THE USPA2 PROTEIN AND SERUM RESISTANCE OF *MORAXELLA CATARRHALIS*

APPROVED BY SUPERVISORY COMMITTEE

Eric J. Hansen, Ph.D. ----------

Simon Daefler, M.D., Ph. D. ----------

Kevin S. McIver, Ph. D. ----------

Jerry Y. Niederkorn, Ph. D. ----------

DEDICATION

TO MY PARENTS, MY WIFE, AND MY KIDS MARIAM AND MOSTAFA

THE USPA2 PROTEIN AND SERUM RESISTANCE OF *MORAXELLA CATARRHALIS*

by

AHMED SHERIF ATTIA

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Ahmed Sherif Attia, Ph.D.

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Supervising Professor: Eric J. Hansen, Ph.D.

Most isolates of *Moraxella* (*Branhamella*) *catarrhalis* are resistant to the bactericidal activity of normal human serum. Several *M. catarrhalis* gene products have been linked to the serum-resistant phenotype but none of them was shown to be directly involved in this phenotype. This study provides the first evidence for the direct involvement of the UspA2 protein of several serum-resistant *M. catarrhalis* strains in the serum-resistant phenotype. This was achieved by using transformation and allelic exchange to introduce hybrid *uspA2* genes into *M. catarrhalis*, together with cloning and expression of different UspA2 proteins in *Haemophilus influenzae.* Using different types of human sera, it was concluded that serum-sensitive *M. catarrhalis* strains

are killed via the classical complement pathway. Analysis of complement deposition on four different serum-resistant *M. catarrhalis* strains and their serum-sensitive *uspA2* mutants showed similar amounts of early complement components binding to these cells, but a significant reduction occurred in the amount of polymerized C9 on the wild-type strains relative to that on the *uspA2* mutants. The binding of the UspA2 proteins of these strains to the complement regulator vitronectin was shown to be responsible for the protection of these strains against complement-mediated killing. This represents the first example of vitronectin-mediated serum resistance on a microbe. In contrast, binding of the complement regulator C4BP by the *M. catarrhalis* strains used in this study did not correlate with serum resistance. Finally, analysis of the untranslated region upstream of the *uspA2* open reading frame showed that the presence of a heteropolymeric nucleotide repeat (AGAT) in this region is necessary for both normal expression of the UspA2 protein and serum resistance. Also, it was shown that changes in the number of AGAT repeats affected transcription of the *uspA2* gene, with 15-18 AGAT repeats yielding maximal levels of transcription. These results indicate that these AGAT repeats play a regulatory role in the expression of the *uspA2* gene.

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LIST OF ABBREVIATIONS

aa	amino acid
Amp	ampicillin
AOM	Acute otitis media
ATCC	American Type Culture Collection
BHI	brain-heart Infusion
bp	base pair
C4BP	C4b-binding protein
CFU	colony-forming unit
Chlor	chloramphenicol
fB	factor B
FITC	fluorescein isothiocyanate
GVBS	veronal-buffered saline containing 0.1% (wt/vol) gelatin
HIS	heat-inactivated serum
HRP	horseradish peroxidase
Kan	kanamycin
kb	kilobase
kDa	kiloDalton
LB	Luria-Bertani
LOS	lipooligosaccharide

MAb	monoclonal antibody
MAC	membrane attack complex
NHS	normal human serum
OMV	outer membrane vesicles
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
rpts	repeats
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecly sulfate
Sm	streptomycin
Spec	spectinomycin
UTR	untranslated region
VBS	veronal-buffered saline
Vn	Vitronectin
Zeo	Zeocin

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CHAPTER ONE

Introduction

Moraxella catarrhalis has gone from being regarded as a relatively harmless commensal organism found in the human nasopharynx to being considered a pathogen that can cause significant disease (156,201,265,292). In the upper respiratory tract, *M. catarrhalis* is an important cause of otitis media in infants and very young children (156). This unencapsulated, Gram-negative bacterium can also cause infectious exacerbations of chronic obstructive pulmonary disease in adults (263,265), and is an infrequent cause of other diseases including pneumonia and tracheitis [reviewed in (201)].

Little is known about the virulence mechanisms used by *M. catarrhalis* to produce disease. The lack of a relevant animal model for otitis media caused by this organism (156) has precluded direct investigation of this process at the experimental level. Numerous *M. catarrhalis* gene products that could be involved in the colonization of the human nasopharynx by this organism or in its ability to spread into other anatomic regions in the human body have been identified [reviewed in (156,292)], and recent studies have highlighted additional gene products (1,171,282) that might participate in these processes. However, the functional significance of these gene products in vivo remains to be determined.

Serum resistance by bacteria is the ability to resist complement-mediated killing by normal human serum. Serum resistance of *M. catarrhalis* is a phenotypic trait that correlates with the virulence of this organism (129). This hypothesis, that serum resistance might be a virulence factor for *M. catarrhalis*, stemmed from observations that complement resistance was more frequently associated with disease isolates than with isolates from healthy individuals (128,152). The *M.*

catarrhalis UspA2 protein, together with several other gene products, has been implicated in the serum-resistant phenotype through mutant analysis. Until now, none of these gene products has been shown to be directly involved in serum resistance. Another important issue regarding serum resistance of *M. catarrhalis* that has not been previously addressed is the mechanism by which *M. catarrhalis* evades complement-mediated killing.

This dissertation provides the first evidence for the direct involvement of the UspA2 protein in the serum-resistant phenotype of several *M. catarrhalis* strains. This was accomplished through analysis of the UspA2 proteins of two *M. catarrhalis* strains, one of them a serum-resistant strain and the other, a serum-sensitive strain. In addition, this dissertation contains a detailed analysis of the interaction of *M. catarrhalis* with complement system components and regulators. These studies indicated that binding of the complement regulator vitronectin by UspA2 is involved in the serum resistance of *M. catarrhalis*; this represents the first example of vitronectin-mediated serum resistance in a microbe. Finally, analysis of the 5'-untranslated region (UTR) of the *uspA2* gene showed that presence of the nucleotide repeat (i.e., AGAT) in this region is necessary for both normal expression of the UspA2 protein and serum resistance.

Much of the text and many of the figures contained in Chapters Three, Four, Five, and Six of this dissertation were previously published in (13,14)

CHAPTER TWO

Review of the Literature

I. *M. catarrhalis*

A. Historical perspective

M. catarrhalis was first described near the end of the nineteenth century [referenced in (23)]. In 1905, *M. catarrhalis* was isolated from children with bronchitis and bronchopneumonia (167). Another report published by Mackey et al in 1919 described *M. catarrhalis* as the cause of chronic colds (176). However, reports suggesting that this microorganism could be the cause of common cold led J. E. Gordon to examine patients with and without cold symptoms and his findings challenged the pathogenic potential of *M. catarrhalis*, as it could be isolated from the nasopharynx of healthy persons (98). Although it was later isolated from patients with acute otitis media (108), it appears that Gordon's report led to the widespread belief that *M. catarrhalis* was strictly a commensal organism and not a pathogen (23).

B. Nomenclature and classification of *M. catarrhalis*

The nomenclature and classification of *M. catarrhalis* were rather confusing for a long time (292). First described in German as *Mikrokokkus catarrhalis* and then in English as *Micrococcus catarrhalis* (52), in 1920 it was classified as a member of the so-called non-gonococcal, non-meningococcal neisseriae (292). However, based on DNA hybridization studies and other analyses,

there was a significant difference between *M. catarrhalis* and *Neisseria* species (295). This led to its transfer to the new genus *Branhamella*, named after Sara Branham (52). However, Bovre proposed a division of the genus *Moraxella* into two subgenera, *Moraxella* and *Branhamella* (120). This sub-division started another controversy in the field. For instance, Murphy favored the *Branhamella* designation because *Moraxella* species are rod-shaped and bacteria belonging to this latter genus rarely cause infections in humans (201), while others thought that DNA hybridization studies showed that *M. catarrhalis* is more closely related to the *Moraxella* species (76). It might be that these many name changes also resulted in further underestimation of the pathogenicity of this organism (295). The latest classification of *M. catarrhalis* describes it as subgenus *Branhamella*, genus *Moraxella*, family *Moraxellaceae*, and order *Pseudomonadales*, within the γ-Proteobacteria (155).

C. Isolation and identification of *M. catarrhalis*

M. catarrhalis is a gram-negative diplococcus and the size of the organism is often described as being larger than both *N. meningitidis* and *N. gonorrhoeae* (295). This organism is non-hemolytic on blood agar, forming small, opaque, gray-white colonies that range in size between 1 mm and 3 mm and can be pushed across the surface of the agar "like a hockey puck on ice" (67). Other phenotypic characteristics used to identify *M. catarrhalis* include oxidase and DNase production, non-fermentation of glucose, maltose, sucrose, lactose and fructose, tributyrin hydrolysis and finally, its ability to reduce nitrate to nitrite (68).

Some phenotypying strategies were developed for typing of *M. catarrhalis* strains; however, they were not used in large-scale studies. For example, when sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to examine the outer membrane proteins (OMPs) of 50 *M.* catarrhalis strains, only minimal variability was observed in the molecular weights of the OMPs

among the tested strains (19). Characterization by isoelectric focusing of β-lactamases produced by *M.* catarrhalis obtained from the sputum of patients with lower respiratory tract disease and from middle ear fluids of children with otitis media indicated that the most common β-lactamase(s) produced by clinical isolates of *M. catarrhalis* in the United States were similar to those produced by the Belgian Ravasio type strain (213). Also, more than 300 strains of *M. catarrhalis* from different parts of the world were serologically typed according to their lipooligosaccharide (LOS) and it was shown that 93.4% of the strains tested expressed one of three possible LOS antigenic types (290).

More recently, modern technologies were applied for rapid identification and classification of *M. catarrhalis* isolates. These technologies included a quantitative bacterial dot method for DNA-DNA hybridization (59), random amplified polymorphic DNA (RAPD) analysis (297), and restriction fragment length polymorphism (RFLP) analysis (41,66,111,299).

D. Carriage of *M. catarrhalis*

M. catarrhalis is found in the upper respiratory tract (i.e., in the nasopharynx) of humans and, infrequently, in animals (pigs, goats, and rabbits) (167). An interesting phenomenon involving the carriage rate of *M. catarrhalis* is the inverse relationship between age and colonization. This observation was recorded as early as 1907 [referenced in (292)] and is also still present today (74). For instance, the carriage rate in infants can be as high as 66% by 1 year of age and 77.5% by 2 years of age (79), whereas it can be as low as 1-5% in adults (153,291). On the other hand, in children between six to nine years of age, the rate of colonization is about 13-17% (45,174). Also, it has been reported that rates of nasopharyngeal carriage of *M. catarrhalis* are significantly higher during winter and autumn than in summer or spring (287). In addition, it has been postulated that a viral trigger

(i.e., infection) might increase the rate of *M. catarrhalis* colonization, but this was not proven conclusively (258).

E. Diseases caused by *M. catarrhalis*

i. Childhood diseases

a. Otitis media

Otitis media is considered the most frequent infection caused by *M. catarrhalis* in children (292). *M. catarrhalis* is one of the three major causes of acute otitis media (AOM) along with *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae* (65). Recognition of *M. catarrhalis* as a likely causative agent of AOM has been documented since 1927 (108). Recurrent episodes of AOM are common among young children and these can interfere with hearing and have been associated with developmental and learning problems (159). *M. catarrhalis* is responsible for 3–4 million cases of otitis media annually in the United States (159,201). In economic terms, AOM infections, in general, cost an estimated $2 billion annually; this includes the direct costs of doctor office visits and medications, plus the indirect costs such as lost work days for parents of sick children (159,201,275).

b. Sinusitis

Sinusitis is a very common infection in early childhood, accounting for ~5-10% of upper respiratory infections (298). *M. catarrhalis* accounts for about 20% of sinusitis cases in acute and subacute episodes of this disease (36,37,298). The association of *M. catarrhalis* sinusitis and allergies

is debatable. One study indicated that there is no increase in the rate of isolation of *M. catarrhalis* among asthmatic children (298) whereas another report indicated that *M. catarrhalis* was the predominant pathogen in subacute and chronic sinusitis in children with respiratory allergy (97). In addition, there is the possibility that there might be an underestimation of isolation efficiency for *M. catarrhalis* during sinusitis and otitis media, as this bacterium stops growing in environments with reduced oxygen concentrations as are usually present in these two diseases (254).

c. Lower respiratory tract infections

Several studies have implicated *M. catarrhalis*, albeit very infrequently, in lower respiratory tract infections such as pneumonia (20,24). In some cases, *M. catarrhalis* pneumonia in children can be complicated by bacteremia (139). In addition, several studies have documented the detection of both local and systemic antibody responses to various *M. catarrhalis* antigens (54,80,96).

d. Other childhood infections

Other childhood infections in which *M. catarrhalis* has been involved include conjunctivitis and keratitis (177). *M. catarrhalis* has been implicated in a case of neonatal meningitis [referenced in (156)]. In addition, *M. catarrhalis* has been associated with cases of tracheitis in children (25,44,77).

ii. Disease in adults

a. Exacerbations of chronic obstructive pulmonary disease

The most common disease associated with *M. catarrhalis* in adults is exacerbations of chronic obstructive pulmonary disease (COPD) (272). For a long time, non-typeable *H. influenzae* and *S. pneumoniae* have been recognized as causes of purulent exacerbations of COPD. However, *M. catarrhalis* has been increasingly recognized as the third most common cause of this condition (265). This recognition of its disease-producing ability was obtained by the isolation of pure cultures of *M. catarrhalis* from transtracheal aspirates from COPD patients experiencing exacerbations and pneumonia (9,103). Also, it has been shown that patients with chronic bronchitis who experience exacerbations caused by *M. catarrhalis* develop a new bactericidal antibody response to the homologous *M. catarrhalis* strain (54).

b. Pneumonia

Although *M. catarrhalis* pneumonia cases are rare, pure cultures of this bacterium have been isolated from patients diagnosed with bacterial pneumonia (318). In this report, the pneumonia occurred in patients with end-stage pulmonary or malignant disease and almost 50 percent of the patients died of their underlying diseases within three months (318).

c. Laryngitis

M. catarrhalis is the most common bacterial species isolated from adult patients with laryngitis (292). One report showed that 55% of isolates collected from adult laryngitis patients were

M. catarrhalis (261). However, the active role of *M. catarrhalis* in the pathogenesis of acute laryngitis was not directly established (126).

d. Other rare infections

M. catarrhalis has been implicated in other rare infections in adults including; endocarditis (141,274), pericarditis (162), septic arthritis (62,192), cellulitis (253), and osteomyelitis (231). In addition, there are indications that *M. catarrhalis* might have been involved in some nosocomial disease outbreaks (47,223).

F. Antimicrobial susceptibility and resistance of *M. catarrhalis*

One of the striking phenomena associated with *M. catarrhalis* is the drastic increase in its rate of resistance to β-lactams. The first β-lactam-resistant *M. catarrhalis* in the United States was isolated in 1976 (300). Subsequently, resistance to β-lactams increased at a rate that had not been demonstrated in other bacteria under similar antibiotic pressure (76). Currently, the rate of β-lactam resistance among *M. catarrhalis* is above 90% and in some cases it reaches 100% (143). *M. catarrhalis* produces two species of β-lactamases: BRO-1 and BRO-2. *M. catarrhalis* strains that express BRO-1 are more resistant than those expressing BRO-2 (90). It has been suggested that these enzymes might be lipoproteins of gram-positive origin, but these predictions have not been confirmed (42). One characteristic of *M. catarrhalis* BRO enzymes is that, via diffusion, they can confer protection from β-lactams on other respiratory pathogens residing in the host (127). This phenomenon is referred to as the indirect pathogenicity of *M. catarrhalis* and, in fact, treatment failures due to this phenomenon have been reported (222,287), underscoring the importance of

reporting mixed cultures that are positive for *M. catarrhalis* (303). On the other hand *M. catarrhalis* remains highly susceptible to amoxicillin/clavulanate, cefdinir and cefixime (142).

M. catarrhalis is intrinsically resistant to trimethoprim (230) and it has been reported that about 10% of *M. catarrhalis* isolates are resistant to trimethoprim/sulfamethoxazole (73). Overall, *M. catarrhalis* remains sensitive to macrolides, fluoroquinolones, and tetracyclines, with few reports of resistance to these antimicrobial agents (156).

G. Potential virulence factors

Having proven its ability as a pathogen, *M. catarrhalis* has drawn the attention of numerous investigators who are now trying to identify the virulence factors of this bacterium that allow it to cause disease. These potential virulence factors could have several functions including: i) mediating binding and colonization; ii) facilitating multiplication within the host, either by avoiding host defenses or by providing access to nutrients and essential elements; and iii) causing damage to the host. These potential virulence factors include, but are not limited to, both outer membrane proteins and lipooligosaccharide.

i. Integral or outer membrane-associated proteins

a. UspA1

One of the *M. catarrhalis* outer membrane-associated proteins that has been extensively studied and implicated as a potential virulence factor is the Ubiquitous Surface Protein A1 (UspA1). UspA1 was initially designated as the UspA protein (116,161), which was observed as a high-

molecular-weight protein that was expressed by 100% of the *M. catarrhalis* strains tested by Helminen et al (116). The UspA antigen was detected by using MAb 17C7 that had been obtained by immunizing mice with *M. catarrhalis* outer membrane vesicles. In fact, the use of this MAb to passively immunize mice enhanced pulmonary clearance of *M. catarrhalis* in a mouse model (116). Furthermore, antibody against UspA, whether developed in mice or guinea pigs, had complement-dependent bactericidal activity toward homologous and heterologous *M. catarrhalis* strains, and active immunization of mice with purified UspA led to more rapid clearance of *M. catarrhalis* from mouse lungs (56).

Subsequent studies showed that UspA is actually composed of two different proteins that share a common epitope that binds MAb 17C7 (5,61). These two proteins were later named UspA1 and UspA2 (5). However, further studies on these two proteins showed that both of them were expressed on the surface of *M. catarrhalis*, that they enhance pulmonary clearance of *M. catarrhalis* when they were used for active immunization, and that antibodies to UspA1 and UspA2 in sera from healthy adults and children were bactericidal and cross-reactive (4,55,181,204). Moreover, a later study indicated that at least two surface-exposed epitopes of both UspA1 and UspA2 are ubiquitously expressed in isolates from nasopharyngeal swabs of young children (190). In addition, both UspA1 and UspA2 were among the *M. catarrhalis* antigens to which antibodies were produced by the majority of adults with COPD who cleared the organism (204), and most COPD patients with *M. catarrhalis* infections made new sputum IgA responses to both proteins (205).

The predicted mass of the UspA1 protein is 80,000-90,000 Da depending on the strain. However, for unknown reasons, its apparent molecular mass in SDS-PAGE is about 120 kDa (5). It has been shown that expression of this protein undergoes phase variation due to changes in the number of G residues contained within a homopolymeric [poly(G)] tract located upstream of the

uspA1 ORF (166). In a more recent study, Meier et al reported that *M. catarrhalis* clinical isolates exhibiting reduced expression of UspA1 and UspA2 belonged to a distinct phylogenetic subpopulation and that antigenic or phase variation was not responsible for reduced levels of UspA1 expression by these strains (191). Moreover, Heiniger el al indicated that cold shock at a physiologically relevant temperature of 26°C increased expression of UspA1 (114).

UspA1 protrudes from the surface of the *M. catarrhalis* cell (125,225) and has been shown to act as an adhesin for several cell lines. First, it was shown that UspA1 is involved in mediating the binding of *M. catarrhalis* to Chang conjunctival epithelial cells and to HEp-2 cells (4). In another study, a single colony derivative of a *M. catarrhalis* isolate was demonstrated to bind to transfected Chinese hamster ovary cells and human respiratory epithelial cells in a carcinoembryonic antigen-related cellular adhesion molecule (CEACAM)-dependent manner (121). Also, purified UspA1 has been shown to bind the extracellular matrix protein fibronectin (185). In a more recent study, recombinant UspA1 was shown to bind fibronectin and a truncated UspA1 protein (aa 299-452) inhibited binding of *M. catarrhalis* to Chang conjunctival epithelial cells to an extent similar to that achieved by anti-human fibronectin antibodies (277). Also, recombinant UspA1 was shown to bind purified C4BP which is a regulator of the complement system (216). In addition, recombinant UspA1 bound both the third component of complement (C3) from EDTA-treated serum and methylamine-treated C3 noncovalently and in a dose-dependent manner (217). However, the physiological significance of the binding of the last two proteins (i.e. C4BP and C3) to UspA1 was not fully addressed in the cited studies.

b. UspA2/UspA2H

UspA2 is the second protein that was a part of what was previously identified as UspA (5). One report also described a high-molecular-weight outer membrane protein (HMW-OMP) that was later shown to be UspA2 (161). The predicted mass of the UspA2 protein is about 62,000 Da, however, it migrates in SDS-PAGE as an aggregate with an apparent molecular mass of more than 200 kDa (5). In an analysis of the outer membrane proteins of *M. catarrhalis*, UspA2 was seen as a band with an apparent molecular mass of 85 kDa, which might be the monomeric form of UspA2 (5). It has been shown that UspA2 forms a very dense layer of relatively short surface projections on the surface of *M. catarrhalis* (125,225). Several studies have shown that UspA2 is expressed by the majority of *M. catarrhalis* strains (116,161,190). UspA2 shares homology with the *Yersinia enterocolitica* protein YadA and DsrA of *Haemophilus ducreyi* and it is considered a putative autotransporter macromolecule (118). Up to this point, very little was known about the regulation of the expression of this protein. However, several papers cited the presence of a tetranucleotide repeat (AGAT) in the region immediately upstream from the translation initiation codon of the *uspA2* ORF (5,61,109) and suggested that these repeats might have a regulatory role in the expression of UspA2.

The direct involvement of UspA2 in serum resistance of *M. catarrhalis* strain O35E was demonstrated recently (13) (these data are described in Chapter Four of this dissertation). The hypothesis that serum resistance might be a virulence factor for *M. catarrhalis* stemmed from observations that the incidence of complement-resistant *M. catarrhalis* strains was higher in samples isolated from ill patients (i.e., adults with lower respiratory tract infections) than in samples from healthy adults or children (128,152). However, isolation of serum-resistant *M. catarrhalis* from the nasopharynges of apparently healthy infants and young children was documented in more recent studies (190,316). As mentioned before, many *M. catarrhalis* strains express UspA2 (116,161,190);

however, this was true also for serum-sensitive strains which raises the possibility that UspA2 expressed by serum-sensitive *M. catarrhalis* strains is structurally different from that of the serum-resistant ones. This possibility was supported by two studies. First, automated ribotyping using the Qualicon RiboPrinter$^{(R)}$ microbial characterization system showed that complement-sensitive and complement-resistant *M. catarrhalis* strains segregated into two lineages within the species (294). Second, the UspA2 protein from a serum-resistant strain caused a significant increase in the serum resistance of a recombinant *H. influenzae* strain while the UspA2 protein derived from a serum-sensitive *M. catarrhalis* strain was not able to produce this effect (13) (these data are presented in Chapter Four of this dissertation).

The UspA2 protein binds to various human proteins that might be involved in allowing *M. catarrhalis* to survive within the hostile environment of the human host. Purified or recombinant UspA2 binds purified human vitronectin (185), purified C4BP (216), the third component of complement (C3) (217), and fibronectin (277). But again, as with UspA1, the physiological roles of these UspA2-mediated binding activities in serum resistance of *M. catarrhalis* were not adequately addressed in the cited studies.

It should be noted that approximately 20% of *M. catarrhalis* strains do not express UspA2. Instead, these strains express a related protein that was designated UspA2H (165). This protein represents a hybrid of both UspA1 and UspA2; much of the N-terminal half of UspA2H is homologous to the N-terminal half of UspA1 while the C-terminal region is homologous to the C-terminal half of UspA2 (165). More interestingly, UspA2H proteins can function both as an adhesin (like UspA1) and a serum resistance factor (like UspA2) (165).

c. Hag (MID)

Another *M. catarrhalis* outer membrane-associated protein that has been proposed to be a virulence factor is the Hag (hemagglutinin) protein, also known as the *Moraxella* IgD-binding protein (MID) (86,225). This protein was first described as the 200 kDa protein and it was associated with the ability of some *M. catarrhalis* strains to mediate hemagglutination (83). Further analysis of this protein using transmission electron microscopy indicated that this protein forms a trypsin-sensitive outer fibrillar coat extending from the bacterial surface (84). Several studies have attributed functional properties to the Hag protein. Besides its ability to cause hemagglutination of human erythrocytes, it was shown to be responsible for the autoagglutination of *M. catarrhalis* strain O35E cells (225). In addition, Hag acts as an adhesin for several cells including cell lines derived from human lung and middle ear tissues such as HMEE (85,131,225). The binding to this latter cell line was shown to directly involve the Hag protein (46). Also, Hag was shown to bind to IgD (86,225) and induce human B lymphocyte activation and Ig secretion in the presence of Th2 cytokines (315). This activation has a strong requirement for signaling through the CD19 molecule (102). Finally, the Hag protein exhibits phase variation due to changes in the number of G residues found in a poly (G) tract within the 5′-end of the *hag* ORF (196,225). In contrast to UspA1, cold shock at a physiologically relevant temperature of 26°C did not increase transcription of the *hag* gene (114).

d. OMP CD

Initial analysis of *M. catarrhalis* OMPs using SDS-PAGE showed two bands in the 60-kDa range that were designated OMP C and OMP D (19,210). Later, it was found that these two bands represent a single heat-modifiable protein that was termed OMP CD (208). The predicted molecular mass of the mature OMP CD is 46 kDa; however, its apparent molecular mass as seen in SDS-PAGE

is ~60 kDa. This migration rate aberration was attributed to the presence of a proline-rich region within the protein (208).

The OMP CD protein is highly conserved among *M. catarrhalis* strains and it contains epitopes that are abundantly expressed on the bacterial surface (19,259). Guinea pig antibodies against OMP CD were bactericidal against various *M. catarrhalis* strains (319). Similarly, mouse antibodies produced against recombinant OMP CD were shown to enhance the pulmonary clearance of *M. catarrhalis* in mice (209). More recently, OMP CD was identified as one of the major targets of antibodies to surface *M. catarrhalis* epitopes in the majority of adults with COPD who cleared the organism (204). OMP CD shares homology with the *Pseudomonas* OprF porin protein (208). The latter protein was shown to act as an adhesin (15), a characteristic that was also shown to be true for OMP CD (130,247). Finally, an *ompCD* mutant was shown to be serum-sensitive (130). However; this same mutant grew very slowly relative to its wild-type parent strain and this difference might contribute to the observed reduction in serum resistance.

e. OMP E

Outer membrane protein E (OMP E) is a 50-kDa protein of *M. catarrhalis* with epitopes on the bacterial surface (203). OMP E is antigenically conserved among *M. catarrhalis* strains; this was shown using immunoblot assays (203,207) and also by using RFLP (26). In spite of the immunogenic potential of OMP E, very low titers of antibodies against OMP E were detected in adults infected with *M. catarrhalis* (27). OMP E exhibits minimal homology to the FadL protein of *E. coli* (26). The latter protein has been shown to be involved in the transport of fatty acids (28); however, it is not known if the *M. catarrhalis* OMP E protein has the same function. In addition, OMP E shares homology with the OMP F porin of *E. coli* (26). OMP E does exhibit a trimeric structure, which is a

characteristic of many porins, raising the possibility that OMP E might act as a porin in *M. catarrhalis* (26). An *ompE* mutant exhibited increased susceptibility to killing by NHS as compared to its wild-type parent strain; however, similar to the OMP CD mutants, this mutant exhibited a growth defect that might be responsible, at least in part, for the observed serum sensitivity (130).

f. Pili (fimbriae)

Several gram-negative bacteria use filaments, termed pili, for attachment to mucosal surfaces. In the case of *M. catarrhalis*, several studies using transmission electron microscopy (TEM) indicated the presence of surface projections that were referred to as pili (7,8,178,249). The expression of pili was decreased by in vitro passage (7). In addition, DNA hybridization using a cloned type IV pilin gene from the related organism *M. bovis* showed that *M. catarrhalis* likely has a type IV pilin gene (178). Also, *M. catarrhalis* showed several phenotypic characteristics that are related to the presence of type IV pili including competence for DNA transformation, autoagglutination, pellicle formation, colony morphology, and pitting of agar. However, *M. catarrhalis* did not exhibit twitching motility (178). A definitive conclusion about the presence and functionality of *M. catarrhalis* pili was not obtained for a long time. In 2004, Luke et al. identified and cloned the *M. catarrhalis* genes encoding PilA, the major pilin subunit, PilQ, the outer membrane secretin through which the pilus filament is extruded, and PilT, the NTPase that mediates pilin disassembly and retraction (173). The construction of relevant isogenic mutants demonstrated that *M. catarrhalis* expresses type IV pili that are essential for natural genetic transformation (173). However, additional efforts are required to elucidate the role, if any, of these type IV pili in the pathogenesis of *M. catarrhalis* disease.

g. Other outer membrane proteins

In recent years, several other outer membrane proteins of *M. catarrhalis* were studied and their potential involvement in virulence or utility for vaccine development was postulated. For example, McaP (*M. catarrhalis* adherence protein) is involved in *M. catarrhalis* adherence to several human cell lines and also exhibits phospholipase activity (282). Outer membrane protein G1 (OMP G1), initially identified as a 26-kDa protein using SDS-PAGE, turned out to be two individual proteins, designated OMP G1a and OMP G1b, which were conserved among *M. catarrhalis* strains and contained epitopes that were displayed on the bacterial surface (1). A subsequent study showed that OMP G1a is expressed during infection of the human respiratory tract and is a target for both systemic and mucosal antibodies (2). M35 is a novel *M. catarrhalis* 36-kDa protein that has structural homology to porins such as OMP C from *E. coli* and OMP K36 from *Klebsiella pneumoniae* and is highly conserved with surface-expressed epitopes (71). Most recently, Hays et al characterized a novel outer membrane protein of *M.* catarrhalis, which existed in two variant forms (i.e., OMP J1 and OMP J2) (112).

ii. Lipooligosaccharide

The *M. catarrhalis* outer membrane contains lipooligosaccharide (LOS) that lacks the long O-polysaccharide side chain, which is a characteristic of enteric bacterial lipopolysaccharide (LPS). However, *M. catarrhalis* LOS shares structural similarities with other non-enteric bacterial LOS molecules, such as those of *N. meningitidis, N. gonorrhoeae,* and *H. influenzae* (132,237). Examination of 302 *M. catarrhalis* strains using serological methods showed that more than 90% of *M. catarrhalis* isolates fall in one of three LOS serotypes that were designated LOS A, B, and C

(290). More recently, Edwards et al described a multiplex PCR assay that differentiated among the three LOS serotypes more rapidly and efficiently than serology (72).

Rabbit immunization with *M. catarrhalis* strains belonging to the different LOS serotypes resulted in the production of serotype-specific antibodies (237). However, the antibodies produced during human infections with different *M. catarrhalis* strains were not specific for the LOS serotype of the infecting *M. catarrhalis* strain (238). MAb 8E7, an immunoglobulin G3 antibody specific for *M. catarrhalis* LOS serotypes A and C, is bactericidal and also inhibits the adherence of *M. catarrhalis* to Chang conjunctival epithelial cells (135). When this MAb was used in passive immunization experiments, it significantly enhanced the clearance of *M. catarrhalis* from mouse lungs in an aerosol challenge model (135). In another study, *M. catarrhalis* LOS detoxified by the use of anhydrous hydrazine was linked to tetanus toxoid. This LOS-protein conjugate could induce the synthesis of bactericidal antibodies in rabbits and an enhancement in the clearance of *M. catarrhalis* from mice lungs (101,134). In a more recent study, detoxified *M. catarrhalis* serotype B LOS was conjugated to tetanus toxoid or a cross-reactive mutant of diphtheria toxin; both conjugates induced the synthesis of bactericidal antibodies in mice or rabbits (321).

At least three genes encoding products involved in LOS biosynthesis, including *galE* (322), *kdsA* (171), and *kdtA* (226), have been shown to be necessary for normal expression of serum resistance by *M. catarrhalis*. However, none of these studies showed the direct involvement of the LOS in the serum-resistant phenotype of *M. catarrhalis*. In addition, in the case of both the *kdsA* and *kdtA* genes, the mutants showed significant growth impairment as compared to that of their respective wild-type parent strains (171,226). Besides the serum-sensitive phenotype, the *kdtA* mutant also showed reduced adherence to human epithelial cells and enhanced clearance from the lungs and nasopharynx of mice (226).

iii. Iron-regulated proteins

Iron is an essential element for several metabolic processes and, within the human body, most of the iron is localized intracellularly. The small amounts of iron that are found extracellularly are sequestered by a group of proteins. These proteins include both transferrin and lactoferrin (305). In the absence of siderophore (i.e., iron-sequestering agent) production by *M. catarrhalis* (49), this bacterium expresses specific proteins that act as receptors for the human iron-sequestering proteins, which are then used as a source for iron (49). These receptors are considered to be potential virulence factors because they are essential for the survival of the pathogen within the hostile, iron-limited environment in the host.

a. TbpA and TbpB

M. catarrhalis transferrin-binding proteins TbpA and TbpB have molecular masses that range between 115-120 kDa (TbpA) and 80-84 kDa (TbpB) (156). TbpB was previously designated as OMP B1 (49). Isogenic mutants of both *tbpA* and *tbpAB* were severely limited in their ability to grow with human holotransferrin as the sole source of iron. However, the *tbpB* mutant was capable of utilizing iron from human transferrin, although not to the same extent as the wild-type parent strain (172). These data indicated that TbpA is capable of binding transferrin and removing iron while TbpB is not essential but may serve as a facilitating protein that functions to optimize this process (172).

Recombinant TbpA and TbpB proteins were capable of eliciting antibodies in vivo (211). An antiserum against TbpA was found not to be bactericidal, but antibodies raised against TbpB were able to kill heterologous *M. catarrhalis* strains within the same family (211). In addition, children

infected with *M. catarrhalis* have antibodies to TbpB in their convalescent sera (48). In a more recent study, it was found that a small proportion of COPD patients made new sputum IgA responses to TbpB (205).

b. LbpA and LbpB

Lactoferrin is another human iron-sequestering protein for which *M. catarrhalis* expresses two receptors (i.e., lactoferrin-binding proteins A and B) (70). Isogenic mutants unable to express LbpA or LbpB were constructed and characterized (39). Both mutants were unable to utilize lactoferrin as the sole source of iron for growth and showed reduced binding activity for human lactoferrin (39). Convalescent human serum from patients infected with *M. catarrhalis* reacted specifically with LbpB but not with LbpA (40). Similar to Tbp, immunization using LbpB produced bactericidal antibodies whereas when LbpA was used, no bactericidal antibodies were obtained (320).

c. CopB

CopB, also known as OMP B2, is an 80-kDa major outer membrane protein (115). The expression of CopB was increased when *M. catarrhalis* was grown under iron-limiting conditions (6,49). An isogenic *copB* mutant was severely impaired in its ability to utilize transferrin and lactoferrin as sole sources of iron for growth whereas this same mutant grew similarly to the wild-type parent strain when ferric citrate was used as the iron source (6). However, the *copB* mutant was able to bind both transferrin and lactoferrin at levels comparable to those bound by the wild-type strain (6). Another phenotype of the *copB* mutant was its increased susceptibility to killing by NHS (117); however, direct involvement of CopB in serum resistance was not addressed in this study. In addition, the *copB* mutant's ability to survive and grow in the lungs of animals was impaired and

genetic restoration of CopB protein expression resulted in re-acquisition of the ability to resist pulmonary clearance in vivo (117). MAbs against CopB were shown to be reactive with about 70% of the tested strains (115,266), indicating that there is moderate heterogeneity in the CopB protein among *M. catarrhalis* strains. Patients with bronchiectasis showed low titers of antibodies against CopB (264). A small proportion of patients made new sputum IgA responses to CopB (205) although salivary antibodies directed against *M. catarrhalis* CopB were also detected in healthy adults (189).

d. HumA

A newly characterized *M. catarrhalis* iron-utilization protein is HumA, an outer membrane protein involved specifically in hemin utilization. HumA expression was clearly increased when *M. catarrhalis* was grown in the presence of hemin (91). In addition, growth analyses revealed that growth of a *humA* mutant in the presence of hemin as the sole iron source was restricted when compared to that of the wild-type parent strain (91). The immunogenic potential of this protein and the possible presence of human antibodies against it remain to be determined.

e. MhuA

The latest *M. catarrhalis* protein to be added to the list of iron-utilization proteins is MhuA. It is a 107-kDa outer membrane protein involved in haemoglobin utilization (92). An isogenic *mhuA* mutant showed a significant lag during growth in the presence of haemoglobin as the sole iron source. Changes in growth conditions seemed to have no apparent effect on the expression of this protein. A MAb raised against MhuA showed that this protein contains highly conserved and surface-exposed epitopes (92).

H. Animal models for *M. catarrhalis* disease

An important tool for the evaluation of the role(s) of the potential virulence factors described above is a reliable and relevant animal model. Several models have been developed for use with *M. catarrhalis*; however, they all suffer from significant limitations.

i. Mouse pulmonary clearance model

The most extensively utilized *M. catarrhalis* animal model is the mouse model for pulmonary clearance. The principle of this model involves measuring the rate at which mice are able to clear *M. catarrhalis* from the lungs after these bacteria have been introduced directly into the lungs (286). The mice do not develop pneumonia, and usually the bacteria are cleared within 24 hours after inoculation. This fact represents the most significant limitation of this model because it cannot be used as model of pulmonary infection (286). The other limitation of this model is the inoculation technique by which the bacteria are introduced directly into the lungs via an incision and intratracheal tube, thus circumventing the natural route of infection. In spite of these limitations, this model was extensively used to evaluate the clearance of bacteria following immunization using different *M. catarrhalis* antigens (57,164,175,185,209). Also, this model has been used to compare the rate at which isogenic mutants are cleared as compared to their respective wild-type parent strains (117). A modified version of this model was used by another group in several studies in which they aerosolized the bacteria instead of introducing them directly into the trachea of the anesthetized mouse (134-136). Rapid clearance of the bacteria from the lung was also observed with this modified model.

ii. SCID mouse model

The severe combined immunodeficient mouse (SCID) was used as a possible animal model for *M. catarrhalis* infection. Multiple routes of infection were used to introduce the bacteria; this included intranasal and intravenous challenges (107). Although different inocula were tested and both clinical and postmortem findings of infection were observed, these symptoms had no resemblance to those seen in human infections with *M. catarrhalis*. The same group tested also SCID/beige mice and they were more affected than SCID mice both clinically and pathologically (107). The authors speculated that natural killer cell and polymorphonuclear cell functions may be important in resolving *M. catarrhalis* infections. The one aspect in which this model had some resemblance to the human situation was that susceptibility to *M. catarrhalis* appeared to be age-dependent (i.e., young mice were more susceptible than somewhat older mice).

iii. Chinchilla model

The chinchilla animal model has been used very successfully with two major bacterial causes of otitis media (i.e., *H. influenzae* and *S. pneumoniae*) (18,93). However, when *M. catarrhalis* was inoculated into the middle ear of the chinchilla, it did not persist (69,89). Together with the rapid clearance of the bacteria, there was no evidence of symptoms consistent with otitis media in humans. In an effort to enhance *M. catarrhalis* infection in chinchillas, adenovirus serotype 1 was co-infected with *M. catarrhalis* to compromise the tubotympanum and facilitate the subsequent induction of middle ear disease (16). However, contrary to what had been seen before with *H. influenzae*, this approach failed to promote the development of otitis media with *M. catarrhalis* (276).

iv. Rat model

Another animal model that was investigated for its potential for studying *M. catarrhalis* infections involved the Sprague-Dawley rat (309). In this study, rats were challenged and rechallenged with four different *M. catarrhalis* strains. However, only viable bacteria in very high concentrations induced purulent otitis media associated with opaque effusions and yielded positive cultures for *M. catarrhalis* after 4 days. In another study, *M. catarrhalis* caused an increase in the number of goblet (mucus-producing) cells within the Eustachian tube (53).

v. Other models

Other animal models that have been investigated for use with *M. catarrhalis* include gerbils and macaques. For gerbils, results similar to those reported for chinchillas were obtained (89). In the case of the macaques, *M. catarrhalis* was found both in healthy rhesus macaques and in possibly immunocompromised rhesus macaques (43). In an earlier study, ~60% of macaques inoculated with *M. catarrhalis* became culture-positive and had mild nasal discharge (289). However, the high cost and technical difficulty that accompanies working with macaques preclude the usefulness of this model.

I. Vaccines for *M. catarrhalis*

With increasing recognition of *M. catarrhalis* virulence and the risks it poses for both young children and adults with COPD, the need for an effective vaccine becomes more important. Currently, there is no vaccine for *M. catarrhalis* (202). However, several candidate immunogens have been proposed (184,186). To be qualified as a vaccine component, the antigen should possess

certain characteristics including surface exposure, conservation among strains, expression in vivo, immunogenicity, and the capability to produce a protective immune response (202). Another point to be considered for an *M. catarrhalis* vaccine candidate is its ability to produce the desired immune response in the target population. The fact that the most susceptible population for otitis media caused by *M. catarrhalis* is infants makes protein antigens the preferable candidates as proteins are likely to be immunogenic in infants (100,202). Most of the potential *M. catarrhalis* virulence factors discussed above could be considered as vaccine components. These include UspA1, UspA2, Hag, OMP CD, OMP E, McaP, LOS, TbpA, TbpB, LpbA, LpbB, and CopB. However, each of these antigens by itself might lack one or more of the mentioned requirements for a vaccine candidate. For instance, one limitation affecting both UspA1 and Hag is their ability to undergo phase variation (166,225). Also, the amino acid sequence of some of the cited antigens is not universally homologous among all strains, a situation that would necessarily require the inclusion of antigens from several selected strains. Finally, since *M. catarrhalis* is a strict human pathogen, the efficacy of a potential vaccine candidate cannot be predicted with complete certainty based on animal model testing. Therefore, designing and optimizing a vaccine for *M. catarrhalis* remains a challenge that needs to be addressed in the future.

II. The Complement System

A. Historical perspective and overview

In the 1880's, von Fodor, Nuttall, and Buchner discovered that serum has a bactericidal effect on bacteria. Later, Nutall showed that this effect was due to a heat-sensitive component in immune human serum. This component turned out to be what it is known now as the complement system. The complement designation was given by Paul Ehrlich in 1899, as it was thought that this component "complements" antibodies in the lysis of target cells [reviewed in (182,183)]. For a long time, the complement system was seen only as an effector mechanism for antibodies, at least until Louis Pillemer introduced the idea of the spontaneous activation of the complement system in the absence of antibodies. This phenomenon was known first as the properdin pathway and was later designated as the alternative pathway [reviewed in (182,183)]

The complement system is comprised of at least 20 plasma proteins that function either as enzymes or as binding proteins (198). It also contains a group of distinct cell-surface receptors that are specific for physiological fragments of some of the complement proteins. In addition, it includes several regulatory membrane proteins that protect host cells against accidental attacks by the complement system (198). Generally, the complement system is involved in three main functions within the human body: lysis of foreign bodies, labeling them to be phagocytosed (opsonization), and induction of inflammatory responses (198).

B. Pathways for complement activation

The complement system can be activated via one of three activation pathways that differ in the triggering event; however, all of the three pathways converge at the level of the fifth complement component (C5). A schematic representation of the organization of the three complement activation pathways and their regulatory proteins is presented in Fig. 1

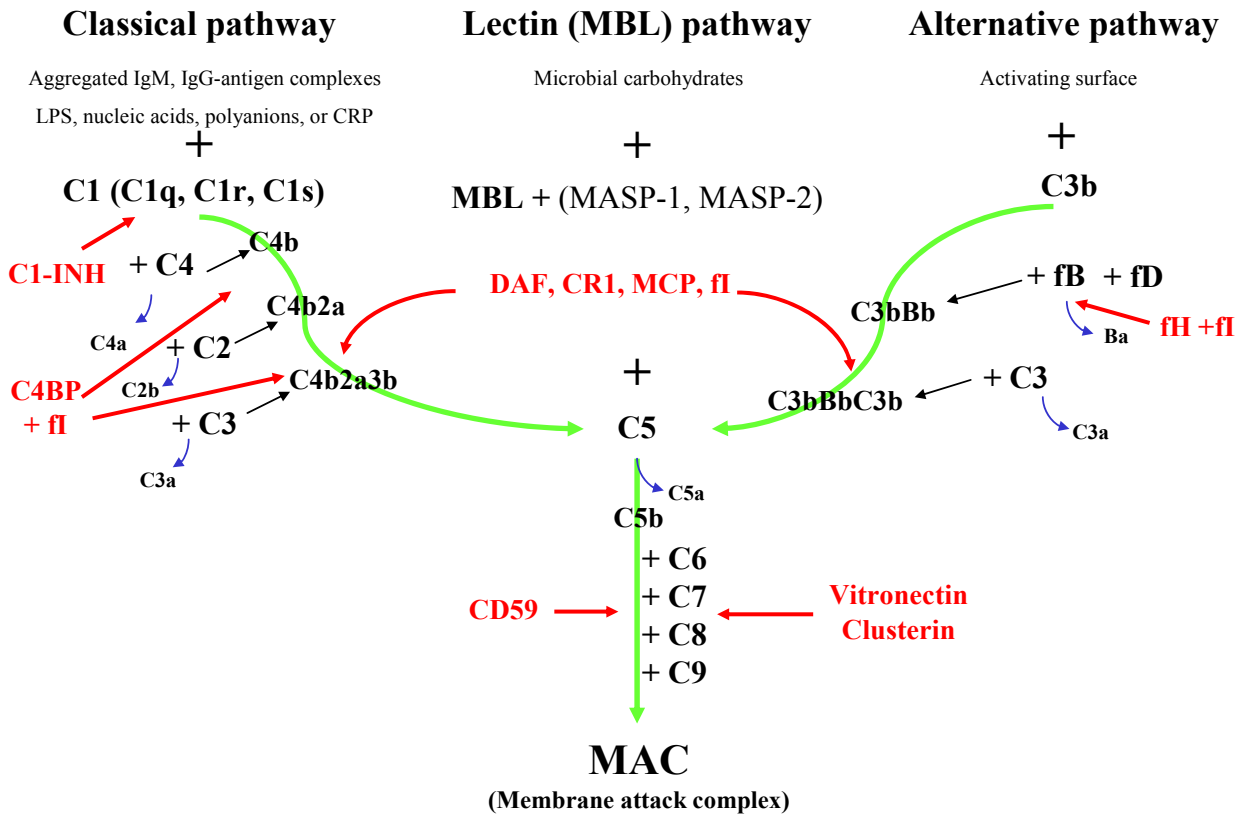

Fig. 1. A schematic representation of the molecular organization of the complement pathways and their regulatory proteins.

i. Classical pathway

The classical pathway can be activated by the binding of the first complement component (C1) to the Fc-portion of an immunoglobulin that can be either one IgM molecule or at least two adjacent IgG molecules (197). The classical pathway can also be activated through the binding of C1 to nucleic acids, bacterial LPS, immune complexes, or C-reactive protein (170). C1 is composed of three subunits (i.e., C1q, C1r, and C1s). C1q is the component that binds to the activator on the surface of the target cell; this binding leads to conformational changes that subsequently lead to the proteolytic activation of both C1r and C1s in the presence of Ca^{2+} (324). The activated C1s would subsequently cleave C4 and C2 (197).

C4 is composed of three polypeptide chains [α (93 kDa), β (75 kDa), and γ (33kDa)] that are held together through disulphide bonds. Upon cleavage by activated C1s, a small fragment (C4a) is released, which acts as an anaphylytoxin (99), leaving behind a larger fragment (C4b) which has a labile binding site through which it binds to the target surface (197).

C2 is a single-chain molecule that is cleaved by the activated C1s into a small fragment (C2b) and a large fragment (C2a). The latter fragment binds to C4b in a Mg^{2+}-dependent reaction to form what is known as the classical pathway C3 convertase (C4b2a) (158).

C3 is the most abundant complement protein and it consists of two polypeptide chains (i.e., α (117 kDa) and β (75 kDa)] that are held together by a disulphide bond. Upon activation, a small fragment (C3a) is released, which acts as an anaphylytoxin (302) and a larger fragment (C3b) that

binds to C4b2a to form the classical pathway C5 convertase (C4b2aC3b) which cleaves C5, leading to triggering of the terminal pathway (197).

ii. Lectin pathway

The lectin pathway was only discovered in the 1980's, making it the most recently described complement activation pathway (317). The first component of this pathway is the mannan-binding lectin (MBL) which mimics C1q in structure. MBL recognizes a range of specific carbohydrate structures which are found on the surfaces of pathogens, such as those containing N-acetyl-glucosamine and mannose (248). MBL is associated with at least two serine proteases known as MBL-associated serine proteases (MASP-1 and MASP-2). These proteases have catalytic activity against both C4 and C2 (281). After C2 cleavage, the activation of the lectin pathway proceeds as in the classical pathway.

iii. Alternative pathway

The third pathway is the alternative pathway, which acts as rapid and antibody-independent route for both activation and amplification of the complement cascade. The key molecule in this pathway is C3 which has an internal thioester bond that is spontaneously hydrolyzed to form C3(H_2O) (221). C3(H_2O) or C3b binds factor B (fB), which is a single-chain 93 kDa protein that is related to C2. This binding is Mg^{2+}-dependent and leads to an increase in the susceptibility of fB to cleavage by the serine protease factor D (fD) (197). Upon cleavage of fB, a small fragment (Ba) is released, leaving behind the large fragment (Bb) attached to C3b, thereby forming C3bBb, the C3 convertase of the alternative pathway (197). The C3bBb is stabilized by the action of a basic glycoprotein called properdin which extends the life time of this convertase by 3- to 4-fold (304).

C3bBb cleaves more C3 molecules and rapidly generates large numbers of C3b that can attach to surface of the target and/or bind to C3Bb to form the C5 convertase of the alternative pathway C3bBbC3b which cleaves C5, leading to triggering of the terminal pathway (197).

iv. Terminal pathway

After complement activation via any of the three pathways mentioned above and the formation of the C5 convertases, the terminal pathway starts. This pathway is also known as the membrane attack complex (MAC) pathway (197). C5 is cleaved, releasing a small fragment (C5a) which has potent anaphylytoxin and chemotaxin properties (60) and a larger fragment (C5b). C5b binds to C6 and forms a stable and soluble bimolecular complex which binds C7 and induces it to express a metastable site in its hydrophobic regions through which the C5b-7 complex can insert itself into membranes (133). Then C8 binds to C5b-7, forming C5b-8 which inserts more deeply into the membrane (197). The C5b-8 acts as a receptor for the last complement component (C9) whose binding initiates a process of C9 polymerization. At least twelve C9 molecules are required to form an elongated channel structure that spans across the membrane; this is known as the MAC (229). These pores or channels in the membrane eventually lead to lysis and death of the target cell (197).

C. Regulation of the complement system

The activation of the complement system has a strong potential for generating inflammation and causing tissue destruction; therefore, it has to be kept under strict control. There are two goals for this regulatory control: preventing damage to host cells during activation and preventing over-

consumption of the complement components. The complement regulators can be categorized into two groups: fluid-phase regulators and membrane-bound regulators (193).

i. Fluid-phase regulators

a. C1-inhibitor

C1-inhibitor (C1-INH) is a single-chain glycoprotein that belongs to a family of serine protease inhibitors called serpins (50). C1-INH regulates the complement systems via two actions: i) inhibiting the protease activity of both C1r and C1s by removing them from the C1-complex (325) and ii) preventing the autoactivation of C1 in the absence of antibodies (323). People who are deficient in C1-INH develop a disease called hereditary angioedema (HAE), associated with edema in the gastrointestinal tract, airways, and skin (64).

b. C4b-binding protein

C4b-binding protein (C4BP) is a potent soluble regulator of both the classical and the lectin complement pathways. C4BP is a multichain protein that consists of seven identical 75 kDa α-chains and a unique 40 kDa β-chain that are held together by disulphide bridges (35). Similar to some other complement regulators, C4BP consists of basic structural units called short consensus repeat units (SRC), also known as complement control protein (CCP) (33). The major ligand for C4BP is the α-chain of C4b and, due to steric hindrance, only four of the C4BP α-chains of C4BP can bind simultaneously to C4b molecules on one surface (326). C4BP functions as a cofactor for factor I (fI) in the cleavage of C4b into C4c (that is released into the medium) and C4d (that stays attached to the target surface) (88,240). Also, C4BP acts as decay-accelerating factor for the classical pathway C3

convertase (C4b2a) by irreversibly displacing C2a from this complex. C4BP also can prevent the assembly of C4b2a by binding nascent C4b (94). Moreover, at very high concentrations, C4BP is able to act as cofactor in degradation of surface-bound C3b and can accelerate decay of alternative pathway C3-convertase (C3bBb) (32).

Besides its regulatory role in the complement system, C4BP binds to the vitamin K-dependent regulator of the coagulation system known as protein S (PS). This binding is mediated through the β-chain of the C4BP molecule (122). The exact role of this complex (i.e., C4BP-PS) is not clear at this time; however, it was observed that this complex, when bound to apoptotic cells, inhibits phagocytosis of these cells (157).

c. Factor H

Factor H (fH) in the alternative pathway is analogous to C4BP in the classical pathway. It was initially described as β1H-globulin (215) and it acts as a regulator of the alternative pathway C3 convertase (C3bBb). fH is comprised of twenty SCR units that are arranged like beads on a string (270). Akin to C4BP, fH carries out its regulatory role via three mechanisms: i) acting as a cofactor for fI in the cleavage of C3b into the inactive form iC3b (220), ii) accelerating the decay of C3bBb by displacing Bb from the complex, and iii) blocking the assembly of the C3bBb complex by competing for the binding sites in C3b with fB (304,311). Using these three mechanisms, fH tightly controls the alternative pathway; otherwise this pathway would be extensively depleted (193). The absence of fH has been associated with a lethal disease in pigs (i.e., membranoproliferative glomerulonephritis (MPGNII)) and a less severe form of this disease has been described in humans who are deficient in fH (308).

Alternative splicing of the fH-encoding gene produces a 42-kDa protein that is called factor-H like protein (FHL-1) (78). Similar to fH, FHL-1 protein displayed cofactor activity in factor I-mediated cleavage of C3b (163). Another group of proteins that share structural similarity with fH but which are products of different genes in the same chromosome as the genes encoding both fH and FHL-1 are called Factor-H related proteins (FHR). At least five of these proteins have been described but their functions are not fully understood (327).

d. Clusterin

Clusterin, also known as SP-40-40, Apo-J, complement lysis inhibitor (CLI), and sulphated glycoprotein 2 (SGP2) (193), is a plasma protein with several proposed functions, including clearing of cell debris and lipid transport (147). Regarding the complement system, it has been shown that clusterin binds to the C5b-7 complex, blocking its attachment to the target surface and that it can also bind to C7, C8, and C9 (284). However, the exact role of this protein in complement regulation is still debatable, and this regulatory role was even challenged due to the fact that physiological levels of clusterin were not able to protect target cells against complement-mediated lysis (124).

e. Vitronectin

Vitronectin, also known as serum spreading factor or site-specific protein (S protein), represents up to 0.5 % of total plasma protein. It is a multifunctional, adhesive glycoprotein that has been implicated in several activities including cell adhesion, cell invasion, and complement regulation (233). The proposed role of vitronectin in complement regulation involves interference with MAC formation at two stages. First, vitronectin occupies the metastable membrane-binding site of the nascent precursor complex C5b-7 such that the formed water-soluble SC5b-7 macromolecule

is unable to insert into the cell membrane (234). However, it does not block the binding of the subsequent complement components C8 and C9. Instead, it leads to the formation of the soluble complex SC5b-9, which cannot be inserted into the membrane of the target cell. The second role is blocking the polymerization of C9, in a concentration-dependent manner, such that ongoing cell-associated pore formation is limited in the presence of vitronectin (149,228).

Vitronectin appears in plasma as a mixture of 65 and 75 kDa forms. It is widespread within the body as it is found in plasma, other body fluids, and also in connective tissue. The protein itself is composed of several domains that are involved in the multifunctional tasks in which vitronectin is involved (193). For instance, its Arg-Gly-Asp (RGD) domain is involved in the binding to the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (235). The binding to terminal complement proteins is mediated partially through its heparin binding domain and a proposed hydrophobic interaction (285). Also, vitronectin has binding domains for polycations, collagen, and somatomedin B (193).

ii. Membrane-bound regulators

a. Complement receptor type 1

Complement receptor type 1 (CR1; CD35) is a single-chain glycoprotein that is expressed by several cells; however, most of the CR1 in the circulation is on the surfaces of erythrocytes (81). CR1 has a cofactor activity for fI in the cleavage of C3b, C4b, and also in the cleavage of iC3b into C3c and C3d (188). In addition, CR1 exhibits decay-accelerating activity towards the C3 and C5 convertases of both the classical and alternative pathways that are formed either on the same cell or

on a nearby target (140). CR1 also is involved in the transport and removal of immune complexes and opsonized targets (193).

b. Decay-accelerating factor

Decay-accelerating factor (DAF; CD55) is a 70-kDa glycoprotein that is widely expressed on various cells and tissues. It blocks the formation of the classical and alternative pathway convertases (C4b2a and C3bBb, respectively). The mechanism of action of DAF involves the displacement of C2a from C4b2a and Bb from C3bBb (87). However, in contrast to CR1, DAF can act only on the convertases formed on the same cell and not on nearby ones (193).

c. Membrane cofactor protein

Membrane cofactor protein (MCF; CD46) is a glycoprotein that is widely expressed on several tissues and circulating cells but not on erythrocytes. It has cofactor activity for fI-mediated cleavage of both C3b and C4b. However, unlike CR1 and DAF, it does not have decay-accelerating activity (268).

d. Protectin

Protectin is known by several names: CD59, p18, membrane inhibitor of reactive lysis (MIRL), and homologous restriction factor 20 (HRF20) (193). It is the most abundant cell surface complement regulator as it is expressed by all circulating cells, endothelial and epithelial cells, and in many tissues (194). CD59 regulates the complement system by binding to the C5b-8 complex and

preventing the polymerization of C9, thereby leading to the blockade of effective MAC formation (251).

D. Bacterial mechanisms used to evade complement

Bacteria and other microbes have developed several mechanisms to evade complement-mediated killing and, in some instances, the same organism has developed more than one mechanism to ensure its survival within the hostile environment of the host. These mechanisms can be grouped into the following categories:

i. Physical interference with complement

Bacteria can use surface structures to interfere with or evade the bactericidal action of the complement system. One of the major complement resistance factors is the presence of a capsule. For instance, the *Staphylococcus aureus* polysaccharide capsule enhances the evasion of opsonsophagocytosis (279). Similarly, the polysaccharide capsule of *Actinobacillus pleuropneumoniae* limits the amount of C9 that binds to this serum-resistant organism, thus protecting it from MAC-mediated lysis (301).

Another bacterial surface structure that is deployed to interfere with the complement system is lipopolysaccharide (LPS). Despite the fact that LPS can function as a complement activator, it plays an important role in protecting some bacteria against complement. The O-polysaccharide of *Salmonella minnesota* seems to physically interfere with MAC insertion into the outer membrane by causing assembly of the MAC away from the bacterial surface (151). In addition, the LPS molecules

of both *Klebsiella pneumoniae* and *Coxiella burnetii* have been shown to mediate their serum-resistance (11,296).

Most bacterial species lack sialic acid on their surface. However, it has been shown that in some pathogenic microorganisms, the presence of sialic acid on their surface can protect them against complement-mediated killing (245). The role of sialic acid in serum resistance has been attributed to the fact that sialic acid can facilitate an interaction with the complement regulator fH, leading to blockade of the alternative pathway (245). This interaction occurs in some serum-resistant *N. gonorrhoeae* strains where a high degree of sialylation was associated with serum resistance and fH binding (243,288). Also, sialic acid might be able to block binding sites for bactericidal antibodies [reviewed in (245)].

ii. Utilization of fluid-phase regulators

The most widespread mechanism for bacterial resistance to complement is the binding of fluid-phase complement regulators (discussed above) and utilizing them to block or interfere with the complement activation process. The list of the fluid-phase regulators that bacterial species bind includes C1-INH, C4BP, and fH. Although several studies have reported the binding of complement regulators to serum-resistant bacterial strains or bacterial moieties, sometimes the physiological significance of this biochemical binding was not proven to be responsible for the serum-resistant phenotypes. Detailed examination of the binding of *M. catarrhalis* to two complement regulators (i.e., C4BP and vitronectin) is included in the Results section of this dissertation.

Tenner et al. reported that some *E. coli* strains are capable of binding the complement regulator C1-INH through the long *O*-polysaccharide side chains of their LPS and that this restricts

complement activation via the classical pathway in very early stages (278). To evade classical pathway activation, several bacteria bind C4BP. For instance, two Ig-binding cell surface molecules that are members of the M protein family of *S. pyogenes* were shown to bind C4BP and this binding significantly increased the resistance of this bacteria to phagocytosis (22,280). Both *N. gonorrhoeae* and *N. meningitidis* were shown to be able to bind C4BP (146,240). Interestingly, blocking of C4BP binding to serum-resistant *N. gonorrhoeae* strains in a serum bactericidal assay, achieved by using FAb fragments against C4BP SCR1, resulted in complete killing of these strains (239). Moreover, *E. coli* K1 was shown to bind C4BP through its OmpA protein (232). Also, it was shown that *M. catarrhalis* strains bind C4BP through both UspA1 and UspA2, but the physiological significance of this binding in relation to serum resistance was not addressed experimentally in a rigorous manner (216).

Several bacteria bind either fH or FHL-1 to evade the alternative complement pathway. *S. pyogenes* binds both fH and FHL-1 through two surface ligands [i.e., M protein and fibronectin-binding protein (Fba)] (29,150,219). Also, *S. pneumoniae* was shown to bind to fH through two surface proteins (i.e., PspC of serotype 2 and Hic of serotype 3) (63,145,242). Non-sialylated *N. gonorrhoeae* strains were shown to bind fH through loop 5 of porin protein 1A (242), while the sialylated strains bound fH through their sialic acid (241).

iii. Other mechanisms

There are other, less common mechanisms through which pathogenic bacteria can evade complement. For instance, some *E. coli* and *Helicobacter pylori* strains incorporate protectin (CD59), released from host cells, into their membrane in a functional way to inhibit formation of the C5b-9 complex on the bacterial surface (244,246). Lipoteichoic acids (LTA) released by gram-

positive bacteria can bind to mammalian cell surfaces, thereby redirecting complement activation away from the bacterial surface (137). Also, some *E. coli* strains can bind the Fc region of human IgG in a nonimmune manner and this binding might be responsible for an increase in serum resistance (256).

CHAPTER THREE

Materials and Methods

I. Bacterial Strains and Culture Conditions

All of the bacterial strains used during the course of these studies are listed in Table 1 together with a brief description and their source or an appropriate reference.

A. *M. catarrhalis* strains

M. catarrhalis strains were grown at 37°C in brain heart infusion (BHI) broth (Difco/Becton Dickinson, Sparks, MD) or on BHI agar plates in an atmosphere containing 95% air-5% CO_2. When necessary, BHI agar was supplemented with kanamycin (15 μg/ml), spectinomycin (15 μg/ml), dihydrostreptomycin sulfate (750 μg/ml) or Zeocin (1 μg/ml). For use with *lacZ* reporter constructs, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was added to a final concentration of 30 μg/ml.

B. *H. influenzae* strains

H. influenzae strains were used as the host for some cloning experiments. These strains were cultured at 37°C on BHI agar plates containing 5% (vol/vol) Levinthal base (10) in an atmosphere containing 95% air-5% CO_2 or in BHI broth containing 10% Levinthal base, both containing ampicillin (10 μg/ml) or spectinomycin (100 μg/ml).

C. *E. coli* strains

E. coli strains were used as the host for some cloning experiments and were grown at 37°C on Luria-Bertani agar plates (255) that were supplemented with ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (50 μg/ml), spectinomycin (100 μg/ml) or Zeocin (25 μg/ml) when appropriate. For use with *lacZ* reporter constructs, X-Gal was added to a final concentration of 30 μg/ml.

D. *N. gonorrhoeae* strains

N. gonorrhoeae strains were used as controls in the complement activation analysis experiments. *N. gonorrhoeae* strains were grown at 37°C in *N. gonorrhoeae* liquid medium (187) or on chocolate agar plates in an atmosphere containing 95% air-5% CO_2.

Table 1. Bacterial strains used in this study

Strain	Genotype or description	Reference or Source
M. catarrhalis		
O35E	Wild-type disease isolate, serum-resistant	(5)
O12E	Wild-type disease isolate, serum-resistant	(3)
O35EΔ2	*uspA2* deletion mutant of O35E, serum-sensitive	This study;(13)
O35E.2ZEO	*uspA2* mutant of O35E, serum-sensitive	(225)
O35E.1	*uspA1* mutant of O35E, serum-resistant	(4)
O35E.12	*uspA1 uspA2* mutant of O35E, serum-sensitive	(4)
O35E-Sm^r	*rpsL* mutant of O35E, streptomycin resistant, serum-resistant	This study;(13)
MC317	Wild-type isolate, serum-sensitive	(190)
MC317.2	*uspA2* mutant of MC317, serum-sensitive	This study;(13)
MC317.1	*uspA1* mutant of MC317, serum-sensitive	This study;(13)
MC317-Sm^r	*rpsL* mutant of MC317, streptomycin-resistant, serum-sensitive	This study;(13)
MC317/35U2	MC317 transformant expressing O35E UspA2, streptomycin-resistant, serum-resistant	This study;(13)
O35E/317U2	O35E transformant expressing MC317 UspA2, streptomycin-resistant, serum-sensitive	This study;(13)
Hybrid 1	MC317 transformant expressing a hybrid UspA2, serum-sensitive	This study;(13)
Hybrid 2	MC317 transformant expressing a hybrid UspA2, serum-sensitive	This study;(13)
Hybrid 3	MC317 transformant expressing a hybrid UspA2, serum-sensitive	This study;(13)
Hybrid 4	MC317 transformant expressing a hybrid	This study;(13)

	UspA2, slightly serum-resistant	
Hybrid 5	MC317 transformant expressing a hybrid UspA2, serum-resistant	This study;(13)
Hybrid 6	MC317 transformant expressing a hybrid UspA2, serum-resistant	This study;(13)
7169	Wild-type disease isolate, serum-resistant	(48)
7169.1	*uspA1* mutant of 7169, serum-resistant, kanamycin-resistant	This study;(14)
7169.2	*uspA2* mutant of 7169, serum-sensitive, spectinomycin-resistant	This study;(14)
7169Δ2	*uspA2* deletion mutant of 7169, serum-sensitive, spectinomycin-resistant	This study;(14)
ETSU5	Wild-type disease isolate, serum-resistant	Stephen Berk
ETSU5.1	*uspA1* mutant of ETSU5, serum-resistant, kanamycin-resistant	This study;(14)
ETSU5.2	*uspA2* mutant of ETSU5, serum-sensitive, spectinomycin-resistant	This study;(14)
ETSU22	Wild-type disease isolate, serum-resistant	Stephen Berk
ETSU22.1	*uspA1* mutant of ETSU22, serum-resistant, kanamycin-resistant	This study;(14)
ETSU22.2	*uspA2* mutant of ETSU22, serum-sensitive, spectinomycin-resistant	This study;(14)
ETSU26	Wild-type disease isolate, serum-resistant	Stephen Berk
ETSU26.1	*uspA1* mutant of ETSU26, serum-resistant, kanamycin-resistant	This study;(14)
ETSU26.2	*uspA2* mutant of ETSU26, serum-sensitive, spectinomycin-resistant	This study;(14)
FIN2344	Wild-type isolate, serum-resistant	Merja Helminen
FIN2344.1	*uspA1* mutant of FIN2344, serum-resistant, kanamycin-resistant	This study;(14)
FIN2344.2	*uspA2* mutant of FIN2344, serum-sensitive, spectinomycin-resistant	This study;(14)

FIN2344Δ2	*uspA2* deletion mutant of FIN2344, serum-sensitive, spectinomycin-resistant	This study;(14)
FIN2406	Wild-type isolate, serum-resistant	Merja Helminen
FIN2406.1	*uspA1* mutant of FIN2406, serum-resistant, kanamycin-resistant	This study;(14)
FIN2406.2	*uspA2* mutant of FIN2406, serum-sensitive, spectinomycin-resistant	This study;(14)
O12E.1	*uspA1* mutant of O12E, serum-resistant, kanamycin-resistant	(165)
O12E.2	*uspA2* mutant of O12E, serum-sensitive, spectinomycin-resistant	This study;(14)
O12EΔ2	*uspA2* deletion mutant of O12E, serum-sensitive, spectinomycin-resistant	This study;(14)
O35E.2	*uspA2* mutant of O35E, serum-sensitive, kanamycin-resistant	(5)
O35E.2Spec	*uspA2* mutant of O35E, serum-sensitive, spectinomycin-resistant	This study;(14)
V1118	Wild-type isolate, serum-resistant	Frederick Henderson
V1118.1	*uspA1* mutant of V1118, serum-resistant, kanamycin-resistant	This study;(14)
V1118.2	*uspA2* mutant of V1118, serum-sensitive, spectinomycin-resistant	This study;(14)
V1120	Wild-type isolate, serum-resistant	Frederick Henderson
V1120.1	*uspA1* mutant of V1120, serum-resistant, kanamycin-resistant	This study;(14)
V1120.2	*uspA2* mutant of V1120, serum-sensitive, spectinomycin-resistant	This study;(14)
V1145	Wild-type isolate, serum-resistant	Frederick Henderson
V1145.1	*uspA1* mutant of V1145, serum-resistant, kanamycin-resistant	This study;(14)
V1145.2	*uspA2* mutant of V1145, serum-sensitive, spectinomycin-resistant	This study;(14)

V1156	Wild-type isolate, serum-resistant	Frederick Henderson
V1156.1	*uspA1* mutant of V1156, serum-resistant, kanamycin-resistant	This study;(14)
V1156.2	*uspA2* mutant of V1156, serum-sensitive, spectinomycin-resistant	This study;(14)
O35E (A429E)	*uspA2* site-specific mutant of O35E, serum-resistant	This study
O35E(A429E)ΔA-N	*uspA2* site-specific mutant of O35E, serum-resistant	This study
MC317(E510A)	*uspA2* site-specific mutant of MC317, serum-sensitive	This study
ATCC25238	Wild-type isolate, serum-resistant	ATCC
ATCC45617	Wild-type isolate, serum-resistant	ATCC
V1171	Wild-type isolate, serum-resistant	Frederick Henderson
O12EΔAGAT	O12E construct that lacks the AGAT nucleotides repeats in the 5′-UTR of its *uspA2* gene, serum-sensitive, streptomycin-resistant	This study
O35EΔAGAT	O35E construct that lacks the AGAT nucleotides repeats in the 5′-UTR of its *uspA2* gene, serum-sensitive, streptomycin-resistant	This study
O12EΔAGAT.2	*uspA2* mutant of O12EΔAGAT, spectinomycin-resistant	This study
O12E-Sm^r	*rpsL* mutant of O12E, streptomycin-resistant, serum-resistant	This study
O35EΔ1	*uspA1* deletion mutant of O35E, serum-resistant, spectinomycin-resistant	This study
O12EΔ1	*uspA1* deletion mutant of O12E, serum-resistant, spectinomycin-resistant	This study
FIN2344Δ1	*uspA1* deletion mutant of FIN2344, serum-resistant, spectinomycin-resistant	This study
7169Δ1	*uspA1* deletion mutant of 7169, serum-resistant, spectinomycin-resistant	This study
O12E-2rpts	O12E construct with 2 AGAT repeats in the	This study

	5′-UTR of its *uspA2* gene	
O12E-6rpts	O12E construct with 6 AGAT repeats in the 5′-UTR of its *uspA2* gene	This study
O12E-8rpts	O12E construct with 8 AGAT repeats in the 5′-UTR of its *uspA2* gene	This study
O12E-9rpts	O12E construct with 9 AGAT repeats in the 5′-UTR of its *uspA2* gene	This study
O12E-10rpts	O12E construct with 10 AGAT repeats in the 5′-UTR of its *uspA2* gene	This study
O12E-11rpts	O12E construct with 11 AGAT repeats in the 5′-UTR of its *uspA2* gene	This study
O12E-12rpts	O12E construct with 12 AGAT repeats in the 5′-UTR of its *uspA2* gene	This study
O12E-15rpts	O12E construct with 15 AGAT repeats in the 5′-UTR of its *uspA2* gene	This study
O12E-18rpts	O12E construct with 18 AGAT repeats in the 5′-UTR of its *uspA2* gene	This study
O12E-23rpts	O12E construct with 23 AGAT repeats in the 5′-UTR of its *uspA2* gene	This study
O12E-2rpts.1	*uspA1* mutant of O12E-2rpts, kanamycin-resistant	This study
O12E-6rpts.1	*uspA1* mutant of O12E-6rpts, kanamycin-resistant	This study
O12E-8rpts.1	*uspA1* mutant of O12E-8rpts, kanamycin-resistant	This study
O12E-9rpts.1	*uspA1* mutant of O12E-9rpts, kanamycin-resistant	This study
O12E-10rpts.1	*uspA1* mutant of O12E-10rpts, kanamycin-resistant	This study
O12E-11rpts.1	*uspA1* mutant of O12E-11rpts, kanamycin-resistant	This study
O12E-12rpts.1	*uspA1* mutant of O12E-12rpts, kanamycin-resistant	This study

O12E-15rpts.1	*uspA1* mutant of O12E-15rpts, kanamycin-resistant	This study
O12E-18rpts.1	*uspA1* mutant of O12E-18rpts, kanamycin-resistant	This study
O12E-23rpts.1	*uspA1* mutant of O12E-23rpts, kanamycin-resistant	This study
O12EΔAGAT.1	*uspA1* mutant of O12EΔAGAT, kanamycin-resistant	This study
O12E-19rpts.1	*uspA1* mutant of O12E-19rpts, kanamycin-resistant	This study
O12E-23*lacZ*	O12E with the 5′-UTR of the *uspA2* gene (with 23 AGAT repeats)-*lacZ* translational fusion inserted in the *hag* gene, kanamycin-resistant	This study
E. coli		
DH5α	Host strain for cloning experiments	(255)
InvαF′	Host strain for cloning experiments	Invitrogen
Top10	Host strain for cloning experiments	Invitrogen
AB1157	Host strain for cloning experiments	(255)
LS1443	Host strain for cloning experiments	(252)
XL10-Gold	Host strain for cloning experiments	Stratagene
HB101	Host strain for cloning experiments	(255)
H. influenzae		
DB117	Host strain for cloning experiments	(267)
N. gonorrhoeae		
FA19	Wild-type strain, serum-resistant, binds C4BP	(195)
UU1	Wild-type strain, serum-sensitive, does not bind C4BP	(310)

II. Plasmids

All the plasmids used and/or constructed in this study are listed in Table 2. These plasmids included: cloning vectors for the expression of various proteins, suicide plasmids for inactivating selected genes, and reporter constructs used for assaying the transcriptional activity of the *uspA2* gene.

Table 2. Plasmids used in this study

Plasmid	Genotype or description	Reference or Source
pAA1	pCR2.1 containing the two flanking regions of the O12E *uspA2* gene ligated together through a *Sal*I site	This study; (13)
pAA2	pAA1 with a spectinomycin resistance cartridge inserted into the *Sal*I site	This study; (13)
pAA3-0rpt	pAA3289 in which the (kan^r-*uspA2* 5′-UTR with 0 AGAT repeats-*lacZ*) fusion is inserted between the *hagC–hagD* fragments, Kan^r, Zeo^r	This study
pAA3-10rpt	pAA3289 in which the (kan^r-*uspA2* 5′UTR with 10 AGAT repeats-*lacZ*) fusion is inserted between the *hagC–hagD* fragments, Kan^r, Zeo^r	This study
pAA3-12rpt	pAA3289 in which the (kan^r-*uspA2* 5′UTR with 12 AGAT repeats-*lacZ*) fusion is inserted between the *hagC–hagD* fragments, Kan^r, Zeo^r	This study
pAA317:16-103	pCR-Blunt II-TOPO containing a fragment of the MC317 *uspA2* gene excluding the translational start codon	This study
pAA-317U2-kp	pGJB103M with the *M. catarrhalis* MC317 *uspA2* gene inserted behind the *kan* promoter in the *Sph*I-*Avr*II sites	This study; (13)
pAA3-18rpt	pAA3289 in which the (kan^r-*uspA2* 5′-UTR with 18 AGAT repeats-*lacZ*) fusion is inserted between the *hagC–hagD* fragments, Kan^r, Zeo^r	This study
pAA3-23rpt	pAA3289 in which the (kan^r-*uspA2* 5′-UTR with 23 AGAT repeats-*lacZ*) fusion is inserted between the *hagC–hagD* fragments, Kan^r, Zeo^r	This study

pAA3289	pAA3906 digested with *Pci*I and *Bae*I, then blunted and religated, Zeo^r	This study
pAA3-2rpt	pAA3289 in which the (kan^r-*uspA2* 5′UTR with 2 AGAT repeats-*lacZ*) fusion is inserted between the *hagC–hagD* fragments, Kan^r, Zeo^r	This study
pAA-35U2-kp	pGJB103M with the *M. catarrhalis* O35E *uspA2* gene inserted behind the *kan* promoter in the *Sph*I-*Avr*II sites	This study; (13)
pAA3-6rpt	pAA3289 in which the (kan^r-*uspA2* 5′UTR with 6 AGAT repeats-*lacZ*) fusion is inserted between the *hagC–hagD* fragments, Kan^r, Zeo^r	This study
pAA3906	pAA5432 digested with *Eco*RV and *Rsr*II then blunted and religated	This study
pAA5432	pCR-Blunt II-TOPO containing the *hagC–hagD* fusion, Kan^r, Zeo^r	This study
pAA7169U2-P2	pGJB103M with the *M. catarrhalis* 7169 *uspA2* gene inserted behind the *ompP2* promoter in the *Sph*I-*Avr*II sites	This study;(14)
pAAFIN2344U2-P2	pGJB103M with the *M. catarrhalis* FIN2344 *uspA2* gene inserted behind the *ompP2* promoter in the *Sph*I-*Avr*II sites	This study;(14)
pAA-kp	pGJB103M with the *kan* promoter cloned into the *Sph*I-*Avr*II sites	This study; (13)
pAAO12E-U2	pACYC184 with the *M. catarrhalis* O12E *uspA2* gene cloned into the *Bam*HI-*Sph*I sites	This study
pAAO12EU2-P2	pGJB103M with the *M. catarrhalis* O12E *uspA2* gene inserted behind the *ompP2* promoter in the *Sph*I-*Avr*II sites	This study;(14)
pAAO12U2-20rpt	pWW115 with the *M. catarrhalis* O12E *uspA2* gene including the 20 AGAT repeats in its 5′-UTR cloned into the *Bam*HI-*Sac*I sites, Spec^r	This study
pAAO12U2-21rpt	pWW115 with the *M. catarrhalis* O12E *uspA2* gene including the 21 AGAT repeats in its 5′-UTR cloned into the *Bam*HI-*Sac*I sites, Spec^r	This study
pAAO35:16-19	pCR-Blunt II-TOPO containing a fragment of the O35E *uspA2* gene excluding the translational start codon	This study
pAAO35EU2-P2	pGJB103M with the *M. catarrhalis* O35E *uspA2* gene	

	inserted behind the *ompP2* promoter in the *Sph*I-*Avr*II sites	This study;(14)
pAA-P2-pro	pGJB103M with the *ompP2* promoter cloned into the *Sph*I-*Avr*II sites	This study;(14)
pAC7	Cloning vector, Kan^r, Amp^r	(306)
pAC7-18	pAC7 with the 5′-UTR region of the *M. catarrhalis* O12E *uspA2* gene with 18 AGAT repeats cloned into the *Sma*I-*Bam*HI sites	This study
pAC7-19	pAC7 with the 5′-UTR region of the *M. catarrhalis* O12E *uspA2* gene with 19 AGAT repeats cloned into the *Sma*I-*Bam*HI sites	This study
pAC7-23	pAC7 with the 5′-UTR region of the *M. catarrhalis* O12E *uspA2* gene with 23 AGAT repeats cloned into the *Sma*I-*Bam*HI sites	This study
pACYC184	Cloning vector, Chlor^r	New England Biolabs
pCR2.1	Cloning vector, Kan^r, Amp^r	Invitrogen
pCR-BluntII-TOPO	Cloning vector, Kan^r, Zeo^r	Invitrogen
pELU2P44SPEC	pBS containing an incomplete *uspA2* gene from *M. catarrhalis* P44 into which a spectinomycin resistance cartridge was inserted	(165)
pGJB103M	Cloning vector, Amp^r	This study; (13)
pLS88	Source of the *kan* promoter	(314)
pSPECR	Source of spectinomycin resistance cartridge	(312)
pUSPA1KAN	pBluescript containing a mutated *uspA1* gene from *M. catarrhalis* O35E	(5)
pWW115	*M. catarrhalis* cloning shuttle vector, Spec^r	Wei Wang

III. Preparation and Detection of Bacterial Antigens

A. Whole cell lysates

Bacterial whole cell lysates (WCL) were normally prepared from bacterial cells that were scraped from freshly grown plates and resuspended in 5 ml of PBS to a cell density corresponding to 300 Klett units, then spun down and resuspended in 1 ml of PBS and digested by boiling with 500 µl of 3X digestion buffer (0.1875 M Trizma base, 30% (vol/vol) glycerol, 6% (wt/vol) SDS, pyronin y for color, pH 6.8). In some cases, WCL were diluted 5- or 10-fold using 1X digestion buffer in order to visualize possible changes in the level of expression of abundant proteins. Proteins in WCL were resolved by SDS-PAGE using 7.5% (wt/vol) polyacrylamide gels and analyzed by using Western blotting as described below.

B. Lipooligosaccharide analysis

For the analysis of the LOS of *M. catarrhalis* strains, a 90 µl portion of the WCL described above was incubated with 100 µg of proteinase K in a 100 µl reaction volume at 56°C for 1 hr. Then the proteinase K activity was stopped by boiling the reaction mixture for 5 min. The LOS samples were resolved by SDS-PAGE using 15% (wt/vol) polyacrylamide gels and analyzed using Western blotting or silver staining by the method of Tsai and Frasch (283). This latter method included the soaking of the polyacrylamide gels in a fixative (40% (vol/vol) ethanol and 5% (vol/vol) glacial acetic acid) overnight, and then the gels were treated with fixative solution containing 0.7% (wt/vol) periodic acid for 10 min and washed three times with distilled water. The staining process was carried out using fresh silver nitrate solution (0.019 M sodium hydroxide, 1.33% (wt/vol) ammonium hydroxide, and 0.67% (wt/vol) silver nitrate) for 15 min, then developed using (0.005% (vol/vol)

citric acid and 0.019% (vol/vol) formaldehyde) until the stain reached the desired intensity. The developing reaction was stopped using stop solution (1% (vol/vol) acetic acid).

C. Outer membrane vesicles

Outer membrane vesicles were extracted from whole cells of *M. catarrhalis* using the EDTA-based method described by Murphy and Loeb (210). *M. catarrhalis* strains were grown to mid-logarithmic phase in a 10 ml BHI culture. A few drops of this were used to inoculate one liter of BHI broth which was incubated at 37°C with vigorous shaking overnight. The next day, the bacterial cells were spun down and resuspended in 30 ml of EDTA buffer (0.05M Na_2HPO_4, 0.15 M NaCl, 0.01 M EDTA, pH 7.4). The collected bacterial cells were homogenized in a 15 ml glass homogenizer, then transferred to a 250 ml flask whose bottom was covered with 3 mm glass beads and shaken vigorously for 45 min at 55°C. The bacterial suspension was transferred to a 50 ml Oak Ridge centrifuge tube together with three 5 ml washes of the glass beads with EDTA buffer. Cell debris was removed by centrifugation at 10,000 x g for 15 min at 4°C and the supernatant was transferred to a new Oak Ridge centrifuge tube and centrifuged at 39,000 x g for 90 min at 4°C. Membrane vesicles in the pellet were then resuspended in 150 μl cold PBS and the protein content was determined using the Bradford assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA). Proteins present in these vesicles were resolved by SDS-PAGE using 7.5% (wt/vol) polyacrylamide separating gels and stained with Coomassie blue [0.1% (wt/vol) Coomassie brilliant blue, 10% (vol/vol) acetic acid, 25% (vol/vol) methanol] for 30 min, and then destained overnight using [10% (vol/vol) acetic acid, 10% (vol/vol) methanol, and 6% (vol/vol) glycerol].

IV. Immunodetection of Bacterial Antigens

A. Western blot analysis

Bacterial proteins and LOS resolved by SDS-PAGE were analyzed by Western blotting by transferring these antigens to nitrocellulose membranes (Schleicher & Schuell BioScience, Keene, NH) and probing them with the appropriate primary antibody. Monoclonal antibody (MAb) 17C7, reactive with both the *M. catarrhalis* UspA1 and UspA2 proteins, and MAb 24B5, which binds only UspA1, have been described elsewhere (5,61). MAb 5D2, reactive with the Hag protein and MAb 10F3, reactive with the CopB protein, have been described (115,117,225). MAb 8E7, which reacts with *M. catarrhalis* serotype A and C LOS, has been described (135). The secondary antibody used for Western blot analysis was polyclonal goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA). Antigen-antibody complexes were visualized by chemiluminescence using the Western Lightning Chemiluminescence Reagent Plus (New England Nuclear, Boston, MA).

B. Colony blot radioimmunoassay

Bacterial cells to be screened for the expression of UspA2 were patched on agar plates and allowed to grow overnight. The next day, the bacterial patches were lifted using sterile Whatman No. 40 filter paper. The patches were allowed to dry at 37°C for 1 hr, then the filter paper was blocked using colony blot buffer [PBS pH 7.4, 1 mM sodium iodide, 0.02 % (wt/vol) sodium azide, and 2 % (vol/vol) FCS] for 1 hr at room temperature. The filter was then probed with the primary antibody

(i.e., MAb 17C7) for 2 hr at room temperature. After washing 3 three times with colony blot buffer, the filter was probed with 10 μl of radioiodinated goat anti-mouse IgG corresponding to 10^6 CPM diluted in 10 ml of colony blot buffer overnight at 4°C with continuous rocking. The next morning, the filters were washed three times with colony blot buffer and then dried at 37°C for 1 hr. The filter was then exposed to an X-ray film in a cassette containing an intensifying screen overnight at -70°C. In some cases, the filter was exposed to a storage phosphor intensifying screen (GE Healthcare, Piscataway, NJ**)** for few hours then scanned using a STORM 820 scanner (GE Healthcare) and the image was analyzed using ImageQuant v.5.2 software (Molecular Dynamics, Sunnyvale, CA).

C. Indirect antibody-accessibility assay

To detect UspA2 proteins on the surface of bacterial cells, MAb 17C7 was used in the indirect antibody-accessibility assay (105). In this assay, bacterial cells were suspended in 5 ml of PBS supplemented with 10% (vol/vol) FCS (PBS/FCS) to a cell density corresponding to 115 Klett units. Portions (100 μl) of this suspension were transferred to 1.5 microcentrifuge tubes containing 900 μl of MAb 17C7 hybridoma culture supernatant fluid and mixed by rotating end over end for 1 hr at 4°C. Bacterial cells were collected by centrifugation at 16,000 x g at 4°C for 10 min. After washing once with PBS/FCS, the bacterial pellets were resuspended in 1 ml of PBS/FCS and 10 μl of radioiodinated goat anti-mouse IgG (corresponding to 10^6 CPM) were added to each tube and mixed by rotating end over end for 1 hr at 4°C. To increase the size of the bacterial pellet, a 50-μl portion of a dense bacterial culture (*E. coli* not expressing *M. catarrhalis* UspA2) was added to each tube before centrifugation at 16,000 x g at 4°C for 5 min. Bacterial pellets were washed three times with 1 ml portions of PBS/FCS then resuspended in 0.5 ml of triple detergent [10 mM Tris-hydrochloride, pH 7.8, 1% (vol/vol) Triton X-100, 150 mM NaCl, 10 mM EDTA, 0.2% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) SDS] (106). Radioactivity associated with the bacteria was measured using a

gamma counter (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, Fullerton, CA). Each sample was analyzed in triplicate and MAb 3F12, a murine IgG MAb specific for the major outer membrane protein of *H. ducreyi* (160), was used as the negative control.

D. Flow cytometry

To compare the levels of the UspA2 protein expressed by different *M. catarrhalis* O12E constructs having varying numbers of AGAT repeats in the 5′-UTR of their *uspA2* gene, flow cytometry was used. Briefly, bacterial cells were suspended in PBS to a final OD_{600} of 0.35. Portions (100 μl) of these suspensions were spun down and resuspended in 100 μl of PBS with 1% (wt/vol) bovine serum albumin (BSA) (PBS/BSA) in which purified MAb 17C7 (3 μg/μl) had been diluted 1:100. The tubes were incubated at room temperature for 20 min and then washed three times with 500 μl PBS/BSA. The bacteria were then incubated with 1 μg of FITC-conjugated goat anti-mouse antibody (Abcam, Cambridge, Mass.) for 20 min at room temperature followed by three washes with 500 μl of PBS/BSA. The final pellet was suspended in 1 ml of PBS and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson). The flow cytometry data were analyzed using CellQuest software (Becton Dickinson). As a negative (isotype) control, bacterial strains were incubated with the secondary antibodies but without prior incubation with MAb 17C7.

V. Human Sera and Related Preparations

A. Normal human serum

This protocol involving human subjects was approved by the Institutional Review Board at UT Southwestern. Blood drawn from healthy adult volunteers was allowed to clot at room temperature. The serum fraction was separated by centrifugation, pooled under aseptic conditions, aliquoted in small volumes, frozen first in a dry ice-ethanol solution and then stored at -70°C. Complement-inactivation was achieved by incubating this normal human serum (NHS) at 56°C for 30 min and this serum was then designated as heat-inactivated serum (HIS). Factor B-depleted human serum was purchased from a commercial source (Quidel, San Diego, CA). An NHS preparation (with measured hemolytic activity values) was also purchased from Quidel for standardization purposes.

B. IgG-depleted serum

To deplete NHS of IgG, a 0.5 ml portion of 40% (vol/vol) NHS diluted in Veronal-buffered saline containing 5 mM $MgCl_2$ and 1.5 mM $CaCl_2$ (VBS^{++}) was mixed with 0.5 ml GammaBind Plus Sepharose suspension (GE Healthcare) in a plastic column for 1 hr at 4°C with gentle agitation. GammaBind Plus Sepharose is described by the manufacturer as not binding other serum proteins including IgM, IgE, IgA, and transferrin. The flow-through from the column was ~20% (vol/vol) NHS depleted of IgG. These IgG-depleted preparations were used immediately in bactericidal assays.

C. Vitronectin-depleted serum

IgG antibody was purified from rabbit polyclonal vitronectin antiserum (Advanced Research Technologies, San Diego, CA) by using Gamma Bind Plus beads (GE Healthcare). Approximately 5 mg of this IgG antibody was coupled to 2.5 ml of Affi-Gel Hz Hydrazide Gel using an Affi-Gel Hz Immunoaffinity Kit (Bio-Rad) at room temperature overnight. To deplete NHS of vitronectin, a 500 µl portion of NHS was incubated with the gel described above in a small chromatography column for 1 hr at 4^{o}C with gentle mixing. The liquid was then allowed to drain from the gel. Low-speed centrifugation was used to collect residual liquid from the gel and all liquid portions were pooled and stored at –70^{o}C until used. This serum was designated vitronectin-depleted serum (Vn-depleted serum). As a control, a 500 µl portion of NHS was incubated with a gel that had been coupled to IgG antibody purified from normal rabbit serum under the same conditions described above; the resultant liquid was designated as mock-treated serum. The extent of vitronectin depletion was more than 90% as assessed by both Western blot analysis and the use of a commercial ELISA test for vitronectin (Innovative Research, Southfield, MI). When a Western blot control assay was used to determine whether this absorption method affected the complement in the serum, there was little difference in the amounts of C1q in the mock-treated serum and the Vn-depleted serum.

D. Zymosan-activated serum

To prepare zymosan-activated NHS (ZAS) (used as a positive control for detection of polymerized C9), 80 mg of zymosan A (Sigma, St. Louis, MO) was boiled in 2 ml of normal saline for 2 hr and then a 10 µl portion of this suspension was added to 90 µl of NHS and incubated for 1 hr at 37°C. Following centrifugation at 10,000 x g for 15 min at 4°C, the supernatant fluid was collected and designated as ZAS.

VI. Serum Bactericidal Assays.

The ability of bacterial strains to resist complement-mediated killing by NHS was assessed using a liquid phase assay. A schematic representation of the standard steps of this assay is presented in Fig. 2. Briefly, bacterial cultures grown to mid-logarithmic phase were diluted in Veronal-buffered saline containing 0.1% (wt/vol) gelatin (GVBS) to a final concentration of 1-2 x 10^5 colony forming units (CFU)/ml. Subsequently, 10 µl (~1-2 x 10^3 CFU) portions were added to 10 µl NHS and 80 µl VBS^{++} in 1.5 ml microcentrifuge tubes on ice. Duplicate 10-µl samples were removed from this mixture and spread on appropriate agar plates. The tubes were incubated in a 37°C water bath without shaking for 30 min and then the tubes were then placed on ice and duplicate 10-µl samples were removed and plated. As a negative control, experiments were carried out using HIS. To block the alternative complement activation pathway, factor B-depleted serum was used. To block the classical pathway, NHS was equilibrated in Veronal-buffered saline containing 10 mM $MgCl_2$ and 10 mM EGTA for 15 min on ice before adding the bacteria. To examine the effect of IgG antibodies on serum resistance, a 45 µl portion of the IgG-depleted NHS (described above) was mixed with 45 µl VBS^{++} to give a final concentration of ~10% NHS for use in the bactericidal assay. To supplement this IgG-depleted serum with IgG antibodies, it was mixed with an equal volume of 20% (vol/vol) HIS and this reconstituted serum was used in the bactericidal assay. For assessing the serum resistance of some of recombinant *H. influenzae* strains, this same method was utilized but NHS and HIS were used at a final concentration of 5% (vol/vol). In testing the bactericidal activity of Vn-depleted serum, the final concentration of the serum was 30% (vol/vol) in a 50-µl reaction volume. In some of these assays, 2.5 µg of purified human monomeric vitronectin (Innovative Research) was added to the Vn-depleted serum. In an additional control assay, 2.5 µg of this purified vitronectin was added to 30% HIS, which was then tested for its bactericidal activity.

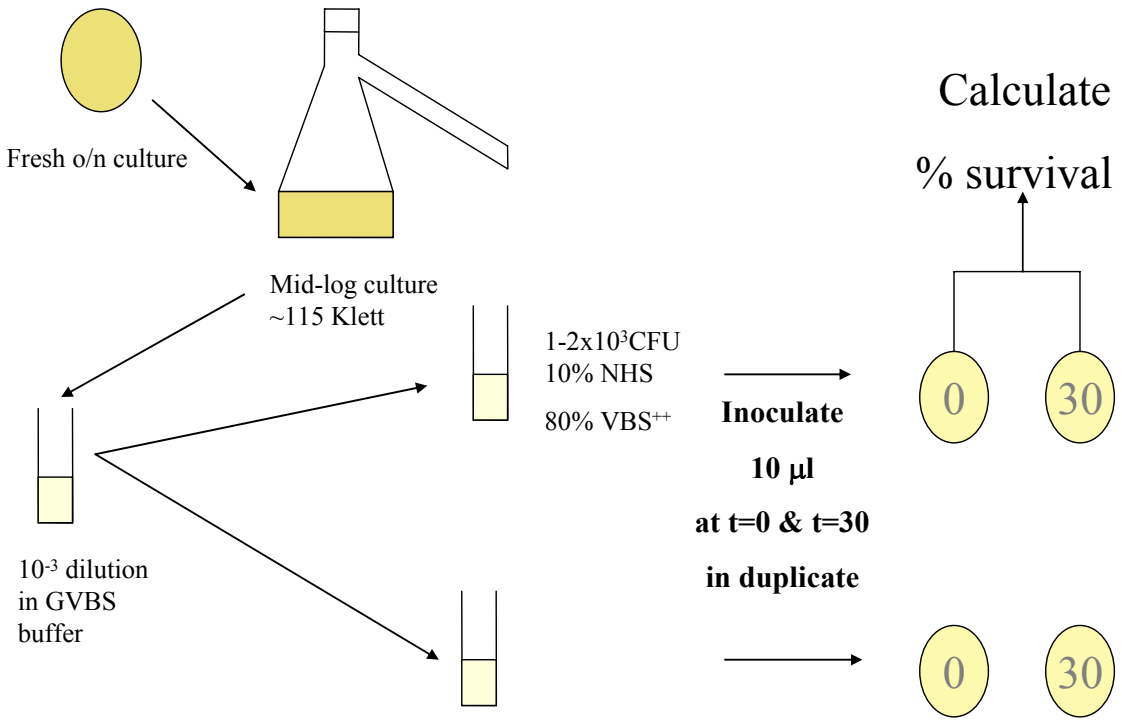

Fig. 2. A schematic representation of the standard steps in the serum bactericidal assay.

VII. Binding of Complement Components and Regulators to Bacterial Cells

A. Early complement components

To monitor the deposition of early complement components on bacterial cells, bacteria were first allowed to grow to mid-logarithmic phase in broth, then spun down and resuspended in 5 ml of VBS^{++} to an OD_{600} of 1.0. Portions (500 µl) of these bacterial suspensions were mixed with 400 µl of VBS^{++} and 100 µl of either NHS or HIS contained in microcentrifuge tubes on ice. Next, the tubes were incubated at 37°C for 20 min. In order to stop complement activation, the tubes were transferred to crushed ice and incubated for 5 min. The bacterial cells were then pelleted by centrifugation at 16,000 x g for 5 min at 4°C. The cells were washed three times using 1 ml of ice-cold GVBS. The final pellets were re-suspended in 100 µl of PBS and digested with 50 µl of 3X digestion buffer by heating at 100°C for 10 min. For detection of C1q, C4, and C3, proteins were resolved by SDS-PAGE using 12.5% (wt/vol) polyacrylamide gels under reducing conditions (i.e., 5% (vol/vol) 2-mercaptoethanol in digestion buffer) and transferred to nitrocellulose membranes. The primary antibodies used in this case were polyclonal goat antibodies against C1q, C4, and C3 (Quidel) and rabbit anti-goat HRP-conjugated antibody (Abcam) was used as a secondary antibody. MAb 10F3, which binds to the *M. catarrhalis* CopB protein (115), was used to probe membrane-bound samples to verify equivalent loading of samples derived from strains O35E, O12E and FIN2344. Due to the lack of reactivity of this MAb with the 7169 CopB protein, MAB 5D2, specific for the Hag protein (225), was used to verify equivalent loading of samples for this strain. To standardize loading for the *N. gonorrhoeae* samples, membranes were probed using MAb 2C3, which recognizes the *N. gonorrhoeae* H.8 lipoprotein (12). This antigen ranges in size from 23.5 kDa to 28.5 kDa among different *N. gonorrhoeae* strains (123).

To measure binding of C3 derived from NHS in a more quantitative manner, bacteria were incubated with 10% NHS in VBS^{++} for 20 min at 37°C, put on ice for 5 min, and then washed three times with ice-cold GVBS. The washed cells were then incubated with polyclonal goat antiserum to C3 (Quidel; 1:200 dilution in a 100 µl reaction volume) for 20 min at room temperature, washed three times, incubated with FITC-conjugated rabbit anti-goat antibody (Abcam), and analyzed by flow cytometry. As a negative (isotype) control, bacterial strains were incubated with both the primary and the secondary antibodies but without prior incubation with NHS.

B. Late complement components

The same WCL used for the detection of the early complement components were used to detect late components, however the samples were analyzed by SDS-PAGE under non-reducing conditions. For detection of C7, proteins were resolved by SDS-PAGE using 12.5% (wt/vol) polyacrylamide gels and transferred to nitrocellulose membranes. In the case of SC5b-9, proteins were resolved by SDS-PAGE using 7.5% (wt/vol) polyacrylamide gels and transferred to PVDF membranes (Millipore, Bedford, MA). Polyclonal goat anti-C7 (Quidel) and a MAb against SC5b-9 (Quidel) were used as the primary antibodies and HRP-conjugated antibodies were used as secondary antibodies. Loading control standardization was done using the same antibodies as described above.

C. Vitronectin

WCL from wild-type and *uspA2* mutants of *M. catarrhalis* prepared as described immediately above were used to detect binding of vitronectin present in NHS or HIS to these bacteria. The samples were subjected to SDS-PAGE in 12.5% (wt/vol) polyacrylamide gels under reducing conditions; the separated proteins were transferred to nitrocellulose membranes, and probed with a

MAb against human vitronectin (Quidel). As a loading control, nitrocellulose membranes were stained with 0.1% (wt/vol) amido black solution and the band corresponding to *M. catarrhalis* CopB protein was used as a control for equivalent loading. For recombinant *H. influenzae* strains, bacterial cells grown to mid-logarithmic phase were incubated with 10% NHS for 20 min at 37°C and WCL were prepared and analyzed as described above. Samples containing equivalent amounts of recombinant UspA2 were used for these latter analyses.

D. NHS-derived C4BP, C4c, and C4d

To measure binding of NHS-derived C4BP, C4c, and C4d, bacteria were incubated with 20% NHS in VBS^{++} for 30 min at 37°C, put on ice for 5 min, and then washed three times with ice-cold GVBS. The washed cells were incubated with MAbs (1 μg each in a 100 μl reaction volume) specific for C4BP, C4c, or C4d (Quidel) for 20 min at room temperature, washed three times, incubated with FITC-conjugated goat anti-mouse antibody (Abcam), and analyzed by flow cytometry. As a negative (isotype) control, bacterial strains were incubated with both the primary and the secondary antibodies but without prior incubation with NHS.

E. Purified C4BP

Binding of purified C4BP to *M. catarrhalis* and *N. gonorrhoeae* strains was assessed by using flow cytometry as described (216). Briefly, 2-3 x 10^8 CFU were incubated with 2.5 μg of purified C4BP (Advanced Research Technologies) in PBS containing 3% (wt/vol) fish gelatin (PBS-FG) in a 100 μl reaction volume at 37°C for 1 hr. The bacteria were then washed three times with 500 μl PBS-FG followed by incubation with 1 μg of a MAb to C4BP (Quidel) in a 100 μl final volume at room temperature for 30 min. After three washes with 500 μl PBS-FG, the bacteria were incubated with 1

μg of FITC-conjugated goat anti-mouse antibody for 30 min at room temperature. Finally, the bacteria were washed three more times with PBS-FG and the final pellet was suspended in 1 ml of PBS and analyzed by flow cytometry. As a negative (isotype) control, bacterial strains were incubated with both the primary and the secondary antibodies but without prior incubation with C4BP.

F. Radiolabeled purified C4BP

Purified C4BP was labeled (by Jo Latimer in the Department of Microbiology, UT Southwestern) with [^{125}I]-iodine using the chloramine-T method (138) to a specific activity of 805 kcpm/μg. Plate-grown *M. catarrhalis* or *N. gonorrhoeae* cells were suspended in PBS supplemented with 2% (wt/vol) BSA (PBS/2%BSA). Portions of these suspensions containing 2-3 x 10^8 CFU were incubated with ~300 ng of [^{125}I]-C4BP for 1 hr at 37°C in a 200 μl volume of PBS/2%BSA. The bacteria were then washed 3 times using 500 μl of PBS/2%BSA and the radioactivity associated with the bacterial pellet was measured using a gamma counter.

VIII. Use of Hemolytic Assays to Evaluate Complement Activation

A. Classical pathway

M. catarrhalis cells from mid-logarithmic phase broth cultures were harvested by centrifugation and suspended in 5 ml of VBS^{++} containing 0.1% gelatin (wt/vol) ($GVBS^{++}$) to OD_{600}= 0.9. A 75 μl portion of this suspension was incubated with 10% (vol/vol) NHS in $GVBS^{++}$ at a final

volume of 300 µl at 37°C for 1 hr with continuous shaking. The bacterial cells were then spun down and the supernatant fluid was filter-sterilized and used as the serum sample for the hemolytic assay.

Total classical pathway activity of serum samples was assessed using the assay previously described (269). Briefly, serum samples were serially diluted in $GVBS^{++}$. Then, 100-µl portions of each dilution were mixed with 100 µl of sensitized sheep RBCs (EA) (2×10^8/ml) (Colorado Serum, Denver, CO) and incubated for 1 hr at 37°C with continuous shaking. Then 1.2 ml 0.15 M NaCl was added to each tube which was then centrifuged at 1250 x g for 5 min. The OD_{412} of the supernatant fluid was recorded. The data are presented as fractional lysis as compared to the 100% lysis that was obtained by adding 1.2 ml distilled water to EA incubated with $GVBS^{++}$.

B. Alternative pathway

The alternative pathway activity of the serum samples was assessed using a protocol similar to the one described above with some modifications. The serum samples were diluted in VBS containing 10 mM $MgCl_2$ and 10 mM EGTA instead of $GVBS^{++}$. Also, rabbit erythrocytes $E_{(rab)}$ (Colorado Serum) were used instead of the sensitized sheep RBCs and the rest of the procedures were the same as described for the classical pathway.

IX. Nucleic Acids Isolation

A. Chromosomal DNA isolation

Chromosomal DNA was isolated from bacteria by using the Easy-DNA kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, bacterial cells scraped from agar plates were suspended in 350 µl of solution A and incubated for 10 min at 65°C, then 150 µl of solution B were added and the tube was mixed vigorously. Nucleic acids were then isolated by the addition of 500 µl of chloroform/iso-amyl alcohol (24:1 vol/vol) solution followed by centrifugation at 16,000 x g for 15 min at 4°C. The upper aqueous phase was separated and RNA was digested by treating the sample with RNaseA (Invitrogen) at a final concentration of 1 µg/µl for 30 min at 37°C. The DNA was recovered by the addition of 1 ml of 100% ethanol followed by incubation on ice for at least 30 min. The DNA was pelleted out by centrifugation at 16,000 x g for 15 min at 4°C. The pellet was washed once using 1 ml of 70% (vol/vol) ethanol and resuspended in distilled water.

B. Plasmid DNA isolation

Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Overnight liquid cultures of *E. coli* or *M. catarrhalis* were used for plasmid DNA isolation. However, for *H. influenzae,* cells grown to early stationary phase (approx 6 hr) were used for this purpose. The principle of this plasmid isolation procedure involves lysis of the bacterial cells followed by the use of a silica gel membrane that is capable of binding DNA in the presence of a high concentration of salt. The bound DNA can then be eluted using distilled water.

C. RNA isolation

RNA was isolated from bacterial cells grown to mid-logarithmic phase using the RNeasy Midi kit (Qiagen) according to the manufacturer's protocol. The principle of this kit involves the lysis of cells in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer to inactivate RNases. The samples are then applied to a silica-gel-based membrane with selective binding properties for RNA. Finally, the RNA can be eluted using distilled water. RNA samples which were used for applications that are highly sensitive for DNA contamination (i.e. real-time RT-PCR), were treated with DNase I (MesageClean Kit, GenHunter Corp, Nashville, TN) to remove any DNA contamination. The RNA was then cleaned by ethanol precipitation.

X. Transformation Protocols

A. Plate transformation

M. catarrhalis strains were transformed by plate transformation using a modification of a plate transformation method previously described (154). Briefly, approx 0.5 μg of input DNA, which can be a circular or linearized plasmid or a linear PCR fragment, was mixed with 2-3 colonies of the recipient strain on an agar plate in an area approximately 1 cm in diam. The plate was incubated at 37°C in an atmosphere containing 95% air-5% CO_2 for 4-6 hr after which the bacterial growth was suspended in BHI broth, serially diluted, and plated on BHI plates containing the appropriate antimicrobial agent.

B. Transformation by electroporation

Electro-competent cells of either *E. coli* or *H. influenzae* were prepared by growing the bacteria to mid-logarithmic phase in a liquid medium and washing them three times with ice-cold 10% (vol/vol) sterile glycerol. *E. coli* cells were aliquoted and stored at –70°C for later use. For *H. influenzae*, electro-competent cells were prepared fresh immediately prior to the electroporation process. Approximately 100 ng of DNA was mixed with the electro-competent cells which were then transferred to an electroporation cuvette (BioRad). The electroporation process was carried out with an *E. coli* Pulser (Bio-Rad) using a setting of 1.6 kV for *E. coli* and 1.5 kV for *H. influenzae*. Then, 250 µl of SOC medium (Invitrogen) for *E. coli*, or BHI broth containing 10% Levinthal base for *H. influenzae,* were added quickly to the bacterial suspension which was incubated for 1 hr at 37°C and then plated on agar plates containing the appropriate antimicrobial agent.

C. Chemical transformation

Chemically-competent *E. coli* cells were obtained from commercial sources and stored at –70°C. Approximately 100 ng of DNA was mixed with an aliquot of the competent cells and allowed to stay on ice for 15 min. Then the mixture was incubated in a 42°C water bath for 30 sec before being put back on ice for 2 more minutes. A 250 µl portion of SOC medium was added and the bacterial suspension was then incubated for 1 hr at 37°C and plated on agar plates with the appropriate antimicrobial agent

XI. Polymerase Chain Reaction (PCR)

A. Single-step PCR

DNA amplification was carried out using different DNA polymerases depending on the application. *ExTaq* DNA polymerase (PanVera, Madison, WI) was used to obtain amplicons with high fidelity and T/A overhangs. However, *Taq* DNA polymerase (New England Biolabs, Ipswich, MA) was used for screening purposes in colony-PCR. To obtain amplicons with high fidelity and blunt ends, the high-fidelity proofreading *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used. All the oligonucleotide primers used in this study are listed in Table 3. The oligonucleotide primers were designed using MacVector software (version 6.5.3; Oxford Molecular Group, Campbell, CA) and were synthesized by Integrated DNA Technologies (Coralville, IA). DNA templates were chromosomal DNA, plasmid DNA, PCR-generated fragments or part of a bacterial colony. The PCR reactions were carried out using a Peltier-effect cycler with a heated lid (MJ Research, Inc., Incline Village, NV). The general scheme of the PCR reaction included an initial step of denaturation (94°C for 1 min), 30 to 35 cycles of denaturation (94°C for 1 min) followed by annealing (45-55°C for 1 min), then extension (1 min/kb for *ExTaq* and *Taq* or 3 min/kb for *Pfu*). These cycles were then followed by a final extension step (72°C for 10 min for *ExTaq* and *Taq* and 30 min for *Pfu*).

B. Multi-step PCR (PCR-sewing)

Multi-step PCR or PCR-sewing (bridging) (169,218) was used to generate amplicons from 2 or more PCR-generated fragments. The same procedures described above were used with the exception that the oligonucleotide primers were designed so that one oligonucleotide primer at the bridging area would have 12-15 nucleotides that are complementary to the first 12-15 nucleotides of

the other oligonucleotide primer at the bridging area. After the generation of the initial PCR-amplified fragments, these were gel-purified at least once using the Geneclean Turbo kit (Q.Biogene, Vista, CA) to get rid of the original DNA template. Then these two fragments were mixed in a 1:1 ratio and used as the template for the second cycle of PCR amplification. If three DNA fragments were required to be linked to each other, two fragments would be used first to generate a fragment that can be linked to the third one using another cycle of PCR amplification.

Table 3. Oligonucleotide primers used in this study

Primer	Sequence (5′-3′)	Comment[a,b]
A429E-Fw	GCGATTGATG**AA**AA**C**AAAGCATCTGCGGATACC AAGTTTGCA	
A429E-Rev	TGCAAACTTGGTATCCGCAGATGCTTT**G**TT**TT**CA TCAATCGC	
AA101-Fw	GAGCATGATGCTTATGCTGGCTTTTGTC	
AA102	TATGGATCCCTGATGTGATGACTTAACTACCAA	*Bam*HI
AA103	AGGCATGCAAAGATATCCAAAAACCCTAAACC	*Sph*I
AA16	CGCGGATCCTGAAAACCATGAAACTTCTCCC	
AA19	ACATGCATGCGAACTCGTAATTCACACCGATG	
AA26-Rev	GCACCCAAGCCAACAATCATGGCA	
AA3-Rev	ACGCGTCGACTTAGCACTCTCTTTTGGTAG	*Sal*I
AA47-Fw	ACATGCATGCTGTCCGCTGATGCTTTCTG	*Sph*I
AA49-Fw	AACAATATCTATGAGCTG	Used with AA49-Rev to make hybrid 1
AA49-Rev	CAGCTCATAGATATTGTT	
AA4-Fw	CGCGGATCCTCTCATCAAAGACACACC	*Bam*HI
AA52-Fw	GAAAACCATGAAACTTCTCCC	
AA52-Rev	GGGAGAAGTTTCATGGTTTTC	

AA54-Rev	TGCCTAGGAAAGCTTTTATCCATCACTCAC	*Avr*II
AA55-Fw	GATCTTACAAAAGACATCA	Used with AA55-Rev to make hybrids 3 and 6
AA55-Rev	TGATGTCTTTTGTAAGATC	
AA56-Fw	ACAGCAAACCGAAGCGATTGA	Used with AA56-Rev to make hybrid 2
AA56-Rev	TCAATCGCTTCGGTTTGCTGT	
AA5-Fw	ACGCGTCGACGTGGCTTTTGGTTGTGAG	*Sal*I
AA60	ATCATTAAGTGAGCCAATGTCTCG	Used with AA61 to make hybrid 4
AA61	GGCTCACTTAATGATGAGCGTATCGATAAAAAC GAATATGACATTAAAAACGAATATGACATT	
AA62	GGGTGTTATGAGCCTGTCCGCTGATGCTTTCTGC CTGTCACCGAT	
AA63Fw	TAAGATCTCATAGATAGCCACATCAATC	
AA68Fw	CTCTCATCAAAGACACACCAA	
AA69Fw	GAGATTTTTCCATTTATGCCAGCAAAAG	
AA69Rev	CTTTTGCTGGCATAAATGGAAAAATCTC	
AA6-Rev	CGCGGATCCAGCCATCAGTCATCAGCTC	*Bam*HI
AA70-Fw	AAAACTCTGTCTTTTATCTGTCC	
AA70-Rev	GGACAGATAAAAGACAGAGTTTT	
AA71-Fw	ATGCCAGCAAAAGAAAACTCTGTCTTTTATCTGT CCGCTGATG	
AA74-Fw	AGATAGATAAAACTCTGTCTTTTATCTGTCC	
AA75-Rev	ATCTATCTCTTTTGCTGGCATAAATGGAAAAATC TC	
AA81-Rev	TGGGATCCATTTAGCACTCTCTTTTGGTA	*Bam*HI
AA84-Fw	GCGGTGTTTGAGGTGATTGGTC	

AA87-Rev	TGTCGTCGCTATGATAACGGC	
AA88-Fw	GAAAACAATAAACCCGAGCCTAAATTTAACTGC TGTATC	
AA89-Rev	CACGAACGAAAATCGTCCTAATAAATGACTGGG GTCTCC	
AA90	TGTCTAGACCTTTAGTTCAAGCACTCCCCC	*Xba*I
AA91	GTGAGTCGACTGCCCTGTTCTATGTTGCGGCCA	*Sal*I
AA92	CCAGTCGACATGCATAAGTGGGCGTGGATAAAG ACGGCAACGCTA	*Sal*I/*Nsi*I
AA93	CCCCCGGGTGACCGTTGTGGGTGGCAATAC	*Ava*I
AA9-Rev	TCGCAGTAGATGCCATACCC	
BD SMART II A	AAGCAGTGGTATCAACGCAGAGTACGCGGG	
CopB-807F	CAATCGTGCCTTGACGCTAGA	
CopB-915R	GCCAAGTTTGTAACCCTTGCCT	
E510A-Fw	GCAAACAAAACTGCGATTGATG**CC**AA**T**AAAGC ATCTGCGGATA	
E510A-Rev	TATCCGCAGATGCTTT**A**TT**GG**CATCAATCGCAG TTTTGTTTGC	
II A	AAGCAGTGGTATCAACGCAGAGT	
Kan-pro-3'	GGCTCATAACACCCCTTGTATTAC	
Kan-pro-5'-*Sph*I	GCCGGCATGCATCGACCCTGCGTTACTGTTCG	*Sph*I
Kan-pro-*Avr*II-3'	GCCTAGGAACACCCCTTGTATTACTGTTTATG	*Avr*II
P2-pro-3′-AvrII	GTCCTAGGAATTTGTATTCCTTATGGTTG	
P2-pro-3′U2-5′	GTTTCATGGTTTTCATAATTTGTATTCCTTATGGT TG	
P2-pro-5′-SphI	ACATGCATGCAGATTTATGGATAGCCTTAG	*Sph*I
Rps-3′	ACGCCACCAACAGCACAATAAACC	

Rps-5′	TGGCGAACTCAAGCAAACAGC	
Spec-3′	TGCAAGGGTTTATTGTTTTC	
Spec-5′	CGATTTTCGTTCGTGAATAC	
U2-1886F	GTAAGTTTAATGCGACCGCTGC	
U2-1987R	CAGCTTTAAACGCCAGATTTGG	
WW141	ACATGAGCTCCTTGATAAGCTTTTATCCAT	*Sac*I
WW144	TTGGATCCGCTAAGCCGTGGACAGTCGGAT	*Bam*HI

[a]Underlined sequence indicates restriction site.

[b]Underlined bold sequences indicate altered nucleotides for site-directed mutagenesis.

XII. Determination of the Transcriptional Start Point of the *uspA2* Gene

A. Primer extension analysis

A 10 μg quantity of freshly isolated bacterial RNA (either from *M.* catarrhalis or recombinant *H. influenzae*) was subjected to reverse transcription using SuperScript II reverse transcriptase (Invitrogen) and the primers AA52-Rev, AA26-Rev, or AA9-Rev in the presence of [α-^{32}P]dCTP (Perkin Elmer, Boston, MA) for 1 hr at 42°C. The labeled RT product was then electrophoresed on a 6% (wt/vol) polyacrylamide/urea sequencing gel together with a sequencing ladder that had been generated with the same primers using the AmpliCycle sequencing kit (Perkin-Elmer) according to the manufacturer's instructions. The gel was then fixed using [5% (vol/vol) acetic acid and 5% (vol/vol) methanol] and exposed to either an X-ray film overnight or to a storage phosphor

intensifying screen for few hours and the bands were visualized as described above in the colony blot radioimmunoassay.

B. Rapid amplification of cDNA ends (5′RACE)

To identify the transcriptional start point of the *uspA2* gene, the BD SMART PCR cDNA Synthesis kit (Becton Dickinson) was used. Briefly, RNA was isolated from wild-type *M. catarrhalis* strain O12E cells grown to mid-logarithmic phase. First-strand synthesis by reverse transcription was carried out using the oligonucleotide primer AA9-rev, which binds 82-bp inside the *uspA2* open reading frame (ORF), and BD PowerScript reverse transcriptase (Becton Dickinson). When it reaches the 5′-end of the mRNA, this enzyme's terminal transferase activity adds few additional deoxycytidine residues (oligo(C)) to the 3′-end of the cDNA. When this reaction is carried out in the presence of the BD SMART II A oligonucleotide, which has a poly (G) tract at its 3′-end, base pairing occurs, thereby creating an extended template. The reverse transcriptase then switches templates and incorporates sequences complementary to that of the BD SMART II A oligonucleotide into the final single-stranded cDNA. Then the BD Advantage 2 PCR Enzyme System (Becton Dickinson) was used to amplify this cDNA using primer II A, which binds to the BD SMART II A oligonucleotide sequence and AA26-Rev, which binds 65-bp inside the *uspA2* ORF. The resultant PCR product was ligated into the pCR2.1 vector (Invitrogen) and transformed into *E. coli* Top10 (Invitrogen) and plated on LB plates containing kanamycin and X-Gal. Several white colonies were selected and plasmid DNA was isolated from them. Nucleotide sequence analysis of those plasmid inserts was used to identify the transcriptional start point of the *uspA2* gene.

XIII. Transcriptional Analysis of the *uspA2* Gene

A. Real-time RT-PCR

RNA was isolated from *M. catarrhalis* strains grown to mid-logarithmic phase in broth and was treated with DNase I to remove any DNA contamination. The RNA was then cleaned by ethanol precipitation. For real-time analysis, the *copB* gene was used as an endogenous control to normalize the results obtained with the target *uspA2* gene. Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). The primer pair CopB-807F and CopB-915R was used to amplify a 109-bp fragment of the *copB* gene and the primer pair U2-1886F and U2-1987R was used to amplify a 102-bp fragment of the *uspA2*gene. A "master mix" for each gene was prepared so that each well would contain 12.5 µl of 2X SYBR Green Master Mix (Applied Biosystems), 1 µl from a 2.5 µM stock of each primer (forward and reverse), 0.1 µl of MultiScribe Reverse Transcriptase (50 unit/µl) (Applied Biosystems) and the final volume would be adjusted to 20 µl by the addition of RNase free water (Qiagen). Then 5 µl portions of the RNA samples (20 ng/µl) were added to each well. All samples were analyzed in triplicate. As a negative control, wells that had the master mix from which the reverse transcriptase enzyme had been omitted were used to detect any DNA contamination. Data analysis was carried out using the 7500 System SDS software v.13 (Applied Biosystems) applying the relative quantification $^{\Delta\Delta}$Ct method. The level of the *uspA2* message was normalized according to the level of the *copB* message and the data are presented as the fold-increase using the normalized level of the *uspA2* message of strain O12E with no AGAT repeats (O12EΔAGAT) as the calibrator.

B. *β*-galactosidase activity assay

β-galactosidase activity assays were carried out using the method described previously (255) with slight modifications. Briefly, bacterial cells containing the *lacZ* reporter plasmids were grown in kanamycin-containing A^+ medium [0.4% (wt/vol) glucose, 0.1% (wt/vol) yeast extract in A buffer (0.06 M K_2HPO_4, 0.03 M KH_2PO_4, 7.5 mM $(NH_4)_2SO_4$ and 1.7 mM Na citrate.2 H_2O)]. The next morning, the overnight cultures were diluted 1:50 in fresh A^+ medium and allowed to grow to A_{600}= 0.4. Portions of the bacterial cultures were mixed with Z buffer [0.06 M Na_2HPO_4, 0.04 M $NaH_2PO_4.H_2O$, 0.01 M KCl, 0.001 M $MgSO_4.7H_2O$ and 0.05 M 2-mercaptoethanol, pH 7.0] to a final volume of 800 µl. The cells were then lysed using 20 µl 0.1% (wt/vol) SDS, 40 µl chloroform was added, and the whole suspension was vortexed vigorously for 10 sec and then equilibrated to 30°C. An aliquot (200 µl) of *o*-nitrophenyl-*β*-galactopyranoside (ONPG) in A buffer (4 mg/ml) was added to the cell lysates until a yellow color started to develop. The reaction was then stopped by the addition of 500 µl of 1 M Na_2CO_3 and the time at which the yellow color had developed was recorded. The A_{420} of the reaction samples were recorded and the units of the *β*-galactosidase activity were calculated using the formula:

$$\frac{1000 \times A_{420} \text{ of the reaction sample}}{\text{Time}_{(min)} \times \text{Vol of culture}_{(ml)} \times A_{600} \text{ of the bacterial culture}}$$

XIV. Molecular Genetic Techniques

A. Identification of streptomycin-resistant mutants of *M. catarrhalis* strains

Streptomycin-resistant mutants of *M. catarrhalis* strains O35E, MC317 and O12E were obtained by spreading approximately 10^{10} CFU of each strain on BHI agar plates containing a high concentration of dihydrostreptomycin sulfate (750 μg/ml) and incubating overnight at 37°C. The development of streptomycin resistance is frequently due to a single nucleotide change in the *rpsL* gene, leading to a single amino acid change in ribosomal protein S12 (212). Nucleotide sequence analysis of the *rpsL* gene of the streptomycin-resistant O35E mutant (O35E-Sm^r) confirmed that a single nucleotide change at residue 128 (A to C) resulted in a single altered amino acid (i.e., K43T) whereas in case of the streptomycin-resistant MC317 mutant (MC317-Sm^r), there was a single nucleotide change in its *rpsL* gene at residue 263 (A to G) which resulted in a single altered amino acid (i.e., K88R). In the case of the streptomycin-resistant O12E mutant (O12E-Sm^r), a change at residue 128 (A to G) resulted in a single altered amino acid (i.e., K43R). A 3-kb amplicon containing the mutated *rpsL* gene plus flanking sequence was generated by PCR using the oligonucleotide primers Rps-5' and Rps-3' and was used for congression experiments (see below).

B. Congression

The principle of the congression experiments is based on the phenomenon described by Nester et al (214) in that there is an increase in the possibility of the uptake by a bacterial cell of an unmarked fragment of DNA if the same cell took up another marked DNA fragment. This principle was applied to facilitate "gene swaps" by allelic exchange in *M. catarrhalis*. The 3-kb amplicon containing the mutated *rpsL* gene plus flanking sequence was used as the marked DNA fragment and

the unmarked DNA fragment was a mutated gene, hybrid gene, or a gene from another wild-type *M. catarrhalis* strain. The marked fragment and the unmarked one were mixed in the ratio of 1:10 and introduced into the target strain by plate transformation (described above); these transformation mixtures were plated on BHI plates supplemented with dihydrostreptomycin sulfate. The target cells were *M. catarrhalis* strains that had an antibiotic resistance cartridge already inserted into the target gene. Streptomycin-resistant transformants were then screened for the loss of the antibiotic marker in the target gene. Later, nucleotide sequence analysis was used to confirm the occurrence of gene replacement.

C. Construction of *uspA1* and *uspA2* mutants of *M. catarrhalis*

i. Insertion mutants

M. catarrhalis strains were subjected to transformation with the suicide plasmid pUSPA1KAN (4) to inactivate their *uspA1* genes. Similarly, *M. catarrhalis* strains were transformed with the suicide plasmid pELU2P44SPEC (165) or pELU244ZEO (225) to inactivate their *uspA2* genes.

ii. Deletion mutants

a. *uspA2* deletion mutants

The regions immediately flanking the *uspA2* ORF in *M. catarrhalis* strain O12E were amplified by PCR using chromosomal DNA from strain O12E as the template. The oligonucleotide primers AA3-Rev and AA4-Fw were used to amplify a 0.75 kb upstream region while the 0.95 kb

downstream region was amplified using primers AA5-Fw and AA6-Rev. Both amplicons were digested with *Sal*I and then ligated and used as a template in bridging PCR (i.e., PCR-sewing) (169,218) with the primers AA4-Fw and AA6-Rev to generate the 1.7 kb USPA2AB fragment. This latter fragment was ligated into the pCR2.1 vector, transformed into *E. coli* InvαF′ (Invitrogen) and the desired recombinant strain was selected on LB-kanamycin agar. The plasmid containing the USPA2AB insert, designated pAA1, was digested with *Sal*I and then blunt-ended by using the DNA polymerase *Pfu*. The spectinomycin resistance cartridge was excised from pSPECR (312) by digestion with *Eco*RV and ligated to this blunt-ended plasmid, resulting in pAA2. Nucleotide sequence analysis showed that the spectinomycin resistance cartridge was inserted between the two original *uspA2* flanking regions in the same orientation as the *uspA2* gene. Plasmid pAA2 was used to transform the wild-type *M. catarrhalis* strain O35E in the plate transformation procedure described above and transformants were selected for spectinomycin resistance. One of these spectinomycin-resistant transformants, designated O35EΔ2, was shown by nucleotide sequence analysis to have a complete deletion of the *uspA2* ORF. Plasmid pAA2 was also used to construct *uspA2* deletion mutants of *M. catarrhalis* strains O12E, FIN2344, and 7169.

b. *uspA1* deletion mutants

Oligonucleotide primers Spec-5′ and Spec-3′ were used to amplify the spectinomycin resistance cartridge from pSPECR (312). The regions immediately flanking the *uspA1* ORF in *M. catarrhalis* strain O35E were amplified by PCR using chromosomal DNA as the template. The oligonucleotide primers AA84-Fw and AA89-Rev were used to amplify a ~ 0.6 kb upstream region while a ~ 0.9 kb downstream region was amplified using primers AA88-Fw and AA87-Rev. The 5′-

end of primer AA89-Rev was designed to be complementary to the 5′-end of primer Spec-5′ and, at the same time, the 5′-end of primer AA88-Fw was designed to be complementary to the 5′-end of primer Spec-3′. PCR-sewing (described above) was used to link the three PCR fragments into one fragment that was used to transform wild-type *M. catarrhalis* strains O35E, O12E, FIN2344, and 7169. The resultant spectinomycin-resistant transformants were tested for lack of expression of the UspA1 protein and were designated O35EΔ1, O12EΔ1, FIN2344Δ1, and 7169Δ1, respectively.

D. Site-directed mutagenesis of *uspA2* genes

Site-directed mutagenesis of the *uspA2* genes of both *M. catarrhalis* O35E and MC317 was carried out using the QuikChange II XL kit (Stratagene) according to the manufacturer's protocol. For use as templates in the site-directed mutagenesis procedure, PCR fragments containing most of the *uspA2* genes excluding the translational start codons were ligated into pCR-Blunt II-TOPO (Invitrogen). For O35E, primers AA16 and AA19 were used to generate this PCR fragment while for MC317, primers AA16 and AA103 were used. The ligation mixtures were transformed into *E. coli* strain XL10-Gold (Stratagene) and the resultant plasmids were designated pAAO35:16-19 and pAA317:16-103, respectively.

To introduce the A429E change into pAAO35:16-19, the primer pair A429E-Fw and A429E-Rev was used to PCR-amplify pAAO35:16-19. After PCR amplification, the unmutated template was digested using *Dpn*I (which only recognizes methylated sites). Then the undigested plasmid was transformed into *E. coli* XL10-Gold and kanamycin-resistant clones were screened for the occurrence of the desired change. The mutated gene fragment was PCR-amplified using AA16 and AA19 and transformed into O35E.2 by plate transformation/congression.

To introduce the E510A change into pAA317:16-103, the primer pair E510A-Fw and E510A-Rev was used in the same procedure described above, except that the final mutated gene fragment was transformed into MC317.2.

To construct the O35E(A429E)ΔA-N mutant, primers AA52-Fw and AA49-Rev were used to generate a PCR fragment using O35E chromosomal DNA as the template whereas primers AA49-Fw and AA6-Rev were used to generate a PCR fragment using MC317 chromosomal DNA as the template. Later, the two PCR fragments were linked together by PCR-sewing and used to transform O35E.2.

E. Construction of hybrid *uspA2* genes

PCR-sewing was used to construct hybrid *uspA2* genes containing segments from both the O35E and MC317 *uspA2* genes. Briefly, the oligonucleotide primers AA47-Fw and AA54-Rev were designed to bind to the 5'-UTR and to the region 3' from the translation termination codon, respectively, of both the O35E and MC317 *uspA2* genes. Additional primers (Table 3) were designed to allow amplification of fragments of varying lengths from the O35E and MC317 *uspA2* genes. In the case of hybrids 1, 2, and 3, the primers at the bridging region were designed to be completely complementary to each other while in case of hybrid 4, due to the lack of nucleotide sequence identity between O35E and MC317 in the bridging region, only the first 15-nt of the oligonucleotide primers AA60 and AA61 were complementary to each other and the rest of the sequence matched the respective DNA templates. After verifying the nucleotide sequence of each hybrid PCR product, they were introduced into the *uspA2* mutant MC317.2 by congression as described above; streptomycin-resistant transformants were screened for the loss of the spectinomycin resistance cartridge and the presence of the hybrid *uspA2* gene was verified by nucleotide sequence analysis. Hybrid 5 was

obtained as a serendipitous event from a transformation experiment in which the wild-type O35E *uspA2* gene was used to transform MC317.2. This transformant was shown to contain a hybrid *uspA2* gene in which the first 444 nucleotides were derived from the MC317 *uspA2* gene. For the construction of hybrid 6, chromosomal DNA from hybrid 5 was used as the template for the PCR reaction with primers AA47-Fw and AA55-Rev to amplify the 5'-half of the hybrid 6 gene and chromosomal DNA from MC317 was used as the template to amplify the 3'-half of the hybrid gene using primers AA55-Fw and AA54-Rev. It should be noted that primers AA55-Fw and AA55-Rev both bind twice on the O35E *uspA2* ORF (at nt 712-730 and 799-817); the binding of AA55-Rev to the first site (nt 712-730) was used to obtain the hybrid 6 PCR product and the binding of AA55-Fw to the second site (nt 799-817) was used to obtain the hybrid 3 PCR product.

F. Construction of *M. catarrhalis* strains that lack AGAT nucleotide repeats in the 5′-UTR of their *uspA2* genes

Chromosomal DNA from either *M. catarrhalis* strain O12E or O35E was used as template for two PCR reactions. For the first reaction, the oligonucleotide primers AA68-Fw and AA69-Rev were used to amplify an ~ 0.5 kb region that was directly upstream of the AGAT nucleotide repeats. For the second PCR reaction, the region directly downstream from the AGAT nucleotide repeats was PCR-amplified using the oligonucleotide primers AA70-Fw and AA54-Rev. This fragment contained the 5′-UTR of the *uspA2* gene directly downstream from the AGAT nucleotide repeats together with the entire *uspA2* ORF and 100 nucleotides from the 3′-UTR downstream of the ORF. After gel purification, the latter fragment was used as the template for another PCR reaction with the oligonucleotide primers AA71-Fw and AA54-Rev. The oligonucleotide primer AA71-Fw binds to the same region as AA70-Fw except it has an additional 13-nt at its 5′-end that are complementary to the first 13-nt at the 5′-end of AA69-Rev. The AA68-Fw–AA69-Rev-derived PCR product and the

AA71-Fw–A54-Rev-derived PCR product were used together as the template for a PCR reaction using the AA68-Fw and AA54-Rev primers. The final PCR product was gel-purified and its nucleotide sequence was verified. It was then used in a congression experiment together with the 3-kb amplicon containing the mutated *rpsL* gene. In the case of strain O12E, the target strain in the plate transformation was the *uspA2* mutant strain O12E.2 while in the case of strain O35E, the *uspA2* mutant O35E.2 was used. Streptomycin-resistant, spectinomycin-sensitive O12E.2 transformants and streptomycin-resistant, kanamycin-sensitive O35E.2 transformants were subjected to nucleotide sequence analysis to ensure that their AGAT repeats had been deleted and that the *uspA2* ORF was intact. One transformant was selected from each strain and these were designated O12EΔAGAT and O35EΔAGAT, respectively.

G. Construction of *M. catarrhalis* O12E strains that have different numbers of AGAT nucleotide repeats in the 5′-UTR of their *uspA2* genes

The wild-type O12E strain has 19 AGAT nucleotide repeats in the 5′-UTR of its *uspA2* gene. Dr. Thomas Rosche in the Department of Microbiology at UT Southwestern was able to isolate two natural variants of this strain that have either 18 or 23 AGAT nucleotide repeats. These three strains were transformed with the 3-kb amplicon containing the mutated *rpsL* gene to obtain streptomycin-resistant strains. The resultant streptomycin-resistant transformants were checked to ensure that they had the original number of AGAT nucleotide repeats in their *uspA2* genes. For the construction of the O12E strain with two AGAT repeats, the same procedures described above for the construction of the ΔAGAT constructs were used except that the primer AA75-Rev was used instead of AA69-Rev and the primer AA74-Fw was used instead of AA70-Fw. Both AA75-Rev and AA74-Fw have the same sequences as AA69-Rev and AA70-Fw, respectively, except they have an additional two AGAT nucleotide repeats in their 5′-end. For the construction of the rest of the O12E strains with different

numbers of AGAT repeats, a schematic representation of the method used is presented in Fig. 3 Briefly, primers AA69-Fw and AA70-Rev were used to amplify the AGAT nucleotide repeat regions from either *M. catarrhalis* strains or recombinant plasmids in *E. coli* that have different number of AGAT repeats in their *uspA2* genes. Later, these PCR products were PCR-stitched to the AA63-Fw–AA69-Rev and the AA70-Fw–AA54-Rev PCR products that had been obtained by using chromosomal DNA of strain O12E as the template. The final PCR products were sequence-verified and then transformed by congression into strain O12EΔAGAT.2. The streptomycin-resistant, spectinomycin-sensitive transformants were screened first by colony PCR using the primers AA63-Fw and AA52-Rev to detect transformants which exhibited a shift in the size of their PCR products as compared to that amplified from strain O12EΔAGAT. The positive transformants identified in this manner were sequence-verified to ensure that they had the desired number of AGAT nucleotide repeats and that each *uspA2* ORF was intact.

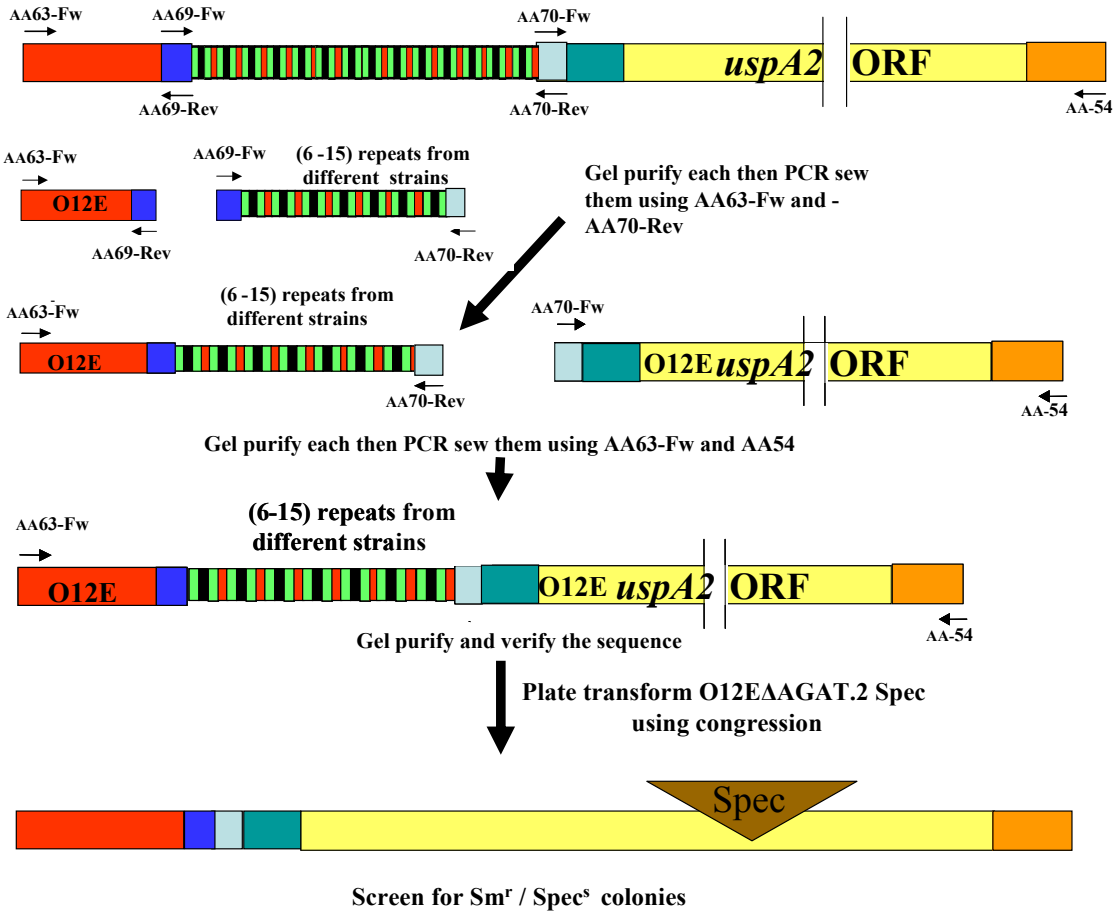

Fig. 3. A schematic representation of the method used to construct O12E strains with different numbers of AGAT nucleotide repeats.

H. Cloning and expression of *uspA2* genes in *E. coli*

Primers AA102 and AA103, respectively, were designed to bind to the 5'-UTR and to the region 3' from the translation termination codon of the *uspA2* gene of *M. catarrhalis* strain O12E. These primers were used to PCR-amplify the *uspA2* gene and then the product was digested with both *Bam*HI and *Sph*I. The digested fragment was ligated into the *Bam*HI/*Sph*I sites of the cloning vector pACYC184 (New England Biolabs) and the resultant plasmid was designated pAAO12E-U2. Several *E. coli* host strains were transformed with this plasmid; these included DH5α, LS1443, AB1157 and HB101. The transformation mixtures were plated on LB agar plates containing chloramphenicol (25 μg/ml). Chloramphenicol-resistant colonies were then screened for UspA2 expression using MAb 17C7 in either Western blots or in the colony blot-radioimmunoassay.

I. Cloning and expression of *uspA2* genes in *H. influenzae*

Plasmid pGJB103M was used as the vector for cloning *M. catarrhalis uspA2* genes in *H. influenzae.* This plasmid is a modified version of pGJB103 (17) and has an additional 1.3 kb insertion containing an *Sph*I site and an *Avr*II site into which the *M. catarrhalis uspA2* genes were cloned. Both the O35E and MC317 *uspA2* genes were PCR-amplified using the primers AA47-Fw and AA54-Rev together with *M. catarrhalis* chromosomal DNA as the template. Each PCR product and pGJB103M were digested with both *Sph*I and *Avr*II; these digested PCR products were individually ligated into pGJB103M and used to electroporate *H. influenzae* Rd strain DB117 (267). Ampicillin-resistant colonies were screened for the expression of UspA2 by using MAb 17C7 in a colony blot-radioimmunoassay. Several MAb-reactive clones were obtained but were found to have very low expression of the UspA2 proteins (data not shown). To increase expression of the *uspA2* genes in *H.*

influenzae, bridging PCR was used to insert a kanamycin (*kan*) gene promoter in front of the O35E and MC317 *uspA2* genes in pGJB103M. The *kan* promoter region was PCR-amplified by using primers kan-pro-5'-*Sph*I and kan-pro-3' with plasmid pLS88 (314) as the template. The *uspA2* genes were PCR-amplified using primers AA62 and AA54-Rev. Primer AA62 binds to the same region in the 5'-UTR region of the *uspA2* genes as AA47-Fw but it lacks a *Sph*I site and contains instead 14-nt that are complementary to the last 14-nt of the *kan* promoter. After PCR amplification, ligation, and restriction enzyme digestion, the *uspA2* genes fused to the *kan* promoter were cloned as described above. The resultant recombinant plasmids expressing relatively high levels of the UspA2 proteins were designated pAA-35U2-kp and pAA-317U2-kp. As a negative control equivalent to a vector-only plasmid, the *kan* promoter region was PCR-amplified using the primers kan-pro-5'-*Sph*I and kan-pro-3'-*Avr*II and cloned into pGJB103M to yield pAA-kp.

To obtain higher levels of *M. catarrhalis* UspA2 expression in *H. influenzae* for studies involving interactions with the complement components, the *uspA2* genes from *M. catarrhalis* strains O35E, O12E, FIN2344, and 7169 were inserted behind the *H. influenzae ompP2* gene promoter (199). Briefly, the *uspA2* ORFs from *M. catarrhalis* strains O35E, O12E, FIN2344, and 7169 were PCR-amplified using the primer pair AA52-Fw and AA54-Rev. Each of these PCR amplicons was ligated by using PCR-sewing to another PCR amplicon that contained the promoter region of the *H. influenzae ompP2* gene (199) that had been amplified using the primers P2-pro-5′-*SphI* and P2-pro-3′U2-5′. The final PCR products were digested with both *Sph*I and *Avr*II, ligated to the *Sph*I/*Avr*II-digested plasmid pGJB103M (13), and used to electroporate *H. influenzae* strain DB117 (267). Ampicillin-resistant clones were screened for UspA2 expression by Western blot analysis of whole cell lysates (13). The plasmid insert from one UspA2-positive clone derived from each *M. catarrhalis* strain was verified by nucleotide sequence analysis; these plasmids were designated pAAO35EU2-P2, pAAO12EU2-P2, pAAFIN2344U2-P2 and pAA7169U2-P2, respectively. As a negative control, the

ompP2 promoter was cloned into pGJB103M after being amplified with P2-pro-5′-*Sph*I and P2-pro-3′-*Avr*II; the resultant plasmid was designated pAA-P2-pro. These recombinant *H. influenzae* strains expressed different amounts of the UspA2 proteins and densitometric analysis with Kodak 1D v.3.5.3 software (Scientific Imaging systems, New Haven, CT) was used to quantitate the differences among these strains. To standardize loading for Western blot detection of UspA2, membranes were stained with Ponceau S dye and a prominent *H. influenzae* protein band of approximately 40 kDa was used as the internal control.

For primer extension analysis, the *uspA2* gene of *M. catarrhalis* strain O12E was cloned into the *M. catarrhalis* shuttle vector pWW115 (obtained from Dr. Wei Wang in the Department of Microbiology, UT Southwestern). Primer WW144 was designed to bind to the 5'-UTR of the *uspA2* gene 286-nt upstream of the beginning of the AGAT repeat region. At the same time primer, WW141 was designed to bind to the region 3' from the translation termination codon of the *uspA2* gene. This pair of primers was used to PCR-amplify the O12E *uspA2* gene, which was then ligated into the *Bam*H1/*Sac*I sites of pWW115 and used to transform *H. influenzae* DB117. Spectinomycin-resistant transformants were screened using the colony blot-radioimmunoassay to identify clones expressing UspA2. Two positive clones were selected for further analysis. One of them contained 20 AGAT repeats and was designated pAAO12U2-20rpt and the other contained 21 AGAT repeats and was designated pAAO12U2-21rpt.

J. Construction of transcriptional reporter systems

i. Multi-copy reporter system

Plasmid pAC7 (306) was used as the vector for cloning the 5′-UTR of the *M. catarrhalis uspA2* gene from different O12E strains containing varying numbers of the AGAT nucleotide repeats. Primers AA101-Fw and AA81-Rev were used together with *Pfu* polymerase to amplify a ~ 0.5 kb fragment from the *uspA2* 5′-UTR with blunt ends. Then this PCR product was digested with *Bam*HI and ligated into pAC7 that had been digested with *Sma*I and *Bam*HI. The primer AA81-Rev was designed so that, after *Bam*HI digestion and ligation, the translation initiation codon (ATG) of the *uspA2* gene will act as the translational start point of the *lacZ* gene. The promoter region of the *uspA2* gene strains O12E-18rpt, O12E-19rpt, and O12E-23rpt was successfully cloned into pAC7 and the resultant plasmids were designated pAC7-18, pAC7-19, and pAC7-23, respectively.

ii. Single-copy *lacZ* reporter system

During attempts to reproduce the experiments with the multi-copy *lacZ* reporter system, it was discovered that the number of AGAT repeats tended to change within a given plasmid in this system and that this led to large variability in β-galactosidase activity measurements. To overcome this technical difficulty, a single copy *uspA2-lacZ* reporter system was integrated into the *M. catarrhalis* chromosome. Briefly, primers AA90 and AA91 were used to amplify a ~ 0.9 kb fragment from the 5′-region of the O12E *hag* ORF; this was designated *hagC*. Also, primers AA92 and AA93 were used to amplify a ~ 0.95 kb fragment from the 3′-region of the O12E *hag* ORF; this was designated *hagD*. These two fragments were linked together by PCR-sewing using AA90 and AA93. Primers AA91 and AA92 were designed so that when the two fragments were linked together, *Sal*I and *Nsi*I

restriction sites would be included between the two fragments. The *hagC-hagD* fragment was then ligated into the pCR-Blunt II-TOPO and this plasmid was designated pAA5432 (Fig. 4A). pAA5432 included two additional *Nsi*I sites in addition to the one engineered between the *hagC-hagD* fragments. To eliminate these two additional sites, pAA5432 was digested with *Eco*RV and *Rsr*II and then blunt-ended and religated to give pAA3906 (Fig. 4B). Plasmid pAA3906 was digested with *Pci*I and *Bae*I and then blunt-ended and religated to give pAA3289 (Fig. 4C). The *Sal*I-*Nsi*I fragment from pAC7-23 was excised and ligated into pAA3289 that had been digested with *Sal*I and *Nsi*I. This ligation mixture was transformed into *E. coli* LS1443 which keeps the plasmids in very low copy number (252). Blue colonies were selected on X-Gal plates and then the *uspA2* 5′-UTR in the recombinant plasmid was sequence-verified; the new plasmid was designated pAA3-23rpt (Fig. 4D). This plasmid was then linearized using *Avr*II and *Pst*I and transformed into O12E by using the plate transformation method. Blue kanamycin-resistant O12E colonies were selected and checked for loss of the expression of the Hag protein. One clone was selected and designated O12E-23*lacZ*. Additional repeated efforts to transform O12E with pAA3 containing the *uspA2* 5′-UTR with 0, 2, 6, 10, 12 or 18 AGAT repeats were unsuccessful.

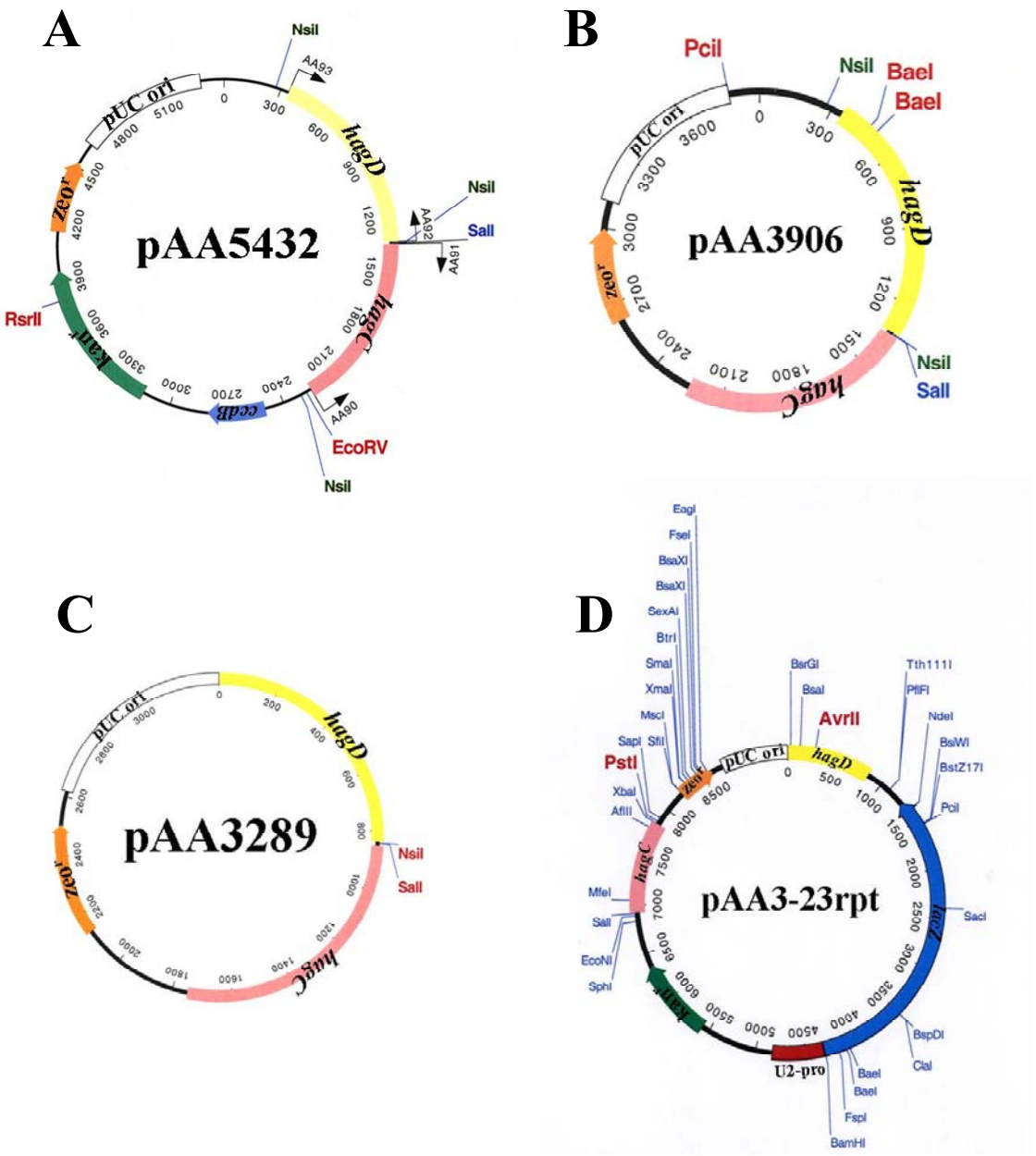

Fig. 4. Schematic maps of the plasmids used to construct the single copy *lacZ* reporter system.

(A) pAA5432 which has the *hagC-hagD* fragments cloned in the pCR-Blunt II-TOPO vector. The position of the *Sal*I site introduced between the *hag* flanks is highlighted in blue, the 3 *Nsi*I sites are highlighted in green and the positions of the *Eco*RV and *Rsr*II sites used to construct pAA3906 are highlighted in red. (B) pAA3906; the positions of the 2 *Nsi*I sites are highlighted in green and the positions of the *Bae*I and *Pci*I sites used to construct pAA3289 are highlighted in red. (C) pAA3289; the positions of the *Sal*I and *Nsi*I sites used to clone the kan^r-*uspA2* 5′-UTR-*lacZ* fragments from pAC7 with different numbers of AGAT repeats are highlighted in red. (D) pAA3-23rpt; the positions of the *Avr*II and *Pst*I sites used to linearize the plasmid prior to transformation into O12E are highlighted in red. All the diagrams were generated using MacVector (v6.5) software.

XV. Nucleotide Sequence Analysis

Nucleotide sequencing was performed by the staff of the McDermott Center for Human Growth and Development, UT Southwestern or by Miss Nikki Wagner in the Department of Microbiology, UT Southwestern. The sequences were analyzed using SeqEd v1.03 (Applied Biosystems) and MacVector v6.5.

XVI. Statistical Analysis

Statistical analysis was carried out by applying the Student's *t*-test using Microsoft Excel 2003 software (Microsoft Corporation, Redmond, WA). *p* values less than 0.05 were considered significant.

CHAPTER FOUR

Direct Involvement of the UspA2 Protein in the Serum-Resistant Phenotype of *M. catarrhalis*

I. Introduction

One phenotypic trait of *M. catarrhalis* that has been proposed to correlate with virulence is the ability of some strains of this bacterium to resist complement-mediated killing by normal human serum (129). First observed about twenty years ago [reviewed in (54,273)], the occurrence of the serum-resistant phenotype has been documented in many subsequent studies of *M. catarrhalis* isolates (54,75,152,190,262,273,316). The hypothesis that serum resistance might be a virulence factor for *M. catarrhalis* stemmed from observations that the incidence of complement-resistant *M. catarrhalis* strains was higher in samples isolated from ill patients (i.e., adults with lower respiratory tract infections) than in samples from healthy adults or children (128,152). More recent studies indicate, however, that most *M. catarrhalis* isolates from the nasopharynges of apparently healthy infants and young children are serum-resistant (190,316).

A number of different gene products of *M. catarrhalis* have been linked to the serum-resistant phenotype. Mutations in four different genes encoding proteins exposed on the surface of the outer membrane including UspA2 (4), CopB (117), OMP CD (130), and OMP E (206) have deleterious effects on serum resistance. At least three genes encoding products involved in LOS biosynthesis, including *galE* (322), *kdsA* (171), and *kdtA* (226), have been shown to be necessary for normal expression of serum resistance by *M. catarrhalis.*

An important question that had not been addressed by any of the cited studies concerning the involvement of different gene products in the serum-resistant phenotype of some *M. catarrhalis* strains is whether any of these gene products is directly involved in or responsible for this phenotype.

Taking advantage of the available tools and information about the UspA2 protein, I conducted a series of experiments to address the involvement of the UspA2 protein in the serum-resistant phenotype of *M. catarrhalis.*

II. Results

A. Identification of a serum-sensitive *M. catarrhalis* strain that expresses UspA2

M. catarrhalis strain MC317 was one of the relatively few isolates obtained from the nasopharynges of infants and children that proved to be serum-sensitive (190). In our hands, strain MC317 was readily killed by NHS (Fig. 5A). Western blot analysis showed, however, that MC317 expressed a UspA2 protein that was reactive with MAb 17C7 (Fig. 5B).

One possible explanation for this observation is that the UspA2 protein of strain MC317 is not on the surface of the bacterium, resulting in the serum-sensitive phenotype of this strain. To address this point, we used the indirect antibody-accessibility assay with MAb 17C7 as the primary antibody. A *uspA1* mutant of MC317 (MC317.1) was constructed for use in this assay to eliminate expression of the UspA1 protein which also binds MAb 17C7. The results of the indirect antibody-accessibility assay showed that the UspA2 protein of MC317.1 is readily accessible to MAb 17C7, albeit at a slightly lower level than the UspA2 protein of the O35E.1 strain (Fig. 6, black columns). Both strains bound much greater levels of MAb 17C7 than did the negative control strain, the *uspA1 uspA2* double mutant O35E.12 (Fig. 6). MAb 3F12, a murine IgG MAb specific for the major outer membrane protein of *H. ducreyi* (160), was used as a control for non-specific antibody binding (Fig.

6, open columns). These results indicate that the serum-sensitive phenotype of the MC317 strain was not caused by either a lack of expression or lack of surface exposure of the UspA2 protein.

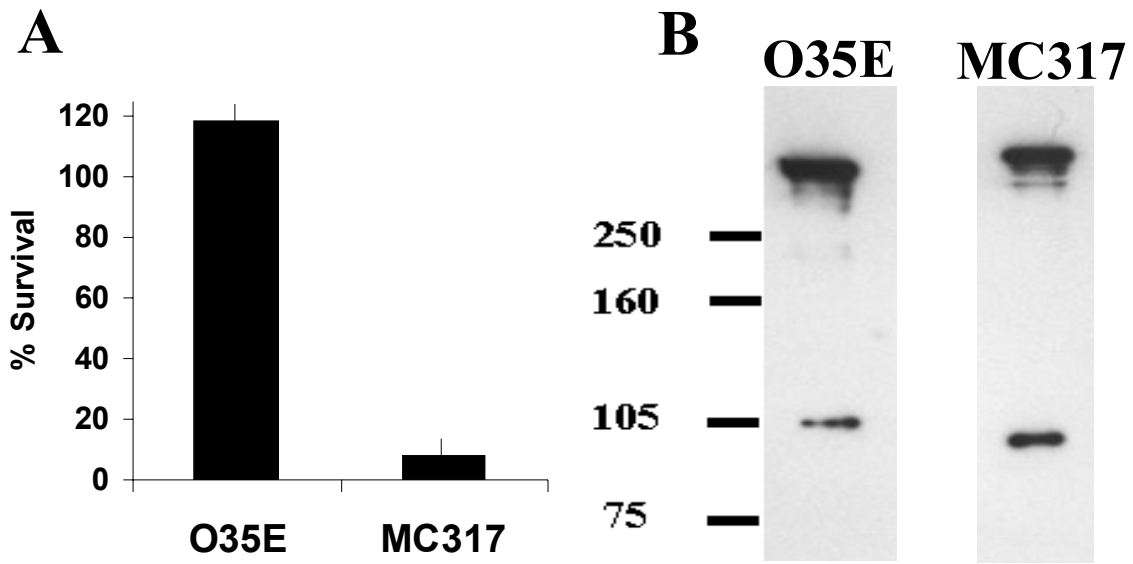

Fig. 5. MC317 is a serum-sensitive *M. catarrhalis* strain that expresses UspA2.

(A) The wild-type strains O35E and MC317 were incubated for 30 min at 37°C in VBS^{++} containing 10% (vol/vol) NHS. Portions of these reaction mixtures were plated at time 0 and after 30 min and the percentage of survival was calculated relative to the original inoculum. The data presented here are the mean of three independent experiments plus the standard deviation. (B) Western blot analysis of whole cell lysates of O35E and MC317 using MAb 17C7 as the primary antibody. Protein molecular mass markers (in kDa) are present on the left side of the panel.

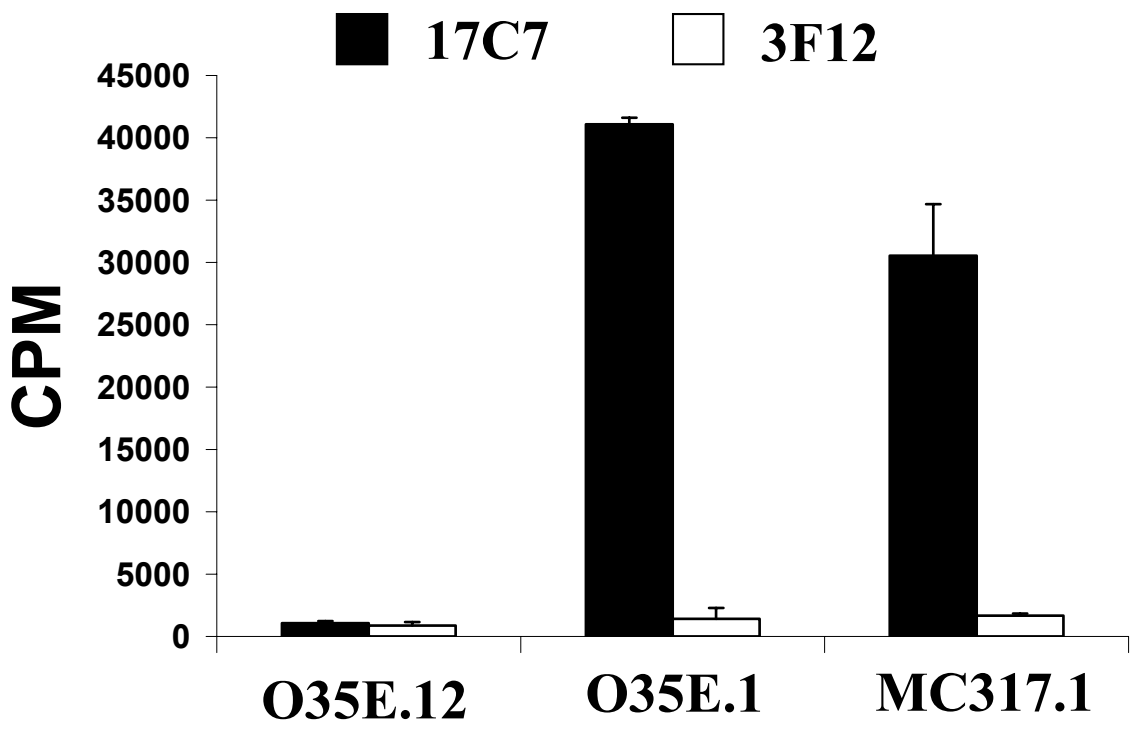

Fig. 6. Indirect antibody-accessibility assay to measure the relative amounts of UspA2 exposed on the surface of *M. catarrhalis* strains.

The *uspA1 uspA2* mutant O35E.12, the *uspA1* mutant O35E.1, and the *uspA1* mutant MC317.1 were tested using the indirect antibody-accessibility assay. CPM of radioiodinated goat anti-mouse IgG bound to MAb 17C7 on the bacterial cell surface (black bars) is plotted on the Y-axis. MAb 3F12, a murine IgG MAb specific for the major outer membrane protein of *H. ducreyi* (160), was used as the negative control (open bars). These data represent the mean of two independent experiments plus standard deviation.

B. Site-directed mutagenesis of the *uspA2* genes of O35E and MC317

Another explanation for the serum-sensitive phenotype of MC317, despite its expression of a surface-exposed UspA2 protein, is that the amino acid sequence of this UspA2 protein is different from that of the serum-resistant strain O35E. Nucleotide sequence analysis of the *uspA2* gene from MC317 revealed an ORF with 1,953 nucleotides that encoded a predicted protein containing 650 amino acids. Alignment of the deduced amino acid sequences of the UspA2 proteins from O35E and MC317 (Fig. 7) revealed regions of significant sequence identity between the two proteins, especially in the putative signal peptide and in the C-terminal portion of these proteins. Overall, these two proteins have 68% identity. However, the MC317 UspA2 protein contains 74 more amino acids than that of strain O35E.

Because the C-terminal region of the UspA2 protein is highly conserved between these two strains, the two differences in this region (Fig. 7, red rectangles) (i.e. A429 in O35E UspA2 is replaced by E510 in MC317) and the missing seven aa (i.e., ALDTKVN)) represented potential sites that might be responsible for the serum-sensitivity of strain MC317. To address this possibility, site-directed mutagenesis was used to construct two strains; O35E(A429E) and MC317(E510A). However, when these mutants were tested in serum bactericidal assays, there was no difference between the wild-type strain and the mutant in each case [i.e. O35E and O35E(A429E)] (Fig. 8, columns 1 and 3) and [MC317 and MC317(E510A)] (Fig. 8, columns 2 and 4). This indicated that aa 429 in the O35E UspA2 protein is not essential for the serum-resistant phenotype of this strain. Moreover, when the seven aa (ALDTKVN) were deleted from the C-terminal region of the UspA2 protein of strain O35E(A429E), the resultant mutant O35E(A429E)ΔA-N was fully serum resistant (Fig. 8, column 5). Taken together, these results indicated that the differences between the O35E and

MC317 UspA2 proteins in the highly conserved C-terminal region most likely are not responsible for the difference in serum resistance of these two strains.

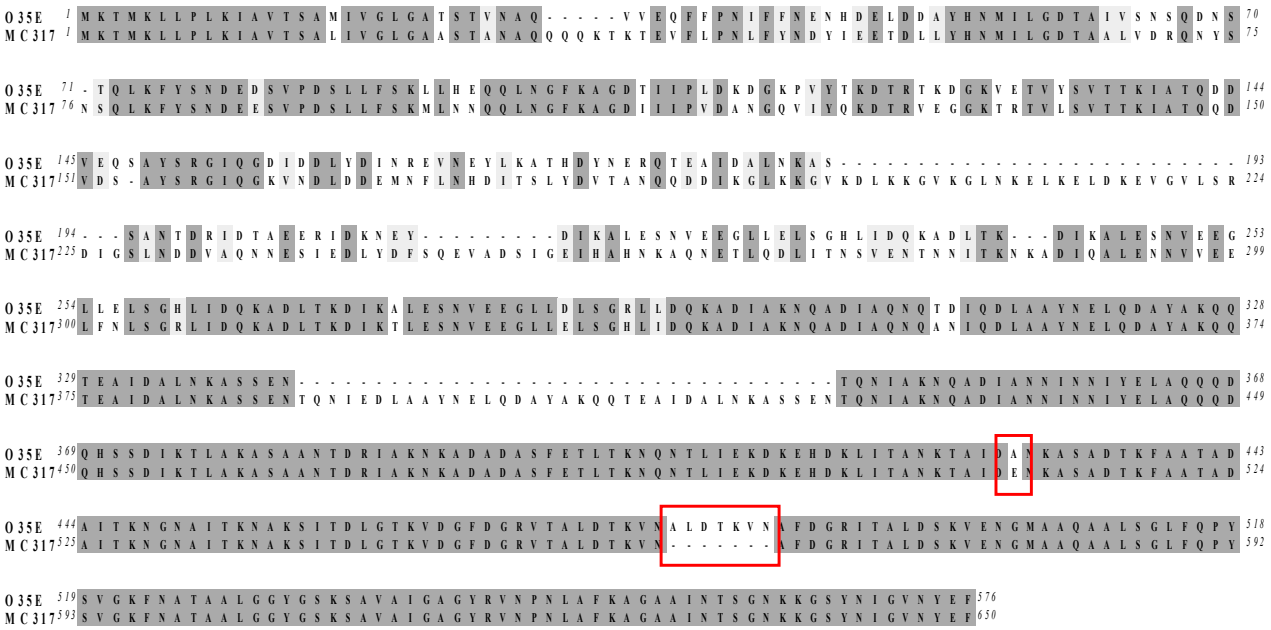

Fig. 7. Comparison of UspA2 proteins from *M. catarrhalis* strains O35E and MC317.

Alignment of the deduced amino acid sequences. Identical amino acids are shaded in dark gray while conserved amino acids are shaded with light gray. Sites of differences in the highly conserved C-terminal region of the UspA2 protein are highlighted with red rectangles. This figure was generated using the ClustalW Alignment program in MacVector (v6.5).

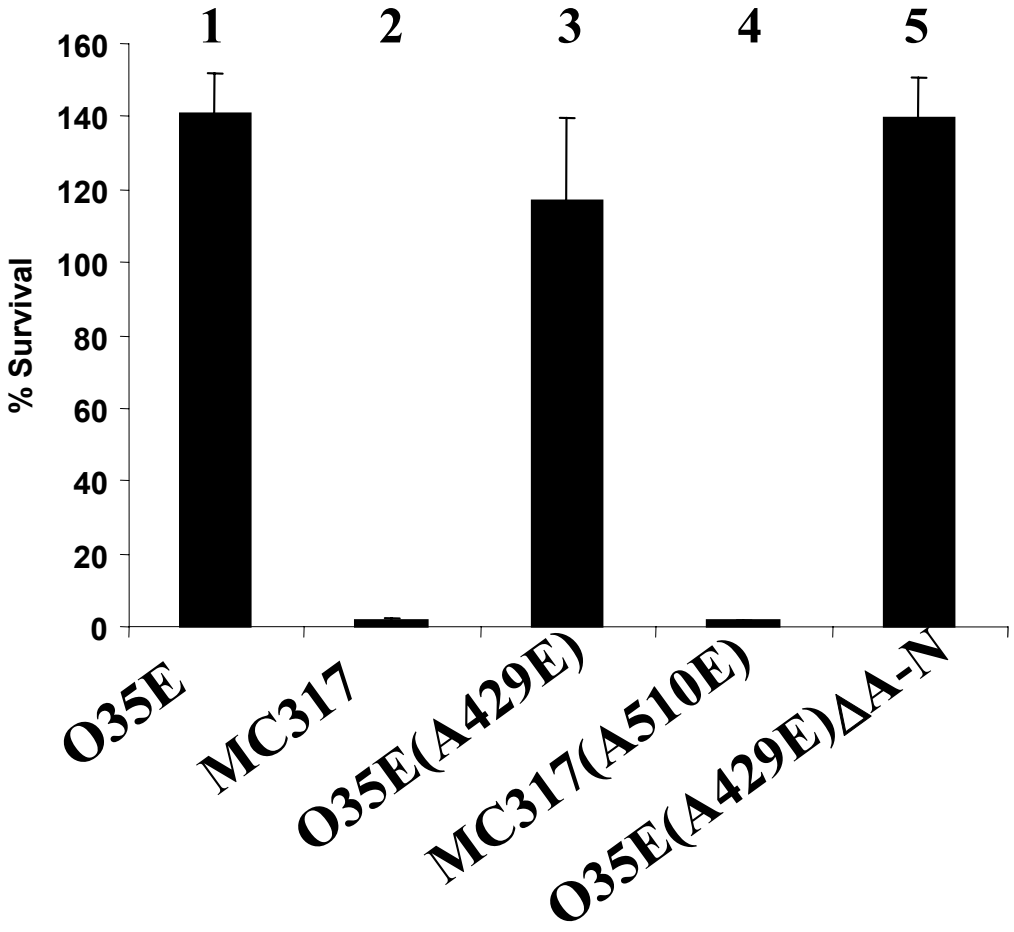

Fig. 8. Effect of site-directed mutagenesis on the serum resistance phenotypes of O35E and MC317.

The wild-type strains O35E, MC317 and their mutants were incubated for 30 min at 37°C in VBS^{++} containing 10% (vol/vol) NHS. Portions of these reaction mixtures were plated at time 0 and after 30 min and the percentage of survival is calculated relative to the original inoculum. The data presented here are the mean of three independent experiments plus the standard deviation.

C. Exchange of *uspA2* genes between O35E and MC317

To determine whether differences in the primary amino acid sequence of the UspA2 proteins from O35E and MC317 were responsible for their different phenotypes with respect to serum killing, we exchanged *uspA2* genes between these two strains using transformation and congression as described in Materials and Methods. Western blot analysis using MAb 17C7 did not reveal apparent differences in the level of expression of UspA2 by the streptomycin-resistant O35E-Smr strain and the streptomycin-resistant MC317-Sm^r strain (Fig. 9A, lanes 1 and 4); these streptomycin-resistant mutants served as positive control strains for this set of experiments. In addition, the transformants O35E/317U2 (i.e., O35E expressing the MC317 UspA2 protein) and MC317/35U2 (i.e., MC317 expressing the O35E UspA2 protein) (Fig. 9A, lanes 3 and 6, respectively) expressed levels of UspA2 protein similar to those of the O35E-Smr and MC317-Sm^r strains.

These two transformants together with their respective control strains and the corresponding *uspA2* insertion mutants (which were used to construct the transformant strains), were tested in bactericidal activity assays. All six of these strains survived equally well in HIS (Fig. 9B, black columns). In the case of NHS (Fig. 9B, open columns), the O35E transformant expressing the MC317 UspA2 protein (O35E/317U2) (Fig. 9B, column 3) was killed as readily as was MC317-Sm^r (Fig. 9B, column 4). On the other hand, the MC317 transformant expressing the O35E UspA2 protein (MC317/35U2) (Fig. 9B, column 6) had a level of serum resistance that was only slightly less than that of the O35E-Sm^r strain (Fig. 9B, column 1). Both *uspA2* mutants were, as expected, sensitive to killing by NHS (Fig. 9B, columns 2 and 5). These results show that differences in amino acid sequence between these two UspA2 proteins are likely responsible for the observed difference in serum resistance of O35E and MC317.

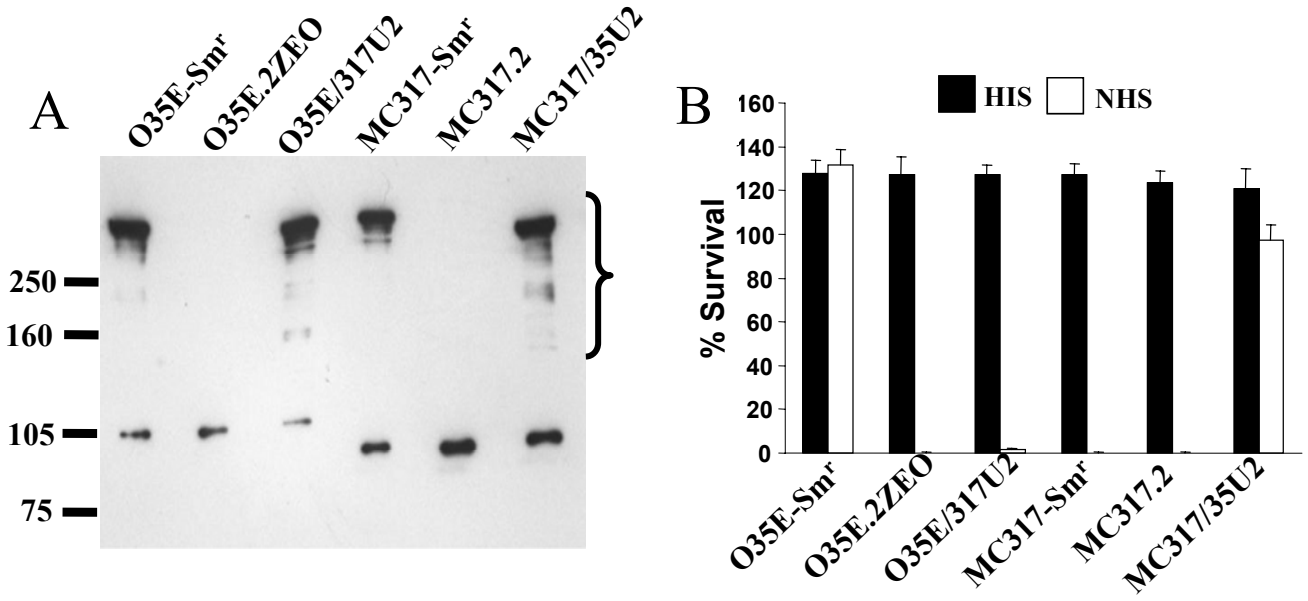

Fig. 9. Effect of *uspA2* gene exchange between *M. catarrhalis* strains on killing by normal human serum.

(A) Western blot analysis using MAb 17C7 to probe whole cell lysates of the *M. catarrhalis* strains: O35E-Sm^r, the *uspA2* mutant O35E.2ZEO, transformant strain O35E/317U2 (i.e., O35E expressing the MC317 UspA2 protein), MC317-Sm^r, the *uspA2* mutant MC317.2 and transformant strain MC317/35U2 (i.e., MC317 expressing the O35E UspA2 protein). The whole cell lysates used in this Western blot experiment were diluted five-fold in order to visualize possible changes in the level of expression of UspA2. Molecular mass markers are shown to the left in kilodaltons. The MAb 17C7-reactive antigen that migrated near the 105 kDa marker is UspA1. (B) Resistance of these six strains (in the same order as above) to killing by either 10% (vol/vol) HIS (black columns) or 10% NHS (open columns). The data presented here are the mean of three independent experiments plus the standard error.

D. Construction and expression of hybrid *uspA2* genes in *M. catarrhalis*

In order to determine whether there is a certain region or domain within the O35E UspA2 protein that is essential for the serum-resistant phenotype, PCR-sewing was used to construct hybrid *uspA2* genes containing different fragments of the O35E and MC317 *uspA2* ORFs. These hybrid *uspA2* genes were introduced into the MC317.2 *uspA2* mutant by transformation and congression. The basic approach involved introducing increasingly greater proportions of the O35E UspA2 protein (starting at the C-terminal end) and then assaying for gain-of-function (i.e., serum resistance) when the hybrid genes were expressed in a serum-sensitive background (i.e., MC317.2). A schematic representation of the six hybrids used in this study is presented in Fig. 10A. All six hybrid UspA2 proteins were expressed at similar levels (Fig. 10B, lanes 2-7) as determined by Western blot analysis. All six hybrid strains survived equally well in HIS (Fig. 10C, black columns 2-7). When NHS was used (Fig. 10B, open columns), the MC317-Sm^r strain (Fig. 10C, column 1) was serum-sensitive and the O35E-Sm^r strain was serum-resistant (Fig. 10C, column 9). When tested in the serum bactericidal assay, the first hybrid UspA2 protein found to increase the serum resistance of MC317.2 was hybrid 4 (Fig. 10C, column 5). The addition of more O35E UspA2 sequence in hybrid 5 (Fig. 10C, column 6) increased the serum resistance of this strain to a level approaching that obtained with the entire O35E UspA2 protein in MC317.2 (Fig. 10C, column 8).

These results suggested that the region of the O35E UspA2 protein between aa 143 and 273 was essential for converting MC317.2 to a serum-resistant phenotype. To confirm this, hybrid 6 was constructed in which the O35E region extending from aa 143-244 was used to replace the equivalent region in the MC317 UspA2 protein. When tested in the serum bactericidal assay, MC317.2 expressing the hybrid 6 UspA2 protein (Fig. 10C, column 7) expressed a level of serum resistance equivalent to that obtained with both MC317.2 expressing the O35E UspA2 protein (Fig. 10C,

column 8) and O35E-Sm^r (Fig. 10C, column 9). To eliminate the possibility that the serum-sensitive hybrid strains did not have surface-exposed UspA2 proteins, I constructed *uspA1* mutants of the serum-sensitive hybrid strains and probed these with the UspA2-reactive MAb 17C7 in the indirect antibody-accessibility assay. The results of these experiments showed that these *uspA1* mutants bound amounts of the radioiodinated goat anti-mouse IgG probe that ranged from 80% to 134% of that bound by the *uspA1* mutant of the fully serum-resistant hybrid 6 (data not shown). Therefore, all of these hybrid strains, regardless of their serum resistance phenotype, expressed comparable amounts of surface-exposed UspA2 protein.

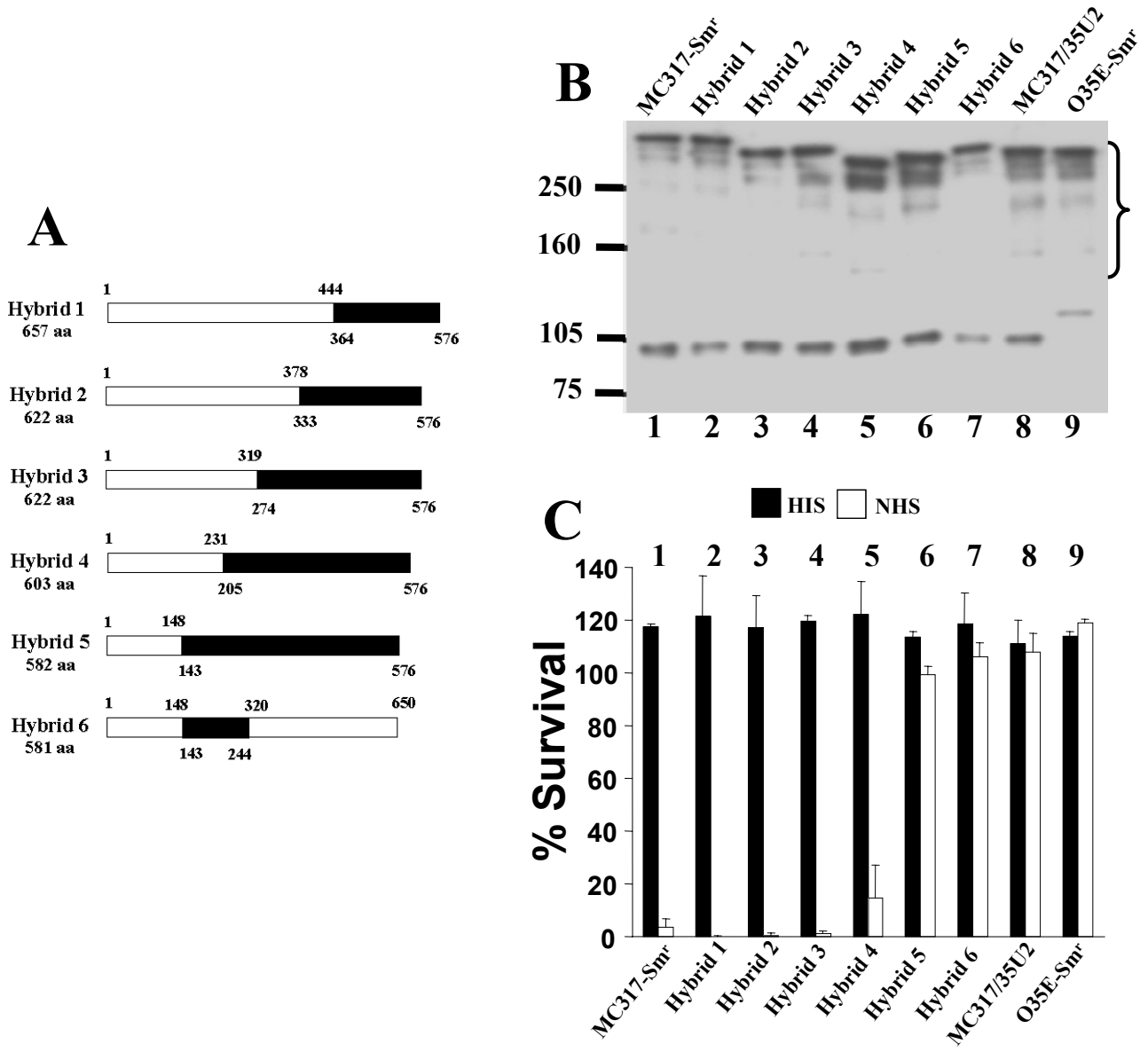

Fig. 10. Comparison and analysis of the MC317/O35E hybrid UspA2 proteins.

(A) Schematic representation of the six hybrids. Open segments and upper numbers represent amino acid sequence from MC317; black segments and lower numbers represent sequence from O35E. Numbering begins with the first residue of the complete protein (containing the signal peptide). All constructs are drawn to scale. (B) Western blot analysis using MAb 17C7 to probe whole cell lysates of the following strains: (1) MC317-Sm^r, (2) hybrid 1, (3) hybrid 2, (4) hybrid 3, (5) hybrid 4, (6) hybrid 5, (7) hybrid 6, (8) MC317/35U2, and (9) O35E-Sm^r. The whole cell lysates used in this Western blot experiment were diluted to be equivalent to 2 x 10^6 cfu in order to visualize any change in the level of expression of UspA2. The bracket indicates the region containing UspA2 and its degradation products. The MAb 17C7-reactive antigen that migrated near the 105 kDa marker is UspA1. Molecular mass markers are shown to the left (in kDa). (C) Resistance of these nine strains (in the same order as above) to killing by either 10% (vol/vol) HIS (black columns) or 10% NHS (open columns). The data presented here are the mean of three independent experiments plus the standard error.

E. Cloning and expression of *M. catarrhalis uspA2* genes in *H. influenzae*

To establish that UspA2 was directly involved in the expression of serum resistance by serum-resistant *M. catarrhalis* strains, I tried to clone and express the UspA2 protein from the serum-resistant strain O12E in several *E. coli* strains. Despite the expression of detectable levels of the UspA2 protein, no substantial increase in serum resistance was observed in this heterologous background. However, it is worth mentioning that some of these recombinant *E. coli* strains showed a very slight increase in serum resistance but these same strains also showed a slower growth rate when compared to the *E. coli* strains carrying the empty vector (data not shown).

To overcome this technical difficulty, I tried to use another heterologous background to express the UspA2 proteins. For this purpose, I used *H. influenzae* strain DB117. An important reason for choosing this particular strain was the reported ability of this strain to express two functional *M. catarrhalis* surface proteins (i.e. UspA1 and UspA2H) (165). The *uspA2* genes from *M.* catarrhalis strains O35E and MC317 were successfully cloned and expressed in *H. influenzae* DB117. A constitutive *kan* promoter was inserted in front of these cloned *M. catarrhalis* genes to increase expression of the UspA2 protein (Fig. 11A, lanes 2 and 3, respectively). Both of these recombinant strains and the *H. influenzae* strain carrying the vector control plasmid pAA-kp survived to the same extent in HIS (Fig. 11B, black columns). When tested for their ability to resist killing by 5% (vol/vol) NHS (Fig. 11B, open columns) the recombinant *H. influenzae* strain expressing the O35E UspA2 protein (Fig. 11B, column 2) was more serum-resistant than the recombinant strain expressing the MC317 UspA2 protein (Fig. 11B, column 3). There was no difference in the level of serum resistance of the *H. influenzae* recombinant strain expressing the MC317 UspA2 protein (Fig. 11B, column 3) and that of the strain which carried only the *kan* promoter within the plasmid (Fig. 11B, column 1).

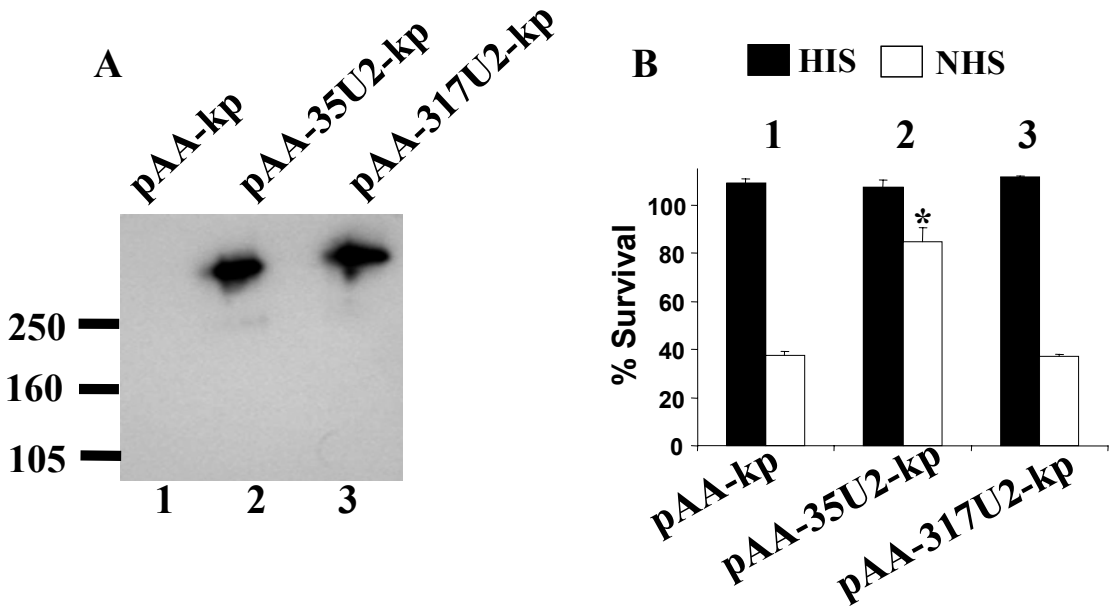

Fig. 11. Effect of expression of the *M. catarrhalis* UspA2 protein on serum resistance of *H. influenzae* DB117.

(A) Western blot analysis using MAb 17C7 to probe whole cell lysates of *H. influenzae* DB117 carrying the following plasmids: (1) pAA-kp (negative control), (2) pAA-35U2-kp, and (3) pAA-317U2-kp. Molecular mass markers (in kDa) are shown to the left. (B) Resistance of these three strains (in the same order as above) to killing by either 5% (vol/vol) HIS (black columns) or 5% NHS (open columns). The data presented here are the mean of three independent experiments plus the standard error. The asterisk indicates that the difference between the strains carrying pAA-35U2-kp and pAA-317U2-kp is significant (p value = 0.002) as determined by the use of a Student t test.

III. Discussion

Previous experiments performed by Christoph Aebi, M. D., and Eric R. Lafontaine, Ph. D., in the Department of Microbiology at UT Southwestern, showed that inactivation of the *uspA2* gene or the very similar *uspA2H* gene by insertion of antibiotic resistance cartridges resulted in sensitivity to killing by complement-sufficient NHS (4,165). Whether the loss of expression of UspA2 (or UspA2H) itself was directly responsible for the altered phenotype or whether the absence of UspA2 affected or altered the expression of some other surface antigen directly responsible for serum resistance could not be determined in these earlier studies. That UspA2 might be involved in serum resistance was also predicted by another independent study using phage antibodies to characterize serum-resistant and serum-sensitive isolates of *M. catarrhalis*. Phage antibodies that selectively bound serum-resistant strains were shown to bind HMW-OMP (161), which is identical to UspA2, in Western blot analysis (38). Whether the serum-sensitive *M. catarrhalis* strains that failed to bind these phage antibodies also did not express a UspA2 protein was not reported (38).

To address this issue, I cloned and expressed the *uspA2* gene from the serum-resistant *M. catarrhalis* strain O12E in a heterologous background (i.e., *E. coli*). However, no substantial increase in serum resistance was observed. One explanation for the serum sensitivity of these recombinant *E. coli* strains might be the improper folding of the UspA2 protein on the surface of the bacterial cells. On the other hand, when the *uspA2* genes from both a serum-resistant *M. catarrhalis* strain (O35E) and a serum-sensitive *M. catarrhalis* strain (MC317) were cloned and expressed in a another heterologous background (i.e., *H. influenzae*), a serum-resistant phenotype was observed. When tested in a serum bactericidal assay, the UspA2 protein from the serum-resistant strain, but not the UspA2 protein from the serum-sensitive strain, conferred increased serum resistance on the *H.*

influenzae recombinant strain. These results show that UspA2 is directly involved in the expression of serum resistance by some strains of *M. catarrhalis*.

Two recent studies have suggested that complement-resistant *M. catarrhalis* strains comprise a distinct subpopulation or lineage within this species (41,294). One group of workers used pulsed-field gel electrophoresis, a nonribosomal PCR restriction fragment length polymorphism (RFLP) procedure, and random amplification of polymorphic DNA analysis to divide 47 serum-resistant and 28 serum-sensitive strains into two groups, with the serum-resistant strains falling into a clonal group (294). The other laboratory applied probe-generated RFLP and single-adapter amplified fragment length polymorphism analyses to characterize 90 *M. catarrhalis* strains, resulting in a dendrogram that had two main branches (41). The vast majority of complement-resistant strains examined in the latter study clustered into one of the two main branches. Interestingly, PCR-based analysis indicated that equal percentages of complement-sensitive and complement-resistant strains had a *uspA2* gene. This finding raises the possibility that these complement-sensitive strains expressed a UspA2 protein similar to that of MC317, which was unable to confer serum resistance on *M. catarrhalis*.

That some *M. catarrhalis* strains express a UspA2 protein that cannot confer serum resistance is reminiscent of the situation with some strains of serum-resistant and serum-sensitive *N. gonorrhoeae* which differ in their expression of a particular type of porin protein (241). Gene exchange experiments involving the *uspA2* genes of strains O35E and MC317 and the consequent change in the serum sensitivity of the transformant strains (Fig. 9) suggested that differences within the primary amino acid sequence of these two UspA2 proteins were responsible for these different susceptibilities to killing by NHS. This hypothesis was confirmed by the finding that the region of the O35E UspA2 protein between aa 143-244 was sufficient to convert strain MC317 to serum resistance when expressed in the equivalent position within the MC317 UspA2 protein. The exact

role of this region of the UspA2 protein in the serum-resistant phenotype of strain O35E represents an interesting area for future research. However, it would be helpful to first determine the mechanism of the UspA2-mediated serum-resistance to know whether this region is directly binding a complement regulator or whether it is affecting the overall structure of the UspA2 protein and thereby affecting its function.

CHAPTER FIVE

Interaction of *M. catarrhalis* with the Complement Regulator C4BP

I. Introduction

UspA2 is present on the *M. catarrhalis* cell surface as a dense layer of short projections (125,225) which allows for its potential interaction with different serum components. Purified or recombinant UspA2 proteins have been reported to bind several proteins normally present in NHS including vitronectin (185) and C4BP (216). In addition, UspA2 has been recently reported to bind to fibronectin (277). The physiological relevance of these binding activities remains to be fully elucidated, but binding of the complement regulators vitronectin or C4BP could affect the serum resistance phenotype. In fact, binding of C4BP has been proposed to be involved in the serum resistance of *M. catarrhalis* (216). However, the reported experiments were performed with purified C4BP in the absence of other serum proteins. Also, this study did not address experimentally the physiological relevance of this binding activity in possibly protecting *M. catarrhalis* from killing by the complement system.

In this chapter, experiments were done to analyze the interaction of twelve different serum-resistant *M. catarrhalis* strains and their *uspA1* and *uspA2* mutants with C4BP. The results of these experiments showed that C4BP binding varies widely among *M. catarrhalis* strains and it is not related to the serum resistance phenotype of the tested strains.

II. Results

A. Construction of a *uspA2* deletion mutant

For further analysis of the interaction of the complement system with the serum-resistant *M. catarrhalis* strain O35E, I constructed a *uspA2* deletion mutant as described in Materials and Methods. Nucleotide sequence analysis of the resultant *uspA2* deletion mutant O35Δ2 confirmed the replacement of the *uspA2* ORF with the spectinomycin resistance cartridge. This analysis also showed that there was a predicted ORF encoding a predicted MetR homolog located a short distance downstream from *uspA2*; this ORF was oriented in the opposite direction from *uspA2*. The nucleotide sequence of this putative ORF was found to have been altered by recombination in this *uspA2* deletion mutant. Therefore, to confirm that this downstream ORF was not involved in conferring serum resistance on *M. catarrhalis*, we tested a mutant of O35E that had a transposon inserted in this ORF and showed that it was as serum-resistant as the wild-type parent strain (data not shown).

B. Phenotypic characterization of O35EΔ2

There was no apparent difference in the colony morphology of the wild-type parent strain O35E and the O35EΔ2 mutant (data not shown). In addition, both the extent and rate of growth of these two strains were very similar if not identical (Fig. 12).

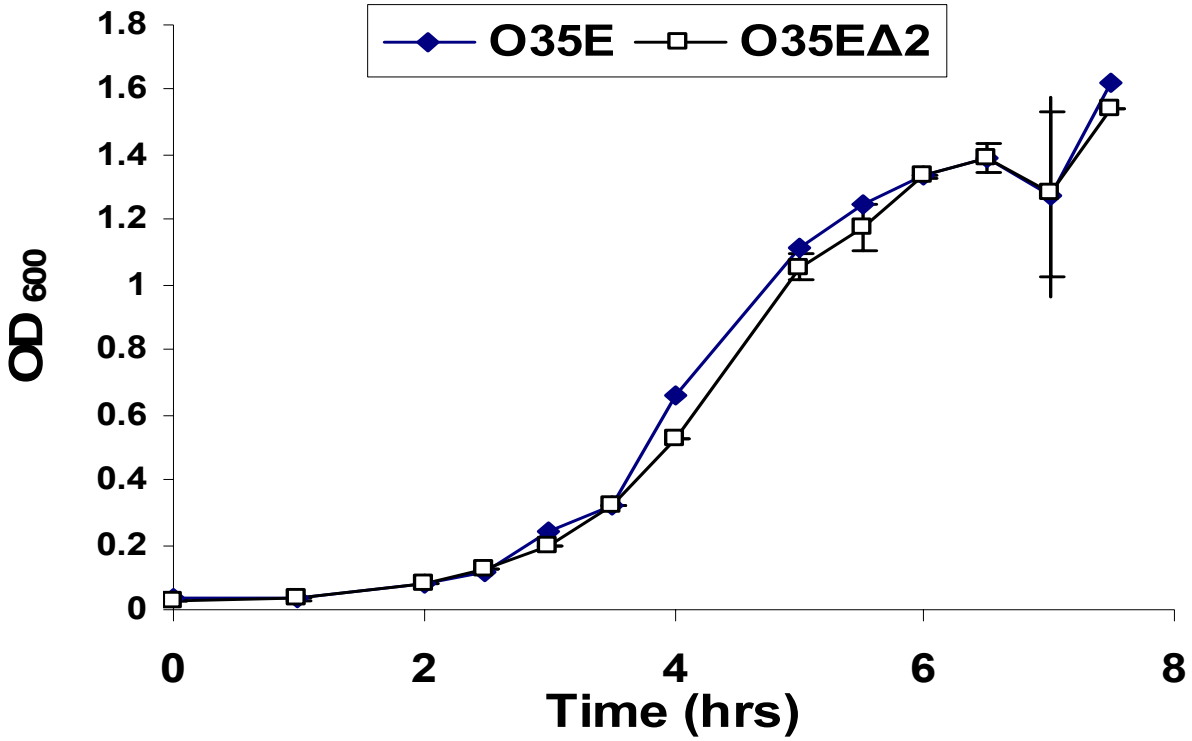

Fig. 12. Growth of *M. catarrhalis* wild-type strain O35E and its *uspA2* deletion mutant O35EΔ2 in BHI broth.

The optical density of the cultures was recorded over time. The data represent the average of two independent experiments and the error bars represent the standard deviations.

Western blot analysis of whole cell lysates of the wild-type strain and the O35EΔ2 mutant (Fig. 13A) with MAb 17C7, which binds both UspA2 and UspA1 (5), showed that the mutant lacked the high molecular weight aggregate that represents the UspA2 protein. At the same time, there was no obvious change in the expression of the UspA1 protein by this mutant (Fig. 13B) as revealed by Western blot analysis using the UspA1-specific MAb 24B5 (61). Examination of the proteins present in EDTA-extracted outer membrane vesicles of both the wild-type and mutant revealed that the mutant lacked the high-molecular-weight UspA2 band that was present in the wild-type parent strain (Fig. 13C). However, no other differences were visible in these outer membrane protein profiles. It has been shown previously that changes in the LOS of *M. catarrhalis* can affect its susceptibility to killing by NHS (322). However, there was no apparent change in the SDS-PAGE migration pattern of the LOS from O35E and O35EΔ2 as detected either by silver staining (Fig. 13D) or by Western blot analysis using the LOS-reactive MAb 8E7 (Fig. 13E).

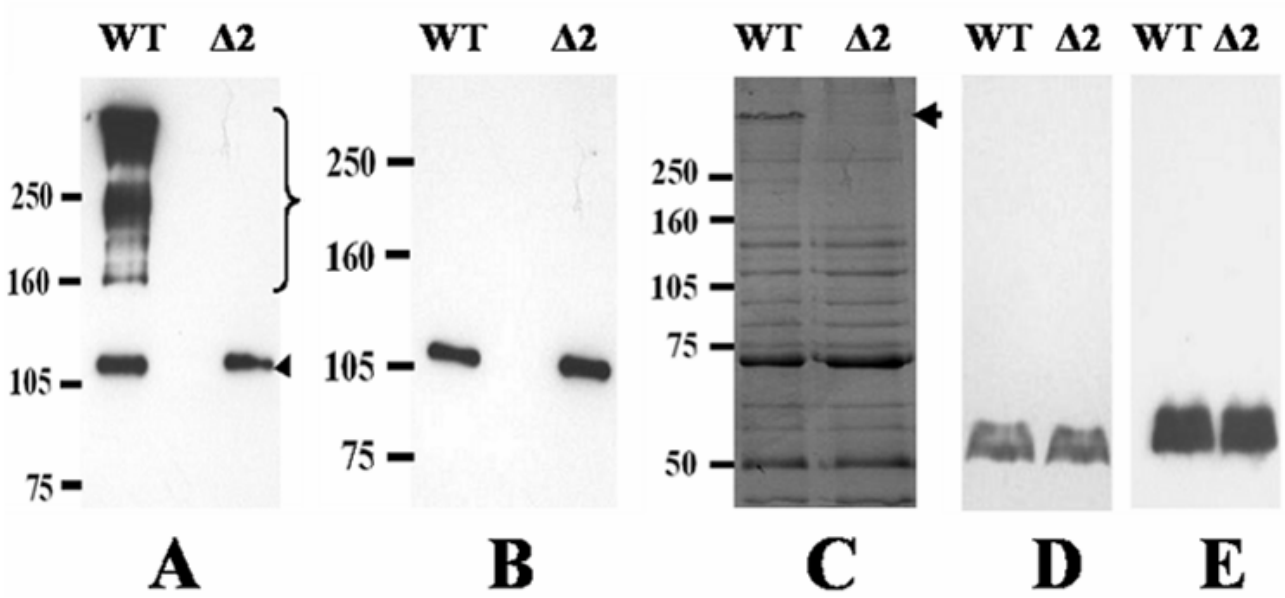

Fig. 13. Comparison of the phenotypes of the wild-type *M. catarrhalis* strain O35E (WT) and the *uspA2* mutant O35EΔ2 (Δ2).

(A) Western blot analysis of whole cell lysates using MAb 17C7 as the primary antibody. This MAb binds both UspA2 (indicated by bracket) and UspA1 (indicated by arrow head). (B) Western blot analysis of these whole cell lysates using the UspA1-specific MAb 24B5 as the primary antibody. (C) Proteins present in outer membrane vesicles from these two strains were resolved by SDS-PAGE and stained with Coomassie blue. The absence of UspA2 in the O35EΔ2 mutant is indicated by the arrow. Whole cell lysates digested with proteinase-K were resolved by SDS-PAGE and LOS was visualized either by staining with silver (D) or by Western blot analysis (E) using the LOS-reactive MAb 8E7 as the primary antibody. Protein molecular mass markers (in kDa) are present on the left of panels A, B, and C.

C. Killing of the *uspA2* deletion mutant O35Δ2 involves the classical complement pathway

As expected from the results obtained with a different *uspA2* mutant (4), the use of 10% NHS in a serum bactericidal assay showed that the *uspA2* deletion mutant O35Δ2 (Fig. 14, column 6) was exquisitely serum-sensitive whereas the wild-type strain O35E (Fig. 14, column 2) was completely resistant. These two strains survived equally well in HIS (Fig. 14, columns 1 and 5). To selectively block the alternative complement pathway, NHS depleted of factor B (271) was used in the serum killing assay. The wild-type parent strain O35E (Fig. 14, column 3) was able to survive in the factor B-depleted serum whereas the O35EΔ2 mutant (Fig. 14, column 7) was readily killed by this same serum. This result suggested that the alternative complement pathway was probably not involved in the killing of this serum-sensitive mutant. To selectively block the classical pathway, the bactericidal assay was carried out in the presence of Mg^{2+} and in the absence of Ca^{2+} (144); NHS was selectively depleted of Ca^{2+} by using the chelating agent EGTA. Strains O35E (Fig. 14, column 4) and O35Δ2 (Fig. 14, column 8) survived equally well under these assay conditions, a result which indicated that killing of this *uspA2* mutant by NHS is accomplished via the classical pathway of complement activation. The serum-sensitive *M. catarrhalis* strain MC317 was readily killed by NHS (Fig. 14, column 10). Also, this same strain was also sensitive to killing by factor B-depleted serum, with 82% of the initial inoculum being killed (Fig. 14, column 11). On the other hand, strain MC317 was resistant to NHS in which the classical pathway was blocked by Mg^{2+}/EGTA (Fig. 14, column 12), indicating that the killing of this serum-sensitive wild-type *M. catarrhalis* strain is mediated by the classical pathway.

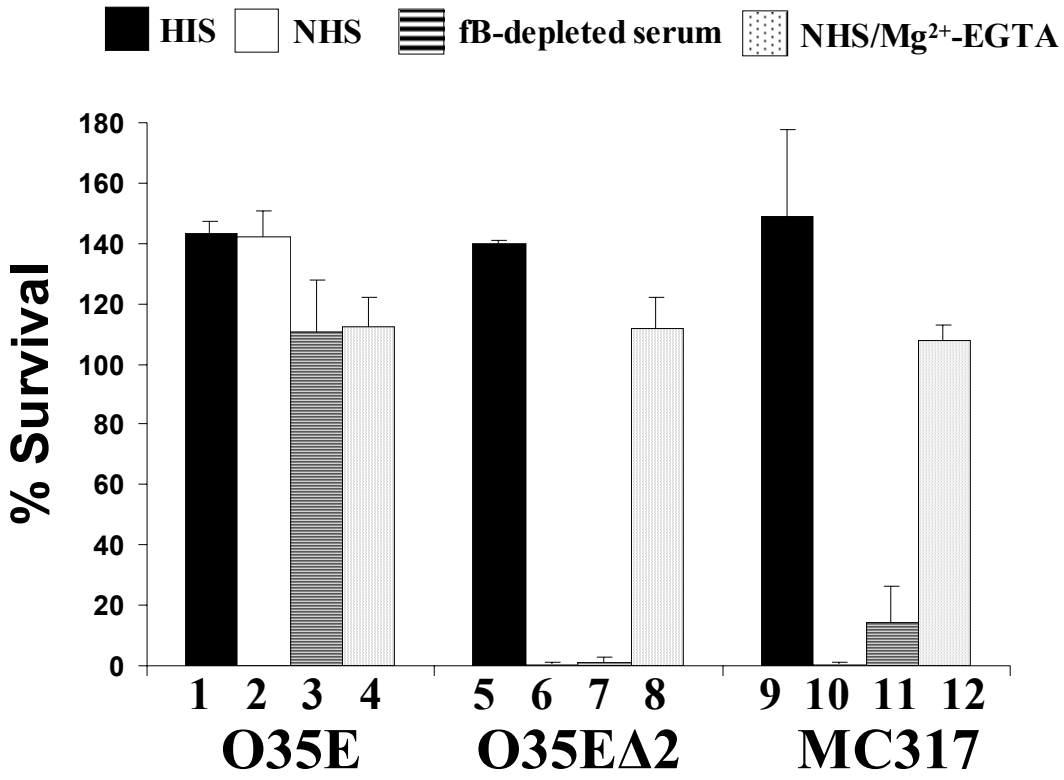

Fig. 14. Killing of wild-type and mutant strains of *M. catarrhalis* by various sera.

The wild-type strain O35E, the *uspA2* mutant O35EΔ2, and the wild-type strain MC317 were incubated for 30 min at 37°C in VBS^{++} containing 10% (vol/vol) HIS (black columns), 10% (vol/vol) NHS (open columns), 10% (vol/vol) factor B-depleted human serum (stripped columns), or in VBS containing 10 mM $MgCl_2$, 10 mM EGTA, and 10% (vol/vol) NHS (dotted columns). Portions of these reaction mixtures were plated at time 0 and after 30 min and the percentage of survival is calculated relative to the original inoculum. The data presented here are the mean of three independent experiments plus the standard error.

D. Killing of the O35EΔ2 *uspA2* mutant by NHS is IgG-dependent

To investigate the possible involvement of IgG antibody in the killing of the *uspA2* deletion mutant, NHS depleted of IgG was used in the serum bactericidal assay. Western blot analysis of this NHS after adsorption with GammaBind Plus Sepharose showed that this treatment removed almost all of the IgG from this serum (data not shown). There was no obvious difference in the survival of the wild-type O35E strain in HIS (Fig. 15, column 1), NHS (Fig. 15, column 2), IgG-depleted serum (Fig. 15, column 3), and IgG-depleted serum supplemented with HIS (as a source of IgG) (Fig. 15, column 4). In contrast, the *uspA2* mutant O35Δ2 survived in both the HIS (Fig. 15, column 5) and the IgG-depleted serum (Fig. 15, column 7) but was readily killed by both NHS (Fig. 15, column 6) and the IgG-depleted serum supplemented with HIS (Fig. 15, column 8). The latter serum mixture was used to confirm that the IgG depletion step had not inactivated the complement components required for serum killing. These results indicated that the killing of the serum-sensitive O35EΔ2 mutant via the classical pathway is IgG antibody-dependent.

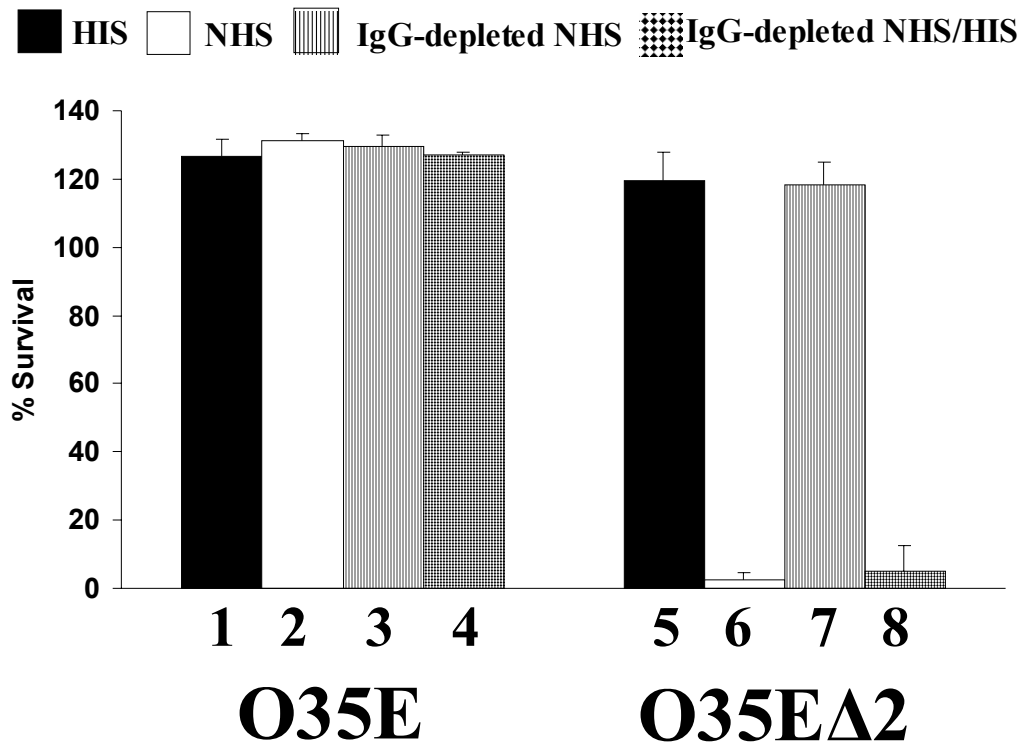

Fig. 15. Involvement of IgG in killing of the *uspA2* mutant O35EΔ2.

Wild-type strain O35E and the *uspA2* mutant O35EΔ2 were incubated with the following sera: 10% (vol/vol) HIS (black columns), 10% (vol/vol) NHS (open columns), 10% (vol/vol) IgG-depleted NHS (vertically striped columns) and 10% (vol/vol) IgG-depleted NHS mixed with HIS as a source of IgG (checked columns). The data presented here are the mean of three independent experiments plus the standard error.

E. *M. catarrhalis* strain O35E binds low amounts of purified C4BP mainly through its UspA1 protein

The experiments in the previous sections demonstrated that the UspA2 protein of *M. catarrhalis* strain O35E is directly responsible for its serum-resistant phenotype and that the killing of the *uspA2* mutant is mediated through the classical pathway. A possible mechanism for serum resistance would involve the binding of the UspA2 protein to a regulator of the classical complement pathway such as C4BP. At this point during this study, it was reported by another laboratory that two serum-resistant *M. catarrhalis* strains bound the complement regulator C4BP mainly through their UspA2 protein (216). Flow cytometry revealed that *M. catarrhalis* strain O35E (Fig. 16A) bound C4BP. However, the *uspA2* mutant O35EΔ2 (Fig. 16B) bound more C4BP than did the wild type strain and the *uspA1* mutant O35E.1 (Fig. 16C) bound only about half as much. Two well-characterized *N. gonorrhoeae* strains (i.e., the serum-resistant strain FA19 and the serum-sensitive strain UU1) (240) were used as positive and negative controls, respectively, for C4BP binding. The geometric mean fluorescence (gmf) of C4BP binding obtained with FA19 (Fig. 16D) was ten-fold greater than that obtained with *M. catarrhalis* O35E whereas binding of C4BP by the negative control strain UU1 (Fig. 16E) was very similar to that obtained with *M. catarrhalis* O35E (Fig. 16A). These results indicated that *M. catarrhalis* O35E bound very small amounts of C4BP mainly through its UspA1 protein. However, this UspA1-mediated C4BP binding did not protect this strain from the bactericidal action of NHS because the *uspA1* mutant O35E.1 (Fig. 16F) was fully serum-resistant. Interestingly, the *uspA2* mutant O35EΔ2 which bound relatively more C4BP than the wild-type strain was serum-sensitive (Fig. 16F).

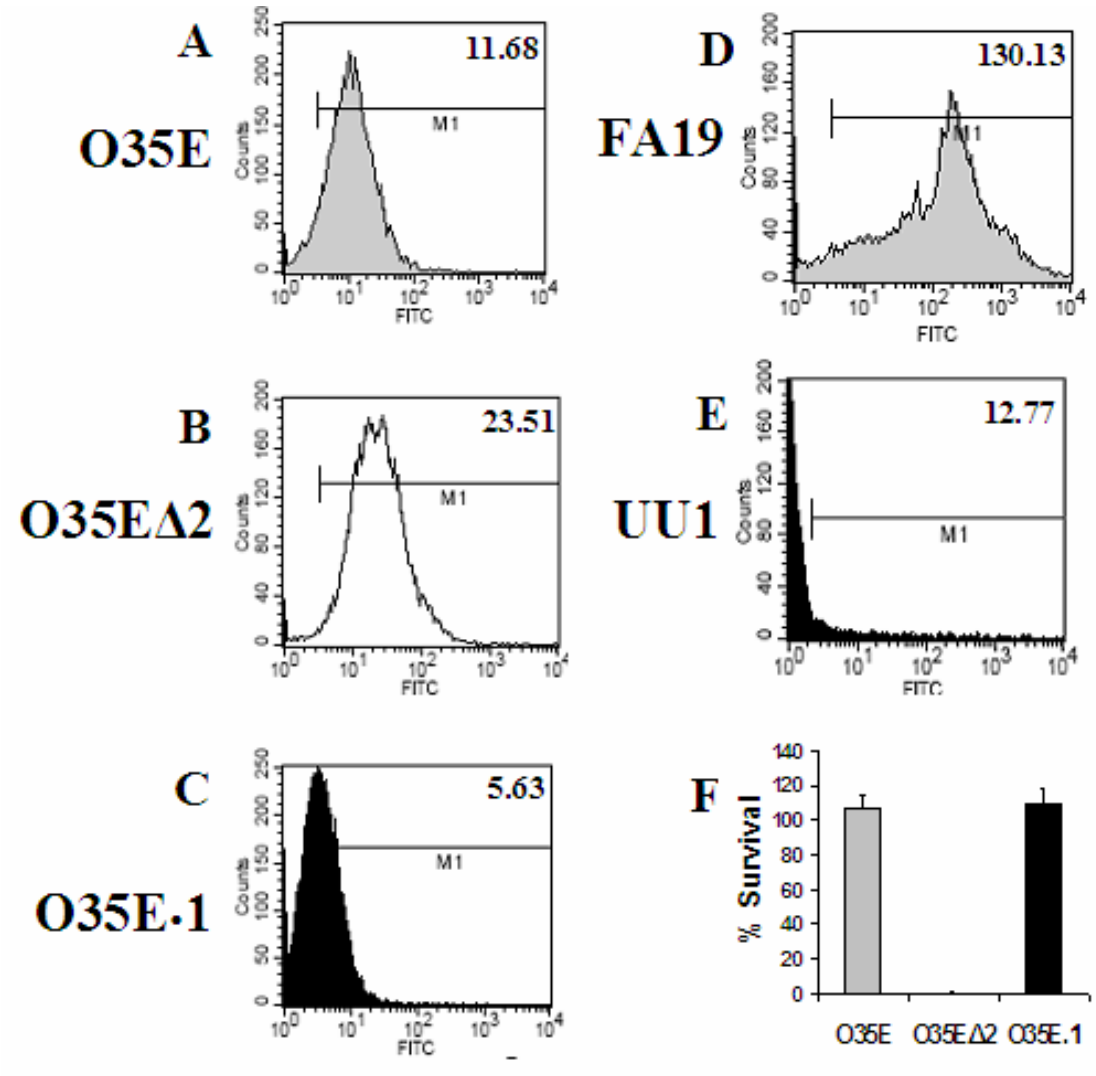

Fig. 16. Flow cytometric analysis of C4BP binding to *M. catarrhalis* and *N. gonorrhoeae* strains.

Suspensions of the *M. catarrhalis* wild-type strain O35E (A), the *uspA2* deletion mutant O35EΔ2 (B), and the *uspA1* mutant O35E.1 (C) were incubated with 2.5 μg of purified C4BP for 1 hr at 37°C. After washing, the bacteria were incubated with a mouse MAb against C4BP followed by washing and incubation with a FITC-conjugated antiserum to mouse IgG. After washing, the cells were analyzed by flow cytometry. *N. gonorrhoeae* strains FA19 (D) and UU1 (E) were included as positive and negative controls, respectively, for C4BP binding. The number in the upper right corner of each panel represents the geometric mean fluorescence (gmf). The M1 gate excludes the majority of events obtained with the negative (isotype) control (i.e., bacteria probed with primary and secondary antibodies in the absence of C4BP). The data presented are representative experiments. (F) Serum bactericidal activity assay using 10% NHS with *M. catarrhalis* O35E and its isogenic mutants. The data represent the mean of three independent experiments and the error bars represent the standard deviations.

F. *M. catarrhalis* strains differ in their binding of purified C4BP

Testing of another eleven different serum-resistant *M. catarrhalis* strains and their *uspA1* and *uspA2* mutants for their ability to bind C4BP revealed that these strains differed markedly in both the amount of C4BP bound and the binding moiety (Table 4). Four strains representing the different C4BP binding phenotypes are presented in Fig. 17. Strain FIN2344 (Fig. 17A) bound a relatively large amount of C4BP mainly via its UspA1 protein because its *uspA1* mutant (Fig. 17B) bound almost no C4BP whereas its *uspA2* mutant (Fig. 17C) bound levels of C4BP only slightly lower than that bound by the wild-type strain. The wild-type V1118 strain (Fig. 17D) bound a small amount of C4BP and a relatively large reduction in C4BP binding was observed with the *uspA1* mutant (Fig. 17E) while a smaller reduction in binding was observed with the *uspA2* mutant (Fig. 17F). The wild-type 7169 strain (Fig. 17G) and its *uspA1* mutant (Fig. 17H) both bound large amounts of C4BP, comparable to those bound by *N. gonorrhoeae* strain FA19 (Fig. 16D), whereas there was a very large reduction in binding observed with the 7169 *uspA2* mutant (Fig. 17I). Finally, the wild-type O12E strain (Fig. 17J) appeared to bind very little C4BP and the *uspA1* (Fig. 17K) and *uspA2* (Fig. 17L) mutations had little effect on this minimal binding activity.

Table 4. Classification of *M. catarrhalis* strains according to their level of binding of purified C4BP and the binding moiety[a].

Major binding moiety = UspA1		**Major binding moiety= UspA2**	**Very low binding**
High level binding	**Low level binding**	**High level binding**	
FIN2344	O35E ETSU5 ETSU22 ETSU26 FIN2406 V1118 V1120	7169 V1145 V1156	O12E

[a]Binding of purified C4BP was measured by flow cytometry

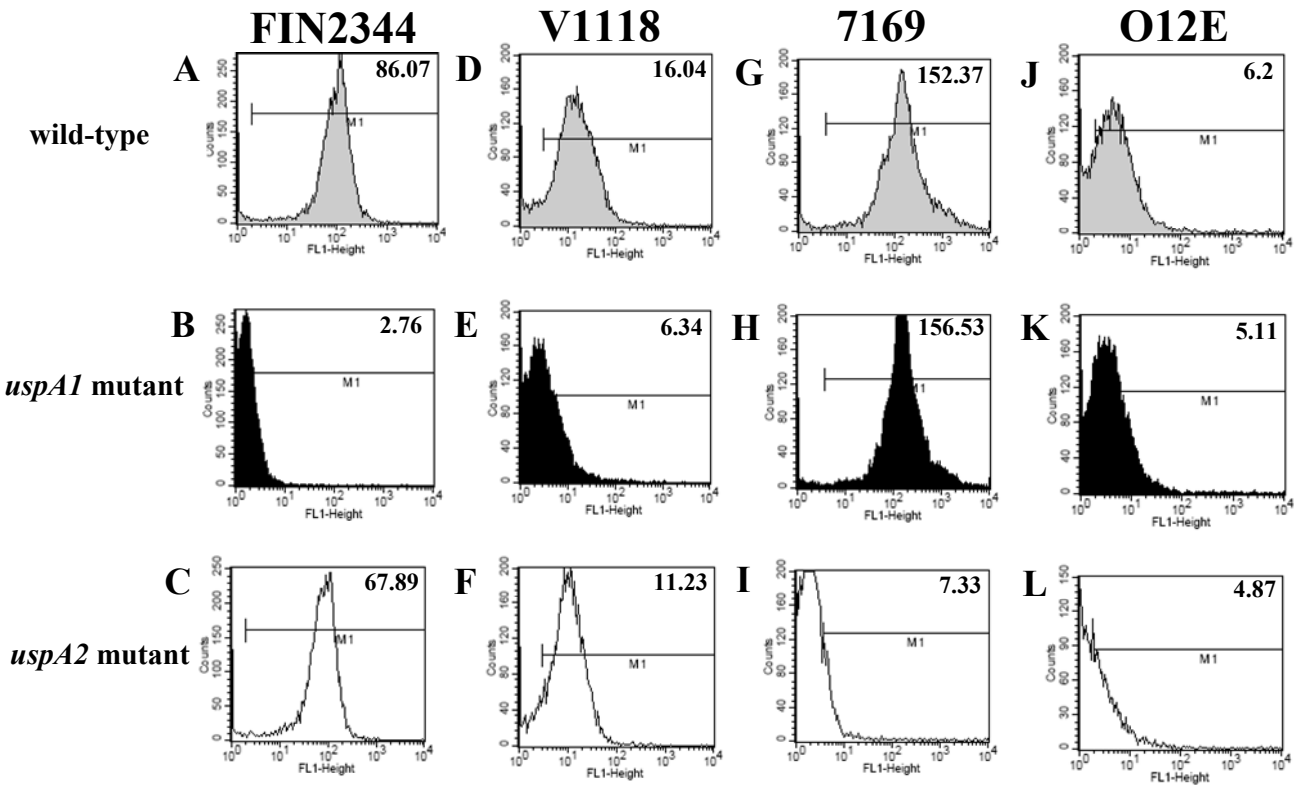

Fig. 17. Binding of purified C4BP to *M. catarrhalis* wild-type strains and mutants.

Flow cytometry-based measurement of C4BP binding to wild-type strains FIN2344, V1118, 7169, and O12E, their *uspA1* mutants, and their *uspA2* mutants was accomplished as described in Fig. 16. The number in the upper right corner of each panel represents the geometric mean fluorescence (gmf). The M1 gate excludes the majority of events obtained with the negative (isotype) control (i.e., bacteria probed with primary and secondary antibodies in the absence of C4BP). The data presented are representative experiments.

G. Binding of radiolabeled C4BP to *M. catarrhalis* strains

Measurement of the binding of radioiodinated C4BP to five of these *M. catarrhalis* strains was used to confirm the flow cytometry-derived data described above. *N. gonorrhoeae* FA19 and UU1 (Fig. 18A) were again used as positive and negative controls, respectively, for binding. *M. catarrhalis* FIN2344 (Fig. 18B) again exhibited high level binding of C4BP (i.e., 49,000 CPM) whereas its *uspA1* mutant had barely detectable levels of C4BP bound and its *uspA2* mutant bound C4BP at wild-type levels. *M. catarrhalis* strains O35E (Fig. 18C) and V1118 (Fig. 18D) both bound levels of C4BP (i.e., 3,000-4,000 CPM) that were more than ten-fold lower than those bound by FIN2344. Both the O35E *uspA1* mutant and the V1118 *uspA1* mutant bound less C4BP than their respective *uspA2* mutants (Fig. 18C and D). Both the wild-type 7169 strain (Fig. 18E) and its *uspA1* mutant bound very high levels of C4BP (i.e., 60,000 CPM) whereas the 7169 *uspA2* mutant bound barely detectable levels. Finally, once again, the O12E wild-type and its respective *uspA1* and *uspA2* mutants bound barely detectable amounts (i.e., 400-800 CPM) of radioiodinated C4BP in this assay.

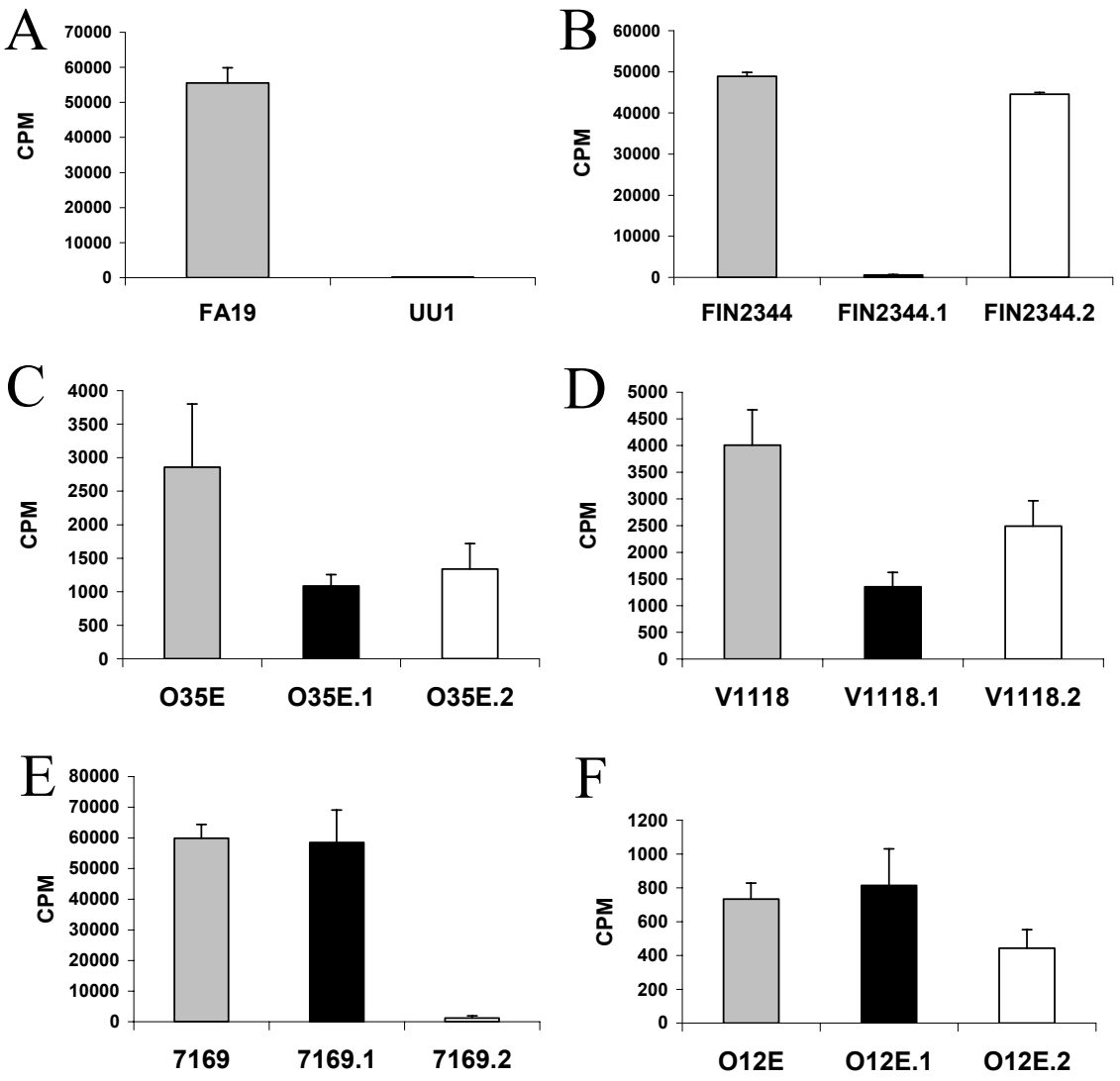

Fig. 18. Measurement of binding of [^{125}I]-C4BP to *M. catarrhalis* strains.

Suspensions of the wild-type strains (e.g., O35E), *uspA1* mutants (e.g., O35E.1), and *uspA2* mutants (e.g., O35E.2) were incubated with ~300 ng of [^{125}I]-C4BP at 37°C for 1 hr. After washing, the radioactivity in the bacterial pellet was measured using a gamma counter and CPM were recorded. These results represent the mean of two experiments and the error bars represent the standard deviations.

H. Effect of *uspA1* and *uspA2* mutations on serum resistance

To evaluate the possible role of C4BP binding in serum resistance, the twelve wild-type strains described above, together with their *uspA1* and *uspA2* mutants, were tested in a serum bactericidal assay using 10% NHS (Fig. 19). In every set of strains tested, the *uspA1* mutant was either as serum-resistant as its parent strain or had only slightly reduced serum resistance, whereas each *uspA2* mutant was exquisitely serum-sensitive. This was true even for FIN2344, which bound very large amounts of purified C4BP via its UspA1 protein (Fig. 18B).

Nordström et al reported that the *uspA1* mutants of the two serum-resistant strains tested in their study exhibited an approximate 50% reduction in serum resistance (216). However, I did not see any substantial decrease in the serum resistance of the *uspA1* mutants of the twelve strains that I tested. As an additional confirmation of my data, I constructed new *uspA1* deletion mutants of four *M. catarrhalis* strains (i.e. O12EΔ1, O35EΔ1, FIN2344Δ1, and 7169Δ1). Upon testing these new deletion mutants in the serum bactericidal assay using 10% (vol/vol) NHS, no obvious reduction in serum resistance was observed relative to their wild-type strains (Fig. 20).

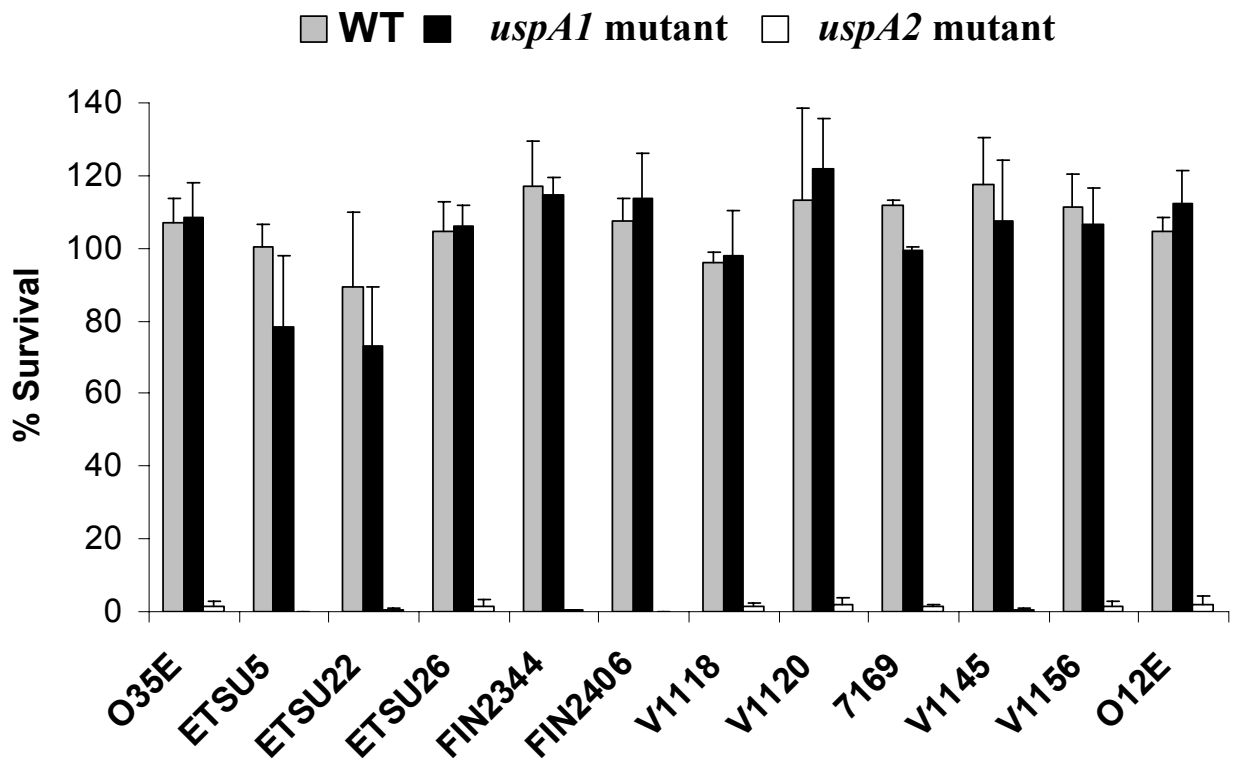

Fig. 19. Serum resistance of wild-type and mutant strains of *M. catarrhalis*.

Wild-type *M. catarrhalis* strains and their respective *uspA1* and *uspA2* mutants were incubated in 10% NHS at 37°C for 30 min. Bacterial aliquots were plated at both t=0 and t=30 min. The % survival was calculated with respect to the original inoculum. These results represent the mean of three independent experiments and the error bars represent the standard deviations.

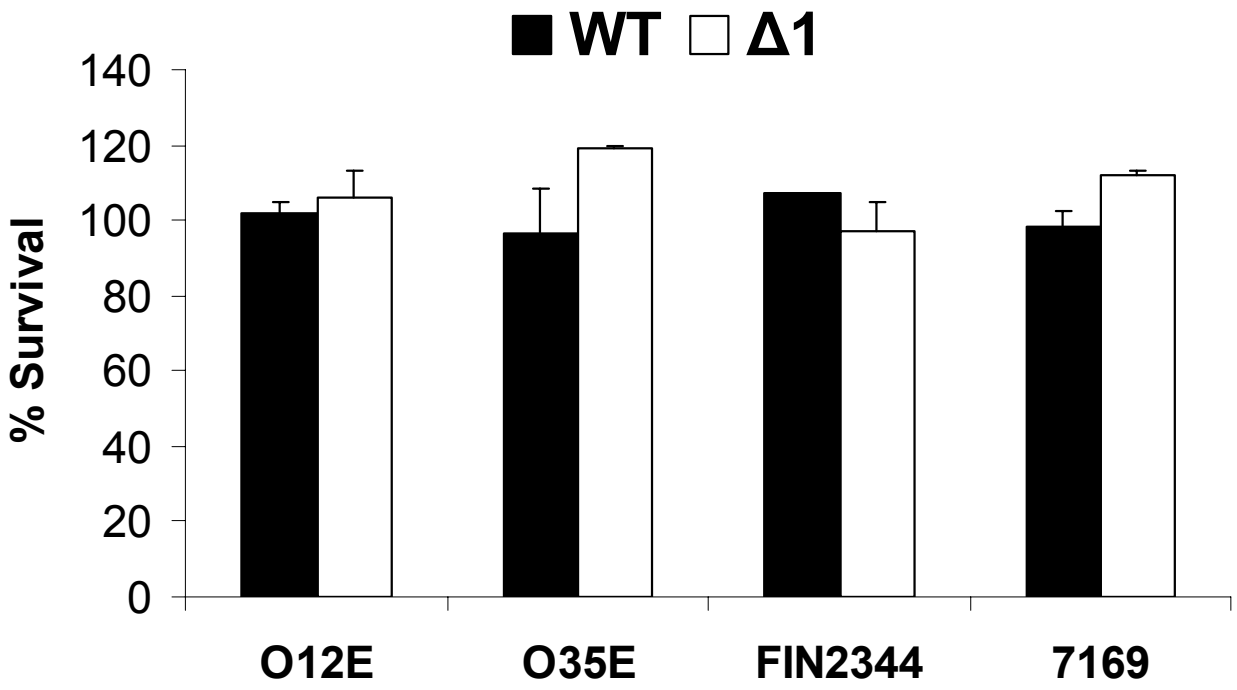

Fig. 20. Effect of *uspA1* deletion mutations on serum resistance of *M. catarrhalis* strains.

Wild-type *M. catarrhalis* strains and their respective *uspA1* deletion mutants were incubated for 30 min in 10% (vol/vol) NHS at 37°C. Bacterial aliquots were plated at both t=0 and t=30 min. The % survival was calculated with respect to the original inoculum. These results represent the mean of two independent experiments and the error bars represent the standard deviation.

I. Measurement of the cofactor activity of C4BP bound to *M. catarrhalis* cells

C4BP can function as a cofactor for factor I to inactivate surface-bound C4b by cleaving it to release the C4c fragment while the C4d fragment remains attached to the surface of the bacterial cell. The C4c MAb used in this study recognizes both C4b and C4c but not C4d (240). In contrast, the C4d MAb used in this study binds both C4b and the C4d fragment (240). Therefore, C4d binding is a reflection of the number of C4b molecules deposited on the bacterium and is not influenced by C4b processing. In contrast, C4BP cofactor activity will result in the release of C4c into solution and a corresponding decrease in binding of the C4c MAb. A higher C4d/C4c ratio indicates more C4BP cofactor function on the bacterial surface (240).

We used this assay in the context of NHS and measured cofactor activity of C4BP bound to two *M. catarrhalis* strains, FIN2344 and 7169, which had been shown to bind large amounts of purified C4BP (Fig. 18) via their UspA1 or UspA2 proteins, respectively. The C4BP-binding positive control strain *N. gonorrhoeae* FA19 had a C4d/C4c ratio of 2.9 (Fig. 21A) whereas the *N. gonorrhoeae* negative control strain UU1 yielded a C4d/C4c ratio of 0.8 (Fig. 21A); this difference was significant ($p = 0.0007$). When the wild-type *M. catarrhalis* strains FIN2344 (Fig. 21B) and 7169 (Fig. 21C) were tested in this assay, it was found that the level of cofactor activity was significantly less ($p = 0.005$ for FIN2344 and $p = 0.002$ for 7169) than that observed with *N. gonorrhoeae* FA19 (Fig. 21A). In addition, when these two wild-type *M. catarrhalis* strains were compared to their respective mutants which did not bind purified C4BP (i.e., the FIN2344 *uspA1* mutant and the 7169 *uspA2* mutant), there was no decrease in the C4d/C4c ratios (Fig. 21B and C, respectively).

These C4BP cofactor activity results suggested that these two wild-type *M. catarrhalis* strains, which readily bound purified C4BP at levels equivalent to those bound by *N. gonorrhoeae* FA19 (Fig. 18), either did not bind much C4BP from NHS or that this NHS-derived C4BP was not functional after binding to *M. catarrhalis*. To address these two possibilities, the amounts of C4BP bound to the same *M. catarrhalis* cells used in the cofactor activity assays were measured by flow cytometry (Fig. 21F-K). It was noted that *N. gonorrhoeae* FA19 (Fig. 21D) continued to bind C4BP in the context of NHS very well while the negative control strain UU1 (Fig. 21E) remained a C4BP non-binder. In contrast, the two wild-type *M. catarrhalis* strains FIN2344 and 7169 (Fig. 21F and Fig. 21I) bound much lower levels of NHS-derived C4BP relative to *N. gonorrhoeae* FA19 (Fig. 21D) and relative to the amounts of purified C4BP bound by these same two strains (Fig. 17 and Fig. 18). Under these conditions, the levels of C4BP bound to the wild-type *M. catarrhalis* strains and their *uspA1* and *uspA2* mutants (Fig. 21F-K) were low and similar. Collectively, these data provide an explanation for the comparably low C4BP cofactor activity seen among these *M. catarrhalis* wild-type strains and mutants (Fig. 21B and C).

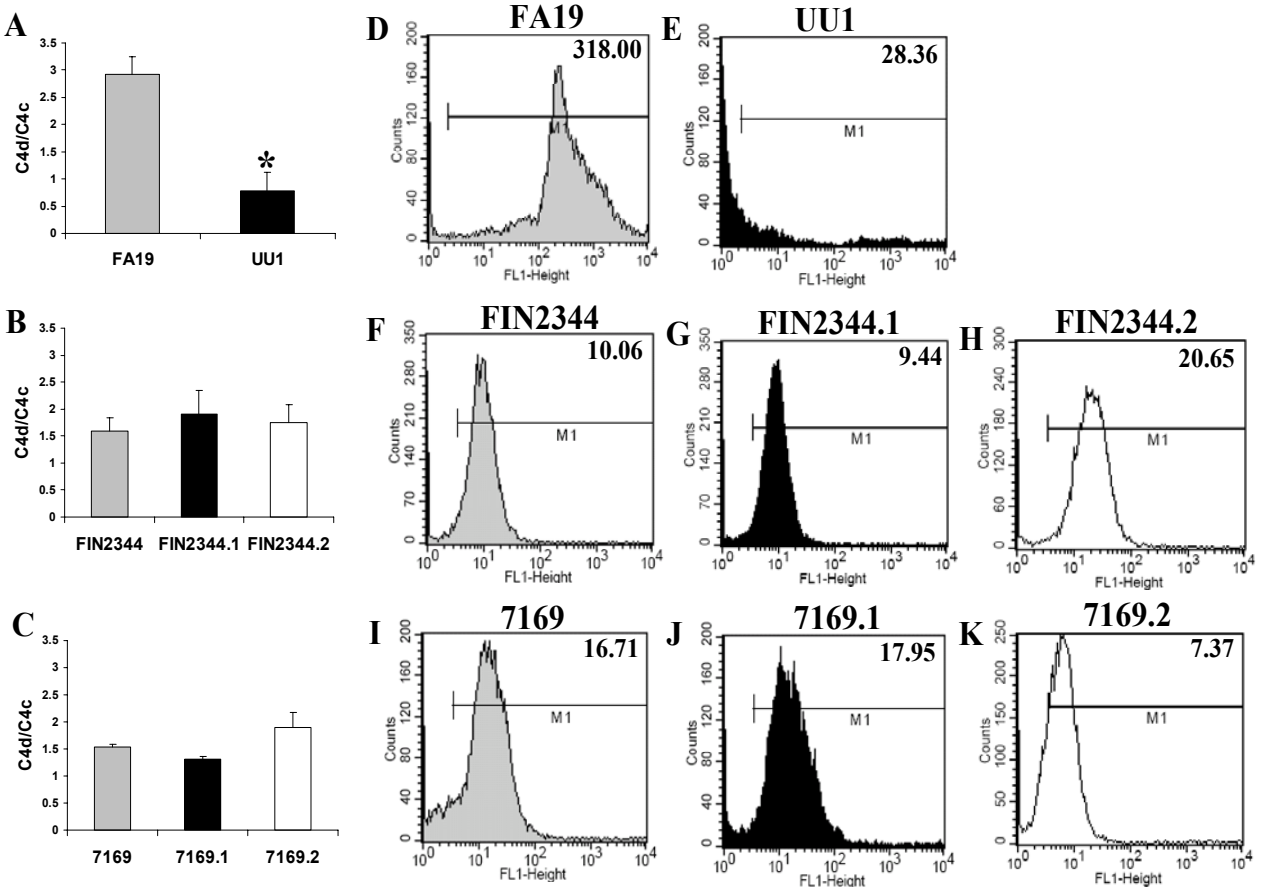

Fig. 21. Interaction of *M. catarrhalis* strains with NHS-derived C4BP.

Cells of *N. gonorrhoeae* FA19 and UU1 (panel A), *M. catarrhalis* FIN2344 and its *uspA1* and *uspA2* mutants (panel B), and *M. catarrhalis* 7169 and its *uspA1* and *uspA2* mutants (panel C) were incubated with 20% NHS for 30 min at 37°C. C4BP cofactor activity was measured by flow cytometry using different MAbs against C4c and C4d and recording the gmf obtained with each strain and the respective MAb and then calculating the C4d/C4c ratio. These data represent the mean of three independent experiments and the error bars represent the standard deviations. (*) indicates that the difference between FA19 and UU1 was significant (p = 0.0007). (Panels D-K) Flow cytometry-based analysis of C4BP binding by these same strains incubated with 20% NHS for 30 min at 37°C; C4BP bound to these bacterial cells was detected as described for Fig. 16. The M1 gate excludes the majority of events obtained with the negative (isotype) control (i.e., bacteria probed with primary and secondary antibodies in the absence of NHS). Representative experiments are shown

III. Discussion

The data presented in the previous chapter showed that the UspA2 protein is directly involved in the serum-resistant phenotype of *M. catarrhalis* strain O35E (13). Mutant analysis of an additional eleven wild-type *M. catarrhalis* isolates proved that expression of UspA2 was essential for serum resistance of these strains (Fig. 19). In addition, the use of various types of sera showed that the *uspA2* deletion mutant of strain O35E is killed via the classical pathway of complement activation in an IgG-dependent manner. Since UspA2 forms a dense layer of projections on the surface of *M. catarrhalis* (125,225), it represents an ideal moiety to interact with components or regulators of the complement system.

Nordström et al (216) recently proposed that *M. catarrhalis* interferes with activation of the classical complement pathway by binding the complement regulator C4BP to its UspA1 and UspA2 proteins. C4BP interferes with the classical complement pathway by both acting as a cofactor with factor I in the cleavage of C4b and inhibiting the formation and accelerating the decay of the C3 convertase (35). In fact, within the past decade, there have been numerous reports describing the binding of C4BP to different pathogens and the association of this binding with either serum resistance or increased virulence. The pathogens that bind C4BP include *S. pyogenes* (280), *N. gonorrhoeae* (240), *E. coli* K1 (232), and *N. meningitidis* (146). A number of these pathogens interact with the second complement control protein domain (CCP2) of the eight CCP domains on the α-chain of C4BP (216). *M. catarrhalis* is the first bacterium described that uses CCP7 as a recognition site for C4BP binding; in addition, it can also bind to CCP2 and, to a lesser extent, CCP5 (216). With the exception of *M. catarrhalis*, CCP1–3 are the major domains of the C4BP α-chain that are used for binding by several bacterial pathogens (21,31,34,232,280).

When twelve serum-resistant *M. catarrhalis* strains were tested for their binding of C4BP in the present study, it was found that these strains differed in both the amount of C4BP bound and in whether this binding involved UspA1 or UspA2 (Table 4). It was expected that differences in total C4BP binding by these wild-type strains might be reflected by differences in serum resistance. However, even those wild-type strains that bound very low amounts of C4BP (i.e., strain O12E) (Fig. 17J) were as resistant to killing by 10% NHS as strains that bound very large amounts of C4BP (i.e., strain 7169) (Fig. 17G). Moreover, it was observed that all of the *uspA1* mutants were essentially as serum-resistant as their wild-type parent strains and that the *uspA2* mutants were consistently serum-sensitive (Fig. 19), regardless of their ability to bind C4BP. A similar phenomenon was previously reported for *Bordetella pertussis* whose filamentous hemagglutinin (FHA) protein binds C4BP; a *B. pertussis fha* mutant that did not bind C4BP was found to still be serum-resistant (82). Similarly, C4BP has been shown to bind the gonococcal type IV pilus (34) but complement regulation and serum resistance as a result of this interaction were not demonstrated.

Taking all these data together, especially those obtained with NHS as the source of C4BP (Fig. 21), it can be concluded that C4BP binding to *M. catarrhalis* does not appear to play a significant role in protecting this bacterium from killing by NHS, at least for those twelve strains included in the experiments described in this chapter. Also, it appears that the extrapolation by Nordström et al (216) of the binding data derived from using purified C4BP to an explanation of the mechanism of serum resistance in *M. catarrhalis* might not be an accurate one because the C4BP binding activity within the context of NHS was completely different. The exact mechanism of serum resistance in the *M. catarrhalis* strains included in this study required further investigation.

CHAPTER SIX

Binding of Vitronectin by the *Moraxella catarrhalis* UspA2 Protein Interferes with Late Stages of the Complement Cascade

I. Introduction

The results obtained in the previous chapter showed that C4BP binding is apparently not responsible for serum resistance of *M. catarrhalis*, at least for the twelve strains included in this study. However, another report in the literature indicated that purified UspA2 protein binds purified vitronectin (185), which is another regulator of the complement system. Also, more than a decade ago, a different laboratory reported that serum-resistant, but not serum-sensitive *M. catarrhalis* isolates, bound vitronectin [Verduin, C. M., M. Jansze, J. Verhoef, A. Fleer, and H. van Dijk. 1994. Complement resistance in *Moraxella (Branhamella) catarrhalis* is mediated by a vitronectin-binding surface protein. Clin. Exp. Immunol. 97S2 (Abst. 143):50]. However, the physiological relevance of this binding in protecting *M. catarrhalis* from killing by the complement system was not addressed experimentally. Extrapolating from these binding data, obtained by using purified proteins or uncharacterized *M. catarrhalis* isolates, to an explanation for the serum resistance mechanism in *M. catarrhalis* might be inaccurate.

In this chapter, detailed analysis of the interaction of four different serum-resistant *M. catarrhalis* wild-type strains and their serum-sensitive *uspA2* mutants with complement components revealed that UspA2 likely interferes with polymerization of C9, thereby interfering with proper formation of the membrane attack complex (MAC) in the bacterial outer membrane. The binding of vitronectin to the UspA2 proteins of three of these strains was shown to be responsible for this effect.

II. Results

A. Wild-type *M. catarrhalis* strain O35E activates the complement cascade

The initial step in the characterization of the interaction of *M. catarrhalis* with the complement system was to determine whether the wild-type serum-resistant strains are capable of activating the complement system. To address this point, I used the well-characterized serum-resistant *M. catarrhalis* wild-type strain O35E. If the wild-type strain activates the complement system, this will lead to the consumption of its hemolytic activity, which will be reflected by a reduced ability to cause hemolysis of a bystander target (sensitized sheep RBCs). Upon testing the hemolytic activity of NHS that had been incubated with either $GVBS^{++}$ (Fig. 22, black columns), wild-type strain O35E (Fig. 22, open columns), or the *uspA2* mutant O35EΔ2 (Fig. 22, grey columns), there was a clear reduction in the hemolytic activity of the NHS incubated with both O35E and O35EΔ2 as compared to that obtained with buffer alone. These results indicate that both the serum-resistant strain O35E and the serum-sensitive *uspA2* mutant O35EΔ2 are capable of activating the complement cascade. Determination of possible differences in the degree of complement activation between these two strains and the stage at which this difference might occur requires further investigation.

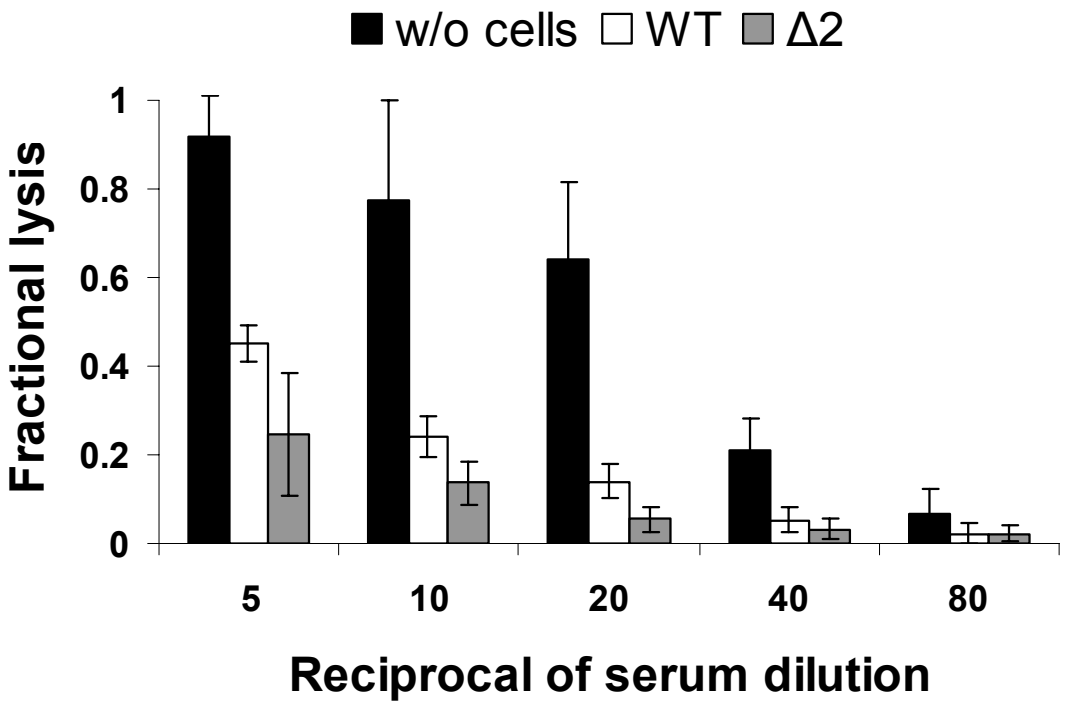

Fig. 22. Wild-type *M. catarrhalis* strain O35E activates the complement system and causes consumption of the hemolytic activity of NHS.

A 75 µl portion of mid-logarithmic cultures of wild-type (WT) O35E and the *uspA2* mutant O35EΔ2 (Δ2) or $GVBS^{++}$ (w/o cells) was incubated with 10% (vol/vol) NHS in $GVBS^{++}$ at a final volume of 300 µl at 37°C for 1 hr with continuous shaking. The bacterial cells were spun down and the supernatant fluid was filter-sterilized and used in the hemolytic assay. The hemolytic assay was performed as described in Materials and Methods. The data are presented as fractional lysis as compared to the 100% lysis that was accomplished using distilled. These data represent the mean of three independent experiments and the error bars represent the standard deviations.

B. Mutants of *M. catarrhalis* are killed via the classical complement pathway

Previous experiments (Fig. 14) showed that the *uspA2* mutant of strain O35E was killed via the classical pathway of complement activation. In order to investigate this phenomenon in other *M. catarrhalis* strains, the newly constructed *uspA2* deletion mutants of strains O12E, FIN2344, and 7169 were used together with the previously described O35E *uspA2* deletion mutant O35EΔ2 (6) in a series of bactericidal activity assays. These four strains were previously shown to differ in the extent and means by which they interacted with the complement regulator C4BP (discussed above). All four wild-type strains and their *uspA2* mutants survived equally in HIS (Fig. 23, black columns). As expected from previous studies (2,6), the four wild-type strains resisted killing by NHS, while their *uspA2* mutants were exquisitely serum-sensitive (Fig. 23, open columns). To investigate the role of the alternative pathway in this bactericidal activity, factor B-depleted serum (which has a non-functional alternative pathway) was tested and it was found that the wild-type strains survived whereas the *uspA2* mutants were readily killed (Fig. 23, grey columns). This result indicated that the classical pathway alone is sufficient for the killing of these *uspA2* mutants. To selectively block the classical pathway while retaining alternative pathway function, these wild-type strains and mutants were incubated with NHS in the presence of $MgCl_2$ and EGTA (Fig. 23, striped columns). With all four strain sets, both the wild-type strains and the *uspA2* mutants survived at equivalent levels, indicating that the classical pathway initiates and sustains killing of these *uspA2* mutants by NHS.

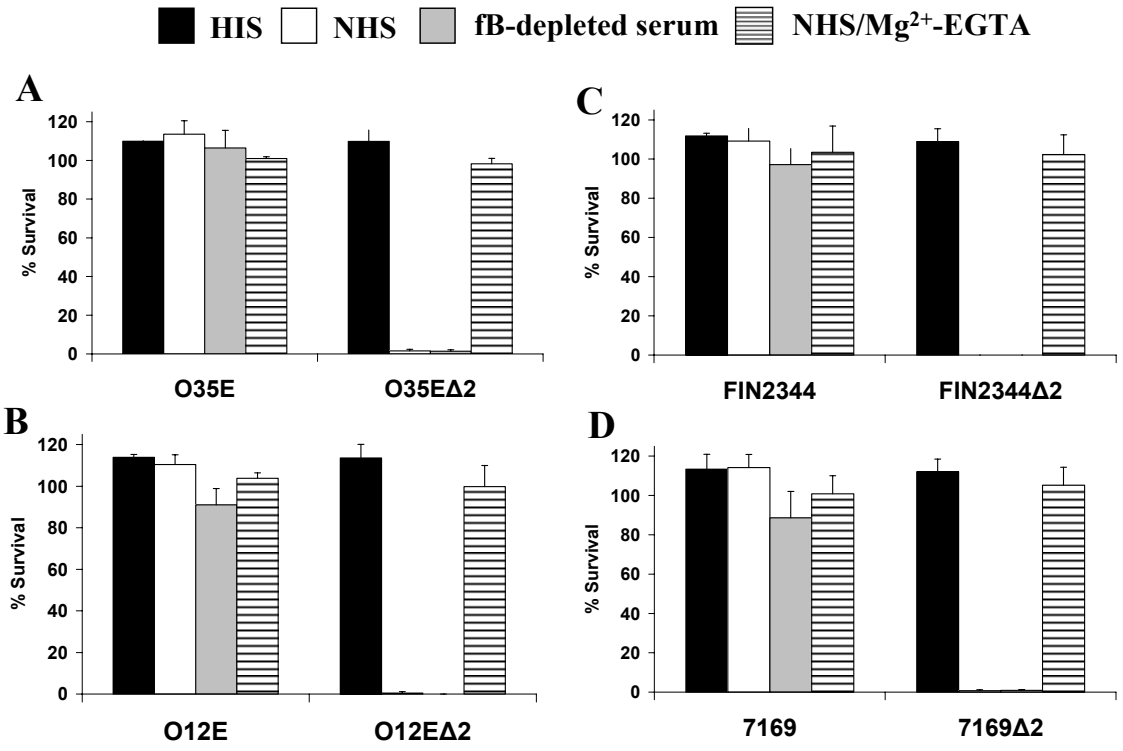

Fig. 23. ***M. catarrhalis uspA2*** **mutants are killed via the classical pathway.**

Serum bactericidal assays were performed as described in Materials and Methods with four *M. catarrhalis* wild-type strains and their uspA2 deletion mutants (Δ2). Panels: (A) O35E, (B) O12E, (C) FIN2344, and (D) 7169. The four different types of sera were: heat-inactivated NHS (HIS) (black columns), NHS (open columns), fB-depleted human serum (gray columns), and NHS containing 10 mM $MgCl_2$ and 10 mM EGTA (striped columns). These results represent the mean of three independent experiments and the error bars represent the standard deviations.

In a control experiment, NHS prepared as described in the Material and Methods, together with commercially available NHS and factor B-depleted sera, were tested in a hemolytic assay with rabbit erythrocytes to determine their level of alternative pathway activity. The results of this assay showed that both NHS preparations have much higher alternative pathway activity than the factor B-depleted serum (Fig. 24).

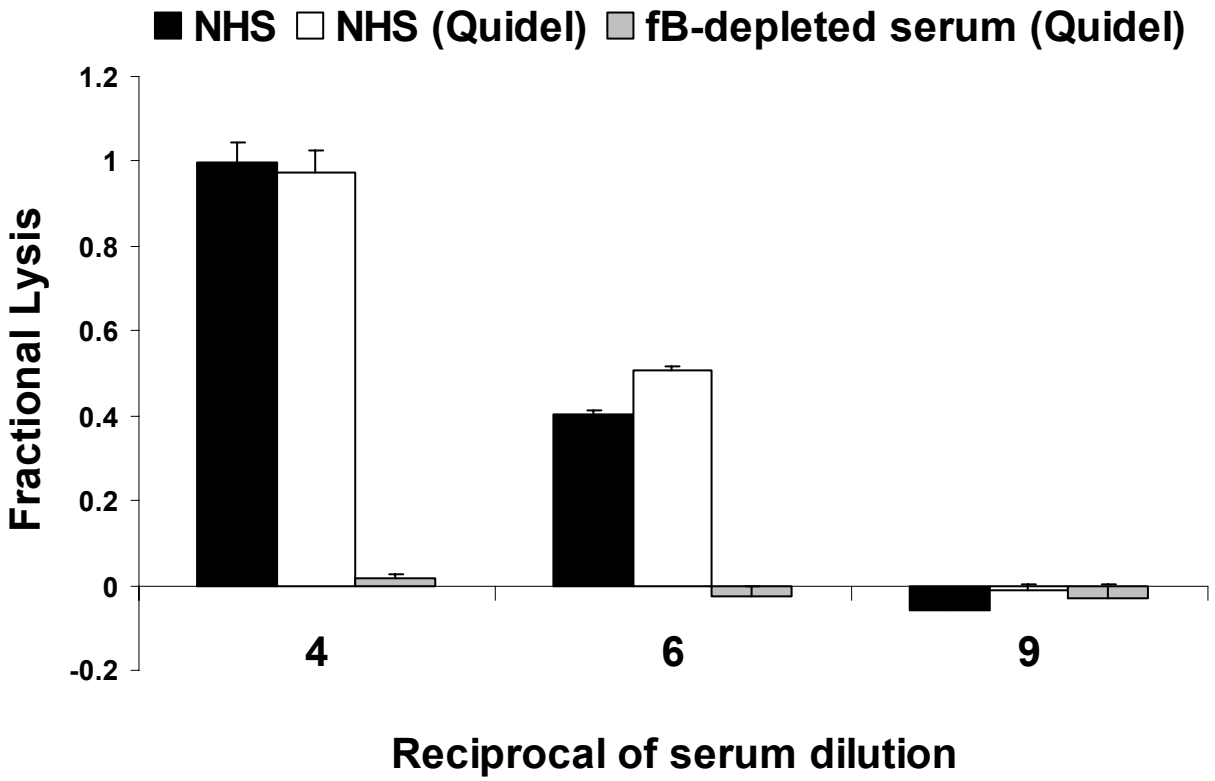

Fig. 24. NHS is sufficient in alternative pathway activity.

The serum samples were diluted in VBS containing 10 mM $MgCl_2$ and 10 mM EGTA. Then 100 µl portions of each dilution were mixed with 100 µl rabbit erythrocytes $E_{(rab)}$ (2×10^8/ml) and incubated for 1 hr at 37°C with continuous shaking. Then 1.2 ml 0.15M NaCl was added to each tube which was then centrifuged at 1250 x g for 5 min. The OD_{412} of the supernatants was recorded. The data are presented as fractional lysis as compared to the 100% lysis that was accomplished by adding 1.2 ml distilled water to $E_{(rab)}$ incubated with VBS containing 10 mM $MgCl_2$ and 10 mM EGTA. These data represent the mean of two independent experiments and the error bars represent the standard deviations.

C. Deposition of the early components of the complement cascade on *M. catarrhalis*

To determine the stage of the complement cascade that was blocked by these four serum-resistant, wild-type *M. catarrhalis* strains, these strains and their *uspA2* mutants were incubated with either HIS or NHS, lysed, and probed by Western blotting to detect complement components deposited on these bacterial cells. To obtain points of reference, these same procedures were performed with the well-characterized *N. gonorrhoeae* strains FA19 (serum-resistant) and UU1 (serum-sensitive) (240) as positive and negative controls, respectively, for classical pathway-mediated activity. Strain FA19 is serum-resistant by virtue of its ability to bind C4BP (240).

i. C1q

The first complement component is C1q which binds to the antibody-antigen complex and triggers the activation of the classical complement pathway (250). It is composed of three chains C1qA (29 kDa), C1qB (26 kDa), and C1qC (22 kDa). The polyclonal antibody used in this experiment recognizes mainly C1qA and C1qB and, to a lesser extent, C1qC (Fig. 25, lane1). No C1q deposition on the *N. gonorrhoeae* strains was observed (Fig. 25, lanes 2-5) and this is consistent with data obtained before using these same *N. gonorrhoeae* strains (personal communication; Peter Rice and Sanjay Ram, Boston University). In contrast, C1q deposition on *M. catarrhalis* was readily detectable. Equivalent amounts of C1q appeared to be deposited on the wild-type parent strain and the respective *uspA2* mutants of O35E, O12E, and FIN2344 (Fig. 25, lanes 8, 9, 12, 13, 16, and 17). The *uspA2* mutant of 7169 bound little or non-detectable C1q (Fig. 25, lane 21) for reasons that are not clear at this time. As expected, no C1q deposition was observed with either the wild-type strains or the *uspA2* mutants incubated with HIS (Fig. 25, lanes 2, 3, 6, 7, 10, 11, 14, 15, 18, and 19).

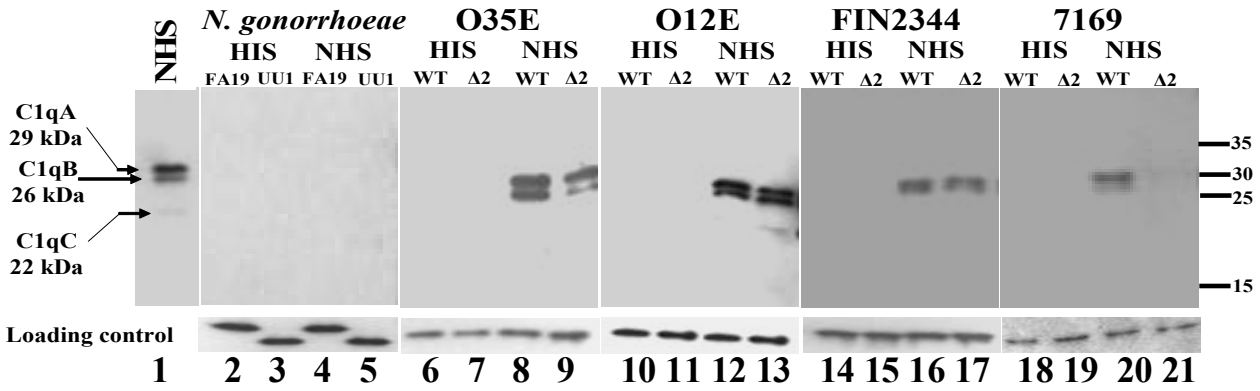

Fig. 25. **C1q deposition on *N. gonorrhoeae* and *M. catarrhalis* strains**.

N. gonorrhoeae strains FA19 and UU1 and *M. catarrhalis* wild-type (WT) strains and *uspA2* mutants (Δ2) were incubated with either 10% HIS or 10% NHS for 20 min, washed three times with ice-cold GVBS, suspended in 100 µl of PBS, and then boiled with 50 µl of 3X digestion buffer. A 5 µl portion of these whole cell lysates diluted 1:10 in 1X digestion buffer was subjected to Western blot analysis. Proteins present in these samples were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The membranes were probed with polyclonal antibody against C1q [antibody recognizes mainly C1qA (29 kDa), C1qB (26 kDa) and, to a lesser extent, C1qC (22 kDa)]. As a loading control, membranes were probed with either MAb 10F3 (3) or MAb 5D2 (225) which recognize *M. catarrhalis* outer membrane antigens. For the *N. gonorrhoeae* samples, membranes were probed with MAb 2C3 (12). The first lane contains a sample of NHS diluted 1:200 that was probed with the C1q antibody. Protein molecular mass markers (in kDa) are presented on the right side of the figure.

ii. C4

The subsequent step in classical pathway activation is C4 binding. C4 is composed of three polypeptide chains [α-chain (93k Da), β-chain (75 kDa) and γ-chain (33 kDa)] linked by disulfide bonds. Upon activation, a small fragment (C4a) is released from the α-chain, allowing the C4b fragment to attach covalently to the target through an internal thioester bond in its α-chain. The polyclonal antiserum to C4 used in the present study reacted primarily with the 75 kDa β-chain of C4/C4b (Fig. 26, lane 1). With the *N. gonorrhoeae* control strains, there was less C4b detected on strain FA19 than on UU1 (Fig. 26, lanes 4 and 5). This result is consistent with the regulatory role of C4BP bound to FA19 in the processing of C4b. There were no appreciable differences in the amount of C4b deposited on the four wild-type *M. catarrhalis* strains compared to their *uspA2* mutants (Fig. 26, lanes 8, 9, 12, 13, 16, 17, 20, and 21), suggesting that UspA2 did not affect the complement system prior to this step. These data also indicate that the extent of C4b processing on the wild-type and *uspA2* mutants was similar because the C4b β-chain is associated only with unprocessed C4b. As expected, C4b was not detected on bacteria incubated with HIS (Fig. 26, lanes 2, 3, 6, 7, 10, 11, 14, 15, 18, and 19).

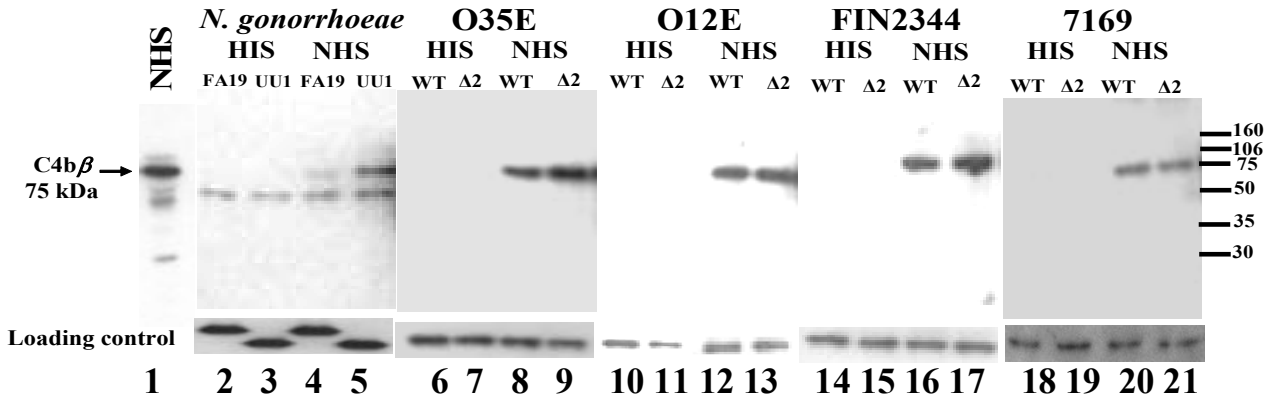

Fig. 26. C4 deposition on *N. gonorrhoeae* and *M. catarrhalis* strains.

The same whole cell lysates described in Fig. 25 were used in this experiment. A 5 µl portion of these whole cell lysates diluted 1:10 in 1X digestion buffer was subjected to Western blot analysis. Proteins present in these samples were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The membranes were probed with polyclonal antibody against C4 [antibody mainly recognizes the C4β chain (75 kDa)]. As a loading control, membranes were probed with either MAb 10F3 (3) or MAb 5D2 (225) which recognize *M. catarrhalis* outer membrane antigens. For the *N. gonorrhoeae* samples, membranes were probed with MAb 2C3 (12). The first lane contains a sample of NHS diluted 1:200 that was probed with the C4 antibody. Protein molecular mass markers (in kDa) are presented on the right side of the figure.

iii. C3

C3 represents the most abundant complement protein and plays a critical role in both the classical and alternative pathways (197). It is composed of a 117 kDa α-chain and a 75 kDa β-chain (Fig. 27A, lane 1) held together by disulfide bonds. Akin to C4, activation of C3 results in covalent binding of C3b via a labile thioester directly to bacterial targets, or to the activating C3-convertases (C4bC2a or C3bBb) already assembled on the bacterial surface, the latter leading to the formation of the C5 convertases that are required for further activation of the terminal pathway (197). Less C3b was deposited on *N. gonorrhoeae* FA19 (serum-resistant and binds C4BP) than on *N. gonorrhoeae* UU1 (serum-sensitive and does not bind C4BP) (Fig. 27A, lanes 4 and 5). Differences in the amounts of C3 deposited on the wild-type *M. catarrhalis* strains and their *uspA2* mutants appeared to be modest at best (Fig. 27A). However, due to the complexity of the banding pattern and crucial role of C3 in the transition from the early stages of complement activation to the late stages, I decided to measure this more quantitatively. Flow cytometry was used to measure C3 binding on the same sets of *N. gonorrhoeae* and *M. catarrhalis* strains. The serum-sensitive *N. gonorrhoeae* strain UU1 bound significantly more C3 than did the serum-resistant FA19 strain (Fig. 27B, group 1) (p value = 0.003), confirming the difference seen by Western blot analysis (Fig. 27A, lanes 4 and 5). For the four pairs of *M. catarrhalis* wild-type strains and *uspA2* mutants, there were no significant differences in the amount of C3 bound to O35E, O12E and 7169 and their respective *uspA2* mutants Fig. 27B, groups 2, 3, and 5). However, the FIN2344 wild-type strain bound significantly less C3 than did its *uspA2* mutant (Fig. 27B, group 4) (p value = 0.04). As expected, C3 was not detected on bacteria incubated with HIS (Fig. 26A, lanes 2, 3, 6, 7, 10, 11, 14, 15, 18, and 19).

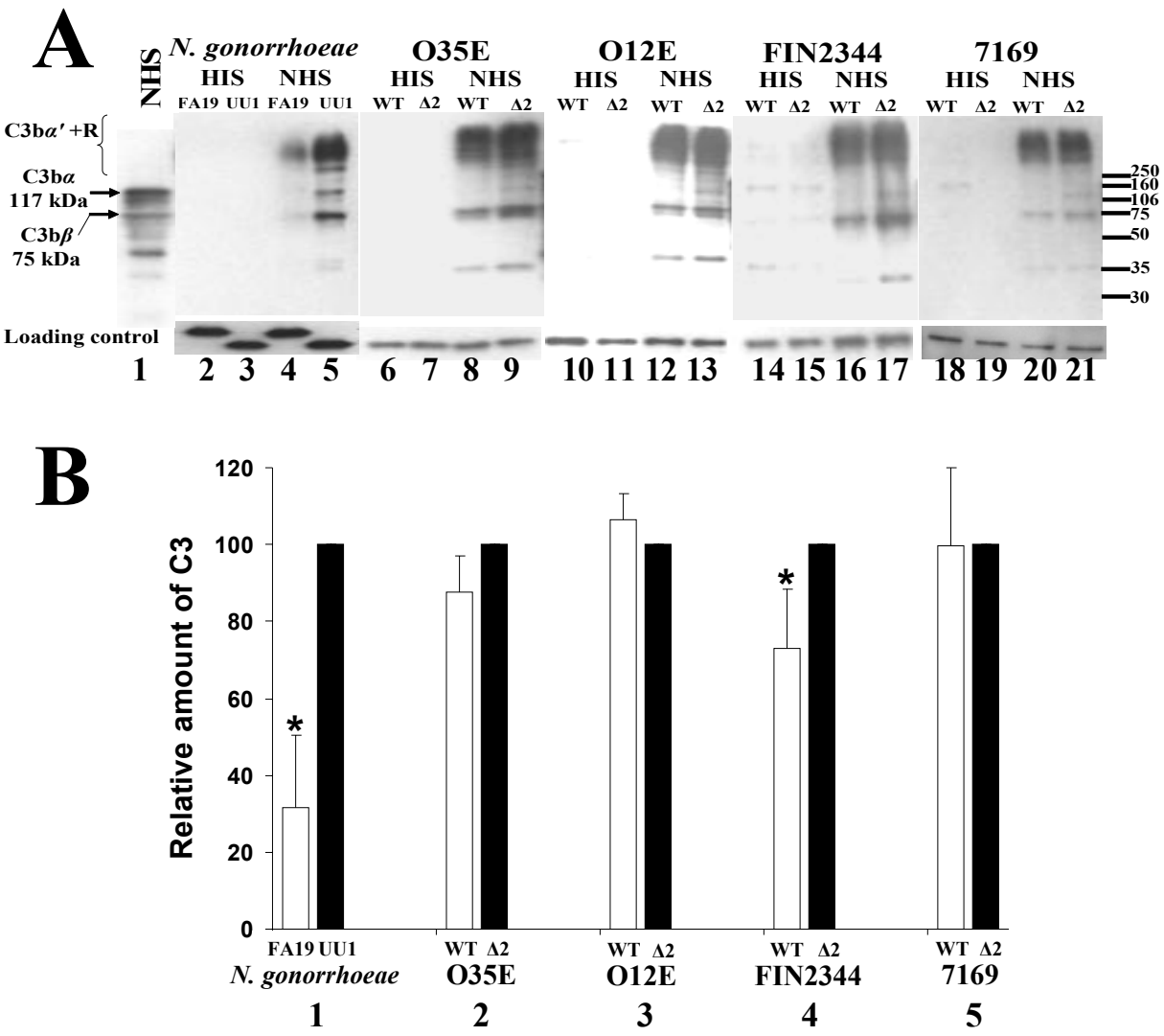

Fig. 27. C3 deposition on *N. gonorrhoeae* and *M. catarrhalis* strains.

(A) The same whole cell lysates described in Fig. 25 were used in this experiment. A 5 μl portion of these whole cell lysates diluted 1:10 in 1X digestion buffer was subjected to Western blot analysis. Proteins present in these samples were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The membranes were probed with polyclonal antibody against C3 [antibody recognizes C3α (117 kDa), C3β (75 kDa), and C3bα' covalently bound to bacteria (C3bα'+R)]. As a loading control, membranes were probed with either MAb 10F3 (3) or MAb 5D2 (225) which recognize *M. catarrhalis* outer membrane antigens. For the *N. gonorrhoeae* samples, membranes were probed with MAb 2C3 (12). The first lane contains a sample of NHS diluted 1:200 that was probed with the C3 antibody. Protein molecular mass markers (in kDa) are presented on the right side of the figure. (B) Relative amounts of C3 bound to these strains as measured by flow cytometry. These experiments involved incubation of bacteria in 10% NHS for 20 min at 37°C. The cells were then placed on ice, washed three times, and subjected to flow cytometry using the same primary antibody as used in Western blot analysis (A). "100%" on the Y-axis represents the relative amount of C3 on the serum-sensitive strain in each pair. The data are the mean from three independent experiments and the error bars represent the standard deviations. The *p* value for the *N. gonorrhoeae* pair was 0.003 and for the FIN2344 pair, 0.04. (*) indicates that the difference between the two strains in each of these two sets was significant. For the other *M. catarrhalis* pairs, the differences were not significant.

D. Deposition of the late components of the complement cascade on *M. catarrhalis*

The late stages of complement activation include the formation of the MAC which starts with the activation of C5, leading to the generation of C5b and the sequential binding of C6, C7, and C8. The last component to participate in this complex is C9 which undergoes a conformational change from a globular to an elongated form and binds to the C5b-8 complex, which traverses the bacterial outer membrane. Finally, more C9 is recruited to form a C9 polymer, leading to the formation of pores in the outer membrane and eventually the death of the target cell (197). The deposition of two components (i.e., C7 and polymerized C9) of the MAC on bacterial cells was analyzed in this study.

i. C7.

C7 is a single-chain 110 kDa molecule (Fig. 28, lane 1) that is involved in the formation of the MAC. Consistent with the results obtained with C4 and C3, there was a reduction in the amount of C7 deposited on *N. gonorrhoeae* strain FA19 relative to that deposited on strain UU1 (Fig. 28, lanes 4 and 5). The *uspA2* mutants of strains O35E, O12E and FIN2344 appeared to bind slightly more C7 than their respective wild-type parent strains (Fig. 28, lanes, 8, 9, 12, 13, 16, and 17). As expected, C7 was not detected on bacteria incubated with HIS (Fig. 28, lanes 2, 3, 6, 7, 10, 11, 14, 15, 18, and 19).

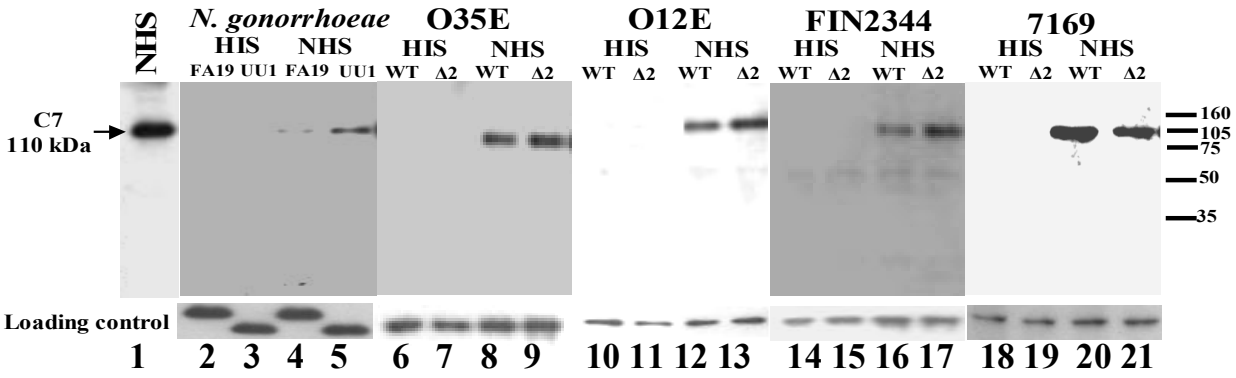

Fig. 28. C7 deposition on *N. gonorrhoeae* and *M. catarrhalis* strains.

The same whole cell lysates described in Fig. 25 were used in this experiment. A 5 µl portion of these whole cell lysates diluted 1:10 in 1X digestion buffer was subjected to Western blot analysis. Proteins present in these samples were resolved by SDS-PAGE under non-reducing conditions. Separated proteins were transferred to nitrocellulose membranes and probed with polyclonal antiserum against C7 (110 kDa). As a loading control, membranes were probed with either MAb 10F3 (3) or MAb 5D2 (225) which recognize *M. catarrhalis* outer membrane antigens. For the *N. gonorrhoeae* samples, membranes were probed with MAb 2C3 (12). The first lane contains a sample of NHS diluted 1:200 that was probed with the C7 antibody. Protein molecular mass markers (in kDa) are presented on the right side of the figure.

ii. Polymerized C9

The MAb used to detect polymerized C9 recognizes a neoepitope that is formed when C9 polymerizes within the MAC; this MAb can detect polymerized C9 in the trimer form (~ 270 kDa) or as a multimer (> 500 kDa) (Fig. 29, lane 1). More polymerized C9 was detected on the serum-sensitive *N. gonorrhoeae* strain UU1 as compared to that bound to the serum-resistant strain FA19 (Fig. 29, lanes 4 and 5). A difference was readily apparent between the wild-type *M. catarrhalis* strains and their *uspA2* mutants, with more polymerized C9 being associated with the mutants (Fig. 29, lanes 8, 9, 12, 13, 16, 17, 20, and 21). Of note, the four strains differed in their "patterns" of C9 polymerization. C9 was distributed equally as trimers and multimers on wild-type FIN2344 (Fig. 29, lane 16) and occurred solely as trimers on wild-type 7169 (Fig. 29, lane 20). A faint multimeric C9 band was seen on wild-type O35E (Fig. 29, lane 8), while no C9 was detected on wild-type O12E (Fig. 29, lane 13). In every instance, the *uspA2* mutants showed strongly reactive C9 multimers. A decrease of the C9 trimer band with intensification of the C9 multimer band was seen with the FIN2344 *uspA2* mutant (Fig. 29, lane 17). Similarly, a shift in C9 reactivity from trimer to multimers was seen with 7169 *uspA2* mutant (Fig. 29, lane 21). These differences that occurred at the level of C9 polymerization raised the possibility that the role of the UspA2 protein in serum resistance might involve interference with the final crucial step in activation of the complement cascade.

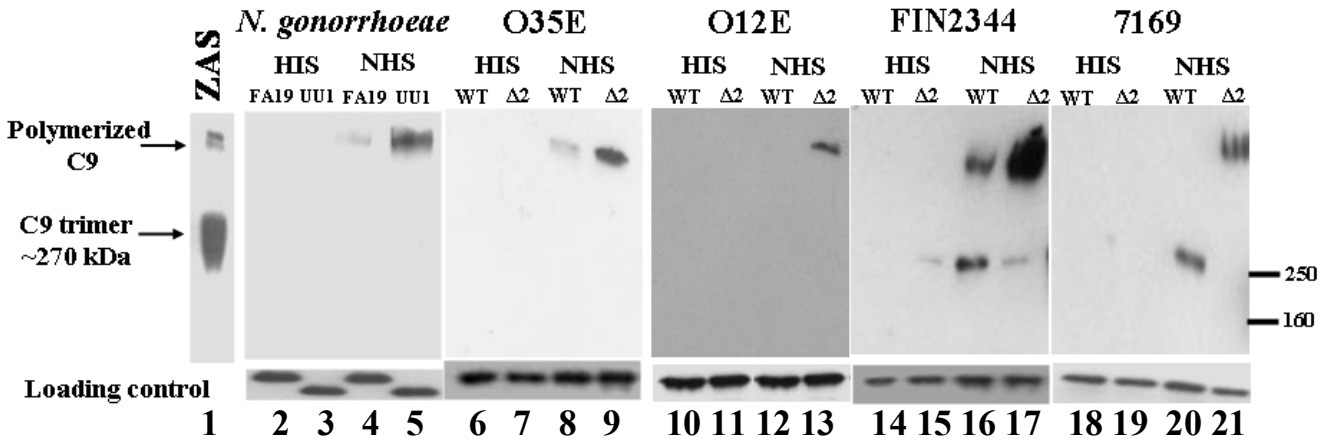

Fig. 29. Polymerized C9 deposition on *N. gonorrhoeae* and *M. catarrhalis* strains.

The same whole cell lysates described in Fig. 25 were used in this experiment. A 12.5 µl portion of these whole cell lysates was subjected to Western blot analysis. Proteins present in these samples were resolved by SDS-PAGE under non-reducing conditions. The separated proteins were transferred to PVDF membranes and probed with a MAb against SC5b-9 which recognizes a neoepitope in polymerized C9 in the MAC. As a loading control, membranes were probed with either MAb 10F3 (3) or MAb 5D2 (225) which recognize *M. catarrhalis* outer membrane antigens. For the *N. gonorrhoeae* samples, membranes were probed with MAb 2C3 (12). The first lane contains a control sample of Zymosan-activated serum (ZAS) that was probed with the MAb against SC5b-9 to detect polymerized C9. Protein molecular mass markers (in kDa) are presented on the right side of the figure.

E. *M. catarrhalis* binds vitronectin from NHS via its UspA2 protein

Several factors have been previously reported to interfere with formation of the MAC. These include vitronectin (also known as S protein), clusterin (also known as SP-40-40) and CD59 (also known as protectin or homologous restriction factor) (224,228,284). It was also previously reported that purified UspA2 protein from *M. catarrhalis* strain O35E bound purified vitronectin (185). Testing of the four wild-type *M. catarrhalis* strains and their respective *uspA2* mutants showed that the wild-type strains O35E, O12E, and 7169 bound more NHS-derived vitronectin than did their *uspA2* mutants (Fig. 30, lanes 4, 5, 8, 9, 16, and 17). The 7169 *uspA2* mutant showed the most dramatic reduction in vitronectin binding relative to its wild-type parent strain (Fig. 30, lanes 16 and 17). Heat-inactivation of the complement system did not eliminate binding of vitronectin to these three wild-type strains (Fig. 30, lanes 2, 6, and 14), suggesting that vitronectin binding occurred directly to the bacterium independent of MAC formation. It should be noted that wild-type strain FIN2344 (Fig. 30, lane 12) bound a much lower amount of vitronectin than did the other three wild-type strains.

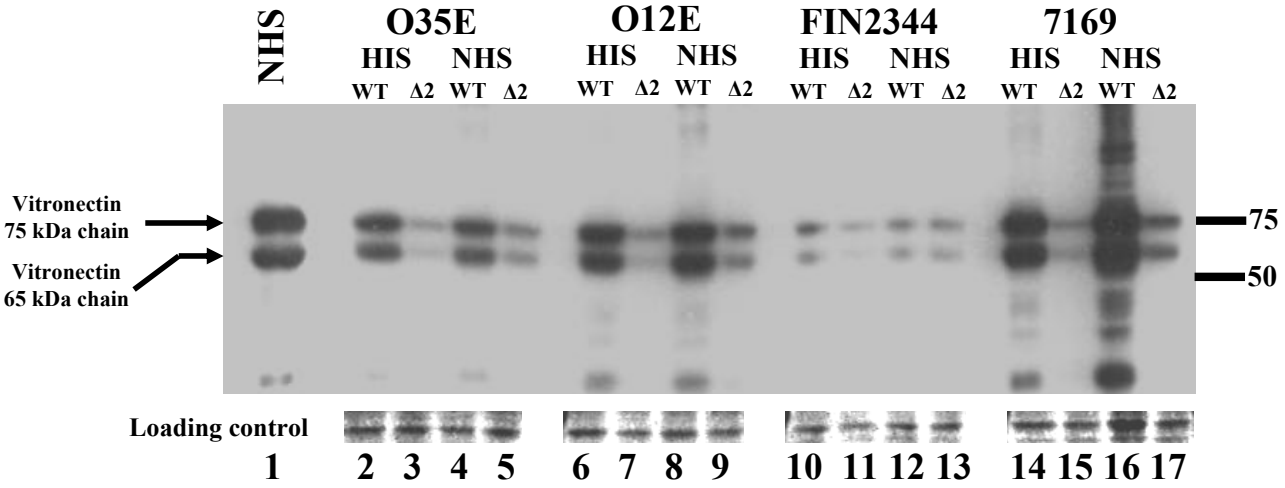

Fig. 30. Binding of vitronectin from NHS to *M. catarrhalis.*

Whole cell lysates were prepared from bacteria that had been incubated with either HIS or NHS as described in Fig. 25. A 10 μl portion of these whole cell lysates was subjected to Western blot analysis. Proteins present in these samples were resolved by SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and probed with a MAb against human vitronectin. As a loading control, membranes were stained with amido black and the CopB protein (117) was used for standardization purposes. The first lane contains a sample of NHS diluted 1:200. Protein molecular mass markers (in kDa) are presented on the right side of the figure.

F. Serum-resistant *M. catarrhalis* strains are killed by vitronectin-depleted NHS

If NHS-derived vitronectin bound to *M. catarrhalis* inhibited polymerization of C9, then removal of vitronectin from NHS should result in an increased susceptibility of the serum-resistant strains to killing by NHS. To test this hypothesis, an immunodepletion technique was used to remove most of the vitronectin from NHS as described in Material and Methods. To assess the degree of vitronectin depletion, Western blots were used. An obvious reduction in the amount of vitronectin was observed in the Vn-depleted serum as compared to the mock-treated serum (Fig. 31A). At the same time, there was little difference between the two sera in the amount of C1q remaining after the immunodepletion (Fig. 31B). To obtain a more quantitative measurement of the degree of vitronectin depletion, a commercial ELISA kit was used and it showed that the degree of vitronectin depletion was 93%.

To test the hypothesis that vitronectin protects *M. catarrhalis* against the bactericidal action of NHS, vitronectin-depleted NHS (Vn-depleted serum) was used in bactericidal assays with the four serum-resistant *M. catarrhalis* strains and their *uspA2* mutants. The wild-type strains O35E, O12E, and 7169 (which had been shown to bind vitronectin through UspA2) all exhibited a significant increase in serum susceptibility when incubated in Vn-depleted serum (columns 2 in Fig. 32A, B, and D, respectively). Moreover, when purified vitronectin was added to the Vn-depleted serum, these three wild-type strains exhibited their normal serum-resistant phenotype (columns 3 in Fig. 32A, B, and D, respectively). To rule out the presence of bactericidal activity in the purified vitronectin preparation, equal amounts of purified vitronectin were added to HIS and shown to have no effect on the survival of these three wild-type strains (columns 4 in Fig. 32A, B, and D, respectively). Interestingly, strain FIN2344, which exhibited very low binding of vitronectin via UspA2 (Fig. 30,

lane 12), did not show any increase in susceptibility to killing by Vn-depleted serum (Fig. 32C, column 2). The *uspA2* mutants of all four strains were exquisitely sensitive to killing by both the Vn-depleted serum and the mock-treated serum (columns 5 and 6 in Fig. 32A, B, C, and D).

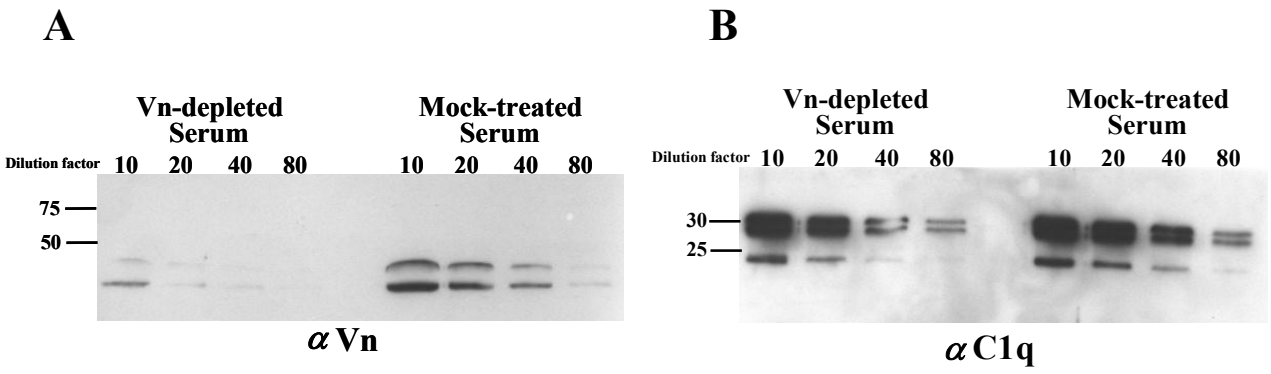

Fig. 31. Assessment of the degree of Vn-depletion using Western blot analysis.

Samples of both Vn-depleted serum and mock-treated serum were serially diluted in PBS and digested using 3X digestion buffer. Proteins present in these samples were resolved by SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and probed with a MAb against human vitronectin (A) or polyclonal antibody against C1q (B). Protein molecular mass markers (in kDa) are presented on the left side of each panel.

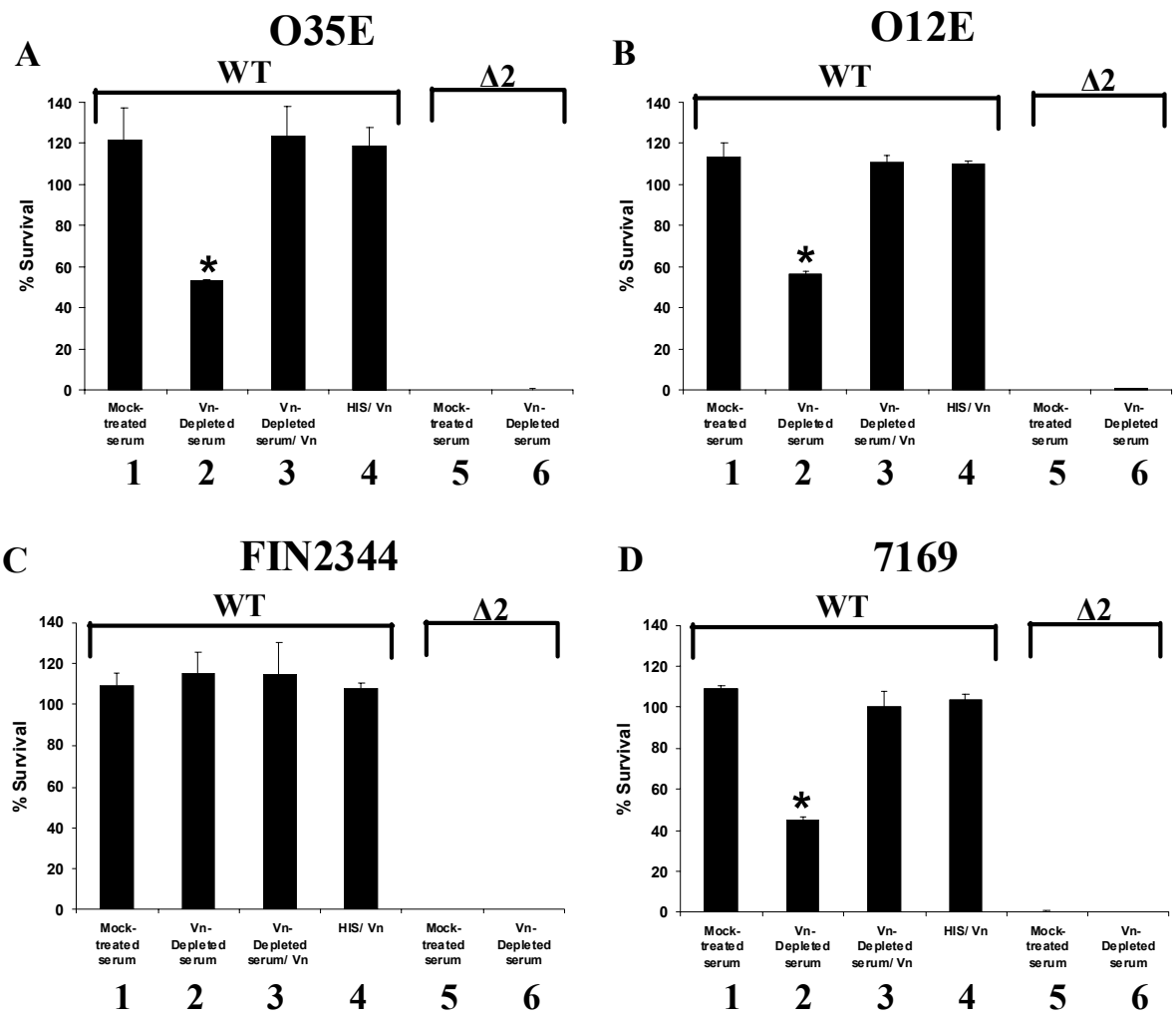

Fig. 32. Vitronectin-depleted NHS has bactericidal activity against serum-resistant *M. catarrhalis* strains.

The serum-resistant wild-type *M. catarrhalis* strains O35E (panel A), O12E (panel B), FIN2344 (panel C) and 7169 (panel D) and their respective *uspA2* deletion mutants (Δ2) were tested in a serum bactericidal assay using both vitronectin (Vn)-depleted and mock-treated sera. These wild-type strains were also tested using vitronectin-depleted serum to which 2.5 μg of purified vitronectin had been added (Vn-depleted serum/Vn) and HIS to which 2.5 μg vitronectin had been added (HIS/Vn). The data represent the mean of two experiments and the error bars represent the standard deviations. The asterisk (*) indicates that the difference between the vitronectin-depleted serum and the mock-treated serum was statistically significant; p = 0.02, 0.007, and 0.0006 for strains O35E, O12E, and 7169, respectively

G. Recombinant *M. catarrhalis* UspA2 proteins expressed in *H. influenzae* confer serum resistance and bind vitronectin

The *uspA2* genes from these four *M. catarrhalis* strains were cloned and expressed in *H. influenzae* strain DB117 under the control of the *H. influenzae ompP2* promoter on a multicopy plasmid. These recombinant *H. influenzae* strains expressed varying levels of UspA2 protein, with the 7169 UspA2 protein being expressed at a level approximately 3-5 fold lower than the others as determined by densitometry (Fig. 33A). However, all four recombinant *H. influenzae* strains expressing UspA2 proteins were fully resistant to the bactericidal activity of 10% NHS whereas the *H. influenzae* strain carrying the vector plasmid with the *ompP2* promoter was fully serum-sensitive (Fig. 33B). Cells of these four recombinant *H. influenzae* strains were also tested for their ability to bind vitronectin from NHS. When cell lysates containing equivalent amounts of UspA2 proteins from these recombinant strains were probed with the vitronectin MAb, it was found that the recombinants expressing the O35E, O12E, and 7169 UspA2 proteins all bound readily detectable amounts of vitronectin (Fig. 33C, lanes 2, 3 and 5). No binding of vitronectin was observed with the vector-only strain (Fig. 33C, lane 1) and barely any binding was observed with the strain expressing the FIN2344 UspA2 protein (Fig. 33C, lane, 4). When the amount of recombinant FIN2344 UspA2 protein was increased in this assay, vitronectin binding was readily detectable (data not shown). These data confirmed that *M. catarrhalis* strains O35E, O12E, and 7169 bound NHS-derived vitronectin through their UspA2 protein while the FIN2344 UspA2 protein exhibited much less vitronectin binding.

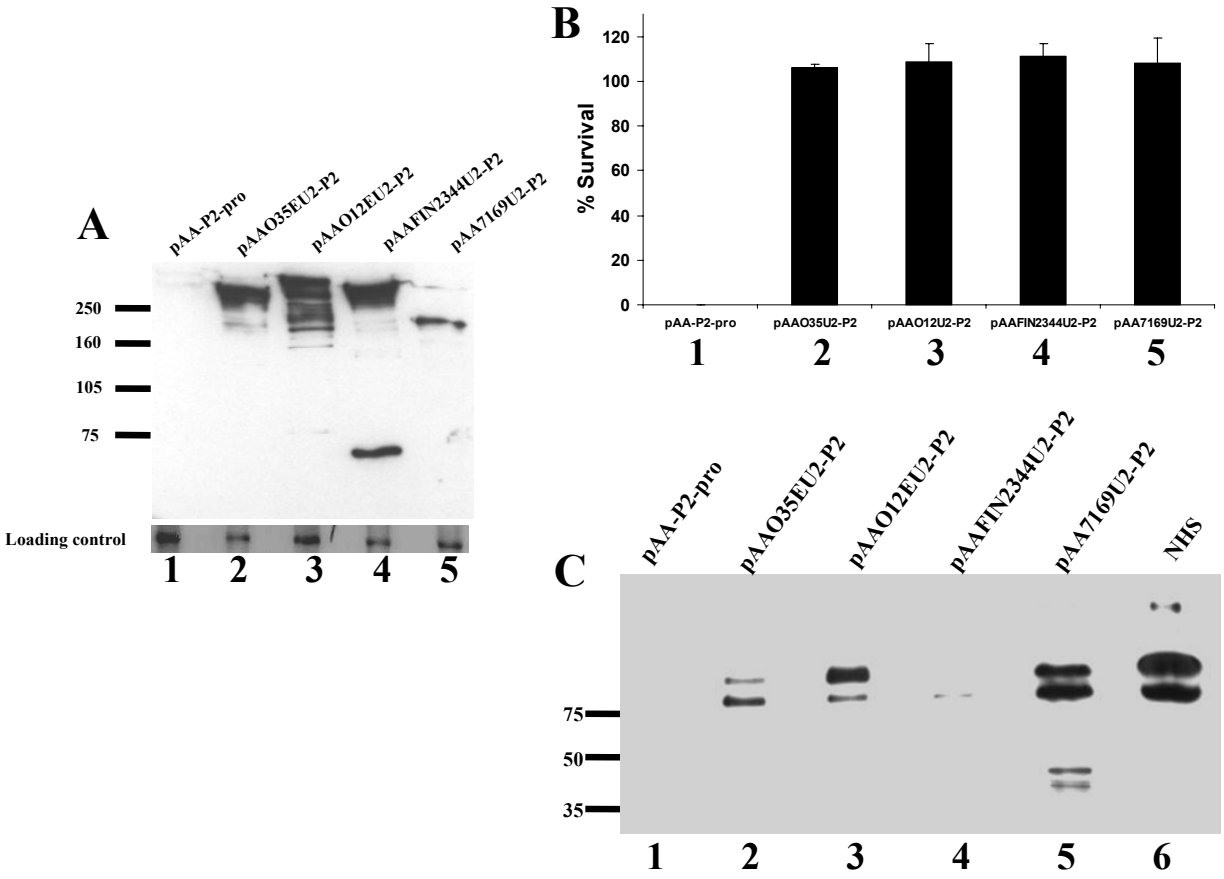

Fig. 33. Recombinant *M. catarrhalis* UspA2 proteins expressed in *H. influenzae* DB117 confer serum resistance and vitronectin binding activity.

(A) Western blot analysis of whole cell lysates of *H. influenzae* strains carrying the empty vector pAA-P2-pro or plasmids encoding UspA2 proteins from strains O35E, O12E, FIN2344, and 7169: pAAO35EU2-P2, pAAO12EU2-P2, pAAFIN2344U2-P2 and pAA7169U2-P2, respectively. The membrane was probed with MAb 17C7 which recognizes the *M. catarrhalis* UspA2 protein (5). The reactive antigen with an apparent mass of approximately 60,000-70,000 in the lane containing the lysate from pAAFIN2344U2-P2 is likely a UspA2 monomer. (B) These same five recombinant *H. influenzae* strains were tested in the serum bactericidal assay using 10% NHS. The data represent the mean of three independent experiments and the error bars represent the standard deviations. (C) Binding of vitronectin from NHS by these recombinant *H. influenzae* strains. Bacterial cells were incubated with 10% NHS for 20 min at 37°C and then washed and processed to prepare whole cell lysates. Samples were then diluted according to the intensity of the UspA2 bands obtained in panel A so that samples corresponding to equivalent amounts of UspA2 were loaded on this gel. The whole cell lysate of the vector-only strain was not diluted. After transfer, the membrane was probed with a MAb against human vitronectin. The last lane contains a sample of NHS diluted 1:200. Protein molecular mass markers (in kDa) are presented on the left side of panels A and C.

III. Discussion

In the experiments described in this chapter, I investigated how *M. catarrhalis* interacts with different components of the complement system in NHS. For this purpose, four serum-resistant strains (O35E, O12E, FIN2344, and 7169) were selected that had already been shown to differ markedly in their binding of purified C4BP (Table 4). Killing of *uspA2* mutants of these four strains by NHS was shown to involve the classical pathway of complement activation (Fig. 23). These data confirmed the results of a previous study from another laboratory comparing serum-sensitive and serum-resistant *M. catarrhalis* isolates that used an indirect method (i.e., a bystander hemolytic assay) to show that *M. catarrhalis* strains activated the classical complement pathway in an IgG-dependent manner (293). Also, it should be noted that a recent study indicated that *M.* catarrhalis is only a weak activator of the mannose-binding lectin pathway of the complement system (110). Analysis of complement deposition on these four *M. catarrhalis* strains and their *uspA2* mutants showed that a detectable difference between each pair occurred at the late stages of complement activation, involving the polymerization of C9 and MAC formation (Fig. 29). These data, based on direct detection of complement components bound to the surface of wild-type and *uspA2* mutant pairs of *M. catarrhalis,* are consistent with an earlier report in which a hemolytic assay was used to measure consumption of complement components by serum-sensitive and serum-resistant isolates of *M. catarrhalis* (293).

The finding that UspA2 appeared to interfere with MAC formation, at least in three of the four serum-resistant strains tested in this study, suggested that UspA2 might either be interfering directly with proper MAC formation or binding a regulator that affects this critical step. Examples of both mechanisms are documented in the literature for other serum-resistant microorganisms. For example, the O-polysaccharide of *Salmonella minnesota* seemed to physically interfere with MAC

insertion into the outer membrane by causing assembly of the MAC away from the bacterial surface (151). *S. typhimurium* resisted complement-mediated killing by using its Rck protein to prevent polymerization of C9 (113) whereas *Borrelia burgdorferi* encodes a CD59-like protein that interferes with MAC formation (224). Other bacteria recruit natural regulators of the complement system that interfere with MAC formation. Rautemaa et al. (244) showed that *E. coli* can incorporate glycophosphoinositol-anchored protectin (CD59) and acquire resistance to NHS-mediated killing. Other pathogens, especially gram-positive organisms including both staphylococci (168) and streptococci (58), can bind vitronectin and clusterin or both, although these organisms are not lysed by the MAC.

Interestingly, *M. catarrhalis* is one of those pathogens that has been reported to bind the MAC regulator vitronectin. *M. catarrhalis* clinical isolates were reported to differ in their binding of vitronectin, although the bacterial gene product responsible for this binding activity was not identified [Verduin, C. M., M. Jansze, J. Verhoef, A. Fleer, and H. van Dijk. 1994. Complement resistance in *Moraxella (Branhamella) catarrhalis* is mediated by a vitronectin-binding surface protein. Clin. Exp. Immunol. 97S2 (Abst. 143):50]. McMichael et al. (185) reported that purified UspA2 from *M. catarrhalis* strain O35E bound purified vitronectin in a dot blot assay. For the first time, a direct correlation was demonstrated between UspA2-mediated binding of vitronectin and the serum-resistant phenotype, at least for three of the four *M. catarrhalis* strains tested in the present study. This was supported by three findings. First, serum-resistant wild-type strains bound more vitronectin from NHS than did their serum-sensitive *uspA2* mutants (Fig. 30). Second, expression of UspA2 from these same three strains in a heterologous genetic background (i.e., *H. influenzae*) resulted in serum resistance concurrent with vitronectin binding (Fig. 33). Finally, vitronectin depletion of NHS resulted in a significant increase in the susceptibility of these three serum-resistant

M. catarrhalis strains to bactericidal activity and addition of purified vitronectin to the vitronectin-depleted NHS restored the serum-resistant phenotype of these strains (Fig. 32).

Vitronectin has been shown to regulate complement at the level of MAC assembly at two stages: (i) it binds to the metastable membrane-binding site of C5b-7 (227,234) and (ii) it binds to the assembling C5b-9 complex and prevents tubular poly-C9 formation (228). The heterogeneity of C9 complexes seen on the different *M. catarrhalis* strains used in this study may be explained by regulation of MAC formation at two levels. Blockade at the level of C5b-7 would lead to the absence of C9 trimer as seen on wild-type strain O12E (Fig. 29, lane 12) and might explain the slight increase in C7 bound by the O35E and O12E *uspA2* mutants (Fig. 28, lanes 9 and 13). Blockade at the level of C5b-9 may permit formation of C9 trimers but not polymers as seen on wild-type strain 7169 (Fig. 29, lane 20). The increased amount of C9 polymerization on the FIN2344 *uspA2* mutant compared to the wild-type strain (Fig. 29, lanes 16 and 17), both of which bound vitronectin very poorly (Fig. 30, lanes 12 and 13), may suggest that UspA2 itself could block further C9 polymerization in the absence of direct vitronectin binding. It is also possible that UspA2 may be involved directly, interfering with insertion of fully formed C5b-9 into the bacterial outer membrane. Vitronectin is a multi-functional protein and another important function it subserves is cell attachment by binding to the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins via its Arg-Gly-Asp (RGD) domain (235). It is possible that vitronectin binding to *M. catarrhalis* may facilitate bacterial attachment to human cells and merits further study.

It is important to note that resistance of the wild-type *M. catarrhalis* strains O35E, O12E, and 7169 to killing by NHS was not completely eliminated by vitronectin depletion (Fig. 32, panels A, B and D), a result which suggests that vitronectin binding is not the only mechanism protecting these strains. However, at least for these three strains which bound vitronectin to avoid serum killing, binding of C4BP appeared not to be involved in serum resistance.

The likely existence of another UspA2-mediated serum resistance mechanism that does not involve vitronectin is reinforced by the results obtained with the FIN2344 and its *uspA2* mutant. Increased binding of C3 by the FIN2344 *uspA2* mutant (Fig. 27B, group 4) suggests that the UspA2 protein of FIN2344 somehow interferes, directly or indirectly, with the early stages of the complement cascade. This increase in complement deposition on the FIN2344 *uspA2* mutant, relative to the wild-type parent strain, was also observed with both C7 and polymerized C9 (Fig. 28 and Fig. 29). Expression of the FIN2344 UspA2 protein in *H. influenzae* resulted in serum resistance but the level of vitronectin binding was much reduced compared to that obtained with UspA2 proteins from strain O35E, O12E and 7169. That this serum resistance phenotype is likely independent of vitronectin binding is reinforced by the observation that Vn-depleted NHS was not able to kill the wild-type FIN2344 strain (Fig. 32, panel C). Exactly how the FIN2344 UspA2 protein confers serum resistance on this strain is not apparent from the available data. It is possible that the FIN2344 UspA2 protein somehow interferes with complement deposition or that it binds a complement inhibitor or regulator present in NHS. This additional mechanism(s) remains to be identified and reinforces the fact that multiple and sometimes redundant mechanisms may be crucial for a bacterial species to survive in a hostile environment.

CHAPTER SEVEN

The Regulatory Role of Nucleotide Repeats in the Expression of the *uspA2* Gene

I. Introduction

Results described in the previous chapters showed the critical role of the UspA2 protein in the protection of *M. catarrhalis* against the bactericidal action of NHS. However, up to this point, very little was known about the regulation of the *uspA2* gene. One characteristic feature of all of the *uspA2* genes sequenced to date is the presence of varying numbers of a small nucleotide repeat (AGAT) in the 5′-UTR of this gene. Because these repeats are located immediately upstream of the *uspA2* ORF, this raised the possibility that changes in the number of these repeats could affect expression of this gene.

The expression of two other surface-exposed *M. catarrhalis* proteins has been shown to be regulated by changes in the number of nucleotide repeats. The UspA1 protein is regulated on the transcriptional level by the changes in the length of a homopolymeric nucleotide repeat [i.e., a poly (G) tract] upstream of the *uspA1* ORF (166). The Hag (MID) protein is also regulated by changes in another homopolymeric nucleotide repeat [i.e., a poly (G) tract] within the 5′-end of the *hag* ORF (196,225). In addition, the UspA2H protein, which is closely related to UspA2, has been shown to be regulated by changes in a homopolymeric nucleotide repeat [i.e., a poly (A) tract] in the 5′-end of the *uspA2H* ORF (Wei Wang et al, unpublished data). These examples show that this microorganism has a tendency to utilize nucleotide repeat motifs in the regulation of the expression of its proteins.

In this chapter, detailed analysis of the 5′-UTR of the *uspA2* gene showed that these AGAT repeats are part of the *uspA2* mRNA. Changes in the numbers of these repeats affected expression of the *uspA2* gene on the transcriptional level, with subsequent effects on both the level of expression of the UspA2 protein and serum resistance.

II. Results

A. *M. catarrhalis* strains vary in the number of AGAT repeats in the 5′-UTR of their *uspA2* genes

The nucleotide sequences of the 5′-UTR of the *uspA2* gene from eleven different *M. catarrhalis* strains were compared. Nucleotide sequence analysis showed that this region was very highly conserved among the tested strains. However, there was considerable variation in the number of AGAT nucleotide repeats (Fig. 34). This AGAT repeat region was located approximately 130-nt upstream of the translation initiation codon of the *uspA2* gene. In the case of the wild-type strain O12E, the number of the AGAT repeats was 19, however, two other naturally occurring O12E variants were isolated that contained either 18 or 23 repeats. No morphological differences were observed among colonies of these latter three strains. The smallest number of AGAT repeats that was observed among the tested *M. catarrhalis* isolates was six and this occurred in strain ATCC43617. It is worth mentioning that strain ATCC43617 is fully serum-sensitive because it cannot express an intact UspA2 protein due to the presence of a premature translation termination codon within its *uspA2* ORF (data not shown).

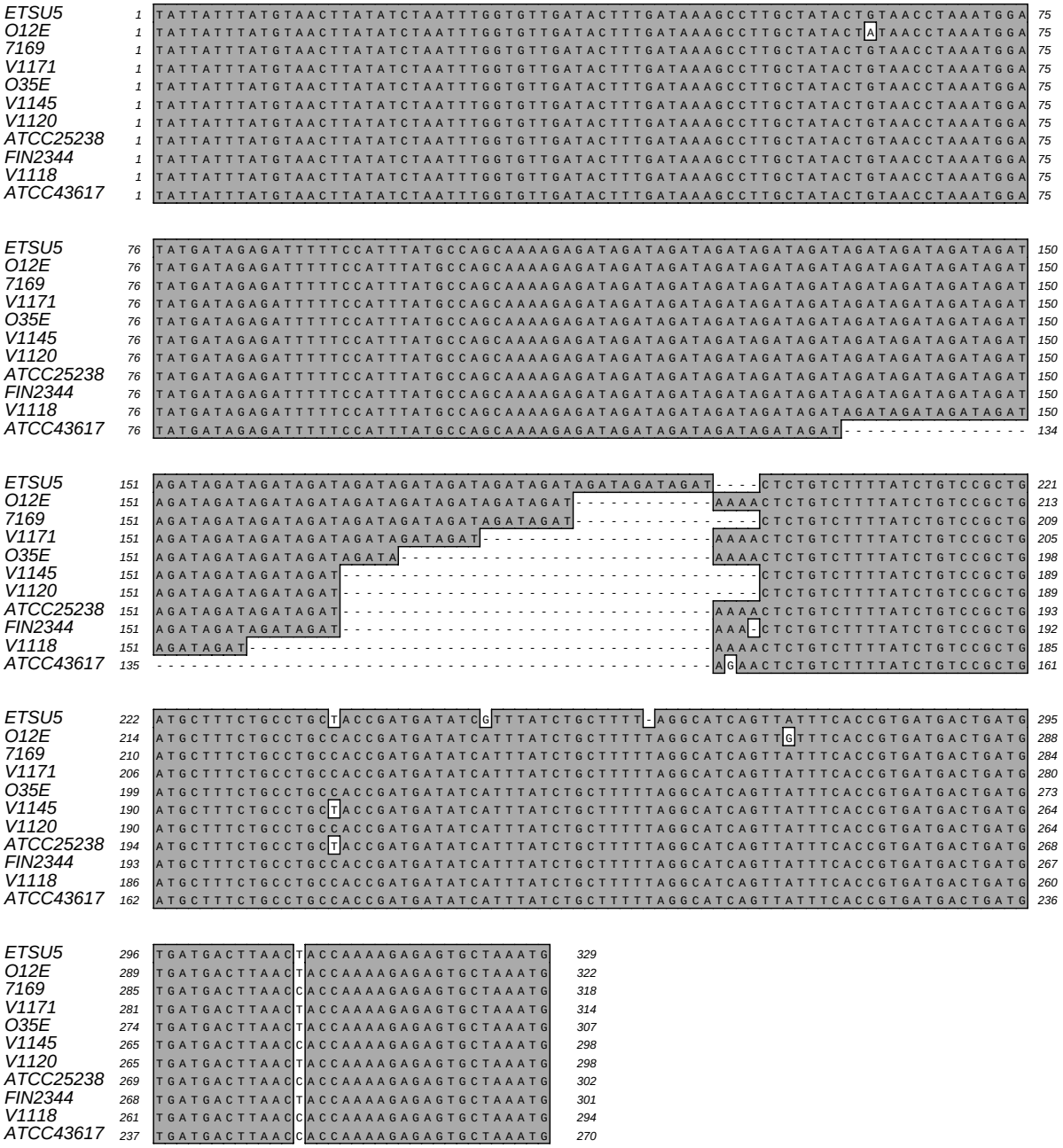

Fig. 34. Alignment of the nucleotide sequences of the 5′-UTR of the *uspA2* genes from eleven different *M. catarrhalis* strains.

Identical nucleotides are shaded in dark gray. This figure was generated using the ClustalW Alignment program in MacVector (v6.5). The nucleotide sequences of the 5'-UTR from strains O35E, ATCC25238, and V1171 were derived from a previous study (61).

B. Deletion of the AGAT repeat region results in reduced UspA2 protein expression and decreased serum resistance

The presence of AGAT repeats upstream from every *uspA2* ORF examined in this laboratory and by others (111) suggested that these repeats might be involved in the expression of the *uspA2* gene. To address this possibility, PCR-sewing and congression were used to delete the entire AGAT repeat region from the *uspA2* genes of *M. catarrhalis* strains O35E and O12E; the resultant transformants were designated O35EΔAGAT and O12EΔAGAT, respectively. No morphological differences were observed between colonies of these mutants and those of their respective wild-type parent strains. Western blot analysis revealed that these two transformants expressed much lower levels of UspA2 than did the streptomycin-resistant transformants of their respective parent strains (Fig. 35A).

We previously used mutant analysis to establish that expression of the UspA2 protein is necessary for serum resistance of both strains O35E and O12E (discussed above). Therefore, it was likely that a reduced level of expression of the UspA2 protein by these strains would be accompanied by a decrease in the level of serum resistance. To test this hypothesis, the two streptomycin-resistant wild-type strains (O35E-Sm^r and O12E-Sm^r) were tested together with the two mutants lacking AGAT repeats (O35EΔAGAT and O12EΔAGAT) in serum bactericidal assays using 10% (vol/vol) NHS. The results of this assay showed that these two mutants lacking AGAT repeats were exquisitely serum-sensitive (Fig. 35B). It was also observed that the O12E-Sm^r strain had reduced expression of the UspA1 protein; this was the result of a spontaneous change [from 10 G to 9 G residues] in the poly (G) tract located upstream of the *uspA1* ORF. However, comparison of the O12E-Sm^r strain with the wild-

type O12E parent strain showed no significant difference between these two strains in the level of serum resistance (data not shown).

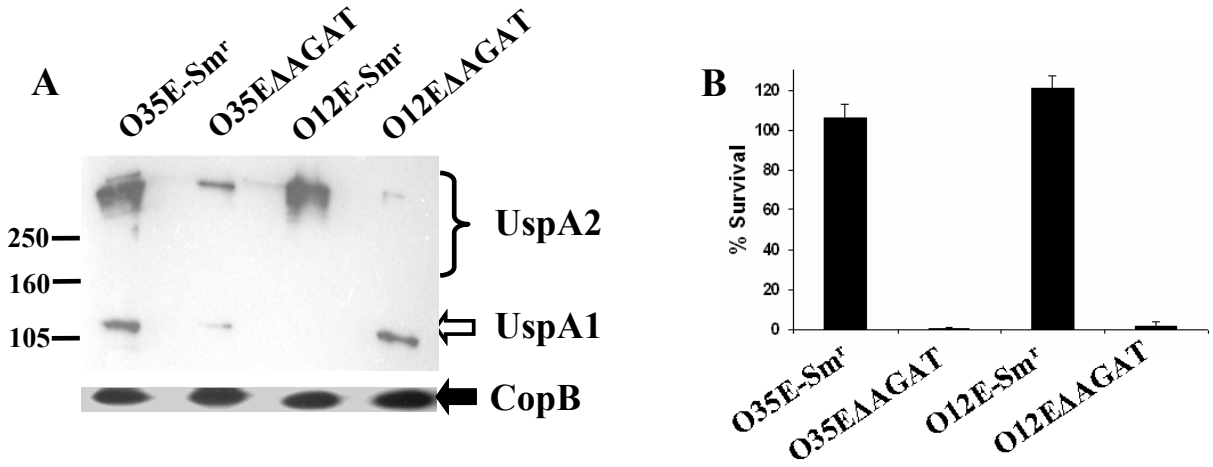

Fig. 35. Deletion of the AGAT repeats from the 5′-UTR of the *uspA2* gene causes a decrease in both UspA2 protein expression and serum resistance.

(A) Western blot analysis of whole cell lysates of *M. catarrhalis* strains O35E-Sm^r, O35EΔAGAT, O12E-Sm^r, and O12EΔAGAT. The nitrocellulose membrane was probed with MAb 17C7 which recognizes both the *M. catarrhalis* UspA2 protein (bracket) and the UspA1 protein (white arrow) (5). Detection of the CopB outer membrane protein with the CopB-reactive MAb 10F3 (115) was used for loading standardization. Protein molecular mass markers (in kDa) are present on the left side of the panel. (B) Serum bactericidal assay with the four strains described in panel A. Bacterial cells were incubated in 10% NHS at 37°C for 30 min. Bacterial aliquots were plated at both t=0 and t=30 min. The % survival was calculated with respect to the original inoculum. These results represent the mean of three independent experiments and the error bars represent the standard deviation.

C. Increasing the number of AGAT repeats results in increased levels of UspA2 protein

The dramatic decrease in UspA2 protein expression that accompanied the deletion of the AGAT repeats (Fig. 35A) raised a question as to how many AGAT repeats were necessary for wild-type levels of synthesis of UspA2. To address this issue, PCR-sewing and congression were used to construct a series of *M. catarrhalis* O12E constructs that have varying numbers of AGAT repeats in the 5′-UTR of their *uspA2* genes. These constructs contained from 2 to 15 AGAT repeats and were analyzed together with the O12EΔAGAT strain with no AGAT repeats, the streptomycin-resistant transformants of the wild-type O12E parent strain with 19 repeats, and the two natural variants of strain O12E with 18 and 23 repeats. No morphological differences were observed among colonies of these twelve strains (data not shown). Western blot analysis of these twelve strains (Fig. 36A) showed that there was an apparent increase in the amount of UspA2 being synthesized with the gradual increase in the number of the AGAT repeats. The two constructs with 15 and 18 AGAT repeats appeared to have the highest level of expression of UspA2 among the tested strains (Fig. 36A). However, a more quantitative analysis was needed to confirm this observation. Therefore, flow cytometry was used to compare the levels of UspA2 protein expressed by *uspA1* mutants of these twelve strains with varying numbers of AGAT repeats (Fig. 36B). Inactivation of the *uspA1* gene was necessary because the UspA1 protein binds MAb (17C7) which was used to detect UspA2 in these flow cytometry experiments (5). There was about a four-fold increase in the gmf value obtained when the number of AGAT repeats was increased from 0 to 2. There was a slight and gradual increase in the gmf values with the gradual increase in AGAT repeat number, with the maximum gmf value being obtained with the construct with 18 repeats. This was followed by a slight decrease in the gmf values obtained with the two constructs with 19 and 23 repeats (Fig. 36B).

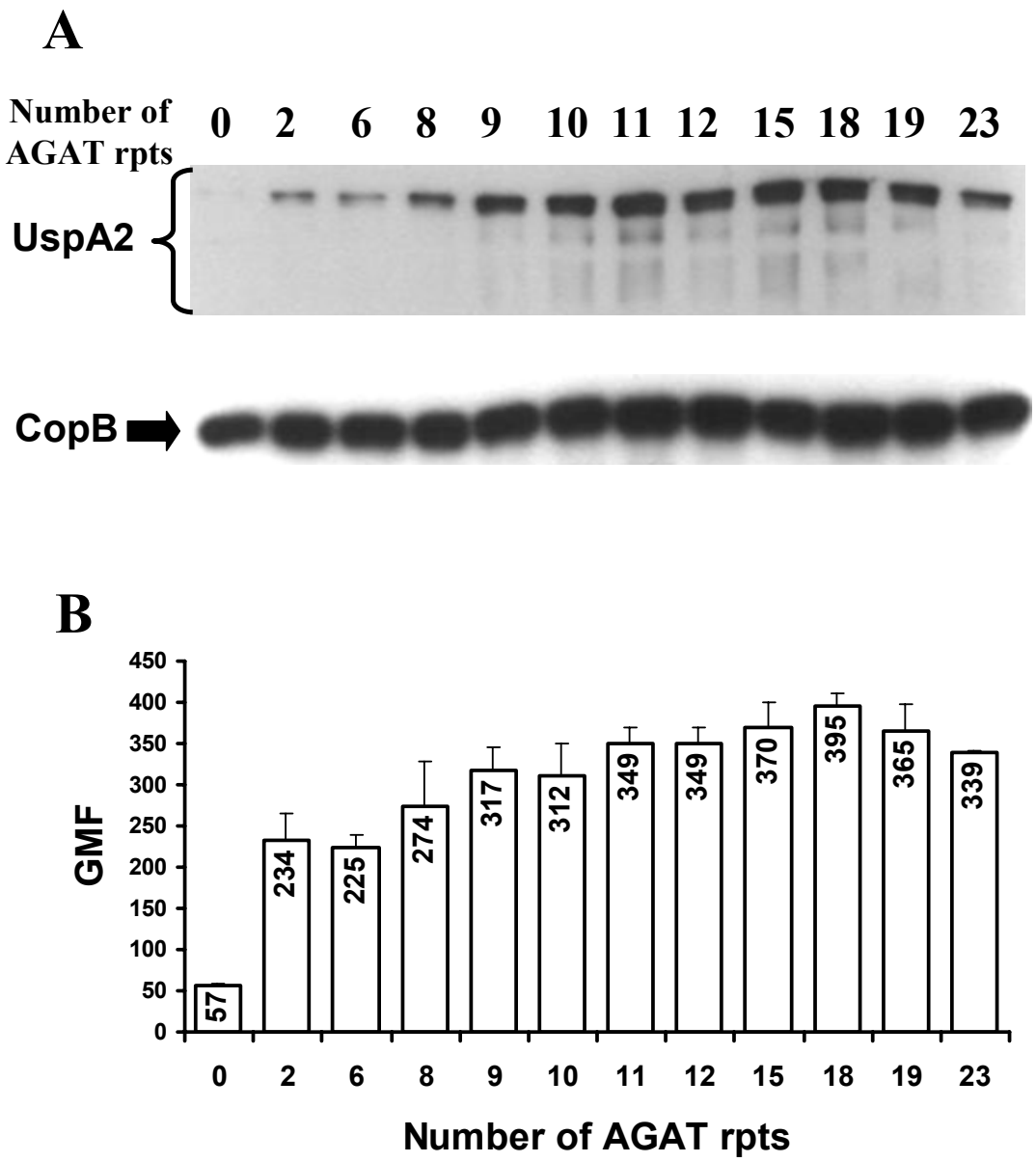

Fig. 36. Effect of increasing numbers of AGAT repeats on expression of the UspA2 protein.

(A) Western blot analysis of O12E-derived constructs with varying numbers of AGAT repeats in the 5′-UTR of their *uspA2* genes. Proteins present in whole cell lysates of these strains were resolved by SDS-PAGE under non-reducing conditions and transferred to nitrocellulose membranes. The membranes were probed with MAb 17C7 which binds the *M. catarrhalis* UspA2 protein. The region of the gel containing the UspA1 protein is not present in this image. As a loading control, membranes were probed with the CopB-reactive MAb 10F3 (115). (B) Flow cytometric analysis of O12E constructs having varying numbers of AGAT repeats in the 5′-UTR of their *uspA2* genes. Whole cells of *uspA1* mutants of these O12E constructs were probed with MAb 17C7 followed by washing and incubation with a FITC-conjugated antiserum to mouse IgG. After washing, the cells were analyzed by flow cytometry and the geometric mean fluorescence (gmf) values were recorded. These results represent the mean of three independent experiments and the error bars represent the standard deviation.

D. Increasing the number of AGAT repeats also results in an increase in serum resistance

The observed increase in expression of the UspA2 protein with the increase in the number of AGAT repeats (Fig. 36) and the demonstrated serum sensitivity of the O12EΔAGAT strain (Fig. 35B) made it important to determine whether there was a minimal or threshold level of UspA2 expression necessary for serum resistance. To address this point, the twelve O12E strains with varying numbers of AGAT repeats in the 5′-UTR of their *uspA2* genes were tested in a series of serum bactericidal assays. When NHS was used at a concentration of 10% (vol/vol) (Fig. 37, open columns), the deletion mutant with no AGAT repeats was again exquisitely serum-sensitive. When the number of AGAT repeats was either 2 or 6, there was a moderate increase in serum resistance, with about 30% of the initial inoculum of these two constructs surviving for 30 min under the conditions of this assay. However, when the number of AGAT repeats was increased to 8, there was a striking increase in serum resistance, with essentially 100% of the initial inoculum surviving. Further increases in the number of AGAT repeats did not cause any substantial increases in the level of serum resistance. To test whether increasing the concentration of NHS used in this assay would cause a shift in the minimum number of AGAT repeats required to obtain full serum resistance, the twelve constructs were also tested using 30% (vol/vol) NHS (Fig. 37, black columns). Again, the deletion mutant with 0 repeats was exquisitely serum-sensitive while the two constructs with 2 and 6 AGAT repeats showed a very slight increase in serum resistance. For the construct with 8 AGAT repeats, only a moderate increase in serum resistance was observed whereas a wild-type level of serum resistance was achieved in the presence of 9 AGAT repeats. Further increases in the number of AGAT repeats beyond 9 did not appear to result in substantial increases in serum resistance in this assay.

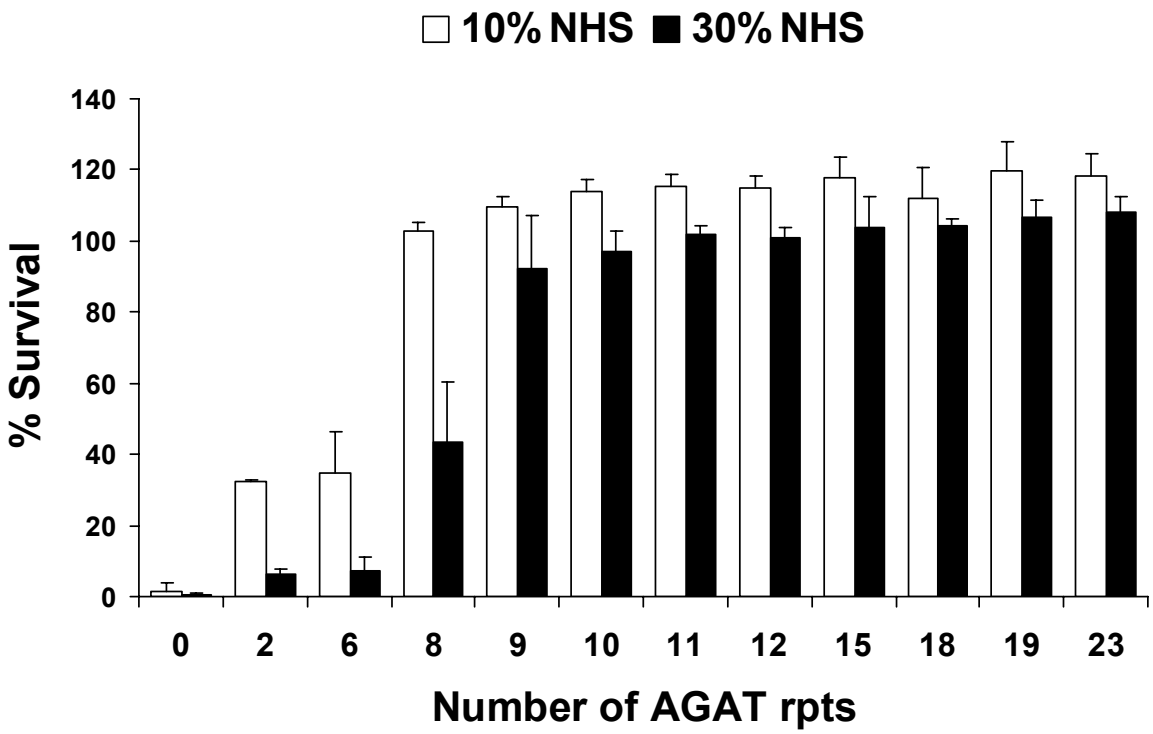

Fig. 37. Serum bactericidal assay with O12E constructs having varying numbers of AGAT repeats in the 5′-UTR of their *uspA2* genes.

Bacterial cells were incubated in 10% NHS (open bars) and 30% NHS (black bars) at 37°C for 30 min. Bacterial aliquots were plated at both t=0 and t=30 min. The % survival was calculated with respect to the original inoculum. These results represent the mean of three independent experiments and the error bars represent the standard deviations.

E. Changes in the number of AGAT repeats affect the level of *uspA2* mRNA

The previous experiments established that the number of AGAT repeats in the 5′-UTR of the *uspA2* gene affected the level of the expression of the UspA2 protein. Because these repeats are located upstream of the *uspA2* ORF, it is most likely that they affect expression of the *uspA2* gene at the level of transcription. To test this hypothesis, total RNA was extracted from the twelve O12E strains with varying numbers of AGAT repeats and was analyzed using real-time RT-PCR. The level of the transcripts of the highly abundant *copB* gene was used to normalize the level of the *uspA2* message among the twelve tested strains. Analysis of the data obtained from the real-time RT-PCR experiments using the $^{\Delta\Delta}$CT method (Fig. 38) showed that increasing the number of AGAT repeats from 0 to 8 had very little if any effect on the level of *uspA2* transcripts. However, an obvious increase (i.e., 2.5-fold) was observed upon increasing the number of AGAT repeats to 9 (Fig. 38). Further increases in the number of AGAT repeats were associated with a gradual increase in the level of the *uspA2* transcripts, reaching a maximum with the strains possessing 15 and 18 AGAT repeats (Fig. 38). This was followed by a decrease in the level of *uspA2* transcripts obtained with the two constructs that had 19 and 23 AGAT repeats (Fig. 38). These data indicate that the number of AGAT repeats does affect expression of the *uspA2* gene at the level of transcription.

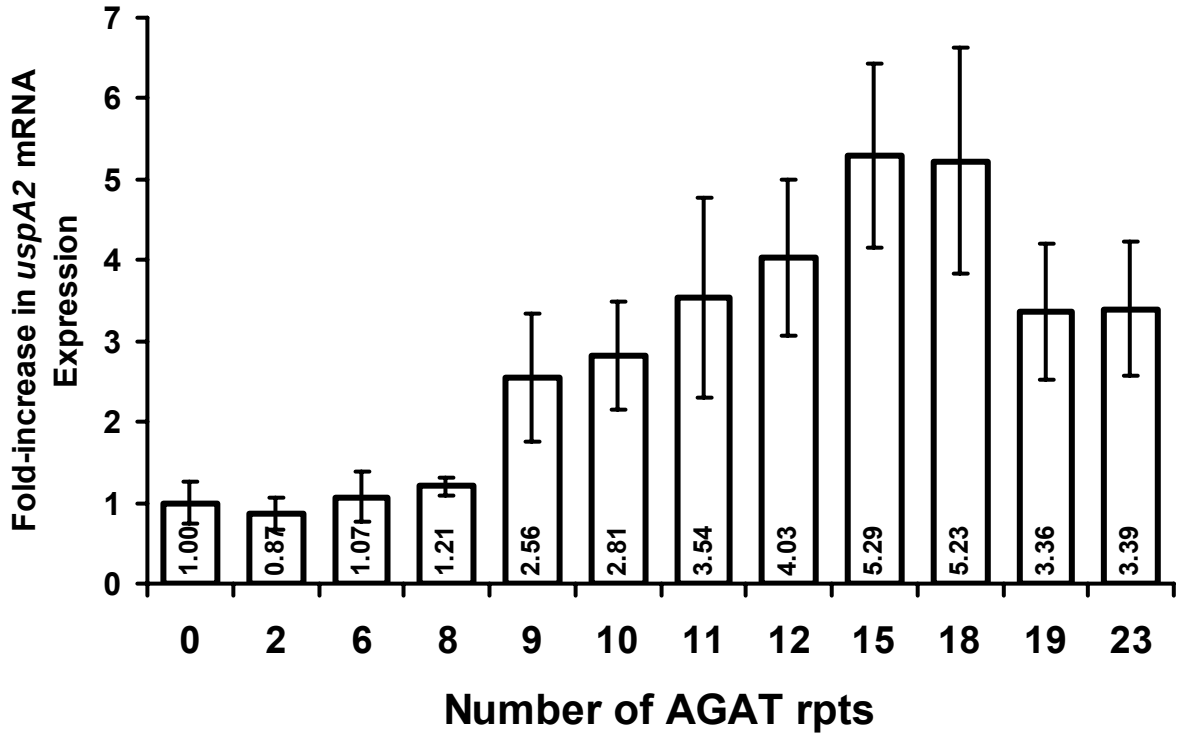

Fig. 38. Real-time RT-PCR analysis of *uspA2* gene expression by O12E constructs having varying numbers of AGAT repeats in the 5′-UTR of their *uspA2* genes.

Total RNA isolated from O12E constructs with varying numbers of AGAT repeats in the 5′-UTR of their *uspA2* genes was used for real-time RT-PCR with primers specific for the *uspA2* and *copB* genes. Data analysis was carried out using the 7500 System SDS software v.13, applying the relative quantification $^{\Delta\Delta}$Ct method. The level of the *uspA2* message was normalized according to the level of the *copB* message and the data are presented as a fold-increase using the normalized level of the *uspA2* gene of O12E with no AGAT repeats (O12EΔAGAT) as the calibrator. These data represent the mean of three independent experiments (each performed with samples in triplicate) and the error bars represent the standard deviations.

F. Mapping of the transcriptional start point of the *uspA2* gene

Having seen the effect of the number of AGAT repeats on the level of transcription of the *uspA2* gene, it became crucial to determine accurately the location of these repeats with respect to the transcriptional start point of the *uspA2* gene. To this end, two approaches were used.

i. Primer extension

Initial attempts to perform primer extension analysis using RNA isolated from wild-type *M. catarrhalis* strain O12E were unsuccessful. As an alternative, a PCR fragment containing the O12E *uspA2* gene and approximately 500-nt upstream of the translation initiation codon was cloned into pWW115 using *H. influenzae* DB117 as the host. Two clones were obtained; one contained 20 AGAT repeats and the other contained 21 AGAT repeats (pAAO12U2-20rpt and pAAO12U2-21rpt, respectively). Primer extension data obtained using these clones were inconclusive, however, due to the presence of sequence "noise" within the sequencing ladder (data not shown). Most probably, the reason for this "noise" was the presence of multiple copies of the transcript with different numbers of the repeats (derived from plasmids with different numbers of AGAT repeats). New experiments were then performed in an *M. catarrhalis* background, with primer extension analyses being carried out with two constructs (i.e., O12E-9rpts and O12E-18rpts). The primers used for these analyses were AA52-Rev, AA26-rev, and AA9-Rev, which bind to three different locations within the *uspA2* ORF [the locations of these primer binding sites are labeled as a, b, and c, respectively (Fig. 39C)]. The results of these experiments showed that the *uspA2* transcript starts at a C residue that is located 43-nt upstream of the start of the AGAT repeat region (Fig. 39). The same results were obtained using RNA from O12E-18rpts with primers AA26-Rev, AA52-Rev (Fig. 39) and AA9-Rev (data not shown) and RNA from O12E-9rpt with primer AA52-Rev (Fig. 39B). These results indicated that the AGAT

repeats are included in the *uspA2* transcript and that changes in the number of AGAT repeats does not affect the location of the transcriptional start point.

ii. Rapid amplification of cDNA ends (5′-RACE)

In an effort to confirm these primer extension data, the BD SMART PCR cDNA Synthesis kit was used to perform 5′-RACE to identify the transcriptional start point of the *uspA2* gene as described in the Material and Methods section. Nucleotide sequence analysis of positive clones indicated that transcription of the *uspA2* gene was initiated at the C residue that is located 10 nucleotides upstream of the start of the AGAT repeat region (Fig. 40). These data, however, are not in agreement with those obtained from the primer extension experiments; the reason for this disagreement is not clear at this time. One possible explanation might be that the RT product obtained in the 5′-RACE experiment is an early termination product. Alternatively, it might represent a transcript that started from an alternative transcriptional start site; this possibility is less likely since no minor bands were seen in the primer extension experiments (Fig. 39A and B). I tend to believe that the data obtained from the primer extension are the more accurate because this technique has been well-established as the standard method for mapping the transcriptional start site of bacterial genes. However, it is worth noting that both methods (i.e., primer extension and 5′-RACE) indicated that the AGAT repeat region is a part of the *uspA2* mRNA.

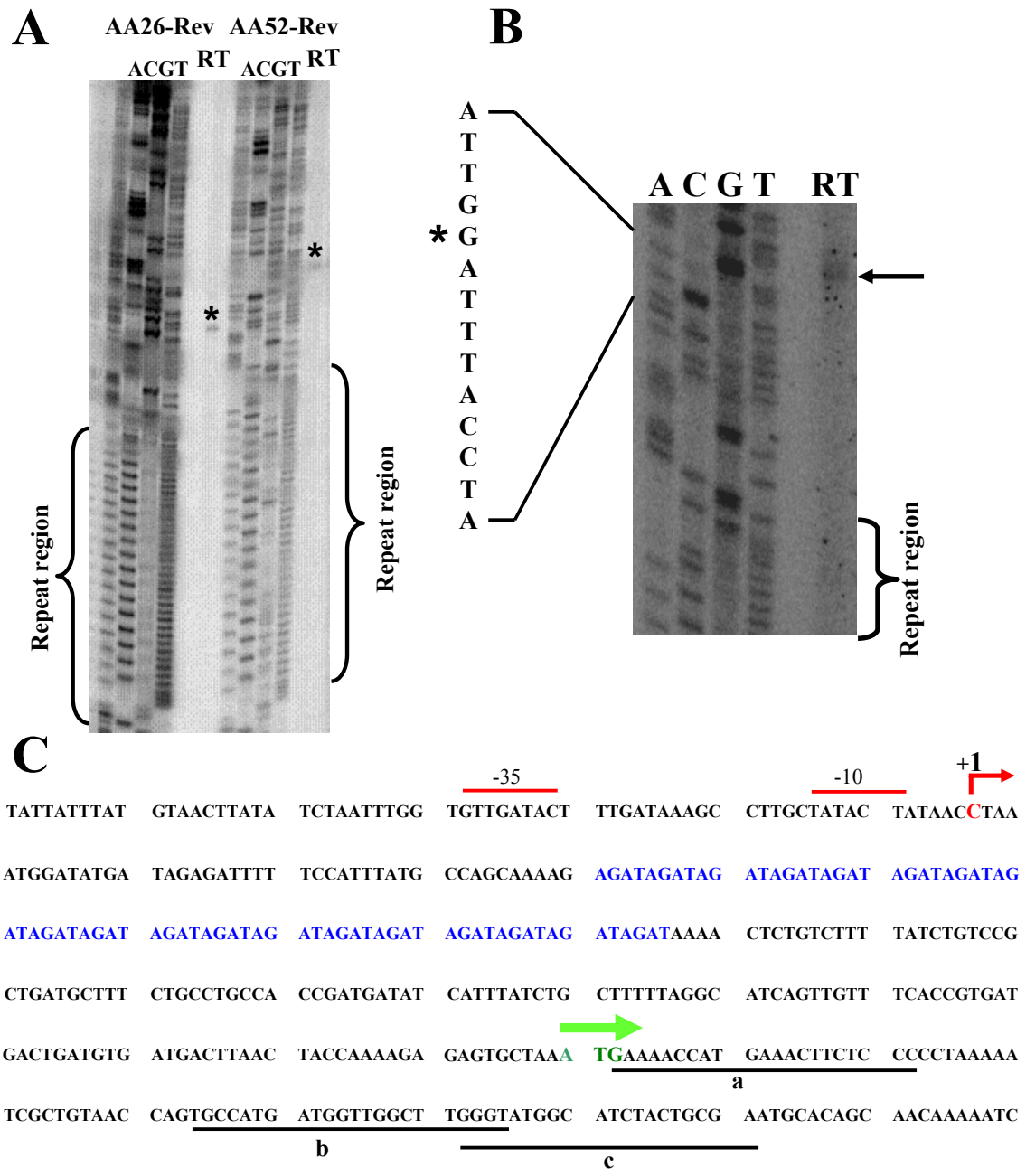

Fig. 39. Determination of the *uspA2* transcriptional start point using primer extension analysis.

(A) Primer extension results for RNA isolated from *M. catarrhalis* O12E-18rpts and using primers AA26-Rev and AA52-Rev. The sequencing ladder and the RT using AA26-Rev were run first for 45 min then those using AA52-Rev were loaded and run. The * indicates the position of the RT products and the repeat regions are indicated with brackets. (B) Primer extension results for RNA isolated from *M. catarrhalis* O12E-9rpts and using AA52-Rev. The arrow indicates the position of the RT product and the repeat region is indicated with the bracket. The * marks the position from which transcription starts. (C) Sequence of 320-nt of the region located upstream of the O12E *uspA2* ORF and 100-nt inside the ORF. The red arrow shows the predicted transcriptional start point that was determined from the data in panels A and B. Sequences similar to the -10 and -35 consensus sequences seen in bacterial gene promoters are marked with red bars. The start of the *uspA2* ORF is marked with the green arrow and the AGAT repeat region is marked with the blue font. The locations of binding sites for primers AA52-Rev, AA26-rev, and AA9-Rev are underlined and labeled as a, b, and c, respectively.

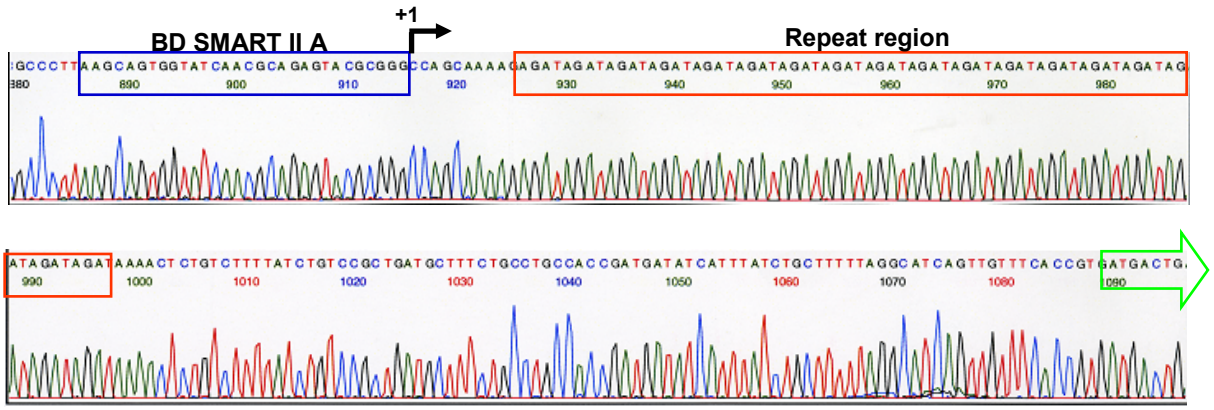

Fig. 40. Determination of the *uspA2* transcriptional start point using 5′-RACE.

Chromatogram showing the sequence of the *uspA2* 5′-UTR region ligated to the BD SMART IIA oligonucleotide (blue box). The putative transcriptional start point is marked by the (+1) sign and is located 10-nt upstream of the AGAT repeat region (red box). The start of the *uspA2* ORF is marked by the green arrow.

G. Development of *lacZ*-based reporter systems for the analysis of *uspA2* gene expression

Efforts were made to develop a reporter system for analyzing the role of the AGAT repeats in the *uspA2* gene. The multi-copy plasmid pAC7 (306) was used to clone the 5'-UTR of the *uspA2* gene from the three natural variants of the O12E strain with 18, 19, and 23 repeats as described in the Materials and Methods section. Upon testing *E. coli* strains carrying the plasmids pAC7-18 (with 18 repeats) and pAC7-19 (with 19 repeats) in β-galactosidase activity assays (Fig. 41), it was found that decreasing the number of the AGAT repeats from 19 to 18 was accompanied by a 1.36-fold increase in β-galactosidase activity. This increase was comparable to the data obtained above using real-time RT-PCR (Fig. 38) where the change in the number of AGAT repeats from 19 to 18 was accompanied by a 1.56-fold increase in the level of the *uspA2* transcript. Attempts to use additional reporter plasmids with varying numbers of AGAT repeats, however, yielded non-reproducible results in the β-galactosidase activity assays. It is likely that this difficulty was the result of spontaneous changes in the number of the AGAT repeats in the 5′-UTR of the *uspA2* gene in this multi-copy plasmid reporter system. For example, nucleotide sequence analysis of plasmid pAC7-23, which was supposed to contain 23 AGAT repeats, showed that it had only 10 AGAT repeats.

In an effort to develop a more stable reporter system, I attempted to construct a single-copy reporter system that was integrated into the *hag* gene in the *M. catarrhalis* chromosome. Plasmid pAA3-23rpt, which has the (kan^r-*uspA2* 5′-UTR with 23 AGAT repeats-*lacZ* fusion) inserted between two fragments of the *hag* gene (*hagC* and *hagD*), was used to transform *M. catarrhalis* strain O12E to obtain strain O12E-23*lacZ* (Fig. 42). This is the first reported *M. catarrhalis* strain to have a reporter system integrated into its chromosome. Unfortunately, subsequent attempts to transform O12E with pAA3 containing *lacZ* fusions with the *uspA2* 5′-UTR with 0, 2, 6, 10, 12, or 18 AGAT repeats were unsuccessful for reasons that are not clear at this time. Additional efforts are required to improve the

efficiency of this reporter system which could become a valuable tool for the characterization of the expression of both the *uspA2* gene and other *M. catarrhalis* genes.

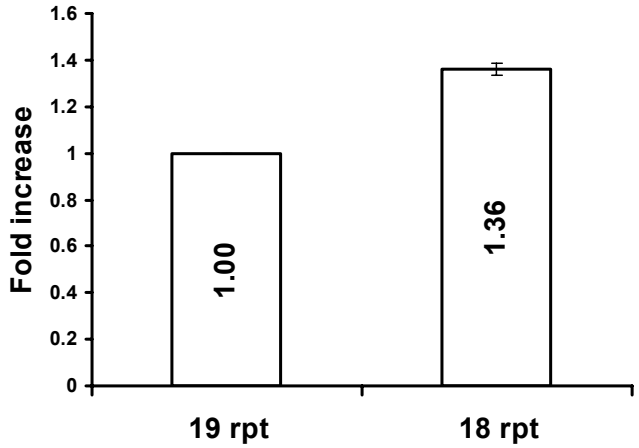

Fig. 41. β-galactosidase activity assay using *E. coli* strains containing the pAC7-derived reporter system.

E. coli DH5α strains carrying plasmids pAC7-18 and pAC7-19 were assayed for their β-galactosidase activity using the assay described in Materials and Methods. The data are presented as fold-increase using the activity of the reporter strain with the 5′-UTR of the *uspA2* gene of the wild-type O12E strain with 19 AGAT repeats (pAC7-19) as the calibrator. The data presented are the mean of two independent experiments and the error bars represent the standard deviations.

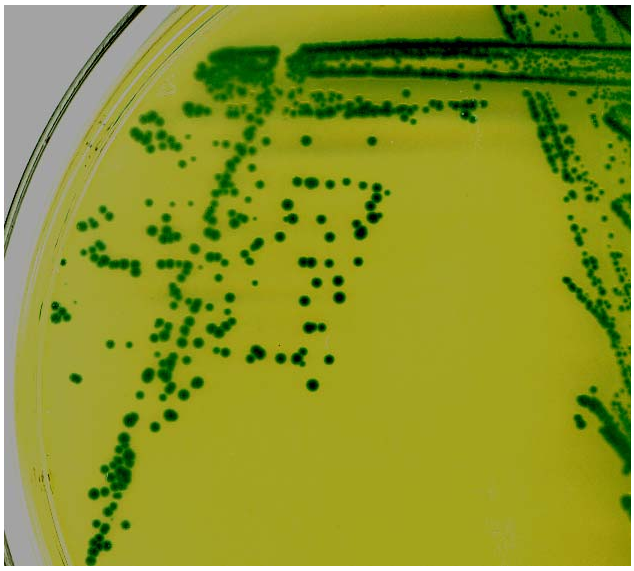

Fig. 42. Photograph of *M. catarrhalis* strain O12E-23*lacZ* grown on an X-Gal plate.

III. Discussion

The data presented in this chapter indicate that the AGAT repeats located in the 5′-UTR of the *uspA2* gene have a regulatory role in the control of this gene. These results add the *uspA2* gene to the growing list of *M. catarrhalis* genes whose expression is influenced or controlled by stretches of nucleotide repeats. This list includes the *uspA1* gene which is regulated on the transcriptional level by changes in the length of a poly (G) tract upstream of the *uspA1* ORF (166). Another member of this list is the *hag* (*mid*) gene which is affected by changes in a poly (G) tract within the 5′-end of the *hag* ORF (196,225). However, what makes the *uspA2* gene different from these two genes is that the nucleotide repeat is not a simple homopolymeric repeat but instead is a heteropolymeric motif consisting of 4 nucleotides (AGAT), which varied in size among the different *M. catarrhalis* strains surveyed in this study from 6 repeats up to 23 repeats.

Gene regulation through changes in the number of nucleotide repeats is a fairly widespread phenomenon in prokaryotic genomes (119). Changes in these repeat units can result in a frameshift leading to a premature translational stop codon if this repeat unit is located within the ORF. Examples of this type of regulation include at least two genes found in *N. meningitidis* that exhibit phase variation in polysaccharide capsule formation caused by changes within a run of 7 C residues in the *saiD* gene (104). Similarly, the *lgtABE* locus of *N. meningitidis*, which is involved in the biosynthesis of the terminal lacto-N-neotetraose structure of meningococcal LOS, has a homopolymeric tract of 14 G residues within the 5'-end of the *lgtA* coding sequence (148). Examples of genes that are regulated on the translational level by variation in the number of heteropolymeric nucleotide repeats include the *lic* loci, responsible for expression of at least two LOS epitopes of *H. influenzae*. The first gene in this system is *lic1* which has, at its 5′ end, a variable number of repeats of CAAT. By shifting upstream initiation codons in- or out-of-frame, these 4-nucleotide units create a translational switch (307).

Another example of translational control can be found in the gene encoding the outer membrane protein II (P.II) of *N. gonorrhoeae* which has a pentanucleotide (CTCTT) repeat within the DNA encoding its signal sequence. Changes in the number of CTCTT repeats lead to frameshifting within the gene and cause changes in P.II expression (200).

The other mechanism for gene regulation by nucleotide repeats involves the presence of these repeat units outside of the coding region of the gene (i.e. upstream of or within the 5′-UTR). In this case, the regulatory role of these repeats would be on the transcriptional level, affecting either transcription initiation or the strength of the promoter (119). Several bacterial genes have been reported to use this type of control mechanism. The gonococcal FetA ferric enterobactin receptor exhibits extremely rapid phase variation between high- and low-expression levels that is controlled by the number of C residues in a string of cytosines located close to the transcriptional start site for *fetA*, between the putative -10 and -35 consensus sequences (51). Similarly the *porA* gene, which encodes the class 1 outer membrane protein (OMP1) in *N. meningitidis*, has a number of G residues in a poly(G) tract located upstream of the -10 region. Isolates that did not express OMP1 had up to nine G residues in the poly(G) tract or an adenosine residue within this poly(G) tract (260). These previous two examples involved homopolymeric nucleotide repeats. Heteropolymeric repeats can also affect transcription. Regulation of NadA, an outer membrane protein and adhesin of *N. meningitidis,* is mediated by changes in the number of TAAA repeats located upstream of the core promoter of *nadA* (180). Also, the pMGA genes of the avian respiratory pathogen *Mycoplasma gallisepticum* encode a family of hemagglutinins that are subject to phase variation that is controlled by a trinucleotide (GAA) repeat that is located upstream of the pMGA transcription start site. The length of the repeat region varies at a high frequency due to changes in the number of repeat units (95). The gene encoding the alpha C protein of Group B *Streptococcus* has a pentanucleotide repeat (AGATT) and a string of A residues located -55 to -78 relative to the start site of the dominant transcript. Any change in the

AGATT motif was associated with a complete null phenotype while deletions in the string of adenine residues were associated with both a decreased-production phenotype and a complete null phenotype (236). What makes the *uspA2* gene different from all of the genes described above is that its tetranucleotide repeat (AGAT) is located downstream of the transcriptional site (i.e., it is included within the *uspA2* mRNA).

Exactly how these AGAT repeats are regulating transcription of the *uspA2* gene is not clear at this point. However, it has been shown that the changes in TAAA repeats upstream of the *nadA* gene of *N. meningitidis* affect the binding of the transcriptional regulatory protein IHF to the *nadA* promoter (179). For the other examples where the nucleotide repeat is located between the -35 and -10 promoters regions, the changes in the length of the repeat tract were suggested to affect the binding of the RNA polymerase to the promoter region (257,313). The fact that the AGAT repeats are located within the *uspA2* mRNA raises the possibility of the binding of a transcriptional regulator yet to be identified.

Another interesting finding obtained from the experiments described in this chapter is the evidence that a threshold level of the UspA2 protein is required to confer serum resistance on *M. catarrhalis*. It was found that *M. catarrhalis* strain O12E needs at least 9 AGAT repeats in the 5′-UTR of its *uspA2* gene to express a level of UspA2 protein that can effectively protect this strain from killing by 30% NHS (Fig. 37). Interestingly, among the *M. catarrhalis* strains surveyed in this study, the lowest number of AGAT repeats found in a serum-resistant strain was 12, while lower numbers were associated with serum-sensitive ones. More specifically, 6 repeats were found in strain ATCC43617, which has a premature stop codon within its *uspA2* ORF, and 8 repeats were found in strain MC317 which expresses a UspA2 protein which is incapable of conferring serum resistance on *M. catarrhalis* (13). However, with the MC317 strain, serum sensitivity was not the result of an

insufficient amount of UspA2 protein (13). These findings might suggest that, in vivo, serum-resistant *M. catarrhalis* strains need to maintain a certain number of AGAT repeats to synthesize the threshold level of the UspA2 protein necessary to evade the bactericidal activity of NHS. The lack of a relevant animal model for use with *M. catarrhalis* (156) precludes direct investigation of this process at the experimental level at this time.

CHAPTER EIGHT

Summary and Conclusions

Within the last three decades, *M. catarrhalis* has established its reputation as a pathogen that is capable of causing disease in both infants and adults (156). One of the phenotypic characteristics of *M. catarrhalis* that had been correlated with its pathogenicity and was considered a virulence factor is the ability of many strains of this species to resist complement-mediated killing when incubated with NHS (129). The main focus of this dissertation was investigating how serum-resistant *M. catarrhalis* strains evade the bactericidal activity of NHS and the role of the UpsA2 protein in this crucial phenotype.

Prior to this study, several *M. catarrhalis* gene products had been linked to the serum-resistant phenotype (3,4,171,226). The common feature of these gene products is that mutants unable to express these products are more susceptible to killing by NHS when compared to the respective wild-type parent strains. However, no direct link has been previously established between any of these gene products and the serum-resistant phenotype. The research contained in this dissertation provided, for the first time, a direct link between the UspA2 protein of *M. catarrhalis* and serum resistance. UspA2, which is a putative autotransporter and forms a dense layer of short projections on the surface of *M. catarrhalis* (125,225), was capable of conferring serum resistance on *H. influenzae* DB117 when it was cloned and expressed in this heterologous background. This was true when the gene encoding the UspA2 protein was derived from a serum-resistant strain (i.e., O35E), whereas when the source of the recombinant UspA2 was a serum-sensitive *M. catarrhalis* strain (i.e., MC317), the recombinant protein did not confer serum resistance on *H. influenzae*.

Further characterization of the UspA2 proteins of both of these strains (i.e., O35E and MC317) and the construction of a series of hybrid *uspA2* genes that contained different proportions of the *uspA2* genes of both O35E and MC317 showed that the N-terminal half of the O35E protein contained a 102 aa region that was involved in the expression of serum resistance. This was confirmed by the experiment in which the region of the O35E UspA2 protein between aa 143-244 was sufficient to convert the serum-sensitive strain MC317 to a serum-resistant one when expressed in the equivalent position within the MC317 UspA2 protein. The exact role of this region of the O35E UspA2 protein in the serum-resistant phenotype still has not been addressed, but represents a target for investigation in the future.

The data described in the two preceding paragraphs have laid the groundwork for new experiments that would increase our understanding of the role of the UspA2 protein in serum resistance of *M. catarrhalis*. For example, it is possible to use software systems that are designed to predict secondary and tertiary protein structures and compare the predicted structures of the UspA2 proteins from both MC317 and O35E. This approach will determine if there are differences in the overall structure of these two proteins on the surface of *M. catarrhalis*, since the indirect antibody-accessibility experiments described in Chapter Four indicated that the MC317 UspA2 protein was slightly less abundant than that of O35E. What this experiment did not address is whether this difference was due to the expression of less UspA2 protein by MC317 or due to less accessibility of its epitope to MAb 17C7, possibly caused by a difference in the conformation of the proteins. Therefore, understanding the overall structure of these two proteins might help address this point.

Another set of experiments is the isolation and the purification of the UspA2 proteins from both strains. This can be done either in native form (i.e., using *M. catarrhalis*) or in recombinant form (i.e., using *E. coli* or *H. influenzae*). I would prefer to use the native form, but in case it did not

yield an acceptable quality and/or purity, the recombinant form would also be useful. Other labs have successfully expressed recombinant UspA2 proteins in *E. coli* (216,277) after omitting the signal peptide in the N-terminal end and excluding the very C-terminal end that is supposedly involved in the formation of the barrel structure involved in getting the UspA2 protein out onto the surface of *M. catarrhalis*. It is noteworthy that we have a preparation of purified native UspA2 from strain O35E (a generous gift from Dr. Bruce Green at Wyeth-Lederle Vaccines, West Henrietta, NY). However, careful examination of this preparation showed that it is contaminated with UspA1. Therefore, future experiments to purify UspA2 should be carried out in an UspA1-null mutant to avoid this problem. The purified UspA2 preparations would be useful for several purposes. For example, they can be used for crystallization to elucidate the structure of the UspA2 protein and determine the potential differences between the UspA2 proteins of O35E and MC317. The purified preparations can be used as binding baits in immunoprecipitation reactions to "pull down" complement regulators from NHS that UspA2 might be binding to help protect *M. catarrhalis* from killing by complement. Furthermore, it would be extremely useful to obtain a group of fusion proteins [i.e., glutathione or histidine fusions] using different fragments of the UspA2 protein of strain O35E. These fusion proteins could also be used in immunoprecipitation experiments and/or the generation of monoclonal antibodies that can be used in serum bactericidal inhibition assays to determine if any of these MAbs is capable of blocking the ability of the UspA2 protein to protect against complement-mediated killing.

Concerning the 102 aa region of the O35E UspA2 protein, the same sort of experiments described above (i.e., peptide purification and MAb generation) would be useful to better understand the role of this fragment in serum resistance. An initial experiment that should be done is the deletion of this region from the UspA2 protein in *M. catarrhalis* strain O35E. There would be two possible outcomes in this experiment. First, there could be no effect of the deletion of this region on

the serum-resistant phenotype of strain O35E. This would mean either that (1) the results obtained with hybrid 6 (the MC317 strain containing this region from O35E within the equivalent position within the MC317 UspA2) were an artifact and the acquisition of serum-resistance was due to another unidentified change within this strain, or (2) this region is not directly involved in the serum-resistant phenotype, but instead affects the overall structure of the UspA2 protein, allowing it to carry out its function. In this latter scenario, this structural modulatory role was essential to allow the MC317 UspA2 protein to confer serum resistance, whereas in O35E this role is not so critical because the serum-resistant phenotype can be still mediated through other regions of the UspA2 protein. A second possible outcome would be that the deletion of this region from the O35E UspA2 protein would lead to complete loss of the serum-resistant phenotype of this strain and this would either mean that the overall structure of the UspA2 protein had been affected, leading to loss of function, or that this region is directly involved in binding of a complement regulator.

Upon deletion of the entire *uspA2* gene from strain O35E, there were no changes between this deletion mutant and the wild-type strain in growth rate, OMP profiles and LOS patterns. Despite the fact that the UspA2 is constitutively expressed on the surface of *M. catarrhalis*, forming a dense layer of short projections, complete elimination of this layer did not detectably affect any of the phenotypes mentioned above. It was expected that such a disruption of the cell surface would result in obvious changes in the phenotype of the whole cell. One explanation for this is that elimination of the UspA2 protein causes an up-regulation of another gene product that stabilized the outer membrane of *M. catarrhalis* without affecting the features tested in this study. One tool for identifying such a macromolecule is DNA microarrays which can be used to compare gene expression by the wild-type strain and the *uspA2* deletion mutant. Also, 2D-gel analysis is another tool that can be used for this purpose.

Different bacterial species employ a wide array of mechanisms to either evade killing by the complement system (i.e., gram-negative bacteria) or evade being opsonized by complement to avoid being phagocytosed (i.e., gram-positive bacteria). These mechanisms range from using surface structures such as LPS and capsules to physically interfere with the complement cascade to the more widespread mechanism of recruiting fluid-phase regulatory proteins of the complement system to block or inactivate the complement system at various stages (30,245).

Prior to this dissertation, little was known about the mechanism(s) through which *M. catarrhalis* resists complement-mediated killing. Some studies proposed potential mechanisms but they generally lacked detailed and rigorous examination of the postulated hypotheses (185,216,217,293). The experiments described in this dissertation provide a detailed analysis of the direct interaction of different *M. catarrhalis* strains with the various components of the complement system. First, it was shown that the serum-sensitive wild-type strain (MC317) and *uspA2* mutants of four serum-resistant *M. catarrhalis* strains are killed via the classical pathway of complement activation. Second, complement deposition analysis showed that serum-resistant wild-type strains differ from their serum-sensitive *uspA2* mutants in the late stages of the complement cascade, more specifically in the stage of C9 polymerization. Third, the ability of serum-resistant *M. catarrhalis* strains to bind vitronectin, a complement regulator affecting C9 polymerization, through their UspA2 proteins was shown to be responsible, at least in part, for the protection of these strains against the bactericidal activity of NHS. Further experiments showed that serum-resistant *M. catarrhalis* strains differed markedly in their binding of the complement regulator C4BP, both in the amount of C4BP bound and in the binding moiety. These binding activities could be detected using purified C4BP, but when these *M. catarrhalis* strains were tested for their binding of this regulator in the context of NHS, C4BP binding was very minimal and seemed to have no functional activity in protecting serum-resistant *M. catarrhalis* strains against complement-mediated killing.

As mentioned in the preceding paragraph, vitronectin binding to *M. catarrhalis* through UspA2 only partially protects this bacterium against complement-mediated killing. This was concluded from the observation that resistance of the wild-type *M. catarrhalis* strains O35E, O12E, and 7169 to killing by NHS was not completely eliminated by vitronectin depletion. Also, it must be noted that vitronectin binding was apparently not involved in the serum-resistant phenotype of *M. catarrhalis* strain FIN2344. These data indicated that there must be an additional mechanism(s) through which *M. catarrhalis* evades the complement system. This additional mechanism(s) could involve direct interference with the complement cascade or binding of other regulators to indirectly block complement activation. This is an area for future research that would lead to a better understanding of the virulence of *M. catarrhalis*, especially considering that previous examples in the literature showed that other bacteria, such as *N. gonorrhoeae*, use multiple mechanisms to evade killing by complement (240,242).

There are experiments that need to be done to obtain a better understanding of the interaction of UspA2 with vitronectin. For example, it is important to determine where in the UspA2 protein vitronectin is binding and, at the same time, where within the vitronectin protein the UspA2 protein is binding. For the first part (i.e., identification of the vitronectin-binding domain within UspA2), the fusion proteins described above would be very useful to localize a vitronectin-binding domain within the O35E, O12E, or 7169 UspA2 proteins. However, we must remember that the interaction might be occurring via a conformational epitope rather than a linear one. In this case, the use of the fusion proteins might not identify the vitronectin-binding domain within the UspA2 protein. On the other hand, for determining the binding domain for the UspA2 protein within vitronectin, human vitronectin fusion proteins are now available (personal communication, S. Ram, Boston University). These constructs can be used in binding assays and/or "pull-down" experiments. Also, it would be

useful to obtain a group of MAbs against the different domains of both UspA2 and vitronectin for use in serum bactericidal inhibition assays to see if any of them would block the binding of UspA2 to vitronectin.

Preliminary experiments using strain MC317 and hybrid 6 showed that both strains bound vitronectin to an extent comparable to that of O35E (data not shown). These experiments need to be repeated; however, if these results are correct, this would argue that the 102 aa region of the serum-resistant *M. catarrhalis* strain O35E UspA2 is not the binding site for vitronectin. However, it might be affecting the overall structure of the UspA2 protein and its ability to interact with vitronectin. Alternatively, it could be mediating its effect on serum resistance through binding of another complement regulator that is responsible for the incomplete serum-sensitivity of strain O35E observed with the Vn-depleted serum.

The mechanism by which *M. catarrhalis* strain FIN2344 resists killing by NHS remains a challenging question. Comparison of the primary aa sequence of the UspA2 proteins from O35E, O12E, 7169, and that of FIN2344 did not show obvious differences between the first three proteins and the latter one that could account for their different serum resistance phenotypes. Protein-modeling software could be used to elucidate possible differences in the overall structures of these four UspA2 proteins. Another approach is to compare complement deposition on both the FIN2344 wild-type strain and its *uspA2* deletion mutant using a more quantitative assay (for example, flow cytometric analysis) instead of Western blots. This might show that the difference between the two strains may have occurred as early as the C1 stage, which would implicate C1-INH as a potential regulator that this strain recruits to evade complement-mediated killing. Alternatively, strain FIN2344 could be binding clusterin or CD59 to interfere with the late stages of the complement pathway. There is only a very slim possibility that strain FIN2344 is binding fH, which is a regulator

of the alternative pathway, because the experiments using different types of human sera indicated that the alternative pathway is most likely not involved in the killing of FIN2344Δ2. However, perhaps the differences seen in the level of C3 binding might have been caused by binding of the regulator fH; therefore this potential binding activity needs to be investigated. Another explanation for the serum-resistant phenotype of strain FIN2344 would be physical interference of the UspA2 protein of this strain with one or more components of the complement system; protein modeling efforts might help in investigating this possibility.

The last group of experiments described in this dissertation focused on an important aspect of the *uspA2* gene which is the regulatory role of the AGAT nucleotide repeats that are located in the 5′-UTR of this gene. Prior to this dissertation, virtually nothing was known about the regulation of this gene. The data presented here showed that the AGAT repeat region constitutes a part of the *uspA2* mRNA and that it is essential for the normal expression of the UspA2 protein and, consequently, the serum-resistant phenotype. Also, it was shown that changes in the number of these repeats affect the level of the transcription of the *uspA2* gene, with the maximum level of *uspA2* transcripts being obtained with 15-18 repeats, although serum resistance achieved wild-type levels in the presence of 9 repeats. However, what remains to be determined is exactly how these AGAT repeats perform this regulatory role.

A useful tool for identifying this mechanism is the reporter strain O12E-23*lacZ*. This strain represents the first *M. catarrhalis* strain that expresses a single copy of the *uspA2-lacZ* reporter system from the chromosome. One approach to identify a possible regulatory mechanism is to use this reporter strain in random transposon mutagenesis experiments, looking for potential regulator(s) that might be regulating the *uspA2* gene through interaction with the AGAT repeats. However, one obstacle is the fact that most *M. catarrhalis* strains are resistant to transposon mutagenesis. Strain

O35E is the only strain that has been shown to be susceptible to transposon mutagenesis. Therefore, I could either transform the reporter system into the O35E background or perform the mutagenesis on O35E and use the mutagenized O35E chromosomal DNA to transform the O12E strain that contains the reporter system. Positive transformants (i.e., those with altered levels of *lacZ* expression) could then be screened to identify potential regulator(s) that might be affecting the expression of the UspA2 protein by interaction with the AGAT repeat region.

Another approach for identifying a possible regulator of the UspA2 protein is the use of DNA fragments with different numbers of AGAT repeats (for example 0 and 18 repeats) in DNA-binding protein "pull-down" assays to identify *M. catarrhalis* proteins that might be binding to this region. The transcriptional regulatory protein IHF has already been shown to be affected by changes in the number of nucleotide repeats in the *nadA* promoter in *N. meningitidis* (179). Alternatively, DNA microarrays and 2D-gels can be used to compare the transcriptomes and the proteomes, respectively, of the O12EΔAGAT strain with those of the O12E-18rpts strain and identify those proteins that might have been induced by the presence or the absence of this repeat region, keeping in mind that the changes in some of these genes or proteins might have been the result of changes in the level of the UspA2 protein affecting the integrity of the outer membrane.

Another mechanism of action of these repeats is that the number of AGAT repeats might affect the secondary structure of the mRNA in this region, leading to an effect on the stability of the transcript or the binding of the transcriptional machinery to the *uspA2* gene. Investigating these possibilities can be carried out using RNA-structure-predicting software or data bases to see what effect changes in the number of the AGAT repeats have on the overall structure of the promoter region of the *uspA2* gene. To determine the effect of the AGAT repeats on the stability of the *uspA2* transcript, S1 nuclease protection assays can be done after rifampin treatment of the *M. catarrhalis*

cells to stop de novo transcription. To look for a possible effect of changes in the number of repeats on optimal binding of transcriptional machinery, DNA-binding experiments can be done using purified components of the transcriptional machinery and analyzed using electrophoretic mobility shift assays (EMSA).

It is also important, as a of proof-of-principle, to see if the changes of the number of AGAT repeats would affect the amount of vitronectin bound to the UspA2 protein and, consequently, the amount of polymerized C9 associated with the bacterial cells when incubated in NHS. This experiment can then be followed by assays to determine the approximate number of the UspA2 protein molecules per *M. catarrhalis* cell together with the number of vitronectin molecules bound to each cell. This would help us to better understand the dynamics and kinetics of the UspA2-vitronectin interaction and should lead to the determination of the minimum number of the UspA2/vitronectin molecules that are required to protect *M. catarrhalis* against complement-mediated killing.

Finally, the observations that there is a threshold level of the UspA2 protein that is required to attain full serum resistance and that this level was achieved in the presence of 9 AGAT repeats in the 5′-UTR of the *uspA2* gene can be further extended by performing two sets of experiments. First, serial passage of the constructs with a small number of AGAT repeats (for example 2 or 6) in low concentrations of NHS might lead to the selection of serum-resistant variants that have increased the number of AGAT repeats to the "threshold" level of 9 repeats. The second approach would require a relevant animal model. This would involve intranasal inoculation of the animals with a *M. catarrhalis* construct containing a small number of AGAT repeats. After successful colonization, colonizing bacteria could be recovered from the nasopharynx and screened for an increase in the number of AGAT repeats.

In conclusion, this dissertation has generated a significant amount of new information that had added to our knowledge about an important surface antigen of *M. catarrhalis*. These data hopefully will lead to a better understanding of the pathogenesis of *M. catarrhalis* disease and, together with the development of a relevant animal model, could lead to better means of both treatment and prevention of diseases caused by this microorganism.

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VITAE

Ahmed Sherif Attia was born in Cairo, Egypt, on December 13, 1974, the son of Mohamed H. Attia and Mona A. Selim. After completing his work at Yehia Al-Rafee Language School, Cairo, Egypt in 1992, he entered Cairo University, Faculty of Pharmacy, Cairo, Egypt. He received the degree of Bachelor in Pharmaceutical Sciences in June, 1997. He was then appointed as a teaching assistant in the Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt. He received a M.S. degree in Microbiology in 2001. In August 2001, he entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center, Dallas, Texas. In February 1999, he married Eman Badr of Giza, Egypt. His daughter Mariam was born on December 13, 1999 and his son, Mostafa, was born on June 26, 2002.

Permanent Address: 1515 Rio Grande Dr #710
Plano, TX, 75075

8 Nabil El-Wakad #13
Ard El-Golf, Heliopilis, Cairo, Egypt