate ATCC 43617 identified genes (MCORF 174 and 700) predicted to encode a phosphatidylserine synthase (PssA) and a phosphatidylserine decarboxylase (Psd), respectively. Generation of *pssA* and *psd* mutant strains of *M. catarrhalis* is underway. We plan to utilize HP-TLC to determine if CL is present in these mutant strains.

This study established that the lysine residue within the HKD motif is required for CLS activity. Site-directed mutagenesis of the lysine residue in the C-terminal HKD motif rendered MclS enzymatically inactive and unable to catalyze the synthesis of CL (Figure 3.4). Previously, Tan et al. generated a point mutation in the histidine residues of both HKD motifs of YmdC/ClsC that abolished the phospholipolytic activity of the CLS [159]. Together, it appears that the amino acid residues in HKD motifs of CLS are crucial for activity. This appears true for all members of the PLD superfamily considering human PLD, yeast PLD, and a vaccinia viral protein were rendered catalytically inactive through mutagenesis of the HKD motif. The lysine to arginine mutation within the HKD motif was originally described by Sung et al. using human PLD. The mutation, however, did not alter expression or localization of the protein [180].

We also conclude that *M. catarrhalis* requires CL to exhibit WT levels of adherence to human epithelial cells (Figure 3.7) and that MclS itself is not an adhesin. Adherence to both A549 and HEp-2 human epithelial cell lines was significantly reduced in the *mclS* mutant strains of isolates O35E, O12E, and McGHS1 compared to the WT and repaired strains (Figure 3.7). In O35E, adherence to human epithelial cells was reduced when MclS was knocked-out (*mclS* insertion mutant) and rendered enzymatically inactive (*mclS* point mutant). These results suggest that MclS is not an adhesin itself. The one instance in which adherence was not reduced in the CL-deficient strain (McGHS1 to HEp-2 cells, Figure 3.7E) may be explained by variation in the usage, expression, or composition of adhesins in different isolates of *M. catarrhalis*.

The adherence phenotype of the *mclS* mutant strains may be explained by specific interactions between CL and proteins within *M. catarrhalis* membranes. In *E. coli*, CL is known to impact the localization and activity of several proteins, including the SecYEG and SecA components of the Sec translocon. Researchers have shown that CL associates with and stimulates the SecYEG components of the Sec translocon and is required for proper subcellular distribution of the SecYEG complex [190]. It is conceivable that, as in *E. coli*, *M. catarrhalis* requires CL for the proper localization and activity of many proteins, including ProP and the Sec translocon. In the absence of CL, the Sec translocon may no longer efficiently transport proteins, including adhesins, through the inner membrane. It is possible that the expression, localization, or display of a known adhesin is altered in the CL-deficient strains, thereby causing the adherence phenotype. According to data obtained by western blot and flow cytometry, the adhesins Hag, UspA1, McaP, OMP CD, and MhaB1/2 are all expressed and localized to the outer membrane. Adherence is a multifactorial process that involves many adhesins so it is possible that the expression or localization of another adhesins not examined in this study (e.g.

lipooligosaccharide) is altered in the CL-deficient strains [57, 61-62, 65, 81, 85, 89]. A better understanding of the relationship between CL and these proteins require characterization of the Sec translocon in *M. catarrhalis*.

In summary, we reveal that *M. catarrhalis* expresses a single CLS, MclS, which contributes to the ability of the bacterium to adhere to human epithelial cells. The *mclS* gene is present in all *M. catarrhalis* isolates and conserved at greater than 99% identity. The *mclS* gene product is predicted to be lipid-modified, likely promoting association with the membrane. While MclS displays significant similarity to YmdC/ClsC of *E. coli*, we have yet to determine if it utilizes PE in addition to PG as a substrate for CL synthesis. Like other members of the PLD superfamily, MclS requires intact HKD motifs for catalytic activity. The reduction in adherence observed in the *mclS* mutant strains can be attributed to the absence of CL. The MclS protein itself is not an adhesin, but instead may be necessary for expression, localization, and display of other *M. catarrhalis* adhesins. This study marks the first instance in which CL is shown to contribute to virulence-associated traits.

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Tables

Table 3.1: Strains and plasmids used in this study

Strain or Plasmid	Description	Selectable Marker	Source
M. catarrhalis:			
O35E	WT isolate	none	[127]
O35E.mclS	*mclS* isogenic insertion mutant strain of O35E	spec	This study
O35E.mclS repaired	Repaired strain of O35E.mclS	stm	This study
O35E.mclS.K438R	*mclS* isogenic point mutant strain of O35E	stm	This study
O35E.ZCSM	*hag*, *uspA1*, *uspA2*, and *mcaP* isogenic mutant strain of O35E	zeo, cat, spec, kan	[61]
O35E.SM100	*rpsL* isogenic mutant strain of O35E	stm	[54]
O35E.CD1	*ompcd* isogenic mutant strain of O35E	kan	[65]
O35E.MhaB	*mhaB1 and mhaB2* isogenic mutant strain of O35E	spec, zeo	[62]
O12E	WT isolate	none	[89]
O12E.mclS	*mclS* isogenic insertion mutant strain of O12E	spec	This study
O12E.mclS repaired	Repaired strain of O35E.mclS	stm	This study
O12E.12Hg	*hag*, *uspA1*, *uspA2* isogenic mutant strain of O12E	zeo, cat, spec	Unpublished
McGHS1	WT isolate	none	[230]
McGHS1.mclS	*mclS* isogenic insertion mutant strain of McGHS1	spec	This study
McGHS1.mclS repaired	Repaired strain of McGHS1.mclS	stm	This study
McGHS1.Hag	*hag* isogenic mutant strain of McGHS1	spec	[230]
E. coli:			
EPI300™	Cloning strain	none	Epicentre®
Plasmids:			
pSPEC^{R}	Source of spectinomycin resistance cassette	spec	[243]
pCC1™	Cloning vector, replicative in *E. coli*	cat	Epicentre® Illumina®
pCC1.mclS	pCC1™ containing *mclS* from O35E	cat	This study
pCC1.mclS.spec	pCC1.mclS with spectinomycin resistance cassette inserted into *mclS*	cat, spec	This study

Table 3.2: Oligonucleotide primers

Primer	Sequence (5' → 3')	Gene	Direction
P1	ATTTGCCCGATACGCCTCACTTAC	*mclS*	Forward
P2	AATCGATAGGCATCAGTCCAGCCA	*mclS*	Reverse
P3	TGGGATCCATTTGATAACAATCATCGCC	*mclS*	Forward
P4	GGATCTGAGTGACCGTTTAATTT	*mclS*	Reverse
P5	GCCAACCAATTACCCAGCCAGCA	MCORF 821	Forward
P6	ACCGCCCAAGGGTCTAGCAA	MCORF 821	Reverse
P7	ACCAAGCAAGTGGCCAGTGCT	MCORF 819	Forward
P8	CCAAAAGCGGGCATTCGGCG	MCORF 819	Reverse
P9	TGGCTGGCTGGACTGATGCC	*mclS*	Forward
P10	AGGCGTATCGGGCAAATTTTTGCA	*mclS*	Reverse
P11	TGCCAATGACCAAGCCAATT	*mcaP*	Forward
P12	TCAGATGCTGGGGTAGTTGA	*mcaP*	Reverse
P13	CGCGGATCCGCGACTCAAGTGAAAATACGCA	*rpsL*	Forward
P14	CCGGAATTCCGGACACGACGTCTTGGCATAA	*rpsL*	Reverse
P15	AGTCTACACGCCAGAGCCTTTGCGGTAGAT	*mclS*	Forward
P16	ATCTACCGCAAAGGCTCTGGCGTGTAGACT	*mclS*	Reverse
P17	CACACTCTGAGCGGTCATTT	MCORF 821	Forward
P18	GAAACTCAATTGCTGGCAGA	MCORF 821	Reverse
P19	GATGCCAATGATGGAACAAC	*mcaP*	Forward
P20	GATTTGGGTTTGTGCTGATG	*mcaP*	Reverse

Figures

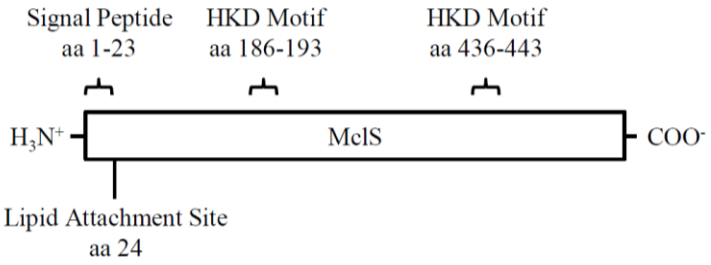


Figure 3.1. The predicted structure of MclS from *M. catarrhalis*. Bioinformatic data predicts that MCORF 820 (renamed *mclS*) encodes a CLS. The presence of two HKD motifs (aa 186-193 and aa 436-443) indicates that MclS is a member of the PLD superfamily. The MKD motifs are thought to associate to form the active site of the enzyme. A signal peptide (aa 1-23) and lipid attachment site (aa 24) were predicted at the N-terminus, thereby characterizing MclS as a putative lipid-anchored protein.

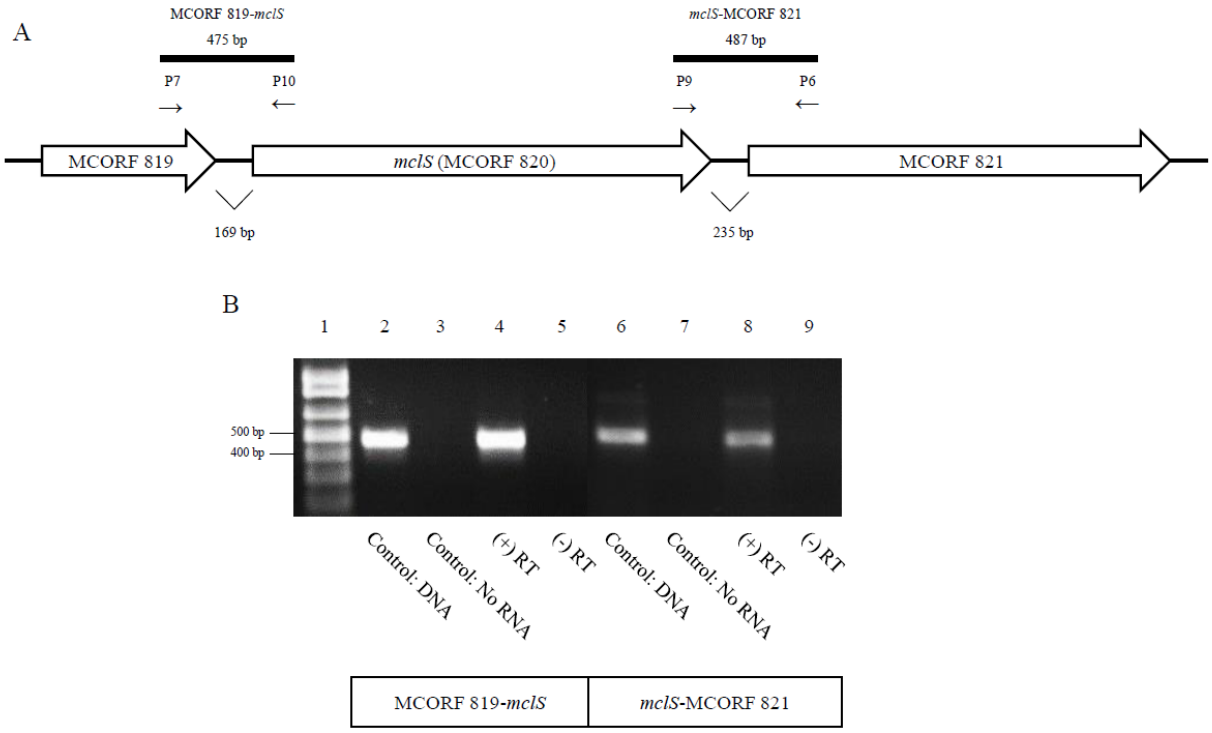


Figure 3.2. A transcriptional-linkage exists between MCORF 819, *mclS*, and MCORF 821. (A) Schematic representation of the *mclS* genetic locus including MCORF 819 and MCORF 821. Primers used in PCR are indicated by arrows. The solid black bars represent the expected PCR products. (B) Image of an agarose gel displaying the results of RT-PCR examining the organization of the *mclS* locus. Primers P7 and P10 were used to amplify the region between MCORF 819 and *mclS* while primers P6 and P9 were used to amplify the region between *mclS* and MCORF 821. Lane 1 contains the DNA size marker. Lanes 2 and 6 contain PCR products from genomic DNA of *M. catarrhalis* isolate O35E. Lanes 3 and 7 contain RT-PCR products from a reaction with no RNA added. Lanes 4 and 5 (MCORF 819-*mclS*) and lanes 8 and 9 (*mclS*-MCORF821) contain RT-PCR products from RNA of *M. catarrhalis* isolate O35E. During cDNA synthesis, reverse transcriptase enzyme was either added (+, lanes 4 and 8) or withheld (-, lanes 5 and 9).

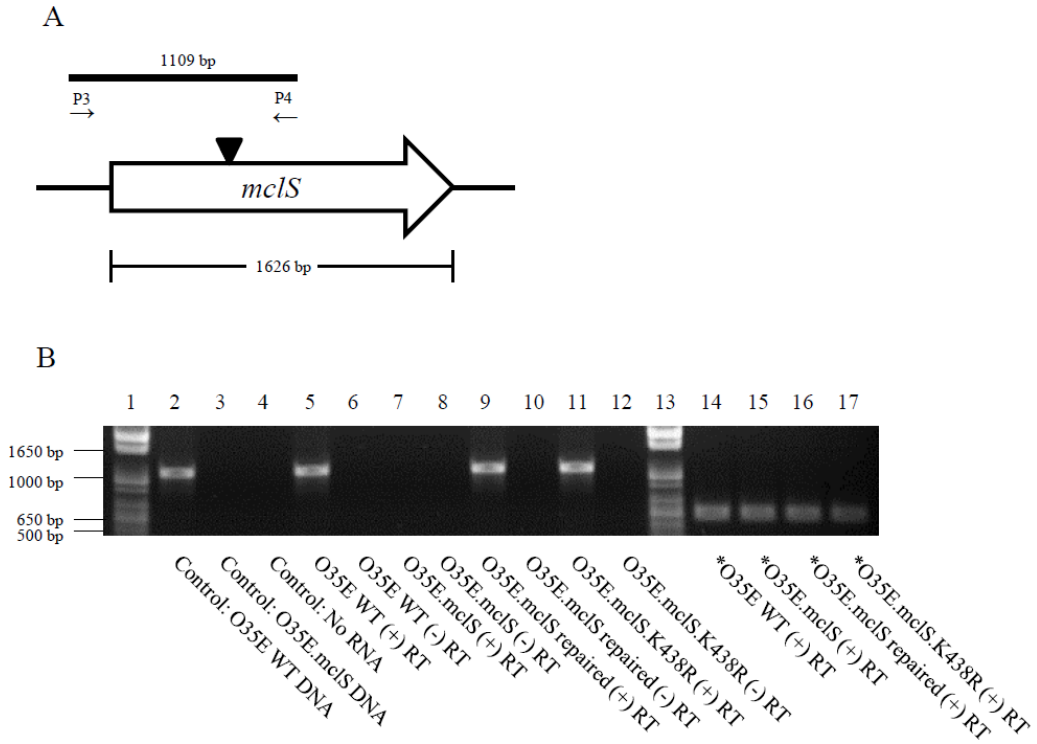


Figure 3.3. **RT-PCR validates the *M. catarrhalis* O35E isogenic *mclS* mutant strains.** (A) Schematic representation of the *mclS* genetic locus. Expression of *mclS* was examined by RT-PCR using the indicated oligonucleotide primers. The solid black bars represent the expected PCR product in the WT strain of *M. catarrhalis* isolate O35E. The black triangle indicates the location of the spectinomycin resistance cassette which interrupts the *mclS* ORF in the *mclS* insertion mutants. (B) Image of an agarose gel displaying the results of RT-PCR examining the transcription of *mclS* in the WT, *mclS* insertion mutant, *mclS* repaired, and *mclS* point mutant strains of *M. catarrhalis* isolate O35E. Oligonucleotide primers P3 and P4 were used to amplify a portion of *mclS*; in the *mclS* insertion mutant strains, this region was interrupted by a spectinomycin resistance cassette. Lanes 1 and 13 contain DNA size markers. Lanes 2 and 3 contain PCR products from the genomic DNA of the *M. catarrhalis* O35E isolate WT strain and the *mclS* insertion mutant strain, respectively. Lane 4 contains RT-PCR product from a reaction with no RNA added. Lanes 5-12 contain the RT-PCR products derived from WT *M. catarrhalis* isolate O35E (lanes 5 and 6), the *mclS* insertion mutant strain (O35E.mclS; lanes 7 and 8), the *mclS* repaired strain (O35E.mclS repaired; lanes 9 and 10), and the *mclS* point mutant strain (O35E.mclS.K438R; lanes 11 and 12), respectively. During cDNA synthesis, reverse transcriptase enzyme was either added (+; lanes 5, 7, 9, and 11) or withheld (-; lane 6, 8, 10, and 12). Lanes 14-17 contain RT-PCR product derived using the *mcaP*-specific primers P11 and P12 to ensure that equal amounts of RNA were analyzed in lanes 5-12. Asterisks signify that *mcaP*-specific primers were used for PCR amplification of cDNA.

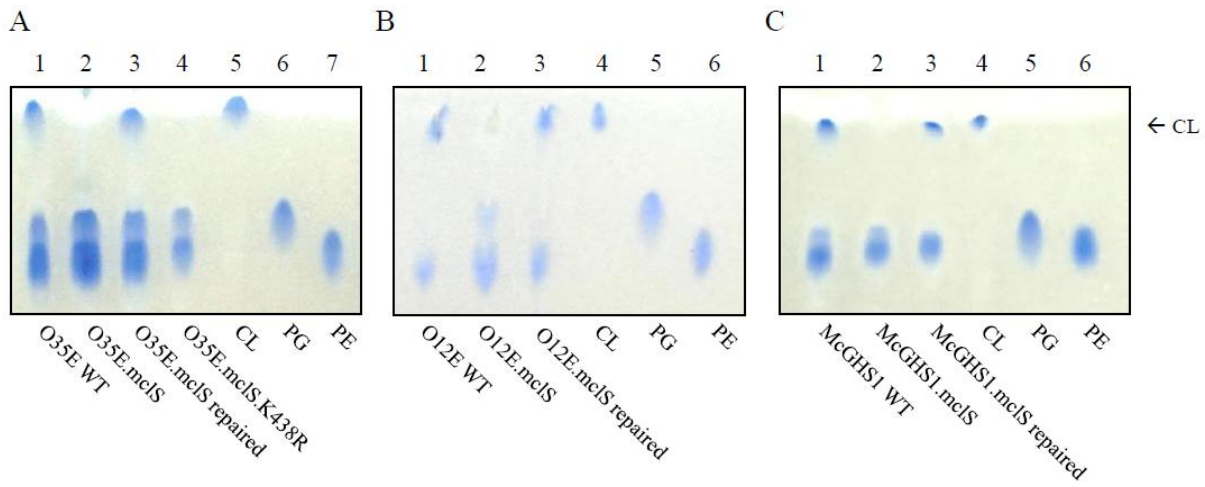


Figure 3.4. *Moraxella catarrhalis* requires *mclS* for synthesis of CL. Analysis of the PL profile of WT, *mclS* insertion mutant, *mclS* repaired, and *mclS* point mutant strains of *M. catarrhalis* isolates O35E (A), O12E (B), and McGHS1 (C) by HP-TLC. Phospholipids were extracted by the method of Bligh and Dyer and developed in 1-D using a mobile phase of chloroform:methanol:acetic acid (65:25:10). Phospholipid spots were visualized by staining with Molybdenum Blue spray reagent. Purified CL, PG, and PE were used as standards to identify the PL of *M. catarrhalis*. The arrow indicates the location to which CL migrates upon HP-TLC analysis.

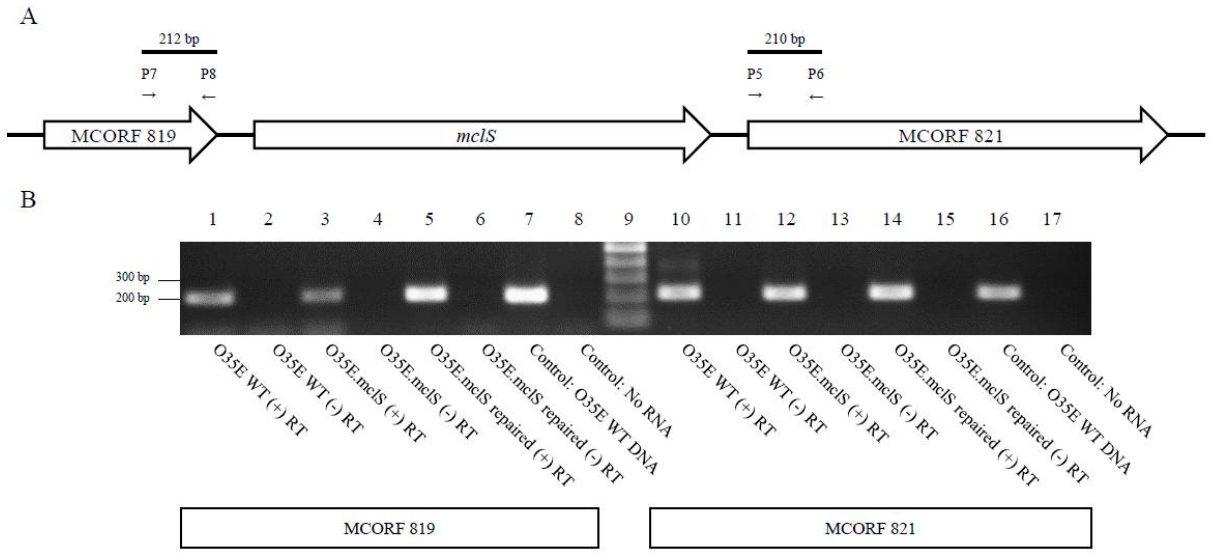


Figure 3.5. Transcription of MCORF 819 and MCORF 821 is not markedly affected by *mclS* insertion mutation in *M. catarrhalis*. (A) Schematic representation of the *mclS* genetic locus including MCORF 819 and MCORF 821. Primers used in PCR are indicated by arrows. (B) Image of an agarose gel displaying the results of RT-PCR examining transcription of MCORF 819 and MCORF 821 in the WT, *mclS* mutant, and repaired strains of *M. catarrhalis* isolate O35E. Primers P7 and P8 were used to amplify a portion of the MCORF 819 while primers P5 and P6 were used to amplify a portion of the MCORF 821. Lane 9 contains the DNA size marker. RT-PCR products of MCORF 819 and MCORF 821 are displayed in lanes 1-8 and lanes 10-17, respectively. For RT-PCR, RNA was extracted from *M. catarrhalis* isolate O35E (WT: lanes 1, 2, 10, 11), the *mclS* insertion mutant (O35E.mclS: lanes 3, 4, 12, 13), and the *mclS* repaired (O35E.mclS repaired: lanes 5, 6, 14, 15) strains. During cDNA synthesis, reverse transcriptase enzyme was either add (+, lanes 1, 3, 5, 10, 12, 14) or withheld (-, lanes 2, 4, 6, 11, 13, 15). Lanes 7 and 16 contain PCR products from the genomic DNA of *M. catarrhalis* isolate O35E. Lanes 8 and 17 contains RT-PCR products from a reaction with no RNA added.

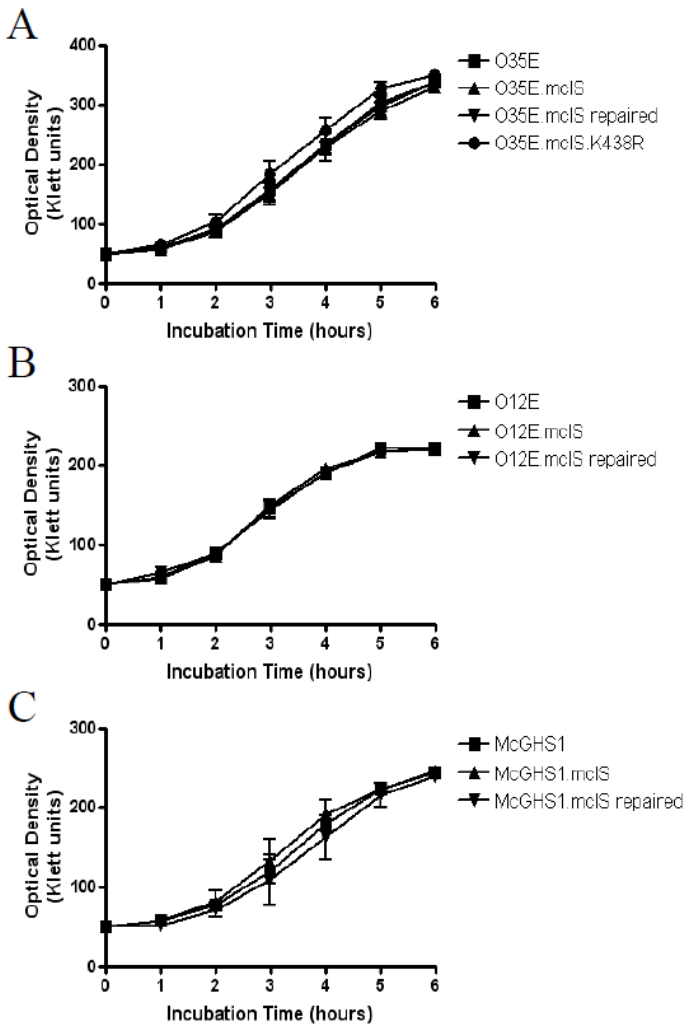


Figure 3.6. The *mclS* mutant strains do not exhibit growth defects *in vitro*. Growth curves of WT, *mclS* insertion mutant, *mclS* repaired, and *mclS* point mutant strains of *M. catarrhalis* isolates O35E (A), O12E (B), and McGHS1 (C).

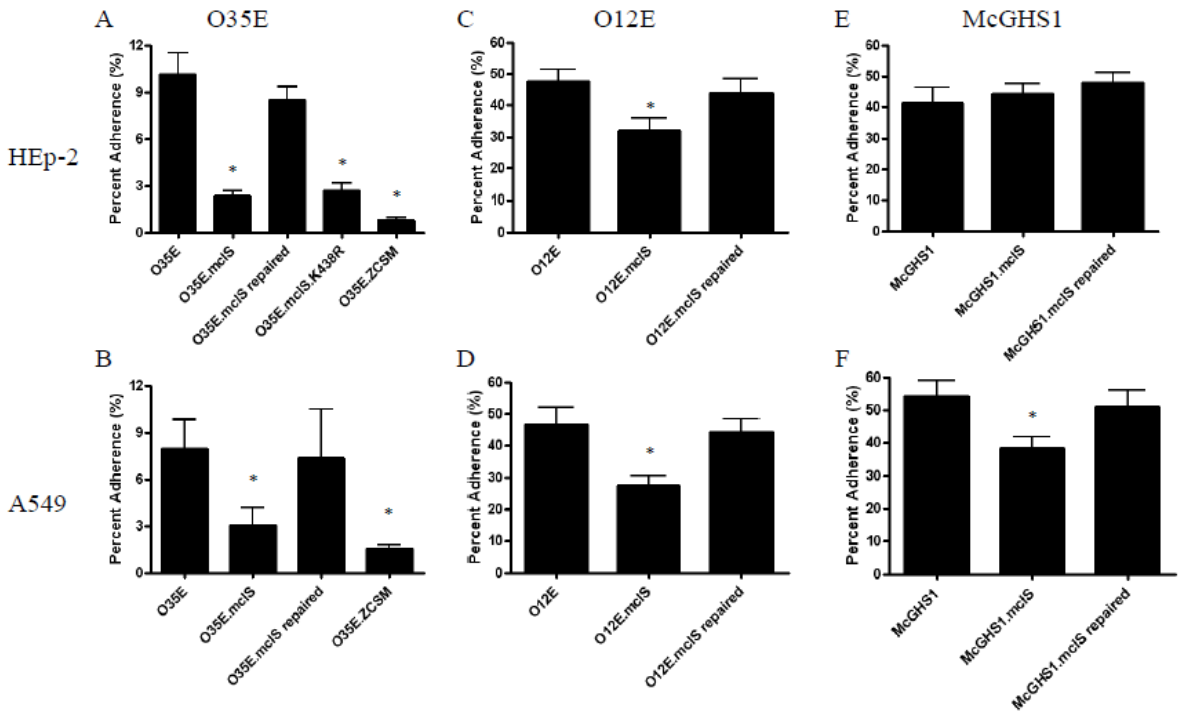


Figure 3.7. *Moraxella catarrhalis* requires CL to exhibit WT levels of adherence to human epithelial cells. Shown are the results of the quantitative adherence assays of *M. catarrhalis* isolates O35E (A, B), O12E (C, D), and McGHS1 (E, F). The results are expressed as the mean percentage (± standard error) of inoculated bacteria binding to HEp-2 (A, C, E) or A549 (B, D, F) epithelial cell monolayers after 30 minute incubation. Asterisks indicate that the reduction in adherence, compared to that of the WT, was found to be statistically significant ($P<0.05$, Mann-Whitney test).

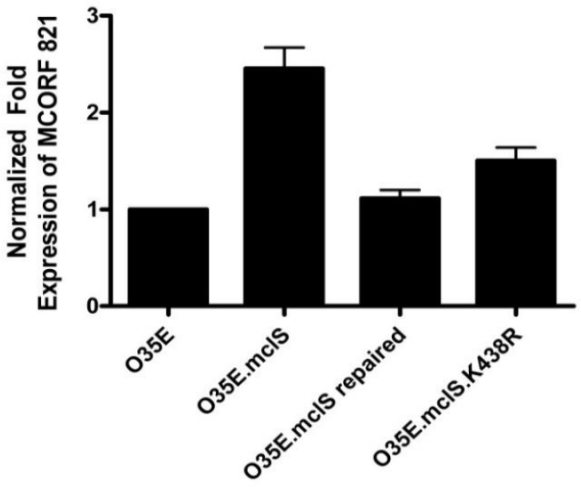


Figure 3.S1. Quantitative RT-PCR analysis confirms that gene expression of MCORF 821 is not decreased in CL-deficient strains of O35E. Shown are the results of the qRT-PCR experiments examining gene expression of MCORF 821, which is located immediately downstream of *mclS*. The results are expressed as the mean fold expression (± standard error) of MCORF 819. Values are normalized to the expression of the reference gene *mcaP*.

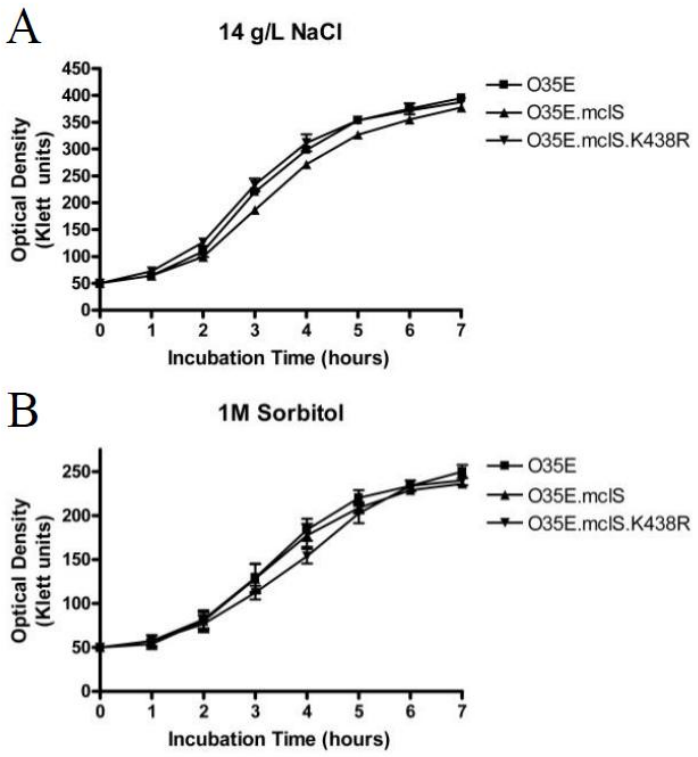


Figure 3.S2. The CL-deficient strains do not exhibit reduced viability in conditions of osmotic stress. Growth curves of WT, *mclS* insertion mutant, and *mclS* point mutant strains of O35E when grown in TH broth containing 14 g/L sodium chloride (A) or 1M sorbitol (B).

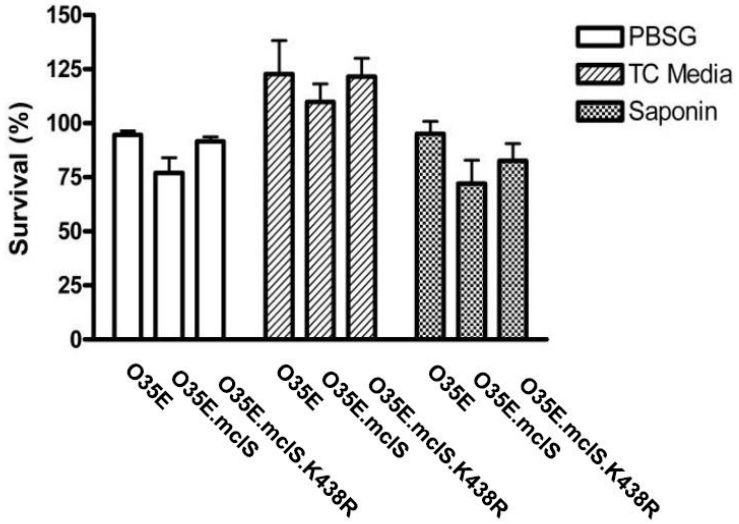


Figure 3.S3. The absence of CL in *M. catarrhalis* does not affect its ability to survive in PBSG, TC media, and saponin solution. WT, *mclS* insertion mutant, and *mclS* point mutant strains of O35E were incubated in PBSG, TC media, and saponin solution for 15-30 minutes. The results are expressed as the mean percentage survival (± standard error) following incubation.

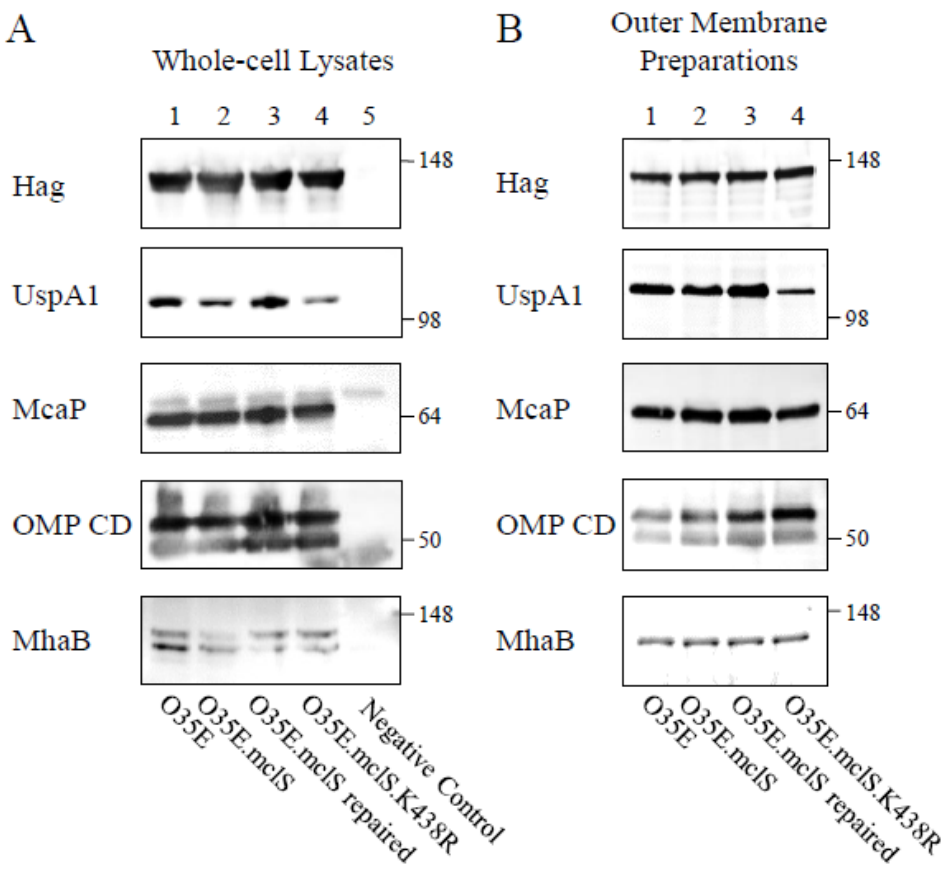


Figure 3.S4. As with WT strains, adhesins are expressed and localized to the outer membrane in CL-deficient strains of *M. catarrhalis*. Western blot analysis of the whole-cell lysate (A) and outer membrane preparation (B) of strains O35E, O35E.mclS, O35E.mclS repaired, and O35E.mclS.K438R demonstrates expression and localization of the following adhesins: Hag, UspA1, McaP, OMP CD, and MhaB1/MhaB2. The negative control in lane 5 of panel A is O35E.ZCSM for Hag, UspA1, and McaP; O35E.CD1 for OMP CD; and O35E.B1B2 for MhaB1/MhaB2. The antibodies used for detection of the adhesins are described in the Materials and Methods.

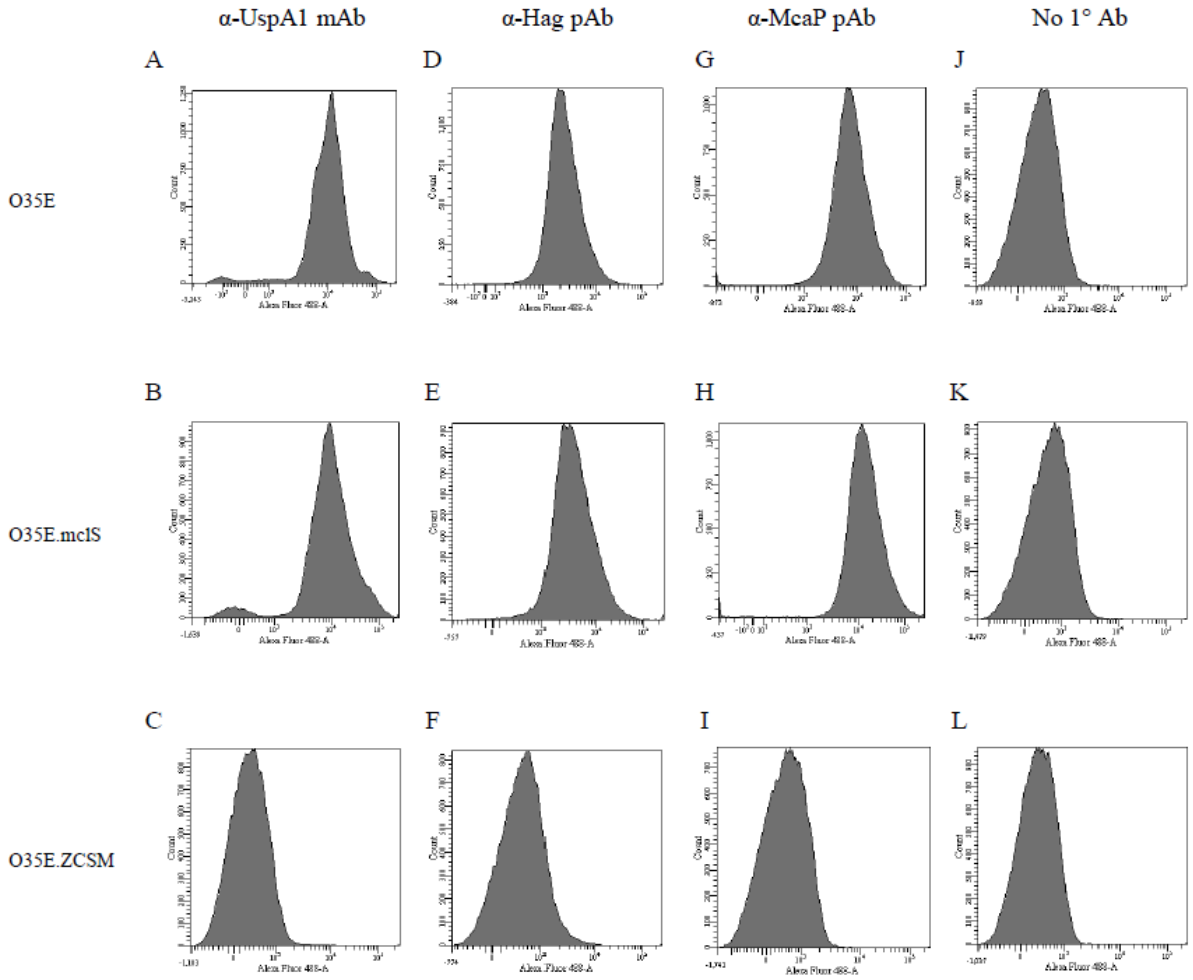


Figure 3.S5. *Moraxella catarrhalis* adhesins are localized to and displayed on the outer membrane of WT and CL-deficient bacteria. Strains O35E (A, D, G, J), O35E.mclS (B, E, H, K), and O35E.ZCSM (C, F, I, L) were incubated with antibodies against UspA1 (A-C), Hag (D-F), and McaP (G-I) followed by incubation with Alexa Fluor 488-conjugated secondary antibodies. As a negative control, *M. catarrhalis* strains were incubated in the absence of primary antibody to determine background fluorescence (J-L). The O35E.ZCSM strain lacks expression of UspA1, Hag, and McaP and therefore was included as a negative control. Display of adhesins was then analyzed by flow cytometry. A detailed procedure and description of the antibodies used for flow cytometry are found in the Materials and Methods. The *x*-axes represent the level of fluorescence, and the *y*-axes indicate the particle count. Shown are representative results of flow cytometry experiments.

CHAPTER 4

CHARACTERIZATION OF MCLS AND CARDIOLIPIN IN *MORAXELLA CATARRHALIS*

Introduction

In previous studies [244], we identified the cardiolipin synthase (CLS) of *Moraxella catarrhalis*, termed MclS, and discovered that cardiolipin (CL) contributes to the adherence of *M. catarrhalis* to human epithelial cells. In this chapter, we characterize MclS and CL to further understand their roles in *M. catarrhalis*.

While MclS does not mediate adherence directly, we hypothesized that its product, CL, may impact the function of adhesins on the bacterial surface. Our initial focus was on five major proteinaceous adhesins, which we ultimately found were both expressed and surface-displayed in the CL-deficient strains [244]. Further analysis of the impact of CL on these adhesins can be found in the Appendix. However, *M. catarrhalis* is known to express additional factors that have been implicated in adherence, and lipoligosaccharide (LOS) is one such factor [81-82]. In Section I, we examined if LOS expression is impacted by the presence of CL.

We were also interested in investigating a possible relationship between CL and the gene located immediately downstream of *mclS* (termed MCORF 821). MCORF 821 is predicted to encode a protein with significant similarity to ProP, an osmoregulator expressed by numerous bacterial species [244]. It has been shown that the localization and function of ProP of *Escherichia coli* is dependent on CL [191]. Therefore, Section II describes several experiments designed to determine if the function of MCORF 821 is dependent or impacted by CL.

During the characterization of MclS in our original study, we uncovered several properties that differentiate MclS from other bacterial CLSs. For example, MclS is predicted to be a lipoprotein, a characteristic that appears unique for a CLS [244]. Therefore, in Section III we determined if *mclS* activity is dependent on lipidation.

While the mechanism by which CL impacts adherence has not yet been elucidated, we hypothesize that CL may impact the function of adhesins found on the outer membrane. For CL to affect adhesins in such a manner, it would likely require the presence of CL within the outer membrane. Through the collection and examination of outer membrane vesicles (OMVs), we examined the phospholipid (PL) composition of the outer membrane of *M. catarrhalis* in Section IV.

Finally, a recent publication described the discovery of a new type of CLS, one which utilizes phosphatidylethanolamine (PE) for synthesis of CL [159]. Based its sequence, MclS is most similar to this new type of CLS. In Section V we utilized a genetic approach to determine if MclS also utilizes PE for CL synthesis.

Section I: Lipooligosaccharide Expression may be Dependent on Cardiolipin in *Moraxella catarrhalis*

We have previously demonstrated that CL contributes to the adherence of *M. catarrhalis* to human epithelial cells and hypothesize that CL is required for the expression or display of known adhesins in the outer membrane. Initially, we tested the CL-deficient strains for expression of five known adhesins (UspA1, Hag, OMP CD, McaP, MhaB1/MhaB2). Each of the five adhesins was expressed in the outer membrane of strains both possessing and devoid of CL (Figures 3.S4, 3.S5). However, *M. catarrhalis* is known to express several other adhesins. Of

note is LOS, which has been linked to adherence to epithelial cells in a variety of bacteria [81-82, 87, 89-91].

LOS is a major component of the outer membrane of *M. catarrhalis*. Though similar to the more-common lipopolysaccharide (LPS), LOS does not possess the repeating O-antigen [245-246]. LOS has been shown to contribute to the adherence of *M. catarrhalis* to human epithelial cells, though the impact appears to be indirect [81-82]. LOS is located on the outer leaflet of the outer membrane and directly interacts with proteins on the bacterial surface [247]. Due to its localization and known role in adherence, we hypothesized that the absence of CL within the *M. catarrhalis* membrane alters the expression and function of LOS leading to a decrease in adherence to epithelial cells.

To test whether expression of LOS is affected by CL, we created whole-cell lysates (WCLs) and LOS preparations from the *wild-type* (WT) and CL-deficient (.mclS) strains previously generated in isolates O35E, O12E, and McGHS1 [244]. WCLs and LOS preparations were then analyzed by western blot to detect LOS in each of the strains.

The WCLs were performed as previously described [230]. Briefly, the *M. catarrhalis* strains were propagated on Todd Hewitt (TH) agar at 37°C for 24 hours and resuspended in NuPAGE LDS Sample Buffer (Novex® Life Technologies™). The LOS preparations were obtained following a procedure adapted from Jones et al [248]. The *M. catarrhalis* strains were propagated on TH agar at 37°C for 24 hours and resuspended in Lysis Buffer containing 0.06 M Tris base (Fisher Scientific®), 10 nM EDTA (Fisher Scientific®), 2% sodium dodecyl sulfate (SDS, vol/vol, Fisher Scientific®) at pH 6.8. The reaction was incubated at 100°C for 10 minutes. After cooling, proteinase K (Fisher Scientific®) was added at a concentration of 750

ng/µL. The reaction was incubated overnight at 37°C. One-tenth volume of 3M sodium acetate (pH 5.2, Fisher Scientific®) and two volumes of ice-cold 100% ethanol were added, and the reaction was placed at -80°C for 1 hour. Following incubation, the reaction was centrifuged at 15,000 x g for 5 minutes. The pellet was washed twice with 70% ethanol and resuspended in distilled water.

Western blots were performed as previously described [230, 244]. Protein loads were equalized based on SimpleBlue™ Safe Stain (Invitrogen™ Life Technologies™) and resolved by SDS polyacrylamide gel electrophoresis (PAGE). The membranes were probed with a 1:100 dilution of either monoclonal antibody (mAb) 4G5 or mAb 3F7, both of which were gifted to us by Anthony Campagnari. The mAb 4G5 binds LOS from all three *M. catarrhalis* serotypes (A, B, and C) while mAb 3F7 exhibits specificity for type B LOS [78, 80]. Following incubation with a 1:10,000 dilution of goat anti-mouse Ig (H+L)-horseradish peroxidase (HRP) secondary antibody (SouthernBiotech). Signals were detected using Luminata™ Crescendo Western HRP Substrate (EMD Millipore) and the Foto/Analyst Luminary/FX imaging system (Fotodyne Inc).

LOS from the O12E and McGHS1 strains was detected in both the WCL (Figure 4.1, left panel) and LOS preparations (Figure 4.1, right panel), suggesting that LOS was successfully isolated and that the mAbs were specific for LOS. Specifically, the O12E strains were recognized by mAb 3F7 (Figure 4.1, top row) while the McGHS1 strains were recognized by mAb 4G5 (Figure 4.1, bottom row). However, the O35E strains were not detected by either mAb 4G5 or mAb 3F7. In the instances in which LOS was detected, the size of LOS did not vary between the WT and CL-deficient strains (Figure 4.1; lanes 1-4, 7-10) suggesting that processing of LOS is not impacted by CL. For O12E, the expression of LOS in the CL-deficient strain appears to be reduced compared to the WT (Figure 4.1; lanes 1-2, 7-8). For McGHS1, this

is not the case; the amount of LOS in the WT and CL-deficient strains appears to be equivalent (Figure 4.1; lanes 3-4, 9-10). Although protein loads were equalized based on staining, a loading control is necessary to draw conclusions based on the intensity of LOS bands.

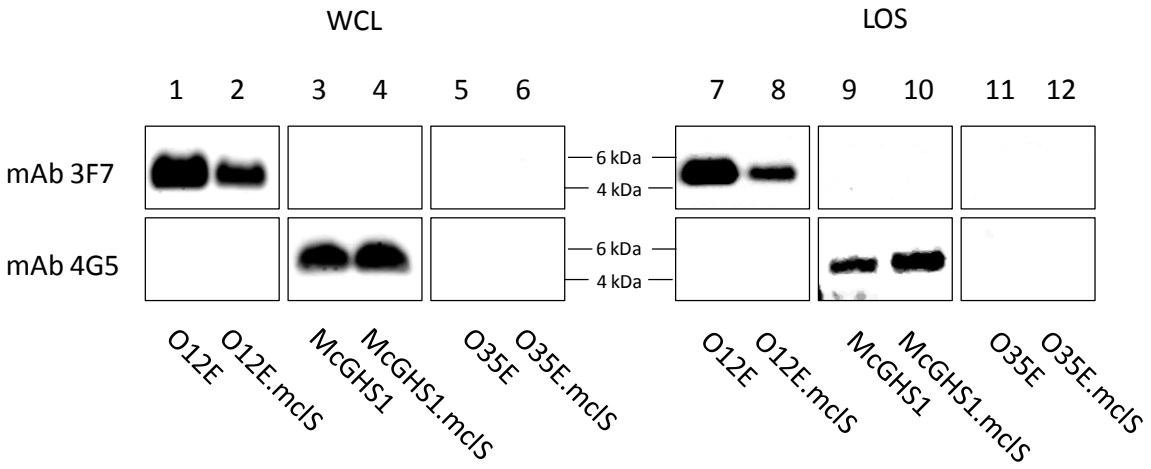


Figure 4.1. CL-deficiency reduces expression of LOS in isolate O12E but not McGHS1. Western blots were performed to detect LOS in WCLs (left panel) and LOS preparations (right panel) of strains O12E (lanes 1-2, 7-8), McGHS1 (lanes 3-4, 9-10), and O35E (lane 5-6, 11-12) using mAb 3F7 (top row) and mAb 4G5 (bottom row).

The serotypes of isolates O12E and McGHS1 have not been previously determined. Based on our data, O12E is of serotype B since the O12E strains reacted with LOS type B-specific mAb 3F7. Since the McGHS1 strains were not recognized by the mAb 3F7, it appears that McGHS1 expressed LOS of serotype A or C. Validation of these findings could be accomplished by use of a multiplex PCR that has been developed for this purpose [249].

Our finding that mAb 4G5 did not react with either the O35E or O12E strains was puzzling. It has been shown previously that LOS from isolate O35E is detected by mAb 4G5 [77, 79]. The contrasting data may be explained by our use of horseradish peroxidase (HRP)-labeled goat α-mouse Ig (H+L) as the secondary antibody, whereas the previous studies utilized HRP-labeled protein A [78]. Interestingly, our secondary antibody still permitted detection of LOS from isolate McGHS1. In future studies, we will use protein A to test if LOS is detected in western blots probed with mAb 4G5.

According to the western blot data, it appears that the CL-deficient strain of O12E expresses LOS at a reduced level compared to the WT isolate (Figure 4.1; lanes 1-2, 7-8). However, the two McGHS1 strains express approximately the same level of LOS (Figure 4.1; lanes 3-4, 9-10). Since we were interested in whether or not the reduction in adherence to epithelial cells in CL-deficient strains of *M. catarrhalis* was attributed to alteration of LOS expression, there does appear to be a correlation. In our previous study, the reduction in adherence was much greater between the WT and CL-deficient strains of O12E compared to the WT and CL-deficient strains of McGHS1 [244]. The greatest decrease in adherence was observed for the WT and CL-deficient strains of O35E, so detection of LOS in these strains would strengthen the case for a role of CL in LOS expression.

Before conclusions can be drawn regarding the level of LOS in any of the strains, a loading control must be performed to demonstrate that equal amounts of extract are present in each lane. The loading control would detect the presence of a constitutively expressed protein in each of the strains (e.g. McaP as demonstrated in Figure 3.S4B) and confirm that any observed difference in LOS level is not due to variation in loading volumes. A method such as flow cytometry could also be used to verify and quantify our data. Overall, more research is

necessary before a definitive conclusion can be reached regarding the impact of CL on LOS expression.

Section II: MCORF 821 is not a Positive Effector of Adherence in *Moraxella catarrhalis*

Previously, we found that MclS is responsible for the synthesis of CL in *M. catarrhalis* and that CL contributes to the ability of the bacterium to adhere to human epithelial cells. However, the direct mechanism by which MclS impacts adherence had not been determined [244]. Here we examine whether or not the phenotypes observed in the CL-deficient strains of *M. catarrhalis* are due to the altered function of a protein encoded by the gene located downstream of *mclS*.

According to BlastP (NCBI), the gene located immediately downstream of *mclS*, termed MCORF 821 due to its location in strain ATCC 43617, is predicted to encode for a member of the Major Facilitator Superfamily (MFS). Proteins within this family transport small molecules across membranes by utilizing the electrochemical potential of the substrates [250]. More specifically, MCORF 821 has significant similarity to ProP of several species including *E. coli* (80% coverage, E-value 6 e-46, 36% identity). ProP is an integral membrane protein that senses osmotic pressure in the environment and transports proline and glycine betaine across the membrane to stabilize the bacterial cell [251]. Interestingly, numerous studies have functionally linked ProP to CL in *E. coli*. The activity of ProP requires polar localization, and this localization is dependent on CL [191]. The functional relationship between CL and ProP may explain why CL-deficient strains of *E. coli* exhibit reduced survival in conditions of osmotic stress [252]. ProP has also been associated with virulence as it is required by uropathic *E. coli* for colonization of the urinary tract [253].

While the locations of *proP* and the CLS-encoding genes are genetically distant in *E. coli*, MCORF 821 is located immediately downstream of *mclS* in *M. catarrhalis*. In our earlier study, we demonstrated that the *mclS* mutations in the CL-deficient strains of *M. catarrhalis* did not reduce transcription of MCORF 821. However, the lack of CL in the CL-deficient strains may affect the localization and/or activity of the MCORF 821 gene product. To examine if changes in the function of the MCORF 821 gene product are responsible for adherence defect observed in the CL-deficient strains of *M. catarrhalis*, we generated an insertion mutation in MCORF 821 of isolate O35E and examined this mutant strain for adherence to human epithelial cells.

To create the MCORF 821 mutant strain in *M. catarrhalis*, we cloned MCORF 821 from strain O35E, inserted it into plasmid pCC1™ (Epicentre® Illumina®), interrupted it with an antibiotic resistance cassette, and incorporated the mutation into O35E via homologous recombination. All bacterial strains were propagated and standard molecular biology techniques were performed as described previously [244]. Oligonucleotide primers P1 and P2 (Table 4.2) were designed to amplify MCORF 821 including 270 base pairs upstream by PCR (*Pfx* DNA Polymerase, Invitrogen™ Life Technologies™). The amplicon was treated with End-It™ DNA End Repair Kit (Epicentre® Illumina®) to generate blunt ends, ligated into pCC1™ using the CopyControl™ PCR Cloning Kit (Epicentre® Illumina®), and transformed into EPI300™ *E. coli* (Epicentre® Illumina®). Next, we linearized pCC1.821 with *XhoI* (New England BioLabs®) and inserted the Zeocin™ resistance cassette extracted from the ZeoCassette™ vector pEM7/Zeo (Invitrogen™ Life Technologies™). The ligation reaction was transformed into EPI300™ *E. coli*. The resulting plasmid, pCC1.821.zeo, was naturally transformed into *M. catarrhalis* isolate O35E, and the insertion mutation was incorporated via homologous

recombination to generate strain O35E.821[244] . The Zeocin™ resistant transformants were selected, and their identity was confirmed by PCR (*Pfx* DNA Polymerase, Invitrogen™ Life Technologies™) and DNA sequencing (University of Michigan Sequencing Core).

Since ProP is required for survival of *E. coli* under osmotic stress, we examined growth of strain O35E.821 in media containing high concentrations of osmolytes. The WT isolate O35E and mutant strain O35E.821 were grown on TH agar, resuspended in TH broth, and used to generate a 50 Klett culture in TH broth supplemented with either sodium chloride (12 g/L or 36 g/L, wt/vol, Fisher Scientific®) or D-sorbitol (0.1 M or 1.0 M, Sigma Life Science™). Broth cultures were incubated at 37°C for up to 7 hours with shaking. The optical density of each culture was determined hourly by use of a Klett™ colorimeter (Scienceware®). Growth curves were performed on two separate occasions.

According to the growth curves, there was no difference between the WT O35E isolate and the mutant strain O35E.821 under conditions of high osmotic stress due to sodium chloride (Figure 4.2A) or sorbitol (Figure 4.2B). The growth curves were extended to 24 hours (data not shown), yet there was still no difference in growth between the strains. It should be noted that growth of both *M. catarrhalis* strains was inhibited as osmolality increased (Figure 4.2). The finding that MCORF 821 is not required for survival under osmotic pressure is in contrast to reports studying ProP in *E. coli*, where ProP is a known osmoregulator. There are several possible explanations for this data. It is conceivable that *M. catarrhalis* expresses other osmosensory transporters that compensate for the loss of MCORF 821. It is also possible that, although its sequence is similar to ProP, MCORF 821 may not be a homolog or perform the same functions. Finally, MCORF 821 may not be expressed under the conditions tested. While we have detected and measured transcript level of MCORF 821, we could develop antibodies

specific for the product of MCORF 821 to examine protein expression since such antibodies are not currently available.

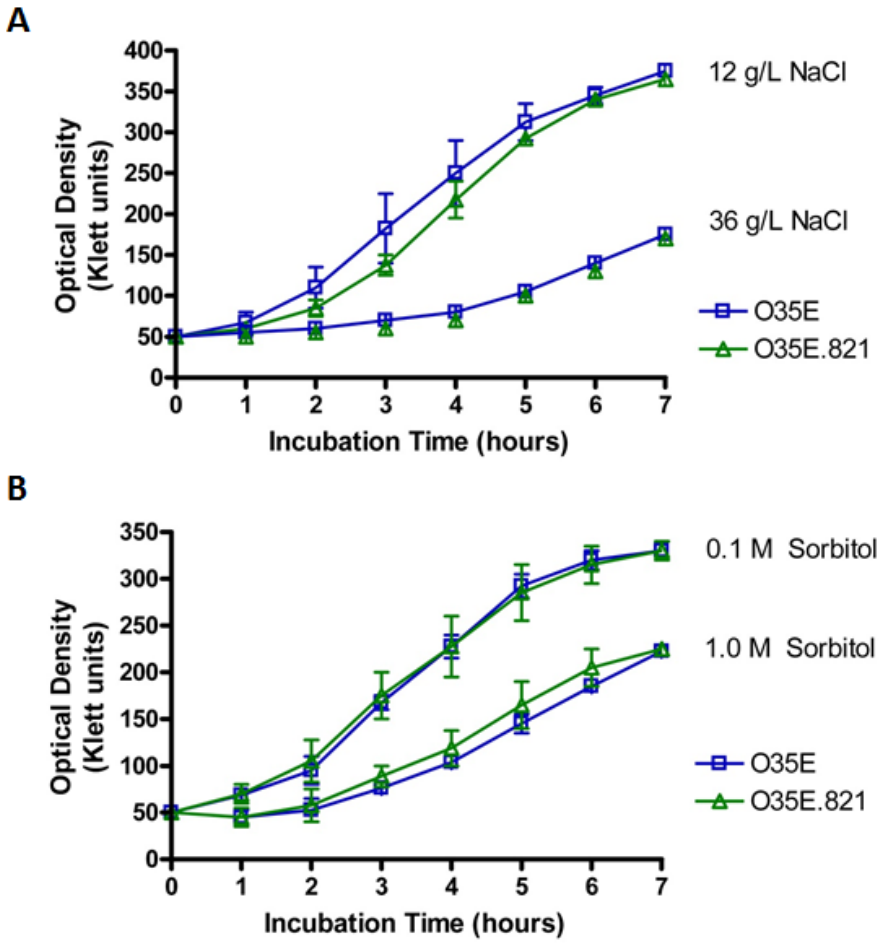


Figure 4.2. Mutation of MCORF 821 does not affect growth of *M. catarrhalis* under osmotic stress. Growth curves of O35E (blue squares) and O35E.821 (green triangles) when grown in TH broth containing 12 g/L sodium chloride (A, upper), 36 g/L sodium chloride (A, lower), 0.1 M sorbitol (B, upper), or 1 M sorbitol (B, lower).

Next, the O35E.821 mutant strain was tested for adherence to human epithelial cells. The quantitative adherence assay was performed as previously described [244]. Briefly, a monolayer of HEp-2 cells (ATCC CCL-23), laryngeal in origin, were seeded onto a 24-well tissue culture plate. The HEp-2 cells were inoculated with either O35E or O35E.821 at a multiplicity of infection of 100:1. Following a 30 minute incubation, the cells were washed to remove non-adherent bacteria. Bound bacteria were released by addition of a saponin-EDTA solution and plated on TH agar for enumeration. Percent adherence was calculated by dividing the number of adherent bacteria by the inoculum. The adherence assay was performed in triplicate on three occasions.

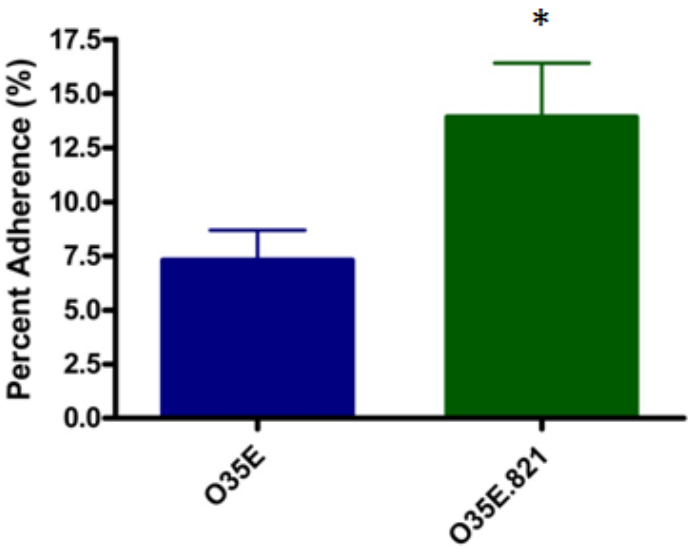


Figure 4.3. Mutation of MCORF 821 leads to an increase in adherence of *M. catarrhalis* to human epithelial cells. Shown are the results of the quantitative adherence assays of *M. catarrhalis* strains O35E (blue) and O35E.821 (green). The results are expressed as the mean percentage (± standard error) of inoculated bacteria binding to HEp-2 epithelial cell monolayers after 30 minute incubation. An asterisk indicates a statistically significant difference in adherence compared to the WT O35E isolate as determined by the Mann Whitney test ($P < 0.05$, GraphPad Prism® 4 software).

If the putative ProP gene product of MCORF 821 does require CL for function, we would expect O35E.821 to exhibit reduced levels of adherence compared to WT O35E since the CL-deficient bacteria exhibit an adherence defect. Surprisingly, however, the O35E.821 strain actually adhered at higher levels than the WT O35E isolate (Figure 4.3). This demonstrates that MCORF 821 is not a positive effector for *M. catarrhalis* adherence to human epithelial cells.

We should note that according to the quantitative reverse transcription PCR performed in our previous study, transcription of MCORF 821 was increased in the CL-deficient strains. Therefore, it is possible that MCORF 821 is a negative regulator of adherence since increased levels of adherence have resulted following up-regulation of MCORF 821 (as in the CL-deficient strains O35E.mclS and O35E.mclS.K438R) while decreased levels of adherence have resulted in the absence of MCORF 821 (as in O35E.821). To determine if MCORF 821 is indeed a negative effector of adherence, we could either overexpress MCORF 821 in *M. catarrhalis* or recombinantly express MCORF 821 in a heterologous bacterium (e.g. non-adherent *E. coli*) by introducing an appropriate promoter preceding the ORF. Analysis of these strains would provide insight into the role of MCORF 821 in both osmoregulation and adherence and its possible interaction with CL.

Section III: MclS does not Require Lipidation of Cysteine for Cardiolipin Synthase Activity

The CLS of *M. catarrhalis*, MclS, is responsible for the biosynthesis of the CL [244]. While CLSs have been studied in a number of bacterial species, MclS has been shown to possess a few unique characteristics. Of particular interest, several bioinformatics programs predict MclS to contain an N-terminal signal sequence followed by a lipid-attachment site [161, 244]. In this study we aimed to determine if lipidation is required for the activity of MclS.

Analyses of the MclS amino acid sequence by the ProSite [254], Phobius [255], and LipoP 1.0 [256] algorithms predict an N-terminal lipoprotein signal sequence spanning amino acids 1-23, a signal peptidase II cleaveage site between amino acids 23 and 24, and lipidation (either N-palmitoyl or S-diacylglyercol) of the cysteine residue at amino acid 24 (C24). Of the prokaryotic organisms in which CLS have been characterized, there has been no mention of a putative lipid-attachment site within any of the CLS proteins. *E. coli*, the prokaryotic model in which CLS enzymes have been best studied, expresses three types of CLS. ClsA is predicted to contain two N-terminal transmembrane helices (TMH); meanwhile, prediction programs do not recognize any sort of membrane attachment domains in ClsB and ClsC [159].

Several lipoproteins have been identified and characterized in *M. catarrhalis*. A total of 88 putative lipoproteins have been identified in *M. catarrhalis* based on the Prosite database [257]. The BRO β-lactamases, which cleave the β-lactam ring and confer resistance β-lactam antibiotics, have been verified as lipoproteins [35] in addition to OMP G1a [258], TbpB [259], and Msp22 [257].

Lipoproteins are formed when a pre-protein containing an N-terminal signal sequence is covalently attached to a lipid through a thioester linkage with a conserved cysteine residue [260]. Following lipid-attachment, the signal sequence is cleaved by a signal peptidase yielding the final product of a lipoprotein [261]. Only cysteine residues have been shown to be lipidated in bacteria, and C24 is predicted to be the only cysteine residue in MclS [262]. To test our hypothesis that MclS requires lipidation for CLS activity, we generated a mutant strain in which C24 was replaced with a serine residue (C24S) that results in the substitution of a thiol group for a hydroxyl group. The resulting strain, O35E.mclS.C24S, would be expected to lack CL if lipidation of MclS is required for its CLS activity [263].

To create a strain of *M. catarrhalis* with the C24S mutation in *mclS,* we cloned the *mclS* gene into the plasmid pCC1, introduced the C24S point mutation into the plasmid-born copy of *mclS,* and swapped the C24S point mutation into the *mclS* locus of O35E.mclS by homologous recombination. All bacterial strains were propagated and standard molecular biology techniques were performed as previously described [244]. A region of the *mclS* gene spanning from 250 base pairs upstream to 175 base pairs downstream of the open reading frame (ORF) was amplified by PCR (*Pfx*) using primers P3 and P4 (Table 4.2). The amplicon was treated with End-It™ DNA End Repair Kit to generate blunt ends, ligated into pCC1™ using the CopyControl™ PCR Cloning Kit, and transformed into EPI300™ *E. coli*. The resulting plasmid pCC1.mclS.fl was sequenced to verify that no mutations were introduced during PCR. A point mutation was incorporated into the putative lipidation site of the *mclS* gene within pCC1.mclS.fl using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Mutagenesis primers P5 and P6 (Table 4.2) were designed to introduce adenine in place of thymine at nucleotide position 70 of the *mclS* ORF, thereby changing the TGC codon (cysteine) to AGC (serine). The mutagenesis reaction was transformed into EPI300™ *E. coli,* and the resulting plasmid pCC1.mclS.fl.C24S was sequenced to verify that only the intended mutation (i.e. cysteine at position 24 to serine, $C^{24}\rightarrow S$) was introduced in the *mclS* gene. The point mutation was then introduced into the O35E.mclS mutant strain by congression as described previously described [62, 244]. Streptomycin resistant and spectinomycin sensitive transformants were screened by PCR (*Taq*) to verify that the C24S version of *mclS* replaced the spectinomycin resistance cassette within O35E.mclS. The *mclS* locus of the resulting O35E.mcls.C24S strain was sequenced to confirm that only the intended mutation was introduced into the gene.

According to our hypothesis, we believe that MclS is lipidated at C24 and that MclS requires this lipidation for synthesis of CL. To test the M. catarrhalis strains for the presence of CL, we extracted phospholipids from WT isolate O35E and the point mutant strain O35E.mclS.C24S and analyzed the samples by thin layer chromatography (TLC). Phospholipids were extracted by the Bligh-Dyer method as previously described [244]. Briefly, solutions were added to the isolated OMVs in the following order: 1:2 (vol/vol) chloroform:methanol (Acros Organics®; Fisher Scientific®), chloroform (Fisher Scientific®), distilled water. The mixture was centrifuged and the bottom layer was collected. The solvent was evaporated over a stream of nitrogen gas (AirGas®), the dried PLs were resuspended in 1:2 methanol:chloroform, and the samples were stored at -20˚C. Phospholipids were separated and visualized by TLC as previously described [244]. Briefly, PLs were spotted onto high performance TLC plates (Analtech), separation by a mobile phase consisting of 65:25:10 chloroform:methanol:acetic acid (Fisher Scientific®), and visualized with Molybdenum Blue spray reagent (Sigma Life Science™). Phospholipids spots were identified by comparison with the following standards: PE, phosphatidylglycerol (PG), and CL (Sigma Life Science™).

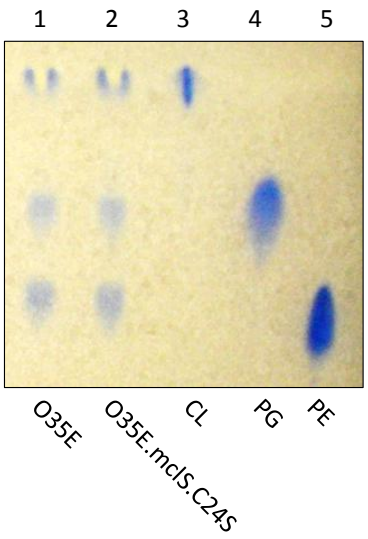


Figure 4.4. Cardiolipin is still synthesized in *M. catarrhalis* despite the C24S mutation of *mclS*. Analysis of the phospholipid profile of *M. catarrhalis* strains O35E (lane 1) and O35E.mclS.C24S by TLC. Spots were identified by comparison to purified CL, PG, and PE standards (lanes 3, 4, and 5, respectively).

As expected, the WT strain contains detectable levels of CL (Figure 4.4, lane 1). However, a similar amount of CL is also detected in the O35E.mclS.C24S mutant strain (Figure 4.4, lane 2). From this data, we conclude that the C24S mutation within MclS did not affect the CL synthesis in *M. catarrhalis*. Our findings demonstrate that lipidation of C24 is not required for the CLS activity of MclS. According to the current literature, only cysteine residues are lipidated in bacteria [262]. Even though O-linked lipoproteins have not yet been identified in bacteria, it is theoretically possible that lipidation could occur at a serine residue. Only a single study has collected data suggesting the possibility of O-linked lipoproteins in bacteria [264]. Therefore, while unlikely, the C24S mutation may still allow for lipidation. In order to eliminate all possibility of lipidation at this site, we could replace C24 with a small amino acid lacking

thiol and hydroxyl side chains (e.g. alanine). Alternatively, we could adapt the procedure from Bootsma et al. that had previously demonstrated that BRO-1 is a lipoprotein. The procedure would involve labeling the O35E and O35E.mclS.C24S strains with [^{3}H] palmitic acid, followed by SDS-PAGE and fluorometery to demonstrate lipidation of *mclS* [35]. Finally, lipidation of MclS could be elucidated using mass spectroscopy following purification of MclS.

It would also be advantageous to determine the localization of MclS. If our future work demonstrates that MclS is not a lipoprotein, membrane attachment could still be achieved through the presence of an N-terminal TMH as predicted by TMHMM 2.0 [265] and TMpred [266]. However, MclS is not necessarily membrane-bound even though MclS acts on PLs. Ideally, we would determine localization of MclS by western blot using antibodies specific for MclS. However, previous attempts to detect MclS in *M. catarrhalis* by western blot using polyclonal sera from mice immunized with His-tagged MclS were unsuccessful (data not shown). Therefore, we have begun to generate constructs which will insert His-, myc-, and FLAG-tags onto the C-terminus of MclS. Commercially available antibodies will then be used to detect the tagged proteins within *M. catarrhalis* following fractionation and OMV preparation. If we find that MclS is lipidated and localized to the membrane, generation of the C24S mutation in one of the MclS-tagged constructs would allows us to determine if lipidation is required for membrane attachment.

Section IV: Cardiolipin is Present in the Outer Membrane of *Moraxella catarrhalis*

CL is a ubiquitous phospholipid found in all domains of life. It is almost exclusively found in energy-transducing membranes including the mitochondrial membranes of eukaryotes and the plasma membrane of prokaryotes. The reason why CL is located predominantly in these

location is unknown, although it has been shown that CL binds to and is required for the function of several proteins involved in ATP production [186-187]. While the majority of CL in bacteria is localized to the plasma membrane, it has been detected in the outer membrane of *Salmonella enterica* serovar *typhimurium* [176] and OMVs of Enterotoxigenic *E. coli* [175].

Adherence of *M. catarrhalis* to human epithelial cells is affected by CL [244], and while the mechanism has not been elucidated, we hypothesize that CL impacts the function of adhesins found on the outer membrane. For CL to impact adhesins in such a manner, it would likely require the presence of CL within the outer membrane. Detection of CL within the outer membrane of *M. catarrhalis* would support further investigation of the localization and function of adhesins in CL-deficient strains.

Like many other Gram-negative bacteria [267], *M. catarrhalis* releases OMVs, 50-150 nm in diameter [93], from its surface into the environment, a process also known as blebbing. *In vitro* studies revealed that release of OMVs confer serum resistance and antibiotic resistance to heterologous species through UspA1/UspA2 and BRO lactamases, respectively [37, 93]. The biological relevance of OMV production has been confirmed by the detection of *M. catarrhalis* OMVs in sputum from patients with respiratory infections [268] and a child with sinusitis [93].

For our purposes, the isolation of OMVs is a convenient method to study the composition of the outer membrane. In 1989, Murphy and Loeb developed a procedure for the isolation of OMVs following exposure of *M. catarrhalis* to heat and EDTA [231]. The purity of the preparations was confirmed through isotopic labeling, and the procedure has been used in several studies since [45, 53, 57, 60, 92, 269]. Using mass spectroscopy, researchers have identified 57 different proteins that compose *M. catarrhalis* OMVs, many of which had predicted to localize

to the outer membrane [270]. In addition, LOS, a major component of the *M. catarrhalis* outer membrane, is also present in the OMV preparations [51, 270-271]. Therefore, if the procedure is effective at selectively isolating outer membrane proteins and LOS through OMVs, then we believed the method may be capable of isolating phospholipid constituents as well.

In our previous study, *mclS* mutants were generated in strain O35E. An insertion mutant (O35E.mclS) and a point mutant (O35E.mclS.K438R) were unable to synthesize CL while the repaired strain (O35E.mclS.repaired) accumulated CL at WT-levels [244]. To examine the phospholipid composition of *M. catarrhalis* OMVs, we subjected each of these strains to the procedure originally described by Murphy and Loeb [231]. Cultures were propagated in 10 mL of TH broth at 37° for 8 hours with shaking (200 rpm). The cultures were then added to 500 mL of sterile TH broth and incubated at 37° for 16 hours with shaking (200 rpm). The next day, the cultures were placed on ice for 15 minutes before being centrifuged at 4°C for 15 minutes at 7,500 x g. Supernatant was decanted, and the pellets were resuspended in 10 mL of EDTA Buffer [50 mM Na_2HPO_4 (Fisher Scientific®), 150 mM NaCl (Fisher Scientific®), 0.01 M EDTA (Fisher Scientific®), pH 7.4]. Clumps were broken up using a glass homogenizer. The bacterial suspensions were added to flasks with glass beads lining the bottom and incubated at 50°C for 45 minutes with shaking (300 rpm). The contents were transferred to a centrifugation tube along with two 5 mL washes of the beads with EDTA Buffer. The tubes were centrifuged at 12,000 x g for 15 minutes at 4°C to pellet the bacteria. The supernatant was transferred to a new tube and centrifuged at 38,000 x g for 90 minutes at 4°C to collect the OMVs. The supernatant was discarded, and the OMV-containing pellet was resuspended in 100 µL phosphate buffered saline (Fisher Scientific®) and stored at -20°C. Upon isolation of the OMVs, phospholipids were extracted and analyzed by TLC as described earlier in this chapter.

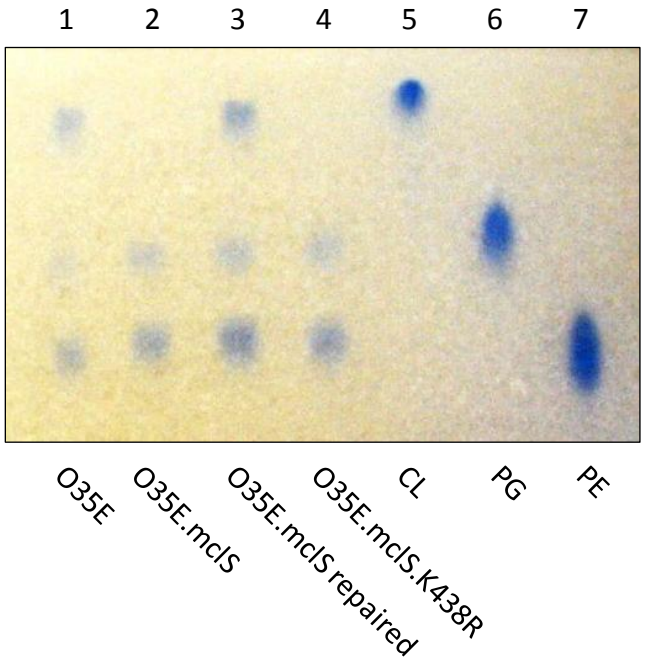


Figure 4.5. CL is present in the OMVs of *M. catarrhalis*. Analysis of the phospholipid profile of OMVs isolated from *M. catarrhalis* strains O35E (lane 1), O35E.mclS (lane 2), O35E.mclS repaired (lane 3), and O35E.mclS.K438R (lane 4) by TLC. Spots were identified by comparison to purified CL, PG, and PE standards (lanes 5, 6, and 7, respectively).

In the OMVs isolated from WT strain O35E, three phospholipids (PE, PG, and CL) were detected (Figure 4.5, lane 1). However, in the CL-deficient strains O35E.mclS (insertion mutant) and O35E.mclS.K438R (point mutant), only PE and PG were detected in the OMVs (Figure 4.5; lanes 2 and 4, respectively). These results suggest that along with PE and PG, CL is a major constituent of the *M. catarrhalis* outer membrane.

Although the OMVs were isolated using a procedure that has been validated in the literature [45, 53, 57, 60, 92, 269], it would be worthwhile to examine the purity of the preparations performed in this particular study. While not performed here, we consistently detect known adhesins in OMV preparations (see figure 3.S4B), thereby demonstrating that the procedure is effective at harvesting outer membrane proteins. To ensure that the OMVs do not contain contaminants from the inner membrane, we could perform a western blot using antibodies specific for a known inner membrane protein. However, we are unaware of any antibodies currently available with specificity for an *M. catarrhalis* inner membranes protein. To obtain such antibodies, we could collect sera from mice immunized with a purified *M. catarrhalis* inner membrane recombinantly expressed in *E. coli*. Potential inner membrane proteins to target would be the SecYEG components of the Sec translocon.

Our data, paired with the previous findings that *E. coli* and *Salmonella* also possess CL in their outer membrane [175-176], bring into question the claim that CL is found almost exclusively in the plasma membrane of bacteria. Additionally, the detection of CL in the outer membrane may help to explain the adherence defect exhibited by CL-deficient strains *M. catarrhalis*. CL may be crucial for the function of adhesins on the bacterial surface. While several major adhesins (UspA1, Hag, McaP, OMP CD, MhaB1/2) have been shown to be expressed and surface-displayed in the CL-deficient strains, further research is needed to examine if these proteins are correctly localized and conformed or if other adhesins may be affected.

Section V: Genetic Approaches to Determine Substrates of MclS are Unsuccessful due to Requirement of Enzymes involved in Phosphatidylethanolamine Synthesis

The enzyme responsible for CL synthesis in *M. catarrhalis*, MclS, has been described previously [244]. While we know that CL is the product of the reaction catalyzed by MclS, the substrates have not been identified. Until recently, all bacterial CLSs were thought to synthesize CL from two molecules of PG [174]. However, a study found that *E. coli* expresses a CLS that instead utilizes a molecule of PG and a molecule of PE as the substrates for the reaction [159]. With this discovery, the authors re-named the three CLS expressed by *E. coli*. The enzyme that utilizes two molecules of glycerol is termed ClsA, and the enzyme that utilizes a single molecule of PG and a molecule of PE is termed ClsC. The substrates for the third type of CLS (ClsB) have not been identified [272].

The discovery of a new type of CLS prompted us to investigate the substrates of MclS. The NCBI BlastP algorithm found that MclS most closely resembles the ClsC of *E. coli* (89% coverage, E value of 3e-108, 39% identity), though all three *E. coli* enzymes have high levels of similarity so it is not possible to identify the substrates simply through sequence analysis. We utilized a genetic approach to determine the substrates of MclS. To do so, we utilized the knowledge that PG and PE are synthesized through independent pathways in bacteria (Figure 1.1). While both ClsA and ClsC utilize PG, only ClsC-type utilizes PE. Therefore, if we generate a PE-deficient strain of *M. catarrhalis*, we should be able to determine the type of CLS by observing the presence or absence of CL. If CL is present in the PE-deficient strain, then MclS is a ClsA-type CLS. If CL is absence in the PE-deficient strain, then MclS is a ClsC-type CLS.

The enzymes responsible for PE synthesis have been well-studied, and we identified the homologs in the *M. catarrhalis* genome. As shown in Figure 4.6, phosphatidylserine (PS) is synthesized from cytidine diphosphate-diacylglycerol (CDP-DAG) by PS synthase A (PssA) and converted to PE by PS decarboxylase (Psd). In *M. catarrhalis*, the *psd* gene, which corresponds to MCORF 700 from strain ATCC 43617, contains an 831 base pairs ORF and is predicted to encode for a PS decarboxylase with a molecular weight of ~31 kDa. According to NCBI BlastP, the closest known homolog is from *Psychrobacter arcticus* strain 273-4 (97% coverage, E value 5e-134, 66% identity). The *pssA* gene of *M. catarrhalis*, which corresponds to MCORF 174 from ATCC 43617, contains an 807 base pairs ORF and is predicted to encode for a CDP-DAG-serine--O-phosphatidyltransferase with a molecular weight of ~29 kDa. According to NCBI BlastP, the closest known homolog is from *Psychrobacter arcticus* strain 273-4 (100% coverage, E value 1xe-119, 65% identity).

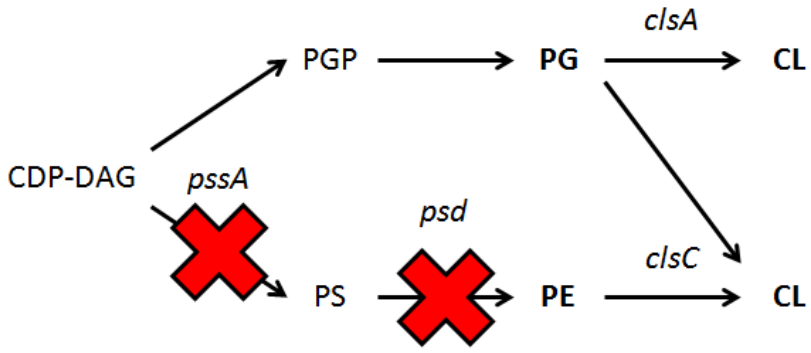


Figure 4.6. Genetic approach to ablate PE synthesis by mutagenesis of *pssA* or *psd*. Shown are the biosynthetic pathways of PG, PE, and CL as studied in *E. coli*.

Our first attempt to generate a PE-deficient strain of *M. catarrhalis* involved creating a *psd* isogenic mutant strain in isolate O35E. The *psd* ORF was cloned into pCC1™ using methods described previously [244]. Briefly, oligonucleotide primers P1 and P2 (Table 4.2) were designed to amplify the *psd* gene from O35E. The amplicon was treated with End-It™ DNA End Repair Kit to generate blunt ends, ligated into pCC1™ using the CopyControl™ PCR Cloning Kit, and transformed into EPI300™ *E. coli*. The reaction was used to transform TransforMax™ EPI300™ *E. coli*, yielding the plasmid pCC1.psd.

Next, the EZ-Tn5™ <KAN-2> Insertion Kit (Epicentre® Illumina®) was used to randomly insert a kanamycin resistance cassette in pCC1.psd. The reaction was used to transform TransforMax™ EPI300™ *E. coli*, yielding various forms of the plasmid pCC1.psd.kan. To identify pCC1.psd.kan in which the kanamycin resistance cassette was inserted into the *psd* ORF, the region was amplified by primers P7 and P8 (Table 4.2). We selected the plasmids which produced an amplicon of ~2.3 kb, the expected size of the *psd* gene plus the kanamycin resistance cassette. Next, primers P7, P8, P9, and P10 (Table 4.2) were used to amplify the region in order to determine the location of the kanamycin resistance cassette within the psd ORF of pCC1.psd.kan. A plasmid was chosen in which the kanamycin resistance cassette is centrally located within the *psd* ORF.

However, transformation of *M. catarrhalis* isolate O35E with pCC1.psd.kan did not result in any kanamycin resistant transformants. It has been reported that *psd* is essential for some organisms and that growth of PE-deficient strains required supplementation [242, 273-274]. Therefore, we supplemented TH agar with $MgCl_2$ (50 µM, 500 µM, 5 mM, and 50 mM; Fisher Scientific®), ethanolamine (100 µM, 1 mM, and 1 mM Fisher Scientific®), or PE (2.5 µg/mL, Sigma Life Science™) [275]. No kanamycin resistant transformants resulted when

plating on supplemented media. The lack of transformants prompted us to conclude that *psd* may be an essential gene for *M. catarrhalis*. It is possible that *M. catarrhalis* requires PE for viability or that the accumulation of PS may be toxic to *M. catarrhalis*.

Since efforts to generate a PE-deficient strain of *M. catarrhalis* by mutating *psd*, we next attempted to generate a *pssA* isogenic mutant strain of isolate O35E. The same procedures utilized to generate pCC1.psd.kan were used to create pCC1.pssA.kan. Oligonucleotide primers P11 and P12 (Table 4.2) were used to amplify the *pssA* gene from O35E. PCR using primers P11 and P12 (Table 4.2) identified pCC1.pssA.kan in which the kanamycin resistance cassette was inserted into the *pssA* ORF. Primers P9, P10, P11, and P12 (Table 4.2) were used to determine the location of the kanamycin resistance cassette within the *pssA* ORF of pCC1.pssA.kan. However, instead of choosing a single pCC1.pssA.kan variant, several plasmids with the kanamycin resistance cassette inserted within various locations of the *pssA* ORF were used to transform *M. catarrhalis*.

Transformation of *M. catarrhalis* isolate O35E with pCC1.pss.kan that contained the kanamycin resistance cassette in the beginning (~250 base pairs upstream of the start codon) or middle (~600 base pairs upstream of the start codon) of the *pssA* ORF did not result in any kanamycin resistant transformants, even using media supplemented with $MgCl_2$, ethanolamine, or PE. However, when the kanamycin resistance cassette was located toward the end of the ORF (~700 base pairs upstream of the start codon), numerous kanamycin resistant transformants resulted. The location of the kanamycin resistance cassette in the resulting O35E.pssA strains was confirmed by PCR using primers P5 and P6 (Table 4.2).

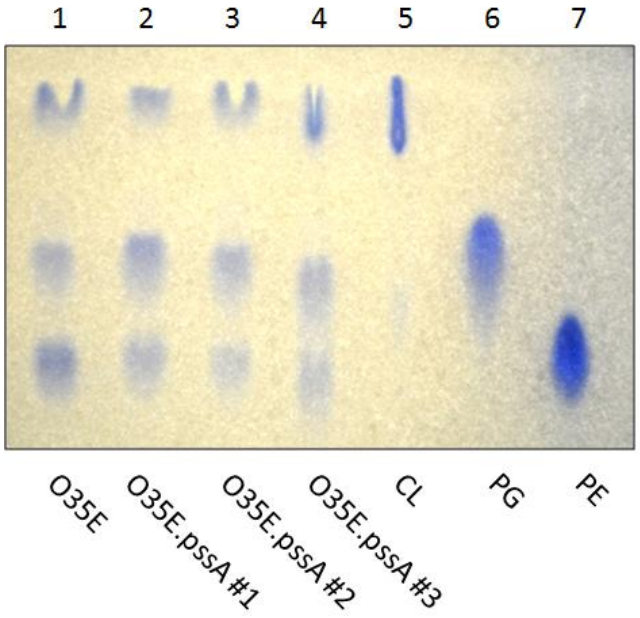


Figure 4.7. The O35E.pssA transformants still synthesize PE. Analysis of the phospholipid profile *M. catarrhalis* strains O35E (lane 1) and three representative transformants of O35E.pssA (lanes 2-4) by TLC. Spots were identified by comparison to purified CL, PG, and PE standards (lanes 5, 6, and 7, respectively).

Phospholipids were extracted from three representative O35E.pssA transformants and their phospholipid composition was compared to that of the WT O35E isolate by TLC (procedures described previously in this chapter). If the enzyme encoded by the *pssA* gene were

knocked-out, we would not expect the O35E.pssA strains to contain PE. However, as shown in Figure 4.7, three representative O35E.pssA transformants (lanes 2-4) possessed PE as well as the WT isolate (lane 1). Based on this data, we believe that when the kanamycin resistance cassette is inserted toward the end of the *pssA* ORF, a significant portion of the gene is still expressed and the enzymatic activity of *pssA* is not eliminated, thereby resulting in the production of PE. Since no transformants resulted following transformation of O35E with constructs containing the kanamycin resistance cassette in the beginning or middle of the ORF, it appears that the *pssA* gene is essential for *M. catarrhalis*. Both enzymes involved in the synthesis of PE from CDP-DAG (PssA, Psd) appear to be essential for the viability of *M. catarrhalis*. It is possible that these two enzymes are required solely for their contribution to PE synthesis, therefore suggesting that PE is an essential phospholipid for *M. catarrhalis*.

Ultimately, we were unable to determine if PE is a substrate for MclS using the genetic approach as described. An alternative genetic approach using a plasmid containing an inducible promoter would beneficial for controlled expression of *psd* and *pssA*; however, such a genetic tool has not yet been developed for use in *M. catarrhalis*. Finally, the substrates for MclS could be identified using a biochemical approach by purifying MclS protein and demonstrating the conversion of PE and/or PG to CL *in vitro*.

Conclusions

In this chapter, we have continued the characterization of CL and the enzyme responsible for its synthesis, MclS. Our research shows that a considerable amount of CL is present in the outer membrane of *M. catarrhalis*, an interesting finding considering that CL is found almost exclusively in the plasma membrane of most bacteria. Interactions between CL and factors

localized to the outer membrane may be responsible for the adherence defects we have observed in CL-deficient strains of *M. catarrhalis* [244]. In fact, we show that there may be a relationship between the presence of CL and expression of LOS, a factor previously associated with adherence [81-82]. In contrast, MCORF 821 appears to be a negative effector of adherence. Regarding MclS, we have found that its activity is not dependent on lipidation of its N-terminus, thereby suggesting that the bioinformatics prediction that MclS is a lipoprotein is incorrect. Genetic attempts to determine the substrate of MclS were unsuccessful because PE, one of the possible substrates, appears to be essential for *M. catarrhalis*. Future efforts to identify the MclS substrates may rely on either an advanced genetic approach or biochemical approach.

Tables

Table 4.1: Strains and plasmids used in this study

Strain or Plasmid	Description	Selectable Marker	Source
O35E	WT isolate of *M. catarrhalis*	None	[127]
O35E.mclS	*mclS* isogenic insertion mutant strain of O35E	Spectinomycin	[244]
O35E.mclS repaired	Repaired strain of O35E.mclS	Streptomycin	[244]
O35E.mclS.K438R	*mclS* point mutant (K438R) strain of O35E	Streptomycin	[244]
O35E.mclS.C24S	*mclS* point mutant (C24S) strain of O35E	Streptomycin	This study
O35E.821	MCORF 821 isogenic insertion mutant strain of O35E	Zeocin™	This study
O35E.pssA	*pssA* isogenic insertion mutant strain of O35E	Kanamycin	This study
O12E	WT isolate of *M. catarrhalis*	None	[89]
O12E.mclS	*mclS* isogenic insertion mutant strain of O12E	Spectinomycin	[244]
McGHS1	WT isolate of *M. catarrhalis*	None	[230]
McGHS1.mclS	*mclS* isogenic insertion mutant strain of McGHS1	Spectinomycin	[244]
EPI 300™	Cloning strain of *E. coli*	None	Epicentre® Illumina®
pEM7/Zeo	Source of Zeocin™ resistance cassette	Zeocin™	Invitrogen™ Life Technologies™
pCC1™	Cloning vector, replicative in *E. coli*	Chloramphenicol	Epicentre® Illumina®
pCC1.821	pCC1™ containing MCORF 821 from O35E	Chloramphenicol	This study
pCC1.821.zeo	pCC1.821 with Zeocin™ resistance cassette inserted into ORF of MCORF 821	Chloramphenicol Zeocin™	This study
pCC1.mclS.fl	pCC1™ containing *mclS* (with flank) from O35E	Chloramphenicol	This study
pCC1.mclS.fl.C24S	pCC1.mclS containing C24S point mutation in ORF of *mclS*	Chloramphenicol	This study
pCC1.psd	pCC1™ containing *psd* from O35E	Chloramphenicol	This study
pCC1.psd.kan	pCC1.psd with kanamycin resistance cassette inserted into ORF of *psd*	Chloramphenicol Kanamycin	This study
pCC1.pssA	pCC1™ containing *psd* from O35E	Chloramphenicol	This study
pCC1.pssA.kan	pCC1.pssA with kanamycin resistance cassette inserted into ORF of *pssA*	Chloramphenicol Kanamycin	This study

Table 4.2: Oligonucleotide primers

Primer	**Sequence (5' → 3')**	**Gene**	**Direction**
P1	TGGCTGGCTGGACTGATGCC	MCORF 821	Forward
P2	TGGGATCCGCGATTCATATCAAGGTCG	MCORF 821	Reverse
P3	TGGGATCCACCAGCATGTTGGGTGA	*mclS*	Forward
P4	TGGGATCCCTTGCAAATCTGATGCTT	*mclS*	Reverse
P5	CTGGTATTACTTGTTGGTAGCAAAAAT	*mclS*	Forward
P6	ATCGGGCAAATTTTTGCTACCAACAAG	*mclS*	Reverse
P7	TGGGATCCGCAGGTGTCATCGGTTATG	*psd*	Forward
P8	TGGGATCCGACAGGTATATTGCCAATT	*psd*	Reverse
P9	ACCTACAACAAAGCTCTCATCAACC	*kan*	Forward
P10	GCAATGTAACATCAGAGATTTTGAG	*kan*	Reverse
P11	TGGGATCCTCTTGGTGAGATGGCAGT	*pssA*	Forward
P12	TGGGATCCCGTTGCAATCAACGACAG	*pssA*	Reverse

CHAPTER 5

IDENTIFICATION OF FACTORS CONTRIBUTING TO THE DEVELOPMENT OF COMPLEMENT RESISTANCE OF *MORAXELLA CATARRHALIS* THROUGH EXPERIMENTAL EVOLUTION[1]

[1] Buskirk S.W. and E.R. Lafontaine. To be submitted to the *Journal of Bacteriology*

Abstract

Moraxella catarrhalis is a human-specific pathogen of the mucosa and causative agent of otitis media in children and exacerbations of chronic obstructive pulmonary disease in adults. To evade immune clearance, *M. catarrhalis* has developed the ability to resist the activity of the complement cascade. UspA2 is the major serum resistance factor expressed by *M. catarrhalis*, and the presence of UspA2 has been correlated with pathogenesis. In this study, we utilize experimental evolution with the objective of identifying additional genes involved in complement resistance of *M. catarrhalis*. We demonstrate that an *M. catarrhalis* strain lacking UspA2 can develop complement resistance following repeated exposure to human serum. The evolved lineages are able to resist complement-mediated killing for short period of time; however, like their ancestor, the lineages are readily killed when incubated with serum for an extended period of time. We intend to extend this study by utilizing whole-genome sequencing techniques to identify the mutations responsible for the development of complement resistance in the evolved lineages.

Introduction

The complement cascade is a major component of the innate immune system, functioning in the recognition and clearance of microbial pathogens. However, many microbes have evolved mechanisms to circumvent complement-mediated killing. The ability to evade the immune response allows pathogens to persist and subsequently cause disease in the host.

Moraxella catarrhalis is a Gram-negative, human-specific pathogen of the mucosa and causative agent of otitis media in children and respiratory infections in adults. Two distinct lineages of *M. catarrhalis* have been identified which diverged approximately 5 million years ago [276-277]. One lineage has been associated with disease in humans and is able to resist the activity of complement as well as adhere to human epithelial cells. The other lineage appears to be avirulent, serum-sensitive, and non-adherent [278]. The complement-resistant phenotype has been associated with the function of a single protein, UspA2. *M. catarrhalis* strains lacking UspA2 are readily killed in the presence of serum, often exhibiting survival rates two orders of magnitude lower than strains expressing UspA2 [45]. UspA2 mediates serum resistance directly by binding C3, C4bp, and vitronectin, thereby blocking both the classical and alternative pathways of the complement cascade [53, 59, 279]. While UspA2 plays a crucial role in complement resistance, several additional factors have been shown to contribute to the survival of *M. catarrhalis* in serum resistance, namely UspA1, CopB [96], OMP CD [65], OMP E [97], and lipooligosaccharide (LOS) [80]. Like UspA2, UspA1 binds C3 and C4bp, albeit at much lower affinities, thereby explaining why UspA1 is only a minor contributor [53, 95]. The remaining factors (CopB, OMP CD, OMP E, and LOS) are required for *M. catarrhalis* strains to exhibit *wild-type* levels of resistance but have not been demonstrated to be mediate serum resistance directly [65, 80, 96-97].

Experimental evolution is the study of how organisms adapt in a controlled laboratory environment. This approach allows researchers to expose organisms to specific conditions and observe the evolutionary changes that occur over a relatively short period of time. Experimental evolution can be used as a genetic screen to select for bacteria that have developed a unique phenotype. Most other genetic screens are limited in that they only select for loss-of-function mutations or, alternatively, gain-of-function-mutations in a heterologous organism. Experimental evolution, on the other hand, can be tailored to select for beneficial mutations in the organism of interest [280]. The approach was first utilized 60 years ago [281], but has recently gained popularity with the advent of high-throughput sequencing that allows for efficient identification of specific mutations at an affordable cost.

Several researchers have utilized experimental evolution to study pathogenic organisms. For example, the approach has been used to examine the trade-offs associated with host-specificity of *Legionella pneumophila* [282], West Nile virus [283], and vesicular stomatitis virus [284-285] and to identify factors that regulate biofilm formation of *Pseudomonas aeruginosa* [286-287] and *Burkholderia cenocepacia* [288]. In fact, experimental evolution has previously been utilized in *M. catarrhalis* to generate a non-clumping variant that was found to be attenuated in a mouse model. In that study, researchers continuously seeded new culture flasks with bacteria that failed to aggregate in broth from the previous passage. The resulting variant exhibited altered display of select surface molecules including UspA1, OMP CD, and LOS [150]. While the variant has not been further characterized, current technology would make it easy to sequence the genome and identify the mutations responsible for the observed phenotypes.

The ultimately goal of this study was to identify factors that contribute to complement resistance in *M. catarrhalis*. To accomplish this objective, experimental evolution was utilized as a genetic screen to select for variants with increased survival in the presence of human serum. We hypothesized that a serosensitive strain of *M. catarrhalis* would eventually develop resistance to complement following recurrent exposure to normal human serum (NHS) through the accumulation of beneficial mutations. The starting culture chosen for this study was the UspA2 mutant strain O35E.2 in which an internal portion of the *uspA2* ORF was replaced by a kanamycin resistance cassette. Using different approaches, we found that lineages resulting from a regimen of short, daily exposures to NHS were significantly more resistant to serum than their ancestor. The evolved lineages were able to survive in serum for short periods of time; however, unlike the UspA2-expressing isolate O35E, prolonged exposure to serum resulted in bacterial death. The genomes of the evolved lineages will be sequenced in order to identify the mutations responsible for the development of complement resistance.

Materials and Methods

Generation of serum-resistant lineages of O35E.2

Three separate approaches were designed to select for serum-resistant variants of O35E.2 through exposure to NHS: Repeated Exposure, Constant Exposure, and Increasing Exposure. The conditions and procedures for each approach are described below. Each approach began by generating a single clonal population of O35E.2 by propagating O35E.2 on Todd Hewitt (TH) agar (Difco™) containing kanamycin (kan, 20 μg/mL, Teknova) at 37°C for 24 hours, then selecting an isolated colony, streaking it onto fresh TH agar supplemented with kan, and incubating at 37°C for 24 hours [289]. The resulting culture, termed the Starting Culture (SC),

was stocked in freezing media composed of 50% glycerol (vol/vol, Fisher Scientific®) and 9.25% TH media (wt/vol).

Repeated Exposure

The SC of O35E.2 was propagated on TH agar supplemented with kan. To prepare the bacteria for exposure to NHS, a portion of the resulting culture was resuspended in phosphate buffered saline with 0.15% gelatin (wt/vol, PBSG, Sigma Life Science™) and used to generate a 150 Klett bacterial suspension (Klett™ Colorimeter, Scienceware®). Next, we created the reaction mixture in which bacteria were exposed to serum. The reaction contained 10% bacterial suspension, 25% NHS (Sigma Life Science™), and 65% Bactericidal Buffer [0.9% NaCl (wt/vol, Fisher Scientific®), 0.1% gelatin (wt/vol, Sigma Life Science™), 0.1% $MgCl_2 \cdot 6H_2O$ (wt/vol, Fisher Scientific®), 0.0375% $CaCl_2 \cdot 6H_2O$ (wt/vol, Acros Organics®), pH 7.2]. The mixture was vortexed vigorously and incubated at 37°C for 90 minutes. Following the incubation period, the mixture was vortexed vigorously and 10% was spread onto TH agar. The plate was incubated at 37°C for 24 hours. The next day, colonies were counted. All colonies from a single plate were collected and resuspended in PBSG. A 150 Klett suspension was generated, a portion was added to freezing media and stocked at -80°C, and the procedure was repeated as previously described. The experiment was conducted with six replicates and each replicate (lineage) was evolved independently and stocked daily. The experiment continued for 25 days.

Constant Exposure

In the pilot experiment, a 230 Klett suspension was generated from a culture of O35E.2 propagated on TH agar. A 1:10 dilution of the suspension was made in PBSG. Next, we

created the reaction mixture in which bacteria were exposed to serum. The reaction contained 10% diluted bacterial suspension, 25% NHS, and 65% Bactericidal TH Broth [Bactericidal Buffer supplemented with 2.5% TH media (wt/vol)], 100 µL of NHS, and kan at 20 µg/mL. The mixture was vortexed vigorously and incubated in a shaker (200 rpm) at 37°C for 24 hours. Aliquots were removed at specific time points, diluted in PBSG, and plated on TH agar to determine culture density.

For the experimental evolution of O35E.2 by constant exposure, the SC of O35E.2 was propagated on TH agar supplemented with kan. The bacteria were resuspended in PBSG and used to generate a 50 Klett bacterial suspension. Next, we created the reaction mixture in which bacteria were exposed to serum. The reaction contained 10% bacterial suspension, 25% NHS, 65% Bactericidal TH Broth, and kan at 20 µg/mL. The mixture was vortexed vigorously and incubated in a shaker (200 rpm) at 37°C for 24 hours. Following the incubation, a small portion was streaked onto TH agar and incubated at 37°C for 24 hours to check for contamination. The remainder was centrifuged for 2 minutes at 17,000 x g, the supernatant was removed, and the pellet was resuspended in 100 µL PBSG. A portion was added to freezing media and stocked at -80°C. A 50 Klett suspension was generated and the procedure was repeated as previously described. The experiment was conducted with four replicates and each replicated (lineage) was evolved independently and stocked daily. The experiment continued for 13 days.

Increasing Exposure

The SC of O35E.2 was propagated on TH agar supplemented with kan. The bacteria were resuspended in PBSG and used to generate a 230 Klett bacterial suspension. Next, we created a mixture containing 10% bacterial suspension, 90% Bactericidal TH Broth, and kan at

20 µg/mL. The mixture was vortexed vigorously and incubated in a shaker (200 rpm) at 37°C for 24 hours. Following the incubation, a small portion was streaked onto TH agar and incubated at 37°C for 24 hours to check for contamination. The remainder was centrifuged for 2 minutes at 17,000 x g, the supernatant was removed, and the pellet was resuspended in 100 µL PBSG. A portion was added to freezing media and stocked at -80°C. A 230 Klett suspension was generated and the procedure was repeated with increasing concentrations of serum (see Table 5.1). The experiment was conducted with three replicates and one control that substituted heat-inactivated NHS (1 hour pre-incubation at 56°C). Each replicated (lineage) was evolved independently and stocked daily. The experiment continued for 13 days.

Growth rate experiments

Cultures propagated at 37°C for 24 hours on TH agar were used to generate bacterial suspensions of 50 Klett. The suspensions were then incubated with shaking (225 rpm) at a temperature of 37°C for up to 9 hours. The optical density of each culture was determined hourly. Growth curves were repeated on two separate occasions.

Bactericidal assays

Bacterial cultures were tested for their ability to resist complement-mediated killing by use of a bactericidal assay. The protocol was adapted from Aebi et al [45]. Bacteria were propagated on TH agar at 37°C for 24 hours. The culture was resuspended in PBSG and used to generate a 150 Klett bacterial suspension. The suspension was then diluted 1:1000 in PBSG. Next, we created the reaction mixture in which bacteria were exposed to serum. The reaction contained 10% diluted bacterial suspension, 10% (or 25%) NHS, and 80% (or 65%) Bactericidal TH Broth. The mixture was vortexed vigorously, and 10% was spread on TH agar (completed in

duplicate). The reaction was incubated at 37°C for up to 30 minutes. Following incubation, the mixture was vortexed vigorously, and 10 µL was transferred to 100 µL PBSG atop a TH agar plate before spreading (again in duplicate). Plates were incubated for 37°C for 24 hours, and colonies were counted the following day. Percentage survival was calculated by dividing the average CFU per plate at each time point by the average CFU per plate at time 0.

Results

The uspA2-mutant strain O35E.2 is confirmed to be serum-sensitive

UspA2 is the major serum-resistance factor expressed by *M. catarrhalis*. Aebi et al. constructed a *uspA2*-mutant strain, O35E.2, in which an internal region of the ~2.7 kb open reading frame (approximately +715 to +935) was replaced by a kan resistance (kan^R) cassette. Following confirmation by PCR and western blot analysis, the authors showed that O35E.2 was readily killed in the presence of NHS [289]. To confirm that O35E.2 is sensitive to serum, we exposed the mutant strain to 25% NHS for up to 1 hour. The WT strain O35E persisted while O35E.2 experienced a drastic decrease in survival (Figure 5.1). From this data, we confirmed that O35E.2 is indeed sensitive to NHS.

Approaches designed to evolve serum-resistance in O35E.2

Even though O35E.2 lacks *uspA2,* we hypothesized that *M. catarrhalis* possesses other genes that may potentially mediate serum resistance. Furthermore, we speculated that recurrent/persistent exposure to serum would promote mutation of these other genes. These mutants would have a fitness advantage in the presence of serum and would be selected for in such an environment, thereby leading to the evolution of serum resistant lineages. Three approaches were developed and utilized to convert the serum-sensitive strain O35E.2 to serum-

resistant (Table 5.2). For each of the three procedures, the Starting Culture (SC) was a clonal population derived from a single isolated colony from a stock of O35E.2. The different approaches were used in order to evaluate the effect of selective pressure and competition on the formation of resistant lineages.

Serum-resistant lineages result from repeated exposure of O35E.2 to NHS

The first approach, termed Repeated Exposure, was completed for six replicates and involved daily exposure of approximately 10^5 bacteria to 25% NHS for 90 minutes. Following exposure, the bacteria were plated and incubated to allow for colony formation. The propagated bacteria were then once again exposed to NHS. The cycle was repeated for a total of 25 days. Each day, an aliquot of each culture was stocked at -80°C for later analysis.

For the first 9 days, CFU counts were taken to monitor the effectiveness of the approach. The SC was highly susceptible to NHS resulting in fewer recoverable CFUs (Fig 5.2). After several days of exposure, however, the lineages developed increased resistance to NHS. All lineages appeared to make a considerable leap in survival within the first couple of days. In the days following, it appears that the level of resistance remained stagnant for most of the lineages. However, the survival of two lineages (RE4 and RE5) increased drastically between days 8 and 9. Overall, survival rates appeared to increase through day 9 so we concluded that the Repeated Exposure procedure was an effective approach for generating serum resistance in *M. catarrhalis*.

While these data are sufficient for monitoring the procedure, they are not appropriate for drawing conclusions in regards to the level of resistance exhibited by the evolved lineages. To obtain quantitative data that directly compares the lineages to the SC, we utilized the bactericidal assay as a direct measure of serum-mediated killing. Lineages from day 12 of the experiment

were resurrected along with the SC. At day 12, five of the six lineages displayed increased serum resistance compared to the SC following a 15 minute exposure to serum (Figure 5.3A). While some lineages (i.e. RE3) were only marginally more resistant than SC, others (RE4, RE5) were drastically more resistant. One lineage in particular, RE5, survived at a level more than seven times of that of the SC.

Since the mutations that conferred serum-resistance may have pleiotropic effects, we decided to examine the other characteristics of the lineages. Specifically, we evaluated the ability of the lineages at day 12 to grow in liquid media. The growth dynamics of the SC and each of the six Repeated Exposure lineages were monitored over a period of nine hours (Figure 5.4). Several lineages, particularly RE5 and RE6, appeared to replicate at increased rates; however, the difference in growth rates between cultures was relatively small and more trials are necessary to determine significance. The SC and all six lineages reached stationary phase by hour 9, and each culture plateaued at an optical density of approximately 420 Klett.

Given that the evolved lineages developed a significant increase in serum resistance by day 12, the experiment was continued for another 13 days. At day 25, each culture was again tested for serum resistance by exposure to 10% NHS for 15 minutes. At this point all of the evolved lineages had developed a significant increase in serum resistance (Figure 5.3B). In fact, many lineages survived at a level near that of the original serum-resistant WT strain O35E. These results demonstrate that the repeated exposure approach was effective at generating serum-resistant lineages from the serum-sensitive SC.

While the evolved lineages are able to resist serum for 15 minutes, they are unable to survive for longer periods of time (Figure 5.5). When the lineages were left to incubate for 30

minutes, very few if any bacteria survived. This was also true of the SC. However, nearly all of the original WT serum-resistant strain O35E survived following a 30 minute incubation. This finding suggests that while the lineages evolved mechanisms to survive in serum for short periods of time, the mechanisms were not as effective as those afforded by the action of UspA2 in the WT strain O35E.

Alternative approaches to generate serum resistance yield mixed results

Two alternative approaches, termed Constant Exposure and Increasing Exposure, were designed to also yield serum-resistant lineages from O35E.2. The Constant Exposure experiment was designed to create an environment where bacteria are in continuous exposure to serum, while also providing nutrients necessary for bacterial growth. To better understand the dynamics of this approach, a pilot experiment was conducted where bacteria were exposed to serum and survival was monitored over a 24-hour period. When a culture with a density of 10^7 bacteria/mL was exposed to 25% NHS, the number of viable bacteria decreased over the first 2 hours (Figure 5.6). However, the culture expanded after reaching the 2-hour time point, ultimately resulting in a density greater than 10^9 bacteria/mL by 24 hours. One explanation for the observed dynamics is that the complement components in serum may become exhausted around the 2 hour time point; however, this has not been investigated further.

After observing the dynamics, the constant exposure approach was implemented with the intention of generating serum-resistant lineages. Approximately $3x10^7$ bacteria were exposed to 25% NHS for 24 hours. The surviving bacteria were collected and again exposed to serum. The experiment was completed in quadruplicate, and the cycle was repeated for a total of 13 days. Each day, an aliquot of each culture was stocked at -80°C for later analysis. Two of the four

replicates (CE1, CE3) did not survive the Constant Exposure experiment through day 13 and were therefore not examined. The two remaining evolved lineages (CE2, CE4) were directly compared to the SC using the bactericidal assay. Neither of the lineages exhibited resistance above the level of the SC (Figure 5.7A). This is in contrast to the lineages generated by the Repeated Exposure experiment (Figure 5.3).

The third and final approach designed to generate serum-resistant variants from the serum-sensitive mutant O35E.2 was through an approach where the SC was exposed to increasing amounts of serum for 13 days. The Increasing Exposure procedure began with approximately $3x10^5$ bacteria growing in Bactericidal TH Broth for 24 hours in the absence of serum. On the second day, the bacteria were collected and exposed to 1% NHS under the same conditions. The serum concentration doubled every other day until the bacteria were exposed to 32% NHS for the second time on day 13 (Table 5.1). Each day, an aliquot of each culture was stocked at -80°C for later analysis. The experiment was completed in triplicate; in addition, a control was included in which bacteria were exposed to heat-inactivated serum. One of the three replicates (IE2) did not survive the Increasing Exposure experiment through day 13 and was therefore not examined.

When the evolved lineages from the Increasing Exposure experiment at day 13 were compared to the SC by bactericidal assay, only of one (IE1) of the two remaining cultures displayed serum resistance above the level of the SC following a 15 minute exposure to NHS (Figure 5.7B). Interestingly, the IE HI lineage that was exposed to HI serum throughout the duration of the evolution experiment also displayed increased levels of resistance. This finding has yet to be explained since we would not expect the SC to evolve serum resistance in the

absence of selective pressure from an active complement cascade. At the least, the experiment will be repeated to rule out human error.

Discussion

In this study, we demonstrate that experimental evolution can be successfully utilized to study serum resistance in *M. catarrhalis*. Of the three approaches that were implemented, daily exposure of *M. catarrhalis* to serum via the Repeated Exposure procedure appeared to be the most effective. Lineages that were evolved under these conditions developed increased serum resistance (Figure 5.3). However, in contrast to the naturally-occurring, serum-resistant isolate O35E, the evolved lineages were readily killed in serum when exposed for longer periods of time (Figure 5.5). It will be worthwhile to determine if the evolved lineages will eventually develop the ability to survive in serum for long periods of time (such as that exhibited by WT isolate O35E) by extending the Repeated Exposure experiment for additional days. The mutations responsible for the development of serum-resistance in the lineages will ultimately be identified by whole-genome sequencing and genomic analysis.

The Repeated Exposure approach was initially monitored for effectiveness by counting the number of bacteria that survived the daily procedure (Figure 5.2). In general, the level of resistance exhibited by the strains remained steady for most of the experiment. However, two lineages (RE4, RE5) demonstrated a striking increase at day 9. It would be worthwhile to track the level of serum resistance of each lineage through the completion of the experiment to see if these leaps in resistance are common for all lineages. If so, it would suggest that the development of serum resistance is dependent on the accumulation of several specific mutations.

We tested the six evolved lineages at day 12 for growth rate and found that two lineages (RE5, RE6) reached log phase quicker than the SC (Figure 5.4). It is extremely unlikely that the difference in growth is responsible for the increased level of survival since the incubation period of the bactericidal assay is so short (15 minutes). While more trials are necessary to validate the change in growth rate, we also plan to test for other pleiotropic effects that the lineages may exhibit. Since *M. catarrhalis* is exposed to many types of selective pressure in the host, colonization and persistence in humans requires more from *M. catarrhalis* than simply resisting complement-mediated killing. *M. catarrhalis* is finely tuned so that it can survive in the face of all pressures present. Therefore, it would be logical to assume that trade-offs have occurred in each of our evolved lineages since the remaining selective forces present in humans were absent from our experiments. We hope to identify these trade-offs by testing for traits that have been associated with virulence such as adherence to epithelial cells, biofilm formation, and nutrient acquisition.

In addition to the Repeated Exposure approach, two other procedures were tested for their ability to generate serum-resistant lineages, although neither appears to be as effective (Figure 5.7). While the Repeated Exposure experiment exposed a lower number of bacteria to serum daily compared to the other two approaches, it was also the only regimen where the bacteria were not in competition with each other for resources. It is possible that the absence of competition between bacteria was a major contributor for the selection of serum resistant variants.

Up to this point in the study, we have generated frozen glycerol stocks of the lineages each day prior to serum exposure. Periodical stocking is crucial for tracking the evolutionary progress of each lineage. However, for a long-term experimental, the process must also be

practical. As a point of reference, during the *E. coli* long-term evolution experiment (LTEE) Lenski stocked cultures every 500 generations, or 75 days [290]. However, the experimental design and research objectives of the LTEE are significantly different than ours. For example, type and amount of selective pressure and even the method of propagation differ between the two experiments. The goal of the LTEE is to answer general questions regarding bacterial evolution and fitness, though specific queries can also be investigated. Meanwhile the aim of this experiment is specific to the identification of genes involved in serum resistance. In order to minimize the possibility that we would miss valuable evolutionary changes within the lineages, we will continue to stock the lineages daily until we analyze sequence data to determine the timeframe of evolutionary change exhibited by the lineages in this study.

To reach our ultimate goal of identifying genes that contribute to complement resistance, we will sequence the genomes of selected lineages. We will utilize whole-genome sequencing to locate mutations and compare the genomes of the evolved variants and the SC. The variants will be sequenced on both a population level as well as the individual level to identify the mutations and quantify mutation frequency. To determine which mutations contribute to complement resistance, we plan to determine the date on which each lineage exhibited a significant increase in resistance and then sequence the lineage both immediately before and immediately after the increase was observed.

There are several locations where we could expect to find mutations within the genomes of the evolved lineages. One possible location is within genes encoding factors previously demonstrated to impact serum resistance, namely UspA1, OMP CD, OMP E, CopB, and LOS. A mutation within the promoter region of these genes will likely increase expression, while a mutation within the ORF may increase the quality of interaction (i.e. binding affinity to a

complement protein). In regards to UspA1, it would not be surprising to find a repeat expansion or reduction within the poly-G tract located upstream of the ORF that regulates its expression. Since the addition or subtraction of a single nucleotide within a repeat is more common than other types of mutations, this may explain the increase in survival of each of the lineages within the first several days of the repeated exposure experiment (Figure 5.2). In addition to sequencing, we will analyze the lineages by western blot to see if the expression and display of any of the factors previously associated with complement resistance have been affected.

While both UspA1 and UspA2 mediate complement resistance by binding vitronectin, C3, and C4bp, there are other mechanisms by which the complement cascade can be averted or inactivated as shown in other bacteria. For example, TraT of *Yersinia enterocolitica* and *Salmonella* species interferes with C5b6 formation [291], Opa of *Neisseria gonorrhoeae* binds heparin [292], and the M protein family of *Streptococcus pyogenes* [293] and Por1A of *Neisseria gonorrhoeae* bind factor H [294]. It is possible that the evolved *M. catarrhalis* lineages have developed the ability to mediate complement resistance through an alternative mechanism. An alternative mechanism may only play a minor role in complement resistance in the ancestral strain, but the accumulation of mutations may have accentuated its role in the evolved lineages. Given how quickly serum resistance developed in the lineages, we would not expect that the increased resistance is due to the development of entirely novel mechanisms of resistance.

Another possible outcome of whole-genome sequencing is the discovery of novel factors such as those that mediate complement resistance directly (i.e. bind/inactivate complement proteins) or those that regulate the mediators. If novel factors are implicated in the complement resistance of the evolved lineages, further investigation would be required to determine their roles within *M. catarrhalis*.

In the ancestral strain O35E.2, a portion of the *uspA2* ORF is replaced with a kanamycin resistance cassette. While O35E.2 is clearly susceptible to complement-mediated killing, a large portion of the genetic material of *uspA2* still remains within the strain. Though improbable, a mutation or recombination event could once again result in the expression of a UspA2-variant that mediates complement resistance. It may be worthwhile to sequence the *uspA2* locus of the evolved lineages to investigate this possibility before sending each for whole-genome sequencing.

Tables

Table 5.1: Percentage of NHS used throughout the Increasing Exposure experiment

Day	NHS (%)
D1	0
D2	1
D3	1
D4	2
D5	2
D6	4
D7	4
D8	8
D9	8
D10	16
D11	16
D12	32
D13	32

Table 5.2: Characteristics of the three approaches designed to evolve serum-resistant lineages

	Repeated Exposure	**Constant Exposure**	**Increasing Exposure**
Selective pressure	High	High	Variable
Competition between variants	No	Yes	Yes
Number of bacteria exposed	$1x10^5$	$3x10^7$	$3x10^5$

Figures

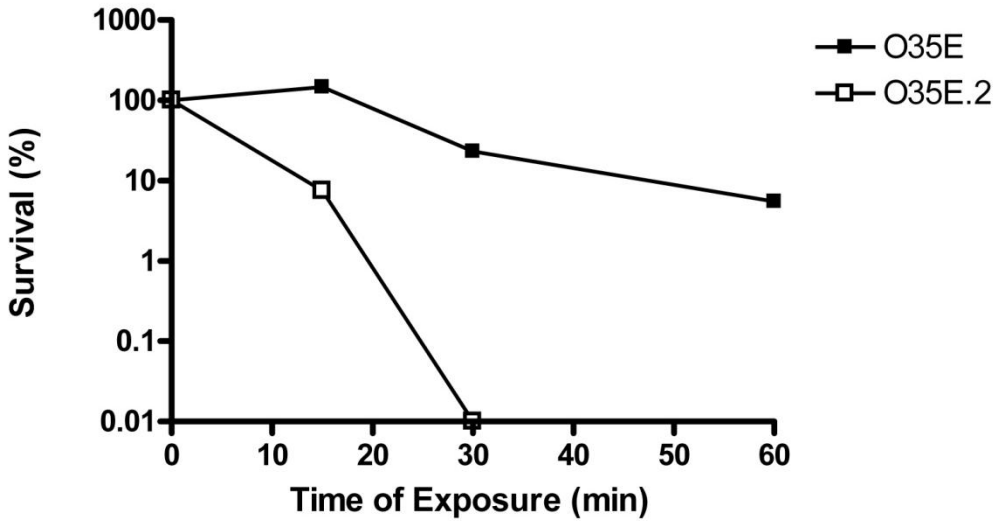


Figure 5.1. WT strain O35E persists in NHS while *uspA2*-mutant strain O35E.2 is readily killed. Strains O35E and O35E.2 were exposed to 25% NHS for up to 1 hour. Time points were taken at 0, 15, 30 and 60 minutes. Percent survival was determined by dividing the CFU at each respective time point by the CFU at time 0. The experiment was performed in duplicate on one occasion.

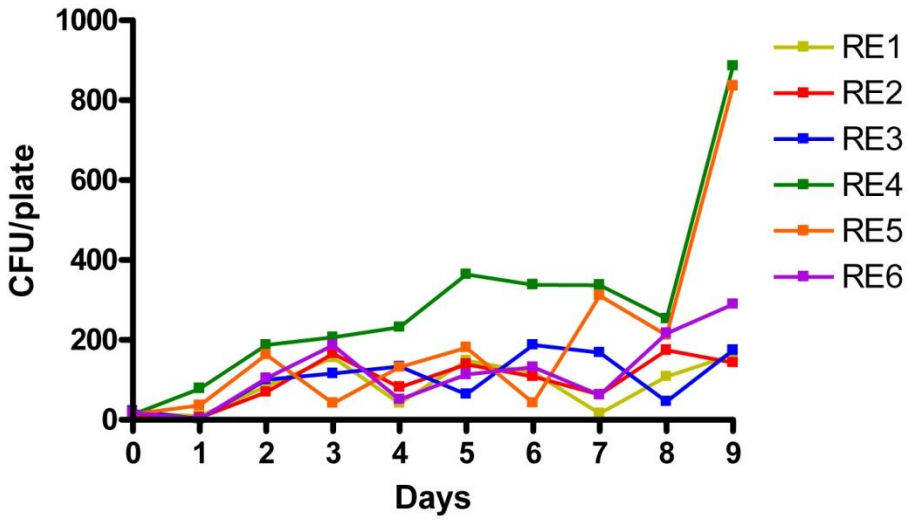
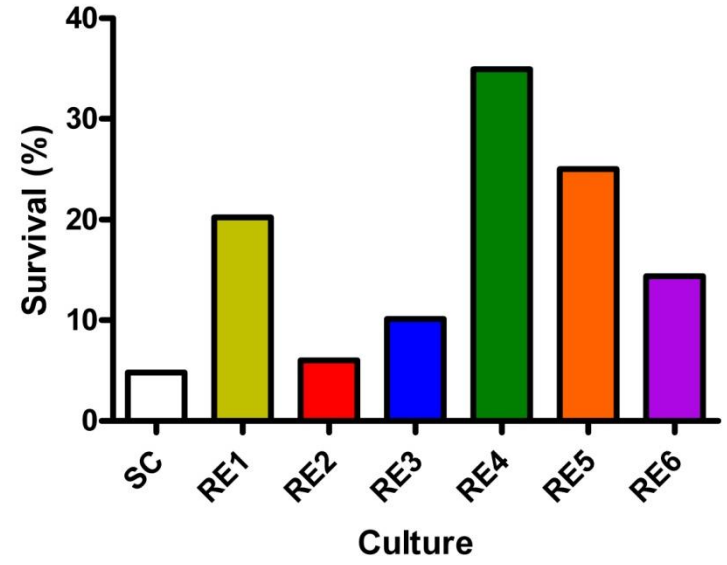
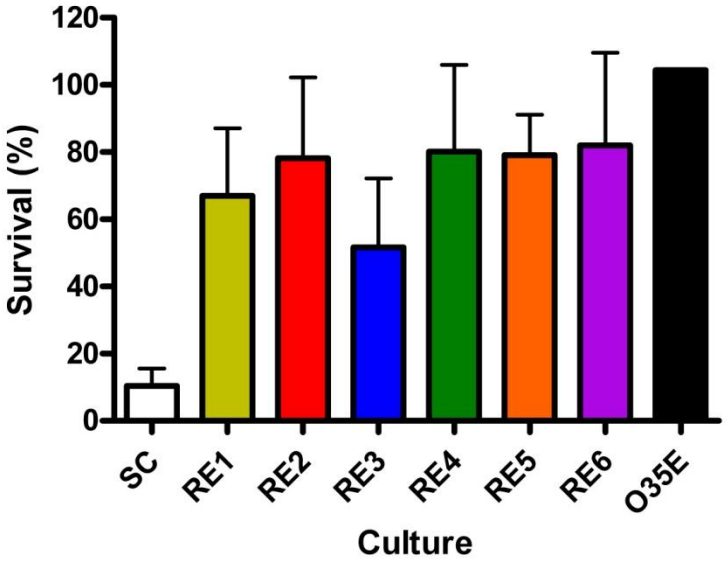


Figure 5.2. Repeated exposure to NHS increases survival of lineages in serum. During the repeated exposure experiment, the number of surviving bacteria appears to increase over the first 9 days of daily exposure. CFU counts were conducted daily once colonies were clearly visible.



A



B

Figure 5.3. Evolved lineages of O35E.2 developed serum resistance following repeated exposure to NHS. Cultures corresponding to day 12 (A) and day 25 (B) lineages RE1 through RE6 were resurrected and directly compared to the SC by bactericidal assay. Cultures were exposed to 10% NHS for 15 minutes. The day 12 lineages were assayed duplicate on one occasion, and the day 25 lineages were assayed in duplicate on two occasions.

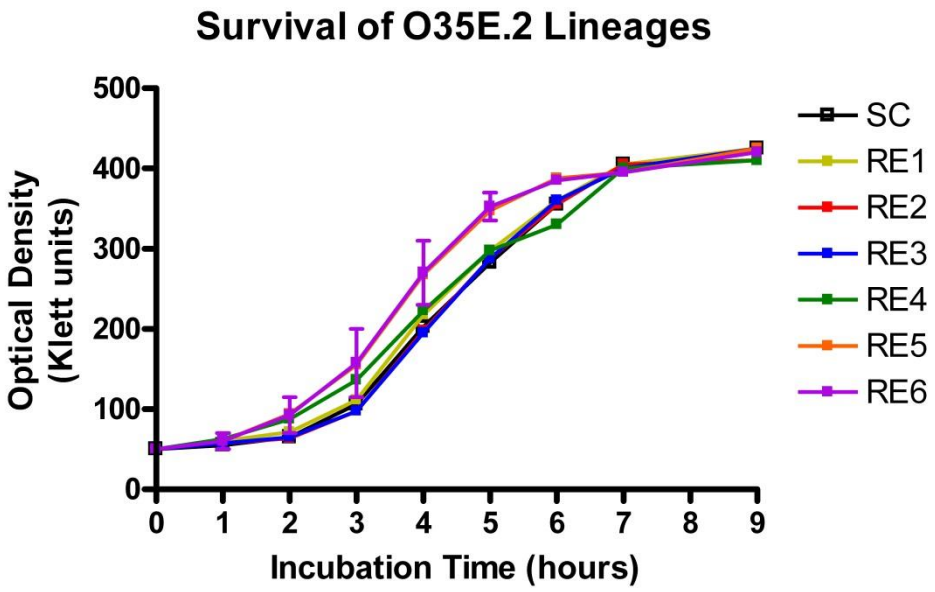


Figure 5.4. Several Repeated Exposure lineages exhibit increased faster growth in broth. The six Repeated Exposure lineages were tested for growth dynamics in liquid media. Cultures were grown for up to nine hours in TH broth. Growth curves were completed on two separate occasions.

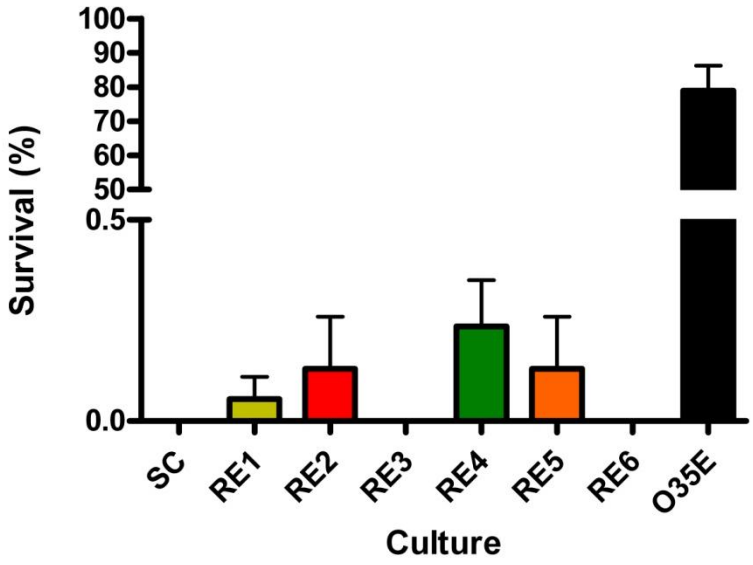


Figure 5.5. Evolved lineages of O35E.2 are only able to survive in the presence of serum for a short period of time. Cultures corresponding to day 25 lineages RE1 through RE6 were resurrected and directly compared to the SC by bactericidal assay. Cultures were exposed to 10% NHS for 30 minutes. The assay was completed in duplicate on two occasions.

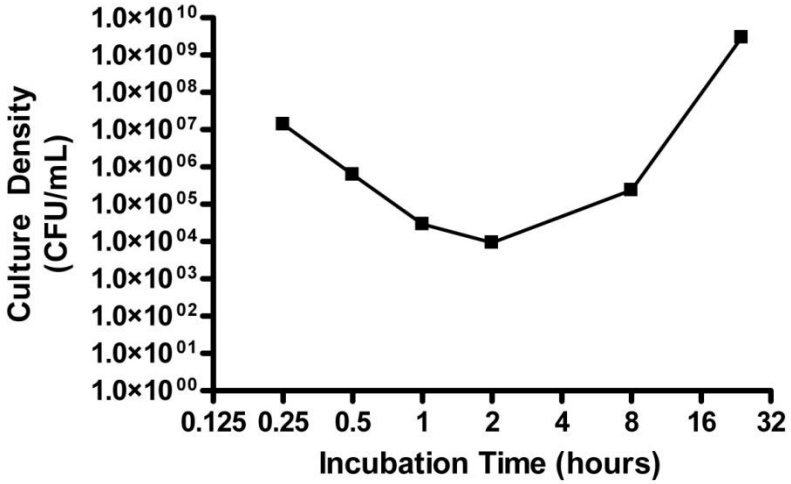
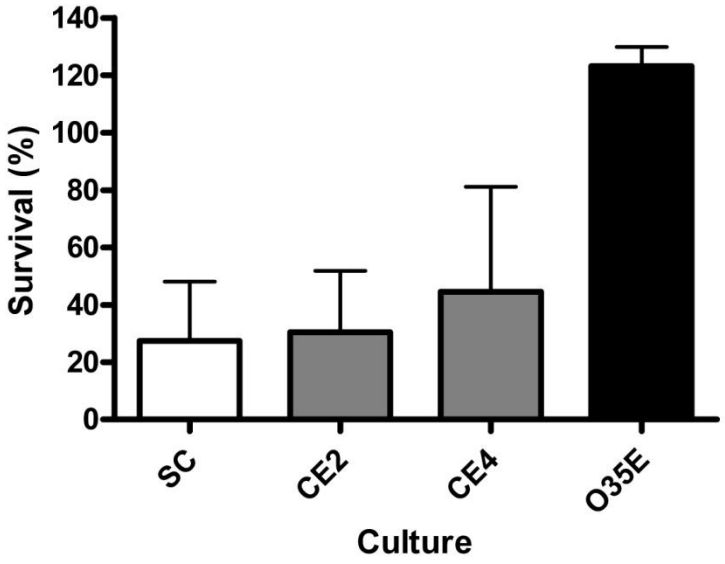
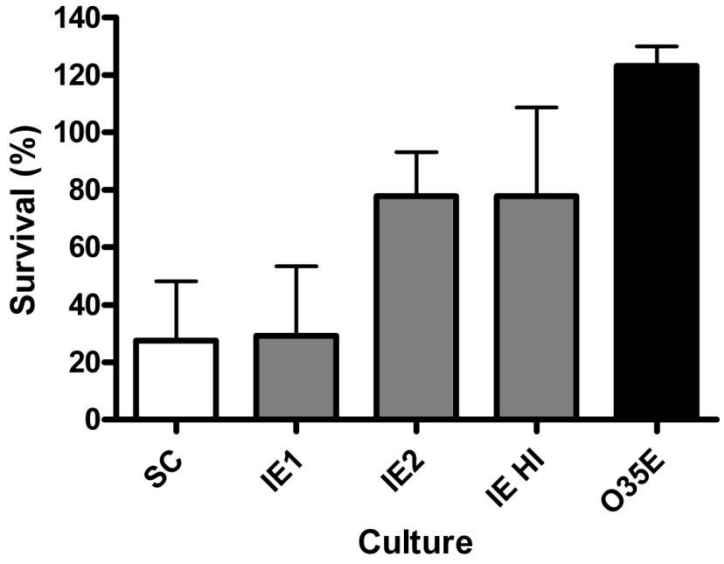


Figure 5.6. A pilot experiment shows that constant exposure of a bacterial culture to serum results in an immediate decrease in bacterial density, followed by expansion. The dynamics of a bacterial culture exposed to NHS were examined. A culture with density of ~10^7 bacteria/mL was exposed to 25% NHS, and the number of viable bacteria was monitored over time. The experiment was conducted on a single occasion.



A



B

Figure 5.7. Evolved lineages of O35E.2 displayed varying levels of serum resistance following constant and increasing exposure to NHS. Cultures corresponding to day 13 lineages evolved through constant exposure to NHS (A) increasing exposure to NHS (B) were resurrected and directly compared to the SC by bactericidal assay. Cultures were exposed to 10% NHS for 15 minutes. The assay was completed in duplicate on two occasions.

CHAPTER 6

CONCLUSIONS

Moraxella catarrhalis relies on an arsenal of virulence traits to infect its human host. Adherence to the mucosal epithelium is crucial for the initial colonization of the host, while complement resistance is necessary for evasion of the host immune system. These two traits in particular have a strong correlation to virulence of *M. catarrhalis*; the serum-resistant, adherent isolates of *M. catarrhalis* are strongly associated with human disease. Numerous *M. catarrhalis* adhesins have been identified and characterized; however, there is still much to learn about the adherence process. Whereas adherence is a multi-factorial process, complement resistance is greatly dependent on expression of a single protein UspA2. Our findings revealed new insight into both the adherence and serum resistance of *M. catarrhalis*.

We have identified a new factor, cardiolipin, that contributes to the adherence of *M. catarrhalis* to epithelial cells. Synthesis of cardiolipin is catalyzed by the *M. catarrhalis* cardiolipin synthase termed MclS. The *mclS* gene is highly conserved throughout *M. catarrhalis* isolates and is transcriptionally linked to the genes immediately upstream and downstream. Neither *mclS* nor cardiolipin is essential for survival of *M. catarrhalis in vitro* as MclS mutant strains we successfully generated in three *M. catarrhalis* isolates. An *M. catarrhalis* strain expressing a catalytically-inactive variant of MclS was unable to synthesize cardiolipin or adhere to epithelial cells, thereby demonstrating that cardiolipin contributes to adherence. While the mechanism by which cardiolipin impacts adherence is yet to be determined, we have demonstrated that cardiolipin is present in the outer membrane of *M. catarrhalis* where it may

interact with adhesins and other factors. While expression and surface display of five major proteinaceous adhesins (UspA1, Hag, McaP, OMP CD, and MhaB1/2) were unaffected in cardiolipin-deficient strains, more research is necessary to determine if LOS, another factor known for mediating adherence [81-82], is influenced by cardiolipin.

It is becoming increasingly evident that adherence not only requires the expression of adhesins, but also other factors that direct the localization and display of the adhesins. This notion is supported by our finding that cardiolipin is required for *wild-type* levels of adherence. Prior to our study, cardiolipin had not been associated with virulence, let alone adherence. On the other hand, phosphatidylethanolamine has been shown to contribute to the pathogenesis of *Escherichia coli* [164] and *Brucella abortis* [163] suggesting that phospholipids in general may be important contributors to virulence.

While the synthesis and role of cardiolipin has been well-studied in *E. coli*, cardiolipin has only been characterized in a few other bacterial species. Based on our findings in *M. catarrhalis*, it may be a mistake to assume that the synthesis and role of cardiolipin is consistent across prokaryotes. Several of our findings were in contrast to those previously described for *E. coli* and other bacteria. In *M. catarrhalis*, cardiolipin constitutes a large percentage of total phospholipids, and cardiolipin levels remain constant despite changes in growth phase. These claims were originally made by Beebe et al. [161] and have been confirmed in our work. It is commonly accepted that cardiolipin is found almost exclusively on energy-producing membranes such as the plasma membrane of bacteria. However, our data show that cardiolipin, along with phosphatidylglycerol and phosphatidylethanolamine, is also present on the outer membrane of *M. catarrhalis*. Whereas the literature suggests that many bacteria contain multiple enzymes with cardiolipin synthase activity, (e.g. *E. coli* and *Bacillus subtilis* each express three),

the *M. catarrhalis* genome only encodes for a single cardiolipin synthase. Indeed, cardiolipin was not detected in *mclS* mutant strains of *M. catarrhalis* under any conditions tested. Finally, as Tan et al. has recently demonstrated, it can no longer be assumed that all bacterial cardiolipin synthases generate cardiolipin from two molecules of phosphatidylglycerol [159]. Therefore, the substrate specificity and type of cardiolipin synthase may vary between bacteria.

In addition to discovering a novel contributor of adherence, we also hope to gain insight into the factors that impact complement resistance. We have shown that a serum-sensitive strain of *M. catarrhalis* can development serum resistance through repeated exposure to serum. Using whole-genome sequencing techniques, we intend to identify the mutations responsible for complement resistance in our evolved lineages. No matter their identity, recognition of the mutations that have occurred in the resistance lineages will advance our understanding of the factors involved in serum resistance of *M. catarrhalis*.

The use of experimental evolution as a genetic screen has increased in recent years due to the feasibility of whole-genome sequencing. In other words, it is now possible to link a phenotypic variant to a genotype. This can be a very powerful tool to gain a deeper understanding of microbial traits. Experimental evolution is unique that, in many cases, it allows for either positive or negative screen. Over a decade ago, Kyd et al. first utilized experimental evolution in *M. catarrhalis* to generate a variant that failed to aggregate following serial passages in liquid media. When tested in the mouse model, the variant was cleared at a significantly slower rate than the *wild-type*. Characterization of the variant revealed that it displays altered expression of several outer membrane antigens that have previously been implicated in virulence [150]. While the authors recognized differences in expression of known virulence-associated factors, the underlying genomic changes were concealed. Today, it would be possible to

complete the study to locate individual mutations in the variant. It is likely that valuable information would be learned regarding the expression and regulation of these outer membrane factors that have been associated with virulence.

It appears that our study is the first its kind to investigate serum resistance by experimental evolution. Based on the successful development of serum resistance in *M. catarrhalis*, we believe that experimental evolution can be utilized to study additional virulence-associated traits in microbes. In fact, experimental evolution has been used to study biofilm formation in *Pseudomonas aeruginosa* [286] and *Burkholderia cenocepacia* [288]. These studies revealed both positive and negative effectors of biofilm formation. Additional research also has the potential to teach us about the trade-offs that a pathogen must balance to successfully infect its host.

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APPENDIX

While examining the role of MclS, it was intriguing to find that CL affects a large portion of adherence exhibited by *M. catarrhalis*. In fact, CL-deficient strains of O35E adhered to human epithelial cell lines at levels similar to the O35E.ZCSM negative control that lacks expression of three major adhesins: UspA1, Hag, and McaP (Figure 3.7). According to our hypothesis that CL impacts expression and/or display of adhesins, it would appear that CL impacts the adhesive properties of either a major adhesin or numerous adhesins since only few mediate such a large percentage of adherence alone. However, this observation may be strain-specific since the reduction in adherence of the CL-deficient strains in isolates O12E and McGHS1 is not as drastic as that exhibited in isolate O35E (Figure 3.7). Not all known *M. catarrhalis* adhesins are expressed in each isolate, and there is often variability in the nucleotide sequence of many adhesins, possibly resulting in differences in binding affinity and adherence levels [44-45, 60, 295].

While we have demonstrated previously that CL does not affect the surface expression of UspA1, Hag, McaP, OMP CD, and MhaB1/2, it is possible that CL is required for the conformation and/or formation of these adhesins. CL is known to impact conformation and localization of proteins and protein complexes including DnaA, MinD [189], ProP [191, 240], and SecYEG [190]. Both UspA1 and Hag are trimeric autotransporters so it is conceivable that CL is required for oligomerization and adherence capabilities of these proteins [89, 296]. In addition, the binding motif of each adhesin must be formed correctly to mediate adherence. Therefore, to determine if CL affects known *M. catarrhalis* adhesins, we would have to examine

the binding motif, conformation, and localization of each of the adhesins. Localization could be examined by fluorescence microscopy using available antibodies to each adhesin. However, the study of the binding motifs and conformation would likely require specific monoclonal antibodies that have not yet been developed for most of the adhesins.