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# Evasion of host recognition by phase variation in Haemophilus influenzae

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**Abstract**
*Haemophilus influenzae* colonizes the human respiratory tract, and is a major source of disease. Antibody and complement contribute to the limitation of *H. influenzae* colonization. This work explores bacterial factors that aid in the evasion of antibody recognition and subsequent complement-mediated lysis. Antibody recognition of *H. influenzae* is affected by the phase variation of lipopolysaccharide (LPS) structures. Phase variation refers to the stochastic, high frequency on/off switching in gene expression. One phase variable gene, *lic1A*, controls the attachment of the small molecule phosphorylcholine (ChoP) to the LPS. We found that ChoP-expressing bacteria had reduced antibody binding and increased survival in the presence of complement. Also, ChoP attachment to the LPS increased the stability of the outer membrane, reducing accessibility to host molecules including antibodies. Next, we conducted a screen of genes required for complement resistance in *H. influenzae*. We found that several LPS phase variable biosynthesis genes are critical for survival in the presence of human antibody and complement. In addition to these, we identified *vacJ* and the associated *yrb* genes, which encode an ABC transporter that increases membrane stability and reduces antibody binding. These findings present two different examples of how alterations in outer membrane stability can affect antibody binding to bacterial surface structures. Finally, we examined the dynamics of the contributions of multiple phase variable LPS biosynthesis genes to bacterial survival. We found that exposure to antibody and complement drives selection for phase variants expressing ChoP, di-galactoside (galα1-4gal), and an alternative glucose structure. Each of these LPS structures, in addition to the phase variable molecule sialic acid, had an independent effect on bacterial survival, and these effects were additive in combination. Bacteria with the maximum number of LPS modifications had the greatest survival, and this correlated with reduced recognition of conserved inner core LPS structures. In summary, LPS phase variation in *H. influenzae* contributes to bacterial evasion of antibody binding and complement-mediated killing. The expression of several LPS phase variable modifications shields conserved surface structures on *H. influenzae* from host recognition to contribute to the successful colonization of this extracellular pathogen.

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# EVASION OF HOST RECOGNITION BY PHASE VARIATION IN *HAEMOPHILUS INFLUENZAE*

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## ABSTRACT

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*Haemophilus influenzae* colonizes the human respiratory tract, and is a major source of disease. Antibody and complement contribute to the limitation of *H. influenzae* colonization. This work explores bacterial factors that aid in the evasion of antibody recognition and subsequent complement-mediated lysis. Antibody recognition of *H. influenzae* is affected by the phase variation of lipopolysaccharide (LPS) structures. Phase variation refers to the stochastic, high frequency on/off switching in gene expression. One phase variable gene, *lic1A*, controls the attachment of the small molecule phosphorylcholine (ChoP) to the LPS. We found that ChoP-expressing bacteria had reduced antibody binding and increased survival in the presence of complement. Also, ChoP attachment to the LPS increased the stability of the outer membrane, reducing accessibility to host molecules including antibodies. Next, we conducted a screen of genes required for complement resistance in *H. influenzae*. We found that several LPS phase variable biosynthesis genes are critical for survival in the presence of human antibody and complement. In addition to these, we identified *vacJ* and the associated *yrb* genes, which encode an ABC transporter that increases membrane stability and reduces antibody binding. These findings present two different examples of how alterations in outer membrane stability can affect

antibody binding to bacterial surface structures. Finally, we examined the dynamics of the contributions of multiple phase variable LPS biosynthesis genes to bacterial survival. We found that exposure to antibody and complement drives selection for phase variants expressing ChoP, di-galactoside (gal$\alpha$1-4gal), and an alternative glucose structure. Each of these LPS structures, in addition to the phase variable molecule sialic acid, had an independent effect on bacterial survival, and these effects were additive in combination. Bacteria with the maximum number of LPS modifications had the greatest survival, and this correlated with reduced recognition of conserved inner core LPS structures. In summary, LPS phase variation in *H. influenzae* contributes to bacterial evasion of antibody binding and complement-mediated killing. The expression of several LPS phase variable modifications shields conserved surface structures on *H. influenzae* from host recognition to contribute to the successful colonization of this extracellular pathogen.

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# CHAPTER 1

## Introduction, Part 1: *Haemophilus influenzae* colonization and the role of oligosaccharide phase variation

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## *H. influenzae* colonization and disease

*Haemophilus influenzae* is a gram-negative coccobacillus that colonizes the human respiratory tract. *H. influenzae* was first described by Richard Pfeiffer, working with Shibasaburo Kitasato under Robert Koch in 1892 (380). At the time, Pfeiffer and his colleagues identified what they called *Bacillus influenzae* from patients with influenza. While *H. influenzae* was initially described as the causative agent of influenza, this theory was disproved by filtration of infectious material from influenza patients during the 1918 pandemic. Since then, it has become clear that *H. influenzae* is an important human pathogen in its own right, and we continue to investigate both asymptomatic colonization and disease caused by this bacterium. The name Haemophilus, or 'heme-loving,' is derived from the nutrient requirements of *H. influenzae*, which is dependent on an environmental source of hemin, as well as nicotinamide adenine dinucleotide (NAD), for growth (87). Humans are the only known reservoir and natural host for *H. influenzae*, and it is therefore important to understand the associations between this bacterium and its native environment, the human nasopharynx.

Colonization with *H. influenzae* occurs early in life, often within the first few months of age, and carriage rates are highest (up to 80%) in infants and children under six years old (89, 113). While individual colonization events are transient, lasting weeks to a few months, exposure to other *H. influenzae* strains in the population can lead to serial colonization. A study comparing healthy patients to those with *H. influenzae* bronchiectasis found that all subjects, including healthy patients, had detectable levels of antibody reactive against several different *H.*

*influenzae* strains, supporting the notion that most people have been exposed to *H. influenzae* by adulthood (201). Transient, asymptomatic colonization can be studied using the murine nasopharyngeal model, where bacteria are introduced intranasally without anesthetics. This model has been used to study the host immune factors that are important for limiting bacterial colonization (440). An experimental human colonization model was recently developed by M. Apicella and colleagues at the University of Iowa, where healthy volunteers were intranasally inoculated with *H. influenzae*, and following six days of colonization all subjects were treated with antibiotics to clear remaining bacteria (303). Study of the commensal state of *H. influenzae* is important to better understand the subsequent processes of disease development and transmission, which are discussed below. Limitation of *H. influenzae* carriage is a critical step toward reducing *H. influenzae*-associated disease.

Infection with *H. influenzae* can lead to invasive diseases including septicemia and meningitis, as well as more localized respiratory tract infections (272, 382). *H. influenzae* is one of the primary pathogens associated with otitis media, which is especially common in children, as well as pneumonia and chronic bronchitis (14, 275). *H. influenzae* is also the bacterial agent most frequently associated with exacerbations of chronic obstructive pulmonary disease (COPD), which is currently the third leading cause of death worldwide (271, 296). Antimicrobials are commonly used to treat patients with *H. influenzae* infections, although many strains carry antibiotic resistance determinants, such as β-lactamase, or express mutant penicillin binding proteins, making bacterial

clearance more difficult (200, 259, 337). It has been suggested that *H. influenzae* can ‘hide’ inside epithelial cells, reducing antibiotic accessibility (14). However, while there is evidence for metabolic activity in intracellular *H. influenzae*, these bacteria do not grow, and there is no evidence for continued replication following cell invasion (258).

There are several studies supporting the proposition that colonization with *H. influenzae* is directly linked to the development of *H. influenzae*-associated disease. For example, a study following infections in COPD patients over time demonstrated that colonization with *H. influenzae* correlated with the timing of disease exacerbations as well as markers of inflammation (296). Also, in children with recurrent otitis media, the frequency of *H. influenzae* colonization matched the frequency of otitis media events (148). For the development of otitis media, it has been suggested that *H. influenzae* spreads to the middle ear through the Eustachian tube, which is shorter in young children (325). An infant rat model of invasive infection showed evidence for direct spread of *H. influenzae* from the nasopharynx to the bloodstream, which was independent of regional lymph nodes (326). Colonization with *H. influenzae* is also thought to be critical for transmission to new hosts. In a study of strain diversity in children, caregivers were colonized by the same *H. influenzae* isolates as the children they cared for (337). Also, high levels of *H. influenzae* DNA can be isolated from both middle ear effusion samples and, importantly for transmission, nasopharyngeal samples (390). These studies highlight the importance of considering both asymptomatic

and disease-related states of *H. influenzae* infection, as the two are intricately linked.

Different types of *H. influenzae* strains are associated with the development of separate disease states: invasive disease versus more localized respiratory tract infections. The separation between these manifestations of *H. influenzae* infections is due to the presence and type of capsule, which is a thick polysaccharide that surrounds the outer membrane of encapsulated strains (57). *H. influenzae* strains with a type-b capsule (Hib) are the most virulent compared to other capsule types, and Hib infection can lead to septicemia and meningitis (10, 441). The advent of conjugate vaccines for Hib, the first of which was licensed in 1987, has led to a dramatic reduction in invasive *H. influenzae* disease (2). Hib vaccines involve conjugation of the type-b polyribosyl ribitol phosphate (PRP) capsule to a protein carrier (61, 119). Conjugation to a protein carrier is necessary because capsule polysaccharide is a T-cell independent antigen, and therefore will not stimulate protective immunity (with memory) in infants (192, 306). The Hib vaccine is highly effective at preventing Hib disease (over 80%), induces long-term antibodies capable of recognizing Hib, and reduces carriage of Hib (119, 233, 260). *H. influenzae* strains without capsule are called non-typeable *H. influenzae* (NTHi), and these are most frequently associated with respiratory tract infections (275). There are other capsule types, from type-a through type-f, although carriage and infections from these are less frequent than for Hib and NTHi strains (156). Of note, the avirulent strain Rd is a capsule-negative derivative of a type-d strain, and is a commonly used laboratory

strain. Rd was the first organism to have its genome fully sequenced, and is very amenable to transformation (102). NTHi strains contain abundant intra- and interstrain diversity, and are genetically distinct from Hib and other encapsulated strains (86, 280, 337).

The burden of disease caused by NTHi strains has necessitated the development of vaccine strategies to target these bacteria. In the absence of a capsule, alternative vaccination approaches are required for NTHi strains. Several methods have been explored, including vaccination with outer membrane vesicles, conjugation of the lipopolysaccharide (LPS) to a protein carrier, and immunization with purified, conserved outer membrane proteins or LPS structures (140, 304, 321, 379). There is one currently licensed vaccine with efficacy against NTHi disease. This vaccine was developed to protect against *Streptococcus pneumoniae* strains, with 10 *S. pneumoniae* capsule types conjugated to protein D, which is a conserved outer membrane protein in *H. influenzae* (105). In addition to protecting against *S. pneumoniae*-associated disease, the protein D conjugate vaccine has 33.5% efficacy for protection against NTHi acute otitis media (307). However, the protein D vaccine does not prevent NTHi colonization (395). Despite the limited efficacy of the protein D vaccine, its ability to reduce NTHi disease serves as a proof-of-principle for the potential to protect against infection through vaccination targeting conserved surface structures. The inclusion of additional targets, or alternative outer membrane structures such as the LPS, are some of the strategies currently being

explored to develop a more successful NTHi vaccine to prevent respiratory tract infections associated with *H. influenzae* infection.

## Host defenses against *H. influenzae* in the respiratory tract

### *Mucociliary clearance*

Successful colonization of the human respiratory tract requires multiple strategies for the subversion of host defenses at the mucosal surface. One of the first challenges is simply remaining at the site of colonization; airway epithelial cells provide the means to literally sweep away a large number of microbes through mucociliary clearance (424). The thick mucus layer present throughout the respiratory tract is the first barrier against infection. Also, exposure to gram-negative bacteria can cause the up-regulation of genes responsible for mucin production (76). To circumvent mucociliary clearance, many microbes have adhesive molecules designed to bind (non-specifically or through direct receptor-ligand interactions) to the host epithelium (21). *H. influenzae* strains express several adhesive proteins, including the HMW1 and HMW2 adhesins, the Hap autotransporter adhesin, protein E, the fimbriae protein P5, and pili (96, 125, 187, 251, 323, 356). Carriage of these adhesins varies between different strains of *H. influenzae*, and some, including the HMW1 and HMW2 adhesins, are only found in NTHi isolates (79). Some adhesins have dynamic interactions with their receptors. For example, P5 binding to one of its epithelial cell receptors, intracellular adhesion molecule 1 (ICAM-1), up-regulates receptor expression

(11). Adhesins can also have dual roles, such as the Hap adhesin, which increases binding to respiratory tract epithelial cells and contains a domain that enables bacterial cell-cell adhesion (249). The aggregation of receptor-bound bacteria has been proposed to be important for the development of microcolonies, which could aid bacterial persistence in the respiratory tract. Multiple adhesins, with different host receptor targets, contribute to the ability of *H. influenzae* to associate with the respiratory tract surface and avoid mucociliary clearance.

*Antimicrobial peptides*

In addition to mucus itself, the respiratory tract contains a number of antimicrobial peptides, both secreted by epithelial cells and present within phagocytic immune cells such as neutrophils (420). Antimicrobial peptides with bactericidal activity have been isolated from human nasal tissue, nasal secretions, and lung airway fluid (56, 229, 355). Members of the two main antimicrobial peptide classes, defensins and cathelicidins, have lytic activity against *H. influenzae* strains (214, 229). Many antimicrobial peptides are positively charged, as their mechanism of action involves binding to and invasion of the negatively charged bacterial cell envelope. A study comparing the binding properties of different antimicrobial peptides found that all peptides that could bind NTHi strains contained positively charged residues (27). Bacterial strategies for antimicrobial resistance, therefore, include protease digestion as well as membrane modifications that reduce the

negative charge of the bacterial surface (75). Examples of membrane charge modifications include the addition of lysine to the cell wall of *Staphylococcus aureus*, and the attachment of aminoarabinose to the lipid A portion of the LPS of *Salmonella enterica*, which both reduce the negative surface charge and increase antimicrobial peptide resistance (142, 300). In *H. influenzae*, an alternative strategy to evade killing by defensins is to import them to the cytoplasm for degradation, through the Sap ABC transporter (242, 349). Antimicrobial peptides can also affect the immune response against invading microbes indirectly. For example, the human cathelicidin LL-37 can down-regulate macrophage activity, and other antimicrobial peptides affect recruitment of neutrophils and T cells (147, 341).

*Cell-mediated immunity*

In addition to cell-free antimicrobial factors, cell-mediated immune responses limit bacterial colonization of the respiratory tract. Alveolar macrophages are the first line of cellular immune defense encountered by microbes at the mucosal surface, and are capable of killing *H. influenzae* (350). Alveolar macrophages from COPD patients have impaired phagocytosis of NTHi strains, suggesting the reduced function of this cell type contributes to *H. influenzae* disease (25). The other major cell type involved in airway immunity is the neutrophil. While neutrophils are initially present in low numbers in respiratory tract tissue, they are recruited to high levels from the respiratory tract vasculature following infection

(439). It has been shown in a murine model of nasopharyngeal colonization that neutrophils help reduce *H. influenzae* colonization (440). Neutrophil recruitment and pro-inflammatory responses induced by *H. influenzae* are initiated in part by the toll-like receptor TLR4, which binds LPS, on epithelial cells and alveolar macrophages (406). TLR4 and TLR2, which recognizes lipoproteins, contribute to the stimulation of a pro-inflammatory immune response and limit *H. influenzae* colonization (22). Other immune cell types, including T and B cell subtypes, are important for the development of protective immunity against *H. influenzae*, such as that observed in patients who have received the Hib conjugate vaccine (192). B cells secrete antibody, establish immunological memory, and undergo affinity maturation to make more antigen-specific antibodies (265). T cells help activate B cells to produce antibody and stimulate phagocytic cells (265). Also, Th17 cells can activate neutrophil recruitment, and were shown to be important in an acute pneumonia model of *S. pneumoniae* infection (205, 237). While it has been shown that adaptive immune cell types are not required for initial recognition and clearance of *H. influenzae* in the naïve host, cell-mediated immune responses may be more important during disease development (108, 440).

*Complement defenses*

In addition to cell-mediated immune clearance, *H. influenzae* is susceptible to direct complement-dependent lysis. There are three main pathways of complement-mediated killing of bacterial pathogens (summarized in Figure 1)

(403). The classical complement pathway involves antibody binding to the bacterial surface, followed by the deposition of complement proteins that form the C1 complex (containing the C1q, C1r, C1s proteins), which cleaves C4 and C2 to form the classical pathway C3 convertase (a C4b2a complex). The mannose-binding lectin complement pathway is initiated by mannose-binding lectin (MBL) and MBL associated proteases binding to the bacterial surface, which leads to the formation of the lectin pathway C3 convertase (a C4b2a complex). It is unclear how much the mannose-binding lectin pathway contributes to control of *H. influenzae* infection, as MBL binding has been observed in very few *H. influenzae* strains (283, 347). Finally, the alternative complement pathway results from the direct binding of C3b (from spontaneous C3 cleavage) to the bacterial surface, which then binds factor B. Factor B is cleaved by factor D to form the alternative pathway C3 convertase (a C3bBb complex). The three complement pathways converge at the level of the formation of a C3 convertase, which cleaves C3 to C3a and C3b. The C3b cleavage fragments bind to the bacterial surface and initiate the formation of the membrane attack complex (MAC) (403). The MAC, which is assembled after cleavage of C5 to make C5a and C5b, contains the complement components C5b, C6, C7, C8, and C9, and together these proteins insert into the bacterial membrane to form a pore, resulting in cell lysis. In addition to direct cell lysis, complement is important for opsonophagocytosis, as C3b deposition acts as an opsonin to increase bacterial recognition and uptake (288). Also, circulating complement cleavage products including C3a and C5a are pro-inflammatory (352).

Several host proteins can interact with components of the complement pathway and affect their activity. For example, C-reactive protein can bind bacteria and initiate classical pathway complement-mediated lysis (in place of antibody binding) (132). Other host proteins, discussed below, can bind the bacterial surface and reduce sensitivity to complement-mediated lysis. The complement system is incredibly complex, involving over 30 proteins, and is a key component of the host immune response to *H. influenzae* infection. Complement proteins are present on the mucosal surface, and levels increase significantly during inflammation (146). Complement depletion experiments in animal models have demonstrated the importance of complement in reducing *H. influenzae* colonization and invasive disease (440, 442). These data are consistent with clinical observations, as people with deficiencies in the activation and regulation of complement have recurrent infections with bacteria including *H. influenzae* (78, 301).

Different strains of *H. influenzae* have varying levels of complement susceptibility based on their repertoire of complement-resistance determinants (423). For example, different capsular types of *H. influenzae* have separate complement resistance profiles (371, 373). Within the same capsular type, duplication of the capsule-encoding locus, which increases the amount of capsule expressed, reduces deposition of C3 and increases survival in the presence of complement (287). For NTHi strains, one example of a complement-resistance mechanism is binding to host C4b-binding protein (C4BP), which reduces susceptibility to classical pathway complement-mediated lysis through

degradation of C3b and C4b (145). While the bacterial receptor is unknown, C4BP binding has been observed for several clinical NTHi isolates (compared to the absence of binding to encapsulated strains). *H. influenzae* protein E binds the host protein vitronectin, which protects against complement-mediated lysis by reducing deposition of MAC proteins on the bacterial surface (354). Also, some NTHi strains bind serum amyloid protein (SAP), which reduces C1q deposition, increasing resistance to classical pathway complement-mediated lysis (70). Other bacteria use similar strategies for the reduction of complement-mediated lysis. For example, sialic acid attached to the outer membrane can bind the host protein factor H, which promotes inactivation of the C3 convertase and C3b proteins, although this does not occur following sialylation in *H. influenzae* (95, 313).

*Antibody recognition of H. influenzae*

Antibodies are another cell-free immune factor critical to the host defense against *H. influenzae*. Antibody recognition of bacteria can lead to the deposition of complement components, resulting in classical pathway complement-mediated killing (404). In addition to complement-mediated cell lysis, antibody binding contributes to the phagocytosis of *H. influenzae* by acting as an opsonin to enhance bacterial uptake, as with some complement proteins (386). Also, antibody binding can cause the agglutination of bacterial cells, which aids in the mucociliary clearance of bacteria not bound to epithelial cells (381). Even without

the Fc-activating portion of antibody, which is required for C1q deposition, antibody-mediated agglutination of *H. influenzae* enhances complement-dependent lysis, likely through increased C3b deposition (65). The importance of antibody for protection against *H. influenzae* has been demonstrated in the murine nasopharyngeal colonization model, where antibody-deficient (μMT) mice have higher levels of bacteria that wild-type mice (440). As mice are not a natural host of *H. influenzae*, the antibodies important for controlling colonization are natural antibodies.

Natural antibodies refer to those produced in the absence of specific antigen stimulation, and typically have poly-reactivity with host and non-host epitopes (12). Natural antibodies present on the mucosal surface provide immediate protection against a wide range of pathogens, although in order to limit potentially damaging responses against self epitopes, these antibodies are not capable of affinity maturation (19). In addition to pre-existing natural antibodies, some antibodies raised in response to other bacteria can cross-react with *H. influenzae* (118). Exposure to *H. influenza* also results in production of antigen-specific antibodies, such as antibodies that bind the outer membrane proteins P6 and protein D (194). While IgA is the most abundant antibody present on the respiratory tract mucosal surface, IgG and IgM are also present and can be detected in human nasal secretions (202). IgG and IgM contribute to killing of *H. influenzae* through classical pathway complement-mediated lysis (53, 282). While IgA does not activate the classical pathway of complement, it can affect bacterial survival by interfering with bacterial adherence and increasing

phagocytosis (198). Although less well characterized, IgD can bind type-b *H. influenzae* strains and is also present in the upper airways (50).

Clinical evidence supports a role for antibody in the defense against *H. influenzae* infection in humans. For example, people with IgM deficiencies have increased susceptibility to NTHi compared to those capable of IgM production and secretion (253). Also, people deficient in the production of all antibody types have persistent infections with *H. influenzae* (330). There are many outer membrane structures of *H. influenzae* that are targets of antibody. For example, antibodies that recognize the HMW1 and HMW2 adhesins increase opsonophagocytic killing in the presence of complement and neutrophils (425). Antibodies against outer membrane proteins including P2, P4, and P6 have been thoroughly investigated due to interest in conserved vaccination targets (32, 175, 386). In addition to these, there are also many bactericidal antibodies targeting LPS structures (85). One important defense mechanism against antibody binding in *H. influenzae* is the expression of IgA1 protease genes, including *iga* and *igaB* (93). However, even with IgA1 protease digestion, *H. influenzae* remains susceptible to IgG and IgM antibody recognition during colonization. Additional defenses against antibody recognition of *H. influenzae* outer membrane structures are the subject of ongoing work and a focus of this thesis.

Surface variation in *H. influenzae*

Host recognition of *H. influenzae* is dependent on the repertoire of surface structures on the bacterial outer membrane. Understanding the composition and variation of these structures is critical to the development of strategies to enhance bacterial clearance. The two main categories of surface structures in *H. influenzae* are the outer membrane proteins and the LPS. The six most prevalent outer membrane proteins in *H. influenzae* are designated P1 through P6, in order of descending molecular weight (221). Of these, the porin P2 is the most abundant outer membrane protein on the surface of *H. influenzae*, and is also a target of bactericidal antibodies (273, 386). Several of these proteins perform critical functions for *H. influenzae*. For example, the lipoprotein P6, which is highly conserved, helps to maintain the stability of the outer membrane (276). Another highly conserved lipoprotein, P4, aids in the utilization of NAD for bacterial growth (315). Other outer membrane proteins include protein D and the multiple adhesins mentioned previously (4). The conservation of these proteins make them good vaccine targets, and several studies have shown that immunization with outer membrane proteins leads to the production of an antibody response against *H. influenzae* (175, 176, 304). However, several outer membrane proteins contain variable domains, and minor outer membrane proteins are often not conserved among *H. influenzae* strains, making vaccination coverage more difficult (79, 123).

The second major surface structure on the outer membrane of *H. influenzae* is the LPS. The LPS of *H. influenzae* is also referred to as the lipooligosaccharide (LOS), because unlike most gram-negative bacteria it does

not contain the extended chain of O-antigen oligosaccharide units (421). Instead, the LPS of *H. influenzae* consists of a truncated oligosaccharide core, similar to that of rough mutants of other bacteria, such as Salmonella (402). The LPS of *H. influenzae* has several variable structures, and these provide a major source of surface antigenic variation. As shown in Figure 2, the LPS is composed of a conserved inner core, which is identical among all strains, and a variable outer core (169, 225). The inner core contains the lipid A portion of the LPS, which has hydrophobic acyl chains that insert into the outer leaflet of the outer membrane. Attached to lipid A is a single 2-keto-3-deoxyoctulosonic acid (KDO) group, with a pyrophosphoethanolamine residue on it. Extending outward (away from the bacterial surface) from the KDO group are three conserved heptose residues, which are referred to as $Hep_{I}$, $Hep_{II}$, and $Hep_{III}$. Aside from the conserved phosphoethanolamine that is attached to the $Hep_{II}$ residue, the remaining LPS extensions from the three inner core heptoses comprise the variable outer core of *H. influenzae* LPS. Outer core LPS structures differ both among separate strains of *H. influenzae* and within a single population of the same isolate (339, 375).

LPS modifications in many bacteria are important for host-pathogen interactions. For example, bacteria with O-antigen extensions have several survival advantages compared to O-antigen-deficient isogenic mutants. In *Francisella tularensis*, O-antigen reduces complement-mediated lysis, and in *Brucella melitensis* the presence of O-antigen aids intracellular survival (54, 94). Also, in Salmonella, O-antigen contributes to the reduction of early innate

immune responses stimulated by bacterial invasion of epithelial cells (77). Modification of lipid A is a mechanism to increase to bacterial survival in the presence of antimicrobial peptides, through reduction of the negative charge of the bacterial membrane, as previously mentioned (82). An additional example of this type of resistance is the de-phosphorylation of lipid A, which increases resistance to cationic antimicrobial peptides in *Helicobacter pylori* and *Porphyromonas gingivalis* (55, 62). Alteration of core oligosaccharide structures can also affect bacterial evasion of host immunity. For example, core oligosaccharide structures, not O-antigen, are required for adherence and invasion of epithelial cells by *S. enterica* (40). In *Escherichia coli*, dendritic cells phagocytose rough LPS mutants more readily than wild-type or deep-rough mutants. Rough mutants contain the oligosaccharide core but no O-antigen, suggesting targeting of core LPS structures (203). LPS modifications affecting host recognition of *H. influenzae* occur primarily in the oligosaccharide core.

Phase variation of oligosaccharide structures in *H. influenzae*

The diversity of core oligosaccharide structures in *H. influenzae* is largely due to the phase variation of LPS biosynthesis genes. Phase variation is defined by high frequency, reversible, switching (off ↔ on) in gene expression. In contrast to spontaneous mutation, which occurs at a frequency of $10^{-8}$ to $10^{-6}$ per generation, phase variation happens at a rate of $10^{-4}$ or higher per generation,

depending on the mechanism (426). There are several mechanisms for phase variation of gene expression. Some of these include gene conversion, which involves gene recombination, site-specific conversion, where recombinases swap out genes at inverted repeat sites, duplication, insertion-excision, and gene deletion. One example from these mechanisms is the duplication of the capsule-encoding locus of *H. influenzae*. The *cap* locus in type b *H. influenzae* is flanked by an insertion element, allowing amplification through locus duplication, which increases the amount of capsule expressed (207). Another mechanism of phase variation is called slipped-strand mispairing (215). Slipped-strand mispairing is caused by the presence of short DNA repeats either within the coding sequence of a gene or in the promoter region. During DNA replication, slipped-strand mispairing can result in either the addition or deletion of DNA repeats, shifting the reading frame of the product. Therefore, slipped-strand mispairing creates a translational on/off switch. Importantly, phase variation is distinct from other forms of gene regulation because it is a stochastic process; rather than responding to an external stimulant, phase variation ensures continuous reversals in gene expression (396). The maintenance of a heterogeneous population is a specific survival strategy for *H. influenzae*, allowing rapid enrichment of the most-fit phase variants amidst constantly changing host conditions.

There are several examples of phase variation by slipped-strand mispairing in different bacterial systems. In *Neisseria gonorrhoeae*, pentamaric repeats in two genes within the *opa*E1 locus are responsible for the phase

variation of opacity proteins (368). In *Neisseria meningitidis*, the presence of a homopolymeric guanosine tract in the gene *pptA* causes phase variation of pili modification with phosphorylcholine (referred to as ChoP) (407). There are also homopolymeric repeat tracts in genes encoding adhesins in *Yersinia pseudotuberculosis* and *Bordetella pertussis* (324, 422). Homopolymeric tracts in the LPS biosynthesis genes of *Campylobacter jejuni* (*wlaN*), *H. pylori* (*futA* and *futB*), and Neisseria species (*lgt* genes) are responsible for phase variation of LPS structures in these bacteria (7, 182, 218).

In *H. influenzae*, there are several LPS biosynthesis genes with tetranucleotide repeats, causing phase variation by slipped-strand mispairing (171). The rate of phase variation for genes containing tetranucleotide repeats in *H. influenzae* is $10^{-2}$ to $10^{-3}$ per generation, and this rate is higher for longer repeat tracts (69, 413). The phase variation of LPS biosynthesis genes affects the attachment of structures to the outer core region of the LPS, and these structures include hexose and non-oligosaccharide modifications. The expression of some outer core LPS structures is dependent on the attachment of proximal structures, which can also be phase variable. As a result, there are examples where the sequential addition of several LPS residues is dependent on the phase variant status of each LPS biosynthesis gene involved in the full extension (136, 339). Phase variation of the outer core creates a mixture of different LPS structures expressed within a single population from the same strain of *H. influenzae*.

Not every strain of *H. influenzae* contains the same repertoire of phase variable LPS biosynthesis genes, so there are different structural possibilities between separate *H. influenzae* strains (305). Also, some phase variable LPS biosynthesis genes have multiple alleles. For example, different alleles of the phosphorylcholine transferase, *lic1D*, control attachment to extensions from separate heptose residues (228). Allelic polymorphisms in the gene *lex2B* determine whether glucose or galactose is attached to the LPS when the associated phase variable gene *lex2A* is expressed (73). Finally, LPS heterogeneity in *H. influenzae* is not solely influenced by phase variation. Several LPS biosynthesis genes compete for substrates, creating another source of random structural variation (340). Unlike phase variation, this type of structural diversity is not heritable, although both are stochastic processes.

*H. influenzae* phase variable LPS biosynthesis genes with tetranucleotide repeats, where the LPS structure affected by phase variation has been characterized, are summarized in Table 1 and discussed below. Aside from the genes listed in Table 1, there are also LPS phase variable genes with other length repeats, such as the *losA1* gene, which has an octonucleotide repeat and controls attachment of a fourth heptose to the outer core of the LPS (84). Phase variation also occurs in genes controlling expression of outer membrane proteins. For example, there is phase variation of the HMW1 and HMW2 adhesins, caused by heptanuclotide repeats in the promoter regions of the *hmw1A* and *hmw2A* genes, and of fimbriae, from dinuclotide repeats in the promoter region of the *hifA* and *hifB* genes (67, 397). Additional genes with repeat tracts of various lengths

have been identified in screens of *H. influenzae* genomes, but these genes remain largely uncharacterized (264, 305). Four of the phase variable genes with tetranucleotide repeats not listed in Table 1 encode hemoglobin-binding proteins, involved in the acquisition of hemin, an essential nutrient required for *H. influenzae* growth (261, 305). There are several host factors involved in sequestering iron, which makes hemin a limited nutrient at the mucosal surface (43, 410). The remaining tetranucleotide repeat-containing gene that has been characterized in *H. influenzae* but is not included in Table 1 is *mod*, which is a type III DNA methyltransferase (69). Phase variation of *mod* affects the expression of two other DNA methyltransferases, which remain uncharacterized, in addition to genes involved in metabolism and hemoglobin acquisition (361).

The phase variable genes with tetranucleotide repeats listed in Table 1 control the attachment of LPS outer core structures. The phase variant status of some of these genes can be determined phenotypically using monoclonal antibodies (mAbs). One of these genes is *lic1A*, which controls the attachment of phosphorylcholine (ChoP) to the LPS of *H. influenzae* (413). ChoP can be detected with the mAb TEPC-15 (417), allowing for the identification of *lic1A* phase-on bacteria. Colony immunoblots using TEPC-15 to distinguish between *lic1A* phase-on and *lic1A* phase-off bacteria are shown in Figure 3. Another pair of LPS phase variable genes, *lic2A* and *lgtC*, controls the attachment of a di-galactoside structure, gal$\alpha$1-4gal, to the LPS (171, 415). This di-galactoside can be detected with the mAb 4C4 (401). Each component of the di-galactoside structure is added separately; *lic2A* controls attachment of the proximal galactose

and *lgtC* controls attachment of the distal galactose (169). Additional phase variable LPS biosynthesis genes included in Table 1 are *oafA*, which controls the addition of O-acetyl groups to the LPS, and *lic3A*, which is associated with LPS sialylation (107, 168). *lic3A* and at least two other non-phase variable genes determine the attachment of sialic acid to different regions of the LPS outer core (173, 186). *H. influenzae* requires an environmental source of sialic acid for expression (345). Finally, *lex2A* expression results in the attachment of glucose or galactose to the LPS, depending on the allele of the associated gene *lex2B* (73).

While all *H. influenzae* strains analyzed to date contain *lic1A*, *lic2A*, *lgtC*, and *lic3A*, several do not encode *oafA* or *lex2A* (305). Also, the location of attachment for each of these LPS structures differs between *H. influenzae* strains (81, 170, 225, 245). In these ways, phase variation of LPS structures creates both intra- and interstrain diversity in the outer core LPS of *H. influenzae*. All of the phase variable genes listed in Table 1 contribute to bacterial survival in serum (107, 138, 168, 415). However, the mechanisms of these effects remain undefined in most cases. One exception to this is *lic3A*, as sialic acid attachment to the LPS reduces deposition of complement components (95). Therefore, expression of *lic3A* increases bacterial survival in the presence of complement. Further research is necessary to characterize the individual and combined effects of LPS phase variable structures on host recognition of bacteria during colonization and disease establishment.

Concluding remarks

*H. influenzae* is an extracellular bacterium that successfully colonizes the human respiratory tract. Understanding the bacterial factors that aid in evasion of mucosal host immune responses is important for the control of *H. influenzae*-associated disease as well as the development of NTHi vaccines. Ideally, vaccination would reduce carriage of NTHi strains in order to prevent the development of diseases including otitis media and chronic bronchitis. However, the effect of eliminating *H. influenzae*, which is carried asymptomatically in a significant portion of the population, remains unknown.

Critical to understanding how the immune system recognizes *H. influenzae* is a thorough knowledge of the bacterial surface. Many structures in the outer membrane of *H. influenzae* are variable, including several LPS outer core structures. Phase variation of LPS structures could influence bacterial survival in the presence of multiple host factors, including antibody and complement. Antibody and complement both limit *H. influenzae* colonization in animal models, and are important for control of *H. influenzae* infections in people. The primary bacterial targets of antibody and complement, however, remain unclear. Antigenic variation of surface structures, including LPS modifications, may help bacteria escape immune recognition by constantly changing the targets of host antibody and complement. Alternatively, phase variable outer core LPS

structures could shield conserved inner core structures from recognition. Understanding the dynamics of LPS phase variation in *H. influenzae* may shed light on how this bacterium evades the host immune system during colonization.

Table 1. Phase variable LPS biosynthesis genes in *H. influenzae*

| Gene[a] | Repeat sequence | Structure |
|---|---|---|
| *lic1A* | CAAT | ChoP[b] |
| *lic2A* | CAAT | Gal[c] |
| *lgtC* | GACA | Gal |
| *lic3A* | CAAT | Neu5Ac[d] |
| *oafA* | GCAA | OAc[e] |
| *lex2A* | GCAA | Gal or Glc[f] |

**a.** Includes only phase variable LPS structures with tetranucleotide repeats for which the LPS modification affected by phase variation is known

**b.** ChoP, phosphorylcholine

**c.** Gal, galactose

**d.** Neu5Ac, sialic acid

**e.** OAC, acetyl group

**f.** Glc, glucose; Gal or Glc dependent on the associated *lex2B* allele

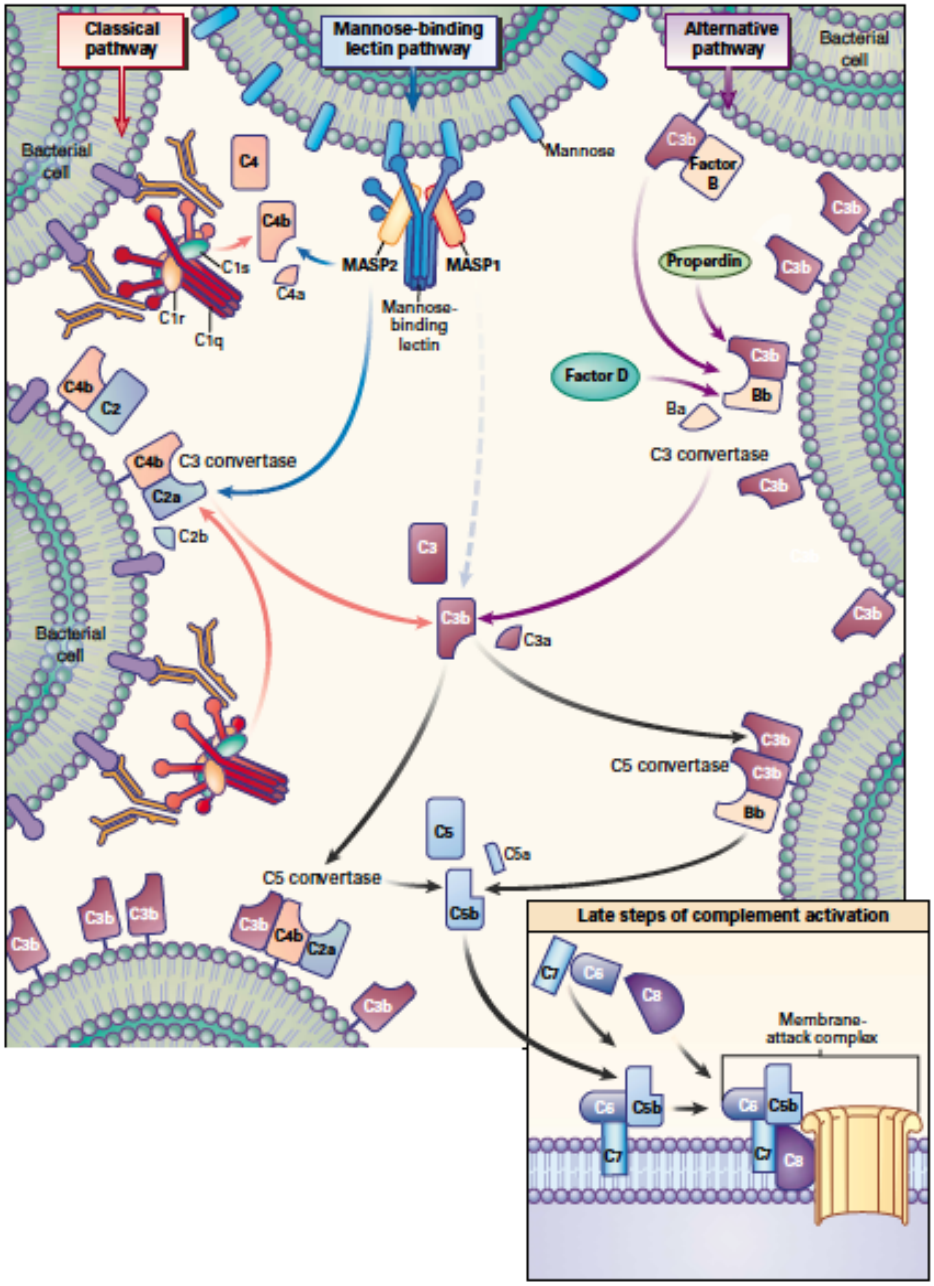


Figure 1. Diagram of complement pathways leading to bacterial lysis. Summary of the classical, mannose-binding lectin, and alternative pathways of complement. All three of these pathways converge at the formation of a C3 convertase, which leads to the formation of the membrane attack complex (MAC), shown in the bottom right, which forms pores in the cell membrane, causing bacterial lysis. (Reproduced with permission from: Walport, M. J., 2001, Complement: first of two parts, *The New England Journal of Medicine*, 344(14), pg 1058-1066, Copyright Massachusetts Medical Society)

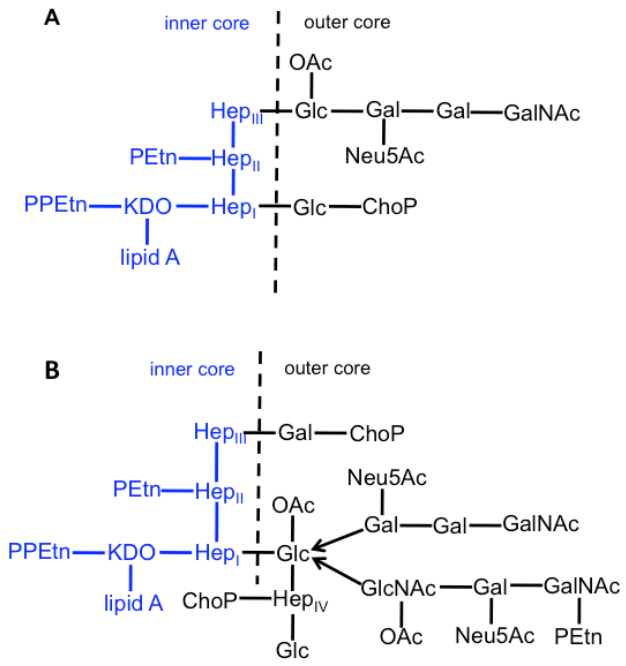


Figure 2. *H. influenzae* LPS structural diagrams. Proposed LPS structures for the *H. influenzae* type-d, capsule- strain Rd (A) and the NTHi clinical isolate R2846 (B). Conserved inner core structures are shown in blue, and are separated by a dotted line from variable outer core structures, shown in black. In B, arrows designate the point of attachment for the two alternative oligosaccharide extensions shown.

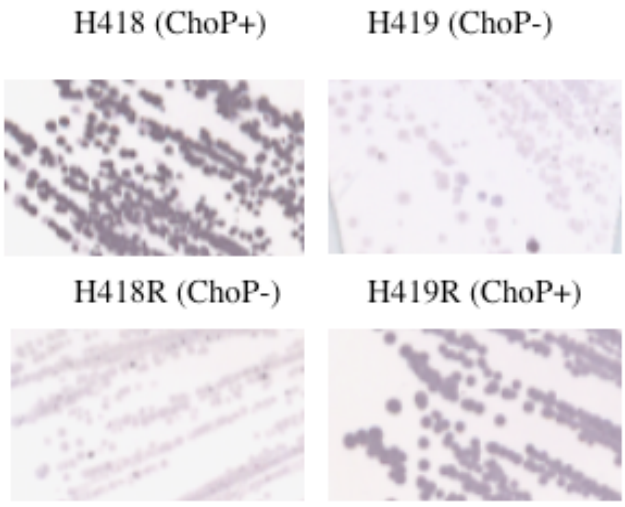


Figure 3. Colony immunoblots of ChoP phase variants. Colony immunoblots, using the mAb TEPC-15 to detect *lic1A* phase-on variants (purple), compared to *lic1A* phase-off variants (light pink). Shown are colony immunoblots of ChoP+ (*lic1A* phase-on) variants, designated H418, and ChoP- (*lic1A* phase-off) variants, designated H419. Also shown are revertants of these, isolated by selective enrichment from the original phase variant populations, designated as H418R (ChoP-) and H419R (ChoP+).

# CHAPTER 2

## Introduction, Part II: Microbial modulation of host immunity with the small molecule phosphorylcholine

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Abstract

All microorganisms dependent on persistence in a host for survival rely on either hiding from or modulating host responses to infection. The small molecule phosphorylcholine, or ChoP, is used for both of these purposes by a wide array of bacterial and parasitic microbes. While the mechanisms underlying ChoP acquisition and expression are diverse, a unifying theme is the use of ChoP to reduce the immune response to infection, creating an advantage for ChoP-expressing microorganisms. In this Minireview, we discuss several benefits of ChoP expression during infection as well as how the immune system fights back against ChoP-expressing pathogens.

## Introduction

Phosphorylcholine [$(CH_3)_3N^+CH_2CH_2P0_4^-$] is a small (0.18 kD) zwitterionic molecule expressed by a number of microbes, across taxonomic kingdoms, which infect humans and other eukaryotic hosts (23, 99, 151, 417). Phosphorylcholine will be referred to here as ChoP, for choline phosphate. Most microbes that express ChoP acquire choline from their host. While choline is not required for growth in most prokaryotes, it is an essential nutrient in eukaryotes. Furthermore, choline is readily available to microbes during infection, as ChoP is a component of the most abundant eukaryotic membrane phospholipid, phosphatidylcholine. The turnover of phosphatidylcholine results in the release of

glycerophospholipids containing choline (44). While microbes can use choline as a nutrient source (328) or as an osmoprotectant in the form of glycine betaine (101, 212) many also modify proteins or glycoconjugates with ChoP. ChoP may be either attached to the surface of the microbe or secreted on modified products.

The advantages of ChoP modification have been explored in both bacterial and parasitic systems. For extracellular bacteria that colonize the respiratory tract, where ChoP-expressing microbes are particularly common, ChoP is always attached to the bacterial surface (99, 120, 411). Surface expression of ChoP can affect epithelial cell adhesion and immune recognition. The intracellular bacteria *Legionella pneumophila*, in contrast, modifies a host protein with ChoP, aiding bacterial survival within host cells (267). In filarial nematodes, a secreted product that contains ChoP moderates the host inflammatory response, creating an environment amenable to parasite persistence (152). Despite the vast differences between these systems, a common theme is the use of ChoP to modulate the host response in order to support microbial survival.

The identification of ChoP on pathogenic microbes has led to the investigation of vaccine approaches to aid the recognition and clearance of ChoP-expressing pathogens. However, these efforts have been limited by the ability of ChoP to stimulate protective immunity, which is affected by the molecule to which it is attached, as well as its accessibility within that molecule. The

prevalence of ChoP expression, both in microbes and their host, demonstrates its importance in the constant interplay between these organisms. In this Minireview, we discuss how bacteria and parasites use ChoP expression to promote survival in their respective hosts. Figure 1 summarizes the most well characterized pathways of ChoP-dependent host modulation during infection. The links between these different mechanisms may provide new avenues of investigation on the role of ChoP expression and how it can be targeted therapeutically.

## Microbial Acquisition and Expression of ChoP

Choline is an essential nutrient in humans, as it is required for the synthesis of several molecules including cell membrane lipids, methionine, platelet activating factor receptor, and the neurotransmitter acetylcholine (436). Choline turnover occurs continuously in eukaryotic cells during the synthesis and recycling of the major membrane lipid phosphatidylcholine (44). As a consequence, many microbes have adapted strategies to take advantage of host-accessible choline for use as a nutrient source, osmoprotectant, and as a mechanism for the evasion of host immune responses. The phosphorylated choline molecule ChoP is associated with a wide range of microorganisms. The two most well characterized groups include bacterial species that colonize the upper respiratory tract and parasitic nematodes that secrete ChoP-modified

products (120, 126, 178, 411). Importantly, ChoP is associated with commensal as well as pathogenic microbes. A summary of the microorganisms that express ChoP-modified molecules can be found in Table 1.

Choline is required for growth in some, but not all, bacterial species. The human respiratory pathogen *Streptococcus pneumoniae*, which cannot synthesize choline, is dependent on choline for growth (312). While *S. pneumoniae* can grow without choline in the presence of a choline structural analog, bacteria under these conditions form extended chains and are unable to autolyse or undergo transformation (383). When environmental choline is available, it is transported into the cell, transformed to ChoP, and incorporated into the cell wall teichoic acid (TA) or lipoteichoic acid (LTA) through the genes encoded by the *lic* operon (437). The *lic* operon was first identified in another respiratory tract bacterial pathogen, *Haemophilus influenzae*, and is also present in commensal Neisseria strains (342, 412). The *lic* operon allows for molecular thievery, whereby microbes utilize a host resource to their own advantage. Parasites, in contrast, utilize the Kennedy pathway for phosphatidylcholine synthesis, similar to most eukaryotes (36, 177, 329). Some bacterial species can also synthesize phosphatidylcholine, either through the Kennedy pathway or by methylation of phosphatidylethanolamine, which is an alternative method for phosphatidylcholine synthesis also used by eukaryotes (1, 9, 223). Pathogenic Neisseria strains contain enzymes that are homologous to those used in the methylation pathway to synthesize ChoP (1).

Most microbes with ChoP-modified molecules have evolved mechanisms to vary ChoP expression and its location. In *Pseudomonas aeruginosa*, for example, ChoP is attached to the elongation factor Tu in a temperature-dependent manner (15). For *S. pneumoniae*, where ChoP is attached to the TA and LTA of the cell wall, the number of ChoP groups per cell wall repeating unit as well as the amount of TA itself are both variable (190, 199). Also, *S. pneumoniae* contains several choline-binding proteins, which can attach to ChoP-modified cell wall (144). While the choline-binding proteins of *S. pneumoniae* are diverse, from the cell wall hydrolytic enzyme LytA to the abundant surface molecule PspA, all have the potential to obscure antibody recognition of the ChoP epitope when bound to the pneumococcal cell wall (331, 433). In a *S. pneumoniae* PspA mutant, for example, there is increased binding of anti-ChoP antibodies as well as serum proteins that recognize ChoP (266).

The location of ChoP attachment itself is variable between different Neisseria species. In the pathogenic species *N. meningitidis* and *N. gonorrhoeae*, ChoP is covalently linked to serine residues on the bacterial pilus, while in commensal species including *N. lactima* and *N. subflava* it is found on the lipopolysaccharide (LPS) (157, 342). Pilus modification is a rare example of the direct attachment of ChoP to a protein residue, as opposed to linkage through glycans, as for the majority of cases. ChoP attachment to the pilus in *N. gonorrhoeae* is in competition with attachment of phosphoethanolamine, and the "winner" is determined by the expression level of the protein PilV (157). In *N. meningitidis*, pilin modification is variable due to the presence of an unstable

homopolymeric repeat in the ChoP transferase gene, *pptA* (407). The on-off switching in expression due to the repeating sequence in *pptA* is an example of phase variation. ChoP attachment to the LPS in commensal Neisseria species and to the LPS in *H. influenzae* is also determined by phase variation.

Phase variation in commensal Neisseria and *H. influenzae* is due to the presence of a track of tetranucleotide repeats in the first gene of the *lic* locus (412, 413). Phase variation in these bacteria occurs through slipped-strand mispairing, resulting in stochastic variation in ChoP attachment (159). Rather than controlling expression in response to an external stimulus, phase variation allows for rapid on-off switching of ChoP expression within the population. This strategy for regulation, or rather the absence of it, allows for the rapid selection of advantageous traits within a heterogeneous population exposed to new environments. In addition, there are multiple alleles of the ChoP transferase gene, *licD*, in *H. influenzae* strains. The *licD* allele carried by a given strain determines the location of attachment to the LPS (228). Finally, some strains of *H. influenzae* contain a duplication of the *lic* locus, resulting in the attachment of two ChoPs per LPS molecule (106).

Parasitic expression of the levels of several ChoP-modified molecules is also variable, albeit through different mechanisms than those found in bacterial species. While there exists a range of different molecules modified by ChoP in *Caenorhabditis elegans*, stage-specific ChoP modification of glycosphingolipids occurs in this as well as other nematodes (220). For example, in the filarial

nematode *Acanthocheilonema viteae*, ChoP is attached to the secretory product ES-62. Secretion of ES-62 is stage-specific, restricted to the latest larval stage and adult worms, and occurs through post-translational control of ES-62 production (367).

The acquisition and control of ChoP expression by so many diverse microorganisms alludes to the importance of this small molecule. This is especially true during host infection, where choline is readily available. Even the human host itself can undergo stage-specific modification of proteins with ChoP, such as during pregnancy. It has been shown that placental polypeptides are modified with ChoP, which has been proposed to help shield the fetus from immune recognition (224). In the following sections the advantages as well as disadvantages of microbial ChoP expression in different host environments are highlighted.

## ChoP Affects Host Recognition

ChoP is found on the surface of several bacterial species, both commensal and pathogenic, that colonize the upper respiratory tract. Despite the constant phase variation of ChoP, ChoP-expressing phase variants of *H. influenzae* are the dominant population isolated from humans and from colonized mice (239, 415, 416). In a six-day colonization study in healthy human adults, inoculation with mixed phase variant populations resulted in selection for ChoP-

expressing phase variant dominant populations following colonization (303). While ChoP is important during colonization, it also plays a role during disease. For example, ChoP-expressing *H. influenzae* are more strongly associated with the development of otitis media in chinchillas (384). Correspondingly, ChoP-expressing *H. influenzae* are correlated with increased persistence in children with otitis media (110). *H. influenzae* expressing ChoP also have delayed clearance from the lungs of infected mice (295). ChoP expression is advantageous for other bacteria as well. *S. pneumoniae* without ChoP-modified TA are unable to colonize the upper respiratory tract in mice and are less virulent in a murine sepsis model (195). ChoP expression in *Histophilus somni* has also been shown to increase colonization of its bovine host (80).

One of the advantages of ChoP expression for survival in the respiratory tract is the ability of ChoP to increase bacterial adhesion to the epithelial cell surface. ChoP is part of the recognition domain of the host protein platelet-activating factor (PAF), which binds to host platelet-activating factor receptor, or PAFr, on epithelial cells. Bacterial species including *H. influenzae*, *S. pneumoniae*, *Aggregatibacter actinomycetemcomitans*, *H. somni*, *P. aeruginosa*, and commensal Neisseria strains exhibit this version of molecular mimicry, allowing binding to PAFr *in vitro* (64, 335, 343, 374). PAFr binding also increases cell invasion for several bacterial species, including *S. pneumoniae*, *P. aeruginosa, A. actinomycetemcomitans,* and *Acinetobacter baumannii* (15, 64, 335, 357). Invasion of epithelial cells allows bacteria to avoid extracellular immune responses, although intracellular survival is dependent on evasion of

anti-bacterial intracellular immunity. Invasion of epithelial cells could also aid bacterial access to the bloodstream, exposing bacteria to yet another host environment. In support of a role for PAFr during bacterial infection, PAFr knockout mice are less susceptible to *S. pneumoniae*-induced pneumonia (317). *S. pneumoniae* also exhibits ChoP-dependent binding to the epithelial receptor asialo-GM1. (370). Although the mechanism for this binding remains unclear, this suggests that *S. pneumoniae* adheres to epithelial cells by binding multiple host receptors through ChoP. A role for ChoP in adhesion has also been proposed for some parasitic species. In the parasite *Plasmodium falciparum*, a var family protein involved in parasite adhesion is modified with ChoP, although whether binding is dependent on ChoP has not been investigated (133). In contrast, a ChoP-modified glycoprotein in *Dictyocaulus viviparus* may inhibit, rather than aid, attachment to the host (206). While several bacterial species have increased adhesion to host cells through ChoP expression, this is not the only example of how ChoP contributes to microbial survival. Also, the *in vivo* impact of PAFr binding remains unclear for some species. For example, the role of PAFr is not always ChoP dependent, as PAFr has also been shown to be important in infection models with bacteria that do not express ChoP (103, 174, 359). In addition, there are examples of ChoP-expressing bacterial infections that are not affected by the absence of PAFr, such as colonization with *H. influenzae* (39).

ChoP expression by microbes can also affect survival in the presence of host immune factors including antibody, complement, and antimicrobial peptides. It was recently shown that ChoP expression in *H. influenzae* reduces antibody

binding to the bacterial surface, resulting in increased survival in the presence of complement (53). ChoP attachment to the LPS in *H. influenzae* alters the physical properties of the outer membrane, resulting in decreased membrane accessibility, reduced membrane permeability, and an altered membrane melting temperature (53). ChoP expression in *H. influenzae* also increases resistance to antimicrobial peptides such as human cathelicidin LL-37 (229). ChoP-expressing *Pasteurella multocida* are also more resistant to cathelicidins produced by their natural host, chickens (153). Increased resistance to antimicrobial peptides has been observed for other phosphorylated amine molecules, including phosphoethanolamine (PEtn) attached to lipopolysaccharide (LPS). When PEtn is expressed on lipid A or the LPS outer core, bacterial species including *Salmonella enterica* and *Citrobacter rodentium* have increased resistance to the antimicrobial peptide polymyxin B (270, 400). These effects may extend beyond LPS modification, as similar effects on polymyxin B sensitivity were also observed for PEtn attachment to flagella in *Campylobacter jejuni* (63). It is possible that phospho-amine associated modifications serve to increase membrane stability in a number of bacterial strains, and the impact of these modifications on host recognition has only recently begun to be explored. ChoP-expressing *H. influenzae* are also associated with biofilms *in vitro*, (418) and in a chinchilla model of otitis media biofilm persistence was correlated with ChoP expression (166). While a cause-and-effect relationship between biofilms and ChoP expression in *H. influenzae* has not been established, these data suggest

that ChoP-expressing phase variants have an advantage during biofilm establishment in the development of otitis media.

ChoP Modulation of the Host Response

In addition to altering host recognition, ChoP is used by microbes to modify host responses during infection. Recently, a mechanism for ChoP-dependent host modification was identified in the intracellular bacteria *Legionella pneumophila*. In this pathogen, a secreted protein reversibly phosphorylcholinates the host GTPase Rab1 (378). The bacterial secreted protein, ankX, is a ChoP transferase, and ChoP modification of Rab1 results in host cell defects in endosome formation (267). It has been proposed that Legionella uses ankX and the dephosphorylcholinase Lem3 as an alternative to the GTP/GDP exchange system used in human cells (131). It is possible that other intracellular bacteria use ChoP to modify the activity of host proteins, although whether this phenomenon is limited to the decoration of host GTPases is unclear. Alteration of endosome processing creates an advantage for *L. pneumophila* during intracellular survival, much as the increased epithelial cell adherence and reduced immune recognition aid extracellular bacteria during host colonization.

Microbes also use ChoP to modify the host immune response. Interestingly, all examples to date of ChoP-mediated immune modulation occur in

parasites. However, there may be common links between the role of ChoP in parasites and in bacteria that have not yet been explored. The most thoroughly studied example of a ChoP-modified immunomodulatory molecule is ES-62, a secretory product of parasitic nematodes. Several of the immunomodulatory properties of ES-62 are dependent on the presence of ChoP. In *A. viteae*, ES-62 induces a Th2 immune response while reducing Th1 immunity. As part of the induction of a Th2-skewed immune response, macrophage and dendritic cell exposure to ES-62 reduces the production of IL-12 and TNF-alpha in response to classic inflammatory signals such as LPS (129). Also, exposure to ES-62 results in the maturation of dendritic cells that promote IL-4 and reduced IFN-gamma cytokine production in T cells (419). ES-62 exposure additionally desensitizes B cell activation and proliferation through the traditional PI3K and Ras MAP kinase signaling pathways (74, 150). Finally, ES-62 can induce a Th2-skewed antibody response, resulting in production of increased IgG1 and decreased IgG2a (238). The filarial nematode *B. malayi* also produces ChoP-modified antigens which suppress B and T cell signaling in a ChoP-dependent manner (208). The suppression of Th1 inflammation allows for parasite persistence within the host.

The phenotypes described above have also been replicated using ChoP-conjugated OVA peptide. ChoP-OVA exposure alone results in the induction of Th2 immunity, demonstrating that ChoP is essential for the immune modulation observed during ES-62 exposure (130). Indeed, even the synthetic oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine, or oxPAPC, molecule, which contains ChoP, has been shown to bind TLR4 and induce IL-8 secretion

(405). Interestingly, PAFr has been found on the surface of macrophages as well as B and T cells, raising the possibility that ChoP-mediated immune modulation occurs through PAFr binding (46, 284). However, while PAFr is functional on macrophages, it is not on B and T cells, suggesting the presence of additional immune activators responsible for ChoP-dependent effects on the immune system (353).

While the exposure or secretion of ChoP-modified molecules creates an advantage for microbes, the immune modulation that results also has the potential to affect immune homeostasis in the host. For example, the anti-inflammatory effect of ES-62 exposure also reduces arthritis in a ChoP-dependent manner (149). Additionally, the importance of ChoP-mediated effects on the immune response to concurrent or subsequent microbial infections remains to be explored.

## Host Responses to ChoP

Microbial expression of ChoP-modified molecules provides several potential advantages during host infection. However, the ability to vary ChoP expression in most microbes suggests that ChoP-modified molecules may only be advantageous in certain host environments. The acute phase reactant protein C-reactive protein (CRP) recognizes ChoP and can initiate classical pathway, complement-mediated killing of *H. influenzae* and increase phagocytosis of *S.*

*pneumoniae* (255, 415). Even the position of ChoP on the LPS of *H. influenzae* affects the level of CRP-mediated bacterial killing (228). A role for CRP has also been demonstrated *in vivo*, as transgenic mice expressing human CRP have increased survival and reduced bacteremia following infection with *S. pneumoniae*, and the majority of these effects are dependent on complement (376, 377). Also, CRP binding to ChoP-modified pilin increases uptake of *N. meningitidis* by phagocytes (48). It was recently shown that in *S. pneumoniae*, CRP binding is inhibited by the surface protein PspA, which is anchored to ChoP (266). Inhibition of CRP binding also reduces complement deposition on the bacterial surface. CRP levels are initially low in both the blood and at the mucosal surface, although these increase dramatically during inflammation (132). When CRP is readily available, such as in rat or human serum during infection, ChoP-expressing *H. influenzae* are more sensitive to complement-mediated killing than bacteria not expressing ChoP (180, 417). In contrast, ChoP-expressing bacteria have the advantage in mouse and rabbit models of bloodstream infection, where CRP levels remain low (85, 116). The pentraxin serum amyloid protein (SAP) can also bind ChoP, although no bactericidal effect has been demonstrated (52). Instead, SAP binding inhibits classical pathway complement activity (70). The difference in the impact of CRP versus SAP binding highlights the concept that ChoP expression may only be advantageous in the presence of certain host conditions.

The second major host factor involved in ChoP recognition is the expression of anti-ChoP antibodies. Anti-ChoP antibodies are B1-lineage, natural

antibodies. B1 antibodies are part of the repertoire of IgM synthesized prior to microbial exposure, and have lower affinity than antibodies induced following infection (19). The major idiotype of anti-ChoP antibodies is T15, originally identified in mice (193, 216). While ChoP modification of host molecules including the major cell membrane lipid phosphatidylcholine is very common, it has been shown that T15 antibodies don't recognize the ChoP epitope in intact human membrane lipids (60, 392). Anti-ChoP antibodies do, however, recognize ChoP on intact or digested microbes, and it has been shown that there are detectable levels of circulating ChoP-containing antigens following infection with ChoP-expressing parasites in the bloodstream (209).

While anti-ChoP antibodies play a role in the recognition of microbes with ChoP-modified surfaces (127), they also are important during clearance of apoptotic cells and may be beneficial during atherosclerosis (348). As a case-in-point, anti-ChoP antibodies that bind to dental plaque bacteria cross-react with oxidized low-density lipoprotein (oxLDL), which contains ChoP (336). Reduced anti-ChoP IgM levels in patients with acute cardiovascular disease corresponds with increased risk of a new cardiovascular event (45, 100). Also, higher anti-ChoP IgM levels correlate with decreased symptomology for systemic lupus erythematosus (SLE) patients (139). These effects, however, aren't limited to anti-ChoP antibodies. CRP, known to play an important role in killing ChoP-expressing microbes, binds to oxLDL as well as apoptotic cells in a ChoP-dependent manner (49). However, ChoP-dependent recognition of LDL may not always be beneficial. A study investigating the effect of anti-ChoP antibodies on

atherosclerosis found that opsonization of *minimally modified* LDL increased inflammation generated by DCs and NK cells (197). These studies suggest that infection with ChoP-expressing microbes can impact non-infectious disease processes in the host.

Evasion of host recognition is critical for microbial survival during infection. However, microbes must also defend themselves, such as bacteria from infection with phages. It has been shown that choline-modified TA in *S. pneumoniae* increases susceptibility to certain phages (222). Several pneumococcal phages contain proteins homologous to the CBPs of *S. pneumoniae*, and choline-binding interactions have been demonstrated for some of these (160, 327). As LPS molecules can serve as phage receptors (181, 432), it is possible ChoP attachment to the LPS affects phage susceptibility in other bacteria as well. In this way, ChoP expression could also impact inter-microbial competition in addition to the ongoing competition between microbe and host.

## ChoP-Based Vaccines

Vaccines that successfully stimulate a protective anti-ChoP response have the potential to be used for immunization against a wide range of ChoP-expressing microbes. Even though B1 anti-ChoP antibodies are low affinity, they provide some protection, as B1-deficient Xid mice (333) are more susceptible to *S. pneumoniae* infection (433). Beyond pre-existing anti-ChoP B1 antibodies,

stimulation of anti-ChoP antibody production occurs during exposure to ChoP-expressing microbes. Carriage of *S. pneumoniae* in young children is associated with increased levels of anti-ChoP antibodies (134). It remains unclear, however, whether these antibodies are able to protect children from additional infections with *S. pneumoniae* or other ChoP-expressing microbes.

While there is no vaccine currently in use that targets ChoP directly, there is one example of a licensed vaccine that induces a response against a protein involved in choline acquisition. One of the vaccines for *S. pneumoniae* contains 10 pneumococcal polysaccharide serotypes conjugated to Protein D. Protein D is a glycerophodiester phosphodiesterase from *H. influenzae* that scavenges host choline (269). In human children, the Protein D-containing vaccine has 35% efficacy against otitis media caused by *H. influenzae* (307). Passive transfer of sera from children immunized with this vaccine also protects chinchillas against *H. influenzae* otitis media with similar efficacy (290). The Protein D-containing conjugate vaccine induces antibodies that inhibit the glpQ activity of Protein D (385), suggesting its efficacy is related to its ability to repress choline scavenging *in vivo*. In recent work it was shown that abrogation of Protein D activity results in reduced ChoP expression and decreased epithelial cell adherence and fitness *in vivo* (185). The success of the Protein D-containing conjugate vaccine against *H. influenzae* infection, while limited, demonstrates the potential for targeting ChoP expression to protect against other ChoP-expressing microbes.

More direct approaches to target ChoP, such as inducing anti-ChoP antibodies, may have a greater impact on stimulating immunity to ChoP-expressing microbes. While the majority of ChoP-specific antibodies are IgM natural antibodies (351), human ChoP-specific IgG is protective against *H. influenzae* and *S. pneumoniae* in mouse models of infection (127). Patients with specific antibody defects, such as IgA-deficient patients, also have elevated levels of anti-ChoP IgG (111). Several vaccine formulations have demonstrated promise for the induction of ChoP-specific responses in animal models of infection with *S. pneumoniae* (387), *N. meningitidis* (20), and *H. influenzae* (379). In *S. pneumoniae*, the level of protection with anti-ChoP IgG is dependent on capsule type (41). ChoP vaccine formulations often involve conjugation of ChoP to a protein carrier, such as keyhole limpet hemocyanin (13, 97). ChoP is an example of a T cell-independent antigen, as with polysaccharides. Conjugation to a protein carrier allows for a repertoire shift in the anti-ChoP idiotype, resulting in the involvement of T cells and the development of higher affinity antibodies (42). The induction of anti-ChoP antibodies may also provide protection against other ChoP-expressing bacteria, including *A. actinomycetemcomitans* and *Treponema pallidum* (29, 308). Interestingly, vaccination with *S. pneumoniae* induces T15 IgM that binds oxLDL, resulting in reduced atherosclerosis (26).

Targeting ChoP surface molecules is an attractive option as a strategy to immunize against multiple pathogens simultaneously. However, this would also affect ChoP-expressing commensal bacteria, and depletion of normal flora due to vaccination is a potential concern. Also, the variable expression of ChoP in many

microbes suggests that even in the presence of protective anti-ChoP antibodies, phase variation or covering the ChoP epitope would allow infection to continue. Given the diversity in ChoP expression, additional targets may be required for vaccines targeting ChoP to have the most significant impact.

## Concluding Remarks

The use of choline beyond a nutrient source has proven to be a useful strategy for microbes as a way to hide from (molecular mimicry), or alter host immune responses. The role of ChoP-modified molecules varies by microbe, although there may be undiscovered functional links. It is unclear, for example, whether ChoP-modified glycans or pilin in bacteria can modulate the immune response like ES-62 does during nematode infection. ChoP modulation of host immune responses may be dependent on the context in which it is presented. Additional studies should give insight to the development of ChoP-targeted vaccines and immune therapies using ChoP-modified molecules. Likewise, surface expression of ChoP on bacteria as demonstrated for *H. influenzae* could impact survival in the presence of antibody, complement, and antimicrobial peptides. ChoP itself may not be the only molecule capable of these and other effects, as other phospho-amine molecule modifications of the LPS increase resistance to antimicrobial peptides. Finally, there may be additional examples of related microbial products that can modify the activity of host proteins, as

highlighted by the discovery of the ChoP transferase of *L. pneumophila*. Regardless of the interplay between different systems, the ability to manipulate ChoP has a marked effect on host recognition and response to microbes during infection.

There has been extensive interest in stimulating immune responses targeting ChoP-expressing microbes. However, the effects of anti-ChoP immunity also impact non-infectious disease conditions, including arthritis, SLE, and atherosclerosis. There is clearly a dual role for anti-ChoP immune responses, which target ChoP-expressing microbes but also respond to host molecules with an exposed ChoP epitope, such as oxLDL. Even in the context of an effective anti-ChoP immune response, the seemingly universal capacity of microbes to vary either ChoP expression or accessibility limits the effectiveness of anti-ChoP immunity in eradicating infections. This Minireview highlights ChoP as a key player in the constant battle between host and microbe during infection. The contribution of this dynamic molecule to microbial survival occurs through several different mechanisms, and the interplay between these and the host response to ChoP-expressing microbes is an important area for ongoing research.

Table 1. Microbes that use ChoP for structural modification*

| Organism group | Taxonomic classification | ChoP-modified structure | Reference |
|---|---|---|---|
| Gram-positive bacteria | *Streptococcus pneumoniae* | TA, LTA | (99) |
| | *Streptococcus oralis* | TA | (121) |
| | *Streptococcus mitis* | TA | (121) |
| Gram-negative bacteria | *Haemophilus influenzae* | LPS | (417) |
| | *Haemophilus haemolyticus* | LPS | (248) |
| | *Pseudomonas aeruginosa* | Elongation factor Tu | (15, 411) |
| | *Neisseria meningitidis* | Pilus | (411) |
| | *Neisseria gonorrhoeae* | Pilus | (411) |
| | *Neisseria lactima* | LPS | (343) |
| | *Neisseria subflava* | LPS | (343) |
| | *Neisseria flavescens* | LPS | (343) |
| | *Histophilus somni* | LPS | (59) |
| | *Aggregatibacter actinomycetemcomitans* | LPS | (126) |
| | *Acinetobacter baumannii* | porinD | (357) |
| | *Pasteurella multocida* | LPS | (362) |
| | *Legionella pneumophila* | Host Rab1 GTPase | (267) |
| | *Proteus mirabilis* | LPS | (109) |
| | *Treponema pallidum* | Membrane lipid | (29) |
| | *Morganella morganii* | O-antigen | (434) |

| | | | |
|---|---|---|---|
| Mollicutes | *Mycoplasma fermentans* | Membrane proteins | (23) |
| Nematode | *Caenorhabditis elegans* | ASP-6, Glycolipids | (117, 220) |
| | *Acanthocheilonema viteae* | ES-62 | (151) |
| | *Brugia pahangi* | Secreted proteins | (232) |
| | *Brugia malayi* | Secreted proteins | (162) |
| | *Dictyocaulus viviparus* | GP300 | (206) |
| | *Ascaris suum* | Glycolipids | (219) |
| | *Ascaris lumbricoides* | Glycolipids | (398) |
| | *Haemonchus contortus* | GP300 | (206) |
| | *Cooperia oncophora* | GP300 | (206) |
| | *Onchocerca volvulus* | Glycolipids, glycoproteins | (154, 430) |
| | *Onchocerca gibsoni* | Glycolipids | (154) |
| | *Trichinella spiralis* | Tsp, Glycolipids | (217, 256) |
| | *Nippostrongylus brasiliensis* | C substance | (299) |
| | *Wuchereria bancrofti* | PC-Ag | (68) |
| Protozoa | *Plasmodium falciparum* | Surface, secreted proteins | (133) |
| | *Eimeria bovis* | Polypeptides | (158) |

*Table does not include microorganisms that contain ChoP-modified phospholipids or those for which the ChoP-modified structure has not been elucidated

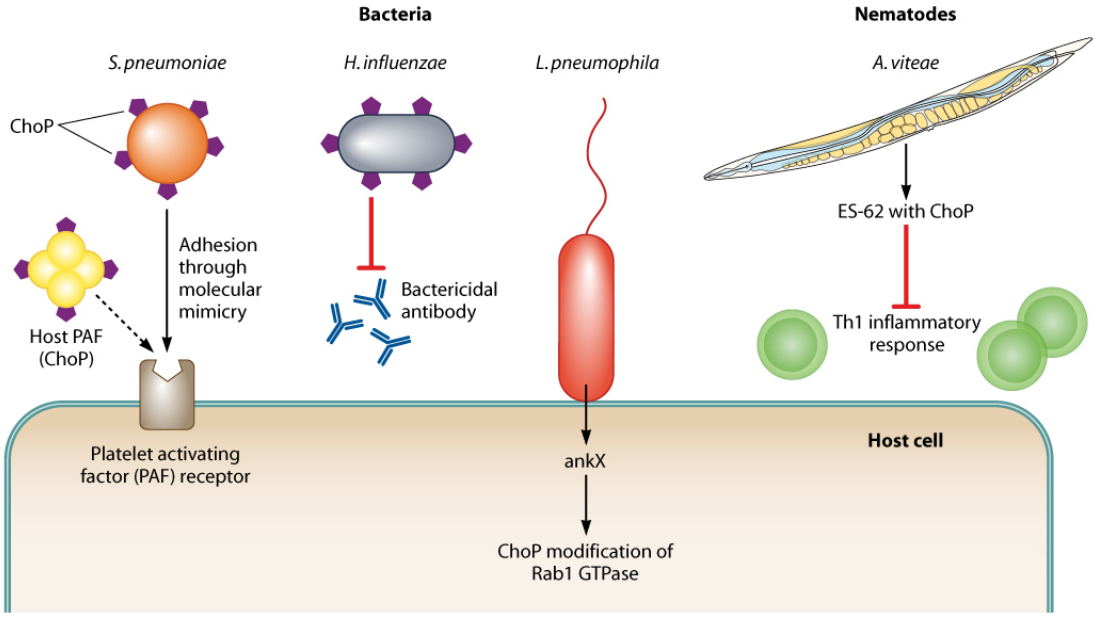


Figure 1. Schematic of the mechanisms used by microbes to modulate host immune responses with phosphorylcholine. In bacteria such as *S. pneumoniae*, the attachment of ChoP to the outer membrane allows for adhesion to the eukaryotic platelet-activating factor receptor (PAFr). This is an example of adhesion through molecular mimicry; in this case mimicry of the natural PAFr binding partner PAF, which also contains ChoP. In *H. influenzae*, ChoP expression reduces binding of bactericidal antibody. In *L. pneumophila*, the secreted protein ankX attaches phosphorylcholine to host Rab1 GTPase in order to modify its activity. The secretion of ES-62 in nematodes results in ChoP-dependent inhibition of pro-inflammatory Th1 immunity.

## DISSERTATION AIMS

*H. influenzae* is an extracellular bacterial pathogen that must evade host immune components present in the respiratory tract. Antibody and complement are present on the mucosal surface, and contribute to the limitation of *H. influenzae* colonization and disease. The overall aim of this study is to understand how *H. influenzae* evades host recognition and complement-mediated killing. Our hypothesis is that the phase variation of LPS structures contributes to bacterial resistance against antibody binding and complement-mediated lysis by shielding inner core structures from host recognition.

*Specific Aim 1: Determine the role of phosphorylcholine (ChoP) expression in bacterial evasion of antibody recognition*

We hypothesize that the attachment of ChoP to the LPS reduces binding of human antibody to the bacterial surface, and increases survival in the presence of complement. We use flow cytometry to compare antibody binding to the surface of ChoP-expressing and non-expressing phase variants. The mechanism for the effect of ChoP on antibody binding is investigated by comparing outer membrane stability between ChoP-expressing and non-expressing bacteria, with the hypothesis that ChoP attachment to the LPS increases the stability of the outer membrane, which reduces membrane accessibility.

*Specific Aim 2: Identify new genes that contribute to bacterial resistance to complement-mediated killing*

We hypothesize that multiple LPS biosynthesis genes are critical for survival of *H. influenzae* in the presence of antibody and complement. We use mariner transposon mutagenesis to screen for genes that are required for bacterial survival in human serum as a source of antibody and complement. Following the identification of critical genes, we investigate the mechanism of the effect of a previously uncharacterized set of genes that contribute to bacterial evasion of complement-mediated killing.

*Specific Aim 3: Determine the collective contribution of multiple LPS phase variable genes to protection against antibody and complement-mediated lysis*

We hypothesize that individual LPS phase variable modifications have independent effects on bacterial survival, and that these effects are additive in combination. We passage bacteria in human antibody and complement and determine whether serial exposure selects for resistant phase variant populations. Also, we determine the effect of single versus multiple LPS modifications on bacterial survival in the presence of human antibody and complement using a series of *H. influenzae* mutants and phase variant enriched populations.

# CHAPTER 3

## Phosphorylcholine allows for evasion of bactericidal antibody by *Haemophilus influenzae*

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Abstract

The human pathogen *Haemophilus influenzae* has the ability to quickly adapt to different host environments through phase variation of multiple structures on its lipooligosaccharide (LPS), including phosphorylcholine (ChoP). During colonization with *H. influenzae*, there is a selection for ChoP+ phase variants. In a murine model of nasopharyngeal colonization, this selection is lost in the absence of adaptive immunity. Based on previous data highlighting the importance of natural antibody in limiting *H. influenzae* colonization, the effect of ChoP expression on antibody binding and its bactericidal activity was investigated. Flow cytometric analysis revealed that ChoP+ phase variants had decreased binding of antibody to LPS epitopes compared to ChoP- phase variants. This difference in antibody binding correlated with increased survival of ChoP+ phase variants in the presence of antibody-dependent, complement-mediated killing. ChoP+ phase variants were also more resistant to trypsin digestion, suggesting a general effect on the physical properties of the outer membrane. Moreover, ChoP-mediated protection against antibody binding correlated with increased resilience of outer membrane integrity. Collectively, these data suggest that ChoP expression provides a selective advantage during colonization through ChoP-mediated effects on the accessibility of bactericidal antibody to the cell surface.

Author Summary

The bacterial pathogen *Haemophilus influenzae* evades immune responses during colonization in its human host. Decoration of the bacterial surface with different structures is one way that *Haemophilus* avoids host recognition. In this study, we show that the attachment of the small molecule phosphorylcholine, or ChoP, to the lipopolysaccharide covering the bacterial surface allows *H. influenzae* to avoid the immune response by inhibiting antibody binding. The presence of ChoP alters the bacterial surface to reduce its accessibility. The ability of ChoP to affect antibody binding is dependent on the positive charge of the molecule, which changes the physical properties of the bacterial membrane. The increased survival of bacteria with ChoP attached to their surface enriches ChoP+ bacteria during colonization. This study reveals a novel mechanism for bacterial evasion of a host immune response.

## Introduction

*Haemophilus influenzae* is an extracellular, gram-negative pathogen that is a primary causative agent of otitis media in children and is also frequently isolated from adults with pneumonia and exacerbations of chronic obstructive pulmonary disease (COPD) (18, 71, 274, 275, 278). Colonization of the upper respiratory tract with *H. influenzae* is common and is the first step in disease development, as *H. influenzae* carriage is associated with recurrent otitis media episodes in

children (88, 148). While the Hib conjugate vaccine has greatly reduced the burden of disease caused by type b *H. influenzae* (119, 188), non-typeable *H. influenzae* (NTHi) strains, which are unencapsulated, remain a common source of respiratory tract infections. Vaccine strategies targeting NTHi strains are complicated by the high variability of outer membrane antigens (17, 123). One of the structurally diverse molecules on the surface of *H. influenzae* is the lipopolysaccharide (LPS).

The LPS of *H. influenzae* is truncated compared to the LPS of other gram-negative bacteria. It contains no repetitive O antigen side chains and is also referred to as lipooligosaccharide (LOS) (184, 298). *H. influenzae* LPS consists of lipid A attached to 3-deoxy-D-manno-oct-2-ulosonic acid (KDO), with three conserved inner core heptoses to which various oligosaccharide extensions, and other non-carbohydrate molecules, can be attached (339). Mass spectrometry (MS) analysis of different *H. influenzae* isolates has revealed a significant level of diversity in LPS structures (81, 225, 227, 235, 236, 244, 319). For example, the length and composition of the hexose extensions from the inner core heptoses, as well as the attachment of molecules such as sialic acid and glycine, varies both between different strains and within glycoforms of the same isolate. A major source of LPS variability in *H. influenzae* is on-off switching, or phase variation, involving LPS biosynthesis genes (305, 375). One of the phase variable molecules expressed on *H. influenzae* LPS is phosphorylcholine.

Phosphorylcholine [$(CH_3)_3N^+CH_2CH_2PO_4^-$], or ChoP, is a small, zwitterionic molecule that is covalently attached to the LPS through its phosphate group. ChoP is a surface structure of a number of bacteria in addition to *H. influenzae*, particularly those found in the respiratory tract, including *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Neisseria* species (120, 204, 411). ChoP is also a component of eukaryotic membrane lipids in the form of phosphatidylcholine. *H. influenzae* must acquire choline from the environment, and turnover of host lipids can be a major source of choline during colonization (44, 91). Choline import, phosphorylation, and attachment to *H. influenzae* LPS is controlled by genes in the *lic1* locus. The choline kinase gene *lic1A* contains a tetranucleotide repeat that is responsible for ChoP phase variation. Slipped-strand mispairing within the repeat region of *lic1A* creates a translational on-off switch controlling ChoP expression (414). As a result, the control of ChoP attachment to the LPS is stochastic, and phase variation occurs at a high frequency (69).

Phase variation of ChoP expression may provide a mechanism for *Haemophilus* to display a variety of phenotypes, allowing rapid adaptation to different host environments. ChoP attachment to the LPS enables recognition by C-reactive protein (CRP), which binds to ChoP and initiates classical pathway complement-mediated killing (416). In host environments with high levels of CRP, such as in the blood, there is a selective advantage for ChoP- phase variants (180). In addition, an antibody response can be initiated against LPS epitopes containing ChoP (41, 379). However, the maintenance of phase

variable ChoP expression predicts that there are also advantages for ChoP+ bacteria in select host environments. During *H. influenzae* colonization there is a strong selection for ChoP+ phase variants. This selection has been observed in several animal models of *Haemophilus* colonization, as well as during human carriage (239, 295, 384, 416). ChoP expression increases adherence to epithelial cells through interaction with platelet-activating factor receptor (rPAF), which normally binds the ChoP-containing molecule PAF. While *in vitro* experiments have demonstrated the capacity of ChoP+ bacteria to bind rPAF, mice deficient in rPAF have no colonization defect (39, 374). These data suggest that there are additional host factors involved in the selection for ChoP+ bacteria during colonization. Here, we show that ChoP attachment to the LPS alters the physical properties of the outer membrane and reduces antibody binding to the surface of *H. influenzae*.

## Results

### *Adaptive immunity is required for the selection of ChoP+ phase variants during colonization*

The role of adaptive immunity in the selection of ChoP+ phase variants was examined *in vivo* using a murine model of nasopharyngeal colonization. The percentage of ChoP+ phase variants was determined by colony immunoblotting following colonization with a mixture of ChoP+/- variants of strain H632, an NTHi

strain that is able to colonize mice (440). The proportion of ChoP+ phase variants in the output (colonizing) population was substantially greater than that in the input (inoculum) after challenge of immune competent, (wild-type) BALB/c mice (Figure 1). In contrast, there was no evidence for a selection of ChoP+ phase variants following colonization in BALB/c mice lacking an adaptive immune system (severe combined immune deficiency, or SCID) (35). These results demonstrate that adaptive immunity is important for the selection of ChoP+ phase variants during colonization.

*ChoP expression reduces antibody binding to the surface of H. influenzae*

The importance of natural, or pre-existing, antibody in limiting *H. influenzae* colonization has been demonstrated previously (440). These data led to an examination of the impact of ChoP expression on antibody binding by flow cytometry. It was found that ChoP+ variants had reduced antibody binding to their surface compared to ChoP- variants (Figure 2). The ChoP+ phase variant of NTHi strain H233 had decreased binding of natural IgG using both serum from (wild-type) BALB/c mice (Figure 2A) and normal human serum (NHS; Figure 2C). Revertants of the originally selected phase variants of strain H233, as well as ChoP+/- phase variants in another NTHi strain, H729, showed a similar effect (Figure 2A, C). The ChoP-mediated reduction of the binding of natural antibody in serum (naïve control) was also observed in the serum of mice previously intranasally immunized with *H. influenzae* (Figure 2B). To confirm the difference

in antibody binding was not dependent on other serum components, the effect of ChoP on binding of IgG purified from NHS was also examined, with the same result (Figure 2D). This binding assay was also conducted using a mutant strain of Rd with constitutive ChoP+ expression (phase locked ChoP+), H491. This strain was grown in chemically defined medium (CDM) with or without choline. The binding of IgG in NHS to H491 was reduced when choline was added to the CDM, compared to CDM without choline (Figure 2E). ChoP expression also reduced binding of IgA from NHS (Figure 2F), from human nasal secretions (Figure 2G), and IgM purified from NHS (Figure 2H).

To examine whether there was an additional effect of ChoP expression on binding of complement component C3, baby rabbit serum (BRS) was used as a source of complement without natural antibody to *H. influenzae*. While there was no difference C3 binding to ChoP+/- phase variants in BRS alone, the ChoP+ phase variant of strain H233 had reduced C3 binding in the presence of BRS with purified IgG from NHS (Figure 2I). Collectively, these data demonstrate that ChoP expression results in decreased antibody binding, which limits complement deposition, on the surface of *H. influenzae*.

*ChoP expression reduces binding of LPS-specific, bactericidal antibody*

The classical pathway of complement-mediated killing can be initiated by binding of CRP or bactericidal antibody to *H. influenzae*. In order to determine if ChoP expression affects classical pathway complement-mediated killing following

antibody binding, NHS depleted of C-reactive protein (CRP) was used as an antibody and complement source for bactericidal assays. ChoP+ phase variants had increased survival in CRP-depleted NHS compared to ChoP- variants of strain H233, as well as revertants of the originally selected phase variants (Figure 3A). There was also increased survival of the ChoP+ phase variant of H233 using IgG purified from NHS (in the same concentration as that used for flow cytometry in Figure 2D) with BRS as a complement source (Figure 3B). The increased survival of ChoP+ bacteria in the presence of CRP-depleted NHS was observed for multiple other NTHi strains, constitutive ChoP+ and ChoP- mutants in Rd, and an *H. influenzae* type b strain (Eagan) (Figure 3C). Serum was IgG-depleted to determine whether the difference in survival of ChoP variants was dependent on antibody. In IgG-depleted NHS there was a recovery of survival for the ChoP- variant of H233, while the addition of purified IgG restored killing (Figure 3D). No differences in survival were detected for bactericidal assays conducted in BRS as a source of complement without antibody, or in NHS with MgEGTA buffer, which allows alternative pathway complement-mediated killing only (not shown). Collectively, these results suggest that ChoP expression increases survival in the presence of bactericidal antibody.

The potential targets on the surface of *H. influenzae* that are protected from antibody binding by ChoP expression were examined using antibody-depleted NHS and mAbs. Purified LPS was used to absorb LPS–specific antibodies from NHS prior to conducting bactericidal assays. LPS antibody pre-absorption with *wt*, constitutive ChoP- LPS resulted in increased survival of the

constitutive ChoP- mutant in Rd, H446 (Figure 4B). In contrast, there was no increase in survival of the constitutive ChoP- mutant after pre-absorption with ChoP- LPS from the Rd *opsX* mutant strain, which is highly truncated with no KDO heptose extensions (Figure 4A, B). To confirm that the LPS pre-absorbed serum retained complement activity, purified IgG was added back to the LPS pre-absorbed serum, and killing was observed (not shown). These results indicate that the majority of the bactericidal antibody affected by ChoP expression in NHS is LPS oligosaccharide-specific. Of note, that there was a greater increase in the survival of the Rd constitutive ChoP- mutant H446 following NHS pre-absorption with ChoP- LPS, compared to pre-absorption with ChoP+ LPS, a result that correlated with the effect of ChoP on antibody binding (Figure 4B). While purified LPS may not accurately mimic the environment of the outer membrane, this result demonstrates that the same decrease in antibody binding observed in ChoP+ whole bacteria is also observed for ChoP+ LPS.

The mAb 6E4, which binds *H. influenzae* LPS (360), was used to examine the effect of ChoP on antibody binding to a specific LPS epitope. ChoP expression reduced 6E4 binding and increased survival following incubation in 6E4 with BRS as a complement source (Figure 4C, D). This was observed for NTHi strain H729 and for the constitutive ChoP+ mutant in Rd, H491. These results confirmed that ChoP expression provides protection against bactericidal antibody binding to LPS oligosaccharide epitopes.

*LPS structural requirements for the effect of ChoP on antibody binding*

A selection of LPS mutants in Rd was used to investigate the importance of the LPS molecular environment for ChoP-mediated protection against antibody binding (Figure 4A). The ChoP+ phase variant of the *lpsA* mutant strain, which no longer has $Hep_{III}$ hexose extensions, maintained reduced antibody binding and increased survival in the presence of CRP-depleted NHS (Figure 5). In contrast, there was no longer a protective effect of ChoP expression on antibody binding and complement-mediated killing for the *orfH* and *lpt6* mutant strains. The *orfH* mutation results in a lack of $Hep_{III}$, while the *lpt6* mutation prevents attachment of a conserved phosphoethanolamine molecule to $Hep_{II}$.

ChoP can be attached to hexose extensions from multiple heptose residues, most commonly $Hep_I$ and $Hep_{III}$ (318-320). The importance of the position of ChoP for its effect on antibody binding was examined using *lic1D* exchange mutants, as the *lic1D* allele dictates the position of ChoP attachment (228). In Rd, ChoP is attached to a hexose extension on $Hep_I$ (HI). Alteration of the position of ChoP in Rd from H1 to a hexose extension on $Hep_{III}$ (H3) resulted in the loss of ChoP-mediated protection against antibody binding and antibody-dependent bactericidal activity (Figure 6). Collectively, these data demonstrate that the constituents of the oligosaccharide and the molecular environment of ChoP are both important for the effect of ChoP on antibody binding and resistance to antibody-dependent, complement-mediated killing.

*ChoP expression alters the physical properties of the outer membrane*

We next investigated the mechanism for ChoP-mediated protection against antibody binding, considering two possibilities; 1) steric hindrance, where ChoP obscures key epitope(s) and 2) ChoP alteration of the physical properties of the outer membrane, resulting in decreased membrane accessibility. In order to test the effect of ChoP expression on the ability of molecules other than antibodies to access the membrane, trypsin sensitivity was compared for ChoP+ and ChoP- phase variants. The fluorescent dye Cy5, which labels lysine residues, was used to quantify the exposure of outer membrane surface proteins by flow cytometry. Following trypsin digestion, there was decreased Cy5 binding to the ChoP- phase variant of strain H233, as well as the ChoP- revertant of the original ChoP+ phase variant (Figure 7A). The same effect was observed for the constitutive ChoP- mutant in Rd, H446 (Figure 7A). The reduction in Cy5 binding following trypsin digestion suggests that ChoP expression affects the general accessibility of molecules, including antibodies, to outer membrane targets.

In order to further examine the effect of ChoP on the physical properties of the outer membrane, membrane barrier function was compared in ChoP+ and ChoP- phase variants. Bacterial uptake of the dye ethidium bromide (EtBr) was used to measure the effect of ChoP on the permeability of the outer membrane. The rate-limiting step for EtBr uptake is transversal of the outer membrane (270). In the presence of a low concentration of polymyxin B, there was an increased rate of EtBr uptake in the ChoP-, compared to the ChoP+, phase variants of H233 (Figure 7B). The same effect was observed for the revertants of the original

variants of strain H233 as well as the constitutive ChoP+ and ChoP- mutants in Rd (Figure 7B). It was necessary to include polymyxin B to cause initial membrane destabilization for dye uptake. While ChoP expression does not affect killing by polymyxin B alone (not shown), the difference in EtBr uptake rates may reflect a difference in outer membrane susceptibility to polymyxin B. The alteration of polymyxin B-induced EtBr uptake demonstrates that ChoP expression strengthens the barrier function of the outer membrane.

Differences in membrane barrier function often correlate with changes in the gel-to-liquid crystalline phase transition temperature, or T*m*, which can be determined by differential scanning calorimetry (DSC) (289). In order to test the effect of ChoP expression on the T*m* of *H. influenzae* LPS, DSC was performed on LPS purified from ChoP+ and ChoP- bacteria. The T*m* of LPS from the Rd constitutive ChoP+ strain H491 was determined to be 29.8±.2°C, while the T*m* of the constitutive ChoP- mutant strain H446 was significantly higher, at 34.3±.1°C ($p<.0001$). Phase transition temperatures were independent of $Mg^{2+}$ concentration.

The effect of ChoP on the integrity of the outer membrane was examined by comparing EDTA sensitivity in ChoP+ and ChoP- phase variants. EDTA treatment chelates the divalent cations that are important for maintaining LPS interactions and membrane stability (58). The ChoP+ variant of strain H233 had increased resistance to EDTA, compared to the ChoP- variant (Figure 8A). ChoP revertants of these variants showed the same trend (not shown). Growth of the

Rd constitutive ChoP+ strain H491 in CDM with choline also resulted in increased EDTA resistance, compared to survival following growth in CDM without choline (Figure 8B). In contrast, the expression of a digalactoside residue (Gal$\alpha$1-4Gal) in strain H233, detected by the mAb 4C4, had no impact on EDTA resistance (Figure 8A).

The importance of the position of ChoP for its effect on outer membrane integrity was also investigated. For the Rd *lic1D* exchange mutant strains, only ChoP in the H1, but not H3, position resulted in increased EDTA resistance (Figure 8C, D). These results correlate with the effect of ChoP position on antibody binding, suggesting that the same structural requirements for ChoP-mediated reduction of antibody binding are necessary for its effect on the integrity of the outer membrane. Next, the effect of divalent cation concentration on antibody binding was explored. Increasing the $Mg^{2+}$ concentration (up to 50 mM) resulted in reduced antibody binding to the ChoP- phase variant of H233 (Figure 8E). These results demonstrate that excess $Mg^{2+}$, which increases the stability of the outer membrane (114, 285, 358), can correct for the difference in antibody binding between ChoP+ and ChoP- variants. Together, these data indicate that ChoP expression alters the physical properties of the outer membrane, and that these effects correlate with the reduction of antibody binding in ChoP+ phase variants.

*ChoP structural requirements for its effect on antibody binding and outer membrane integrity*

ChoP is a zwitterionic molecule with a positively charged quaternary amine group, which may be important for its effect on antibody binding and the physical properties of the outer membrane. The structural components of ChoP that are required for its effect on antibody binding and the integrity of the outer membrane were examined using a strain that incorporated a ChoP analog, dimethylethanolamine phosphate [$(CH_3)_2NCH_2CH_2PO_4^-$]. This ChoP analog differs from ChoP by a single methyl group, which reduces the positive charge on the amine group with minimal alteration of the overall structure. Incorporation of the ChoP analog into the LPS of the Rd constitutive ChoP+ strain H491 grown in CDM + dimethylethanolamine was confirmed by MS (not shown). H491 grown in CDM + dimethylethanolamine had a similar level of antibody binding as when the strain was grown in CDM alone (Figure 2E). In addition, while H491 grown in CDM + choline had increased EDTA resistance compared to when it was grown in CDM alone, H491 grown in CDM + dimethylethanolamine showed the same percent survival in EDTA as when it was grown in CDM alone (Figure 8B). While it cannot be ruled out that the single methyl group impacts the steric inhibition of antibody binding, these results suggest that the positively charged quaternary amine on ChoP is important for the reduced antibody binding and increased outer membrane integrity observed in ChoP+ phase variants.

## Discussion

Understanding the requirements for *H. influenzae* colonization is integral to the effort to reduce the burden of NTHi-associated disease. *H. influenzae* is susceptible to antibody-dependent, classical pathway complement-mediated killing *in vitro*, and this may be an important mechanism for host control of *H. influenzae in vivo*. For example, human patients with primary antibody deficiencies have persistent colonization and higher rates of disease from NTHi strains (330). In this light, bacterial factors that affect antibody recognition could play a major role in *H. influenzae* survival during colonization. In this study, it was found that ChoP+ phase variants have reduced binding of antibody, including antibody binding to LPS epitopes, as well as increased survival in the presence of antibody-dependent, complement-mediated killing. While there was no difference in C3 deposition or bacterial survival for ChoP+/- phase variants in the absence of antibody, the experiments conducted in the present study do no exclude the possibility that ChoP expression affects binding of other classical pathway complement components. The major bactericidal antibody from the serum sources in this study was IgG, which can reach the site of colonization through transcytosis (291, 322). Indeed, nasal lavage fluid from BALB/c mice contains IgG that binds *H. influenzae* targets, including LPS, and a role for complement in limiting *H. influenzae* colonization has also been demonstrated (440). Increased resistance to bactericidal antibody was observed for ChoP+ phase variants of multiple *H. influenzae* strains, despite the heterogeneity

observed in this bacteria (268). These data demonstrate a novel mechanism for evasion of antibody recognition by *H. influenzae* during colonization. It was also shown that adaptive immunity is required for the increase in the selection of ChoP+ phase variants during colonization. While the short-term colonization model used for the *in vivo* experiments demonstrates a role for ChoP expression in protection against natural antibodies, it was also shown that ChoP+ phase variants have reduced binding of antibody from pre-exposed, immune hosts. Taken together, these results suggest that ChoP expression provides a selective advantage at the mucosal surface during colonization, as ChoP+ bacteria are better protected against antibody binding and antibody-dependent clearance.

ChoP is one of several LPS structural determinants whose attachment to the LPS is controlled by stochastic phase variation (264, 414). Other phase variable decorations to the LPS have been shown to have an effect on serum resistance. For example, loss of O-acetylation, sialylation, or the digalactoside residue Gal$\alpha$1,4Gal results in increased serum sensitivity, attributed to different mechanisms (83, 107, 172). The LPS of *H. influenzae* is highly heterogeneous (47), and phase variation of LPS epitopes may allow bacteria to quickly adapt to the repertoire of antibodies present in different host environments. While it was shown in the current study that ChoP+ phase variants have reduced binding of the mAb 6E4, the full scope of the LPS, or non-LPS, epitopes protected from antibody recognition by ChoP expression remains unknown. As mentioned previously, ChoP attachment to surface structures has been observed in several bacterial species. In addition to the LPS, ChoP has been found in bacteria on

teichoic acid, pili, and an elongation factor protein (15, 98, 407). It has also recently been shown that an effector protein injected by *Legionella pneumophila* modifies host regulatory factors with ChoP (15, 98, 407). The current study supplies another example of how the attachment of this ubiquitous molecule modulates the properties of its target.

The importance of the molecular environment of the LPS for ChoP-mediated protection against antibody binding was examined using a set of LPS mutants. It was found that two conserved inner core LPS structures (PEtn and $Hep_{III}$) are required for the reduction of antibody binding in ChoP+ phase variants. This result could be due to the direct loss of epitopes that are normally protected against antibody binding by ChoP expression, or through an indirect effect of these structures on binding of antibody to other LPS epitopes. Previously, it was shown that changing the location of ChoP attachment affects sensitivity to CRP (228). In accordance with this data, we determined that the position of ChoP attachment to the LPS is important for the effect of ChoP on antibody binding in Rd. While the inner core structure of LPS is conserved among *H. influenzae* strains, ChoP can be attached to hexose extensions off of any of the three inner core heptoses or to a fourth heptose present in some NTHi strains (225, 235, 319). There are also NTHi strains with a partial duplication of the *lic* locus, resulting in the attachment of two ChoP residues to the LPS in ChoP+ phase variants (106). Each *H. influenzae* strain may have optimized its LPS structural arrangement to enable ChoP-mediated protection against

antibody binding. The variable position of ChoP also argues against its main function being sterically hindering antibody binding to other LPS epitopes.

The finding that ChoP+ phase variants of *H. influenzae* are also less sensitive to trypsin digestion of outer membrane proteins led to the investigation of the effect of ChoP on the physical properties of the outer membrane. The reduced access of a non-antibody molecule to the membrane suggests that the effect of ChoP on antibody binding is due to a general effect on the outer membrane, rather than direct steric hindrance. This concept is supported by a recent study from this lab demonstrating that mutations in *H. influenzae* that change the distribution of phospholipids in the outer membrane result in decreased outer membrane stability and increased antibody binding to LPS epitopes (282).

In support of the hypothesis that ChoP expression affects the physical properties of the outer membrane, it was shown that ChoP+ phase variants have reduced sensitization to treatments that may compromise the outer membrane, such as polymyxin B-induced EtBr permeability. Polymyxin B is a cationic peptide that targets negatively charged residues in the LPS (438). A previous study demonstrated that ChoP expression reduces sensitivity to killing by the cationic antimicrobial peptide LL-37 (229), further supporting a ChoP-mediated impact on the integrity of the outer membrane. While studies in several bacterial species have shown that lipid A modifications can affect antimicrobial resistance and membrane permeability (24, 270, 358, 400), the data presented in the current

paper demonstrate that modifications outside of the lipid A-KDO inner core region can also impact membrane integrity and barrier function. DSC has been used to determine the phase transition temperatures of phospholipid membrane systems as well as purified LPS from various bacterial strains (38, 179). In the current study, the phase transition temperature for purified LPS from a constitutive ChoP+ strain was found to be reduced compared to that for ChoP- LPS. A similar trend is observed in phospholipid membrane systems, where the addition of lipids containing ChoP reduce membrane permeability and the T*m* (289). In DSC experiments using LPS isolated from *Salmonella minnesota,* it was found that mutants with reduced oligosaccharide extensions had a lower T*m*, demonstrating that changes to the oligosaccharide, in addition to lipid A alterations, can affect the phase transition temperature (37, 114).

It was shown in the current study that ChoP+ phase variants of *H. influenzae* have increased resistance to membrane disruption by EDTA. EDTA chelates the divalent cations that are important for the stabilization of the outer membrane through association with multiple negatively charged phosphate groups on the LPS (6, 285). It has been shown that EDTA treatment causes loss of outer membrane organization and shedding of LPS molecules, resulting in reduced membrane integrity (6, 393). The modification of outer membrane integrity correlated with an effect on antibody binding, as the addition of $Mg^{2+}$ alone resulted in decreased antibody binding. While ChoP is a relatively small structural addition to *H. influenzae* LPS, the presence of the positively charged quaternary amine group may impact the outer membrane by altering charge

interactions. It was shown in this study that bacteria that incorporated dimethylethanolamine phosphate instead of ChoP did not have reduced antibody binding or increased EDTA resistance. These data indicate that the positively charged amine group, rather than the negatively charged phosphate group, is required for the effect of ChoP on outer membrane integrity and antibody binding. The expression of molecules with amine groups on lipid A, as well as KDO, has been shown to increase outer membrane stability in other bacteria (213, 316). Our study suggests a similar effect through modification of the outer core of the oligosaccharide with ChoP. This also supports the notion that a large part of the effect of ChoP on antibody binding is indirect, through alteration of outer membrane accessibility, rather than direct steric inhibition.

In summary, these data indicate that ChoP expression increases the barrier function and integrity of the outer membrane, and these alterations correlate with a reduction in the accessibility and binding of antibody to ChoP+ phase variants. Phase variation of ChoP may be an important consideration in the design of NTHi vaccines targeting LPS epitopes, as selection for ChoP+ phase variants could abrogate vaccine effectiveness. In contrast, vaccines targeting ChoP would select for ChoP- phase variants, and thereby increase effective immune responses to other cell surface epitopes. While phase variation of LPS structures can directly alter the presence of specific epitope(s), these data suggest a novel mechanism whereby ChoP expression affects the ability of antibody to recognize bacterial targets by altering access to outer membrane antigens.

## Materials and Methods

### *Ethics Statement*

This study was conducted according to the guidelines outlined by National Science Foundation Animal Welfare Requirements and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance Number A3079-01.

### *Bacterial strains*

All strains are listed in Table 1. Strains were grown in brain heart infusion media (Becton Dickinson Biosciences, Franklin Lake, NJ) supplemented with Fildes enrichment (Remel, Lenexa, KS) and 20 μg/mL β-Nicotinamide adenine dinucleotide hydrate (Sigma, St. Louis, MO), referred to as sBHI. When specified, strains were grown in CDM, prepared as previously described (252). CDM was supplemented with 300 μM of choline chloride or the choline analog *N,N*-Dimethylethanolamine (Sigma) where indicated. Selection of ChoP and Galα1-4Gal containing phase variants was performed by colony immunoblotting as previously described (417). Revertants of ChoP variants were selected in the same manner. Each ChoP phase variant population was determined to be over

98% ChoP+ or ChoP- by colony immunoblotting, and constitutive ChoP mutants were 100% ChoP+ (H491) or ChoP- (H446). The percentage of ChoP+ and ChoP- bacteria in each phase variant population remained constant during growth to log phase, as there is no selective pressure on ChoP expression *in vitro*. ChoP+ colonies transferred to a nitrocellulose membrane were detected using a 1:10,000 dilution of the monoclonal antibody TEPC-15 (Sigma) followed by a 1:10,000 dilution of alkaline phosphatase-conjugated anti-mouse IgA (Sigma). Colonies expressing the Gal$\alpha$1-4Gal structure were selected using a 1:10,000 dilution of the monoclonal antibody 4C4 (401) followed by a 1:10,000 dilution of alkaline phosphatase-conjugated anti-mouse IgG (Sigma).

*Mouse nasopharyngeal colonization*

Colonization studies were conducted as described previously (440). Briefly, mice were intranasally inoculated with $10^7$ CFU/mL of bacteria that were first washed and diluted in phosphate-buffered saline (PBS). ChoP variants were grown separately, followed by combination at a 3:1 ratio of ChoP- : ChoP+ bacteria by volume prior to inoculation. Nasal lavage fluid was collected in 200 $\mu$l of PBS and plated onto sBHI containing 50 $\mu$g/mL streptomycin following three days of colonization. Bacterial counts obtained by nasal lavage were comparable to those collected by plating nasopharyngeal tissue homogenates. The percentage of ChoP+ colonies was determined through detection of ChoP by colony immunoblotting.

*Flow cytometric analysis*

Antibody binding was detected by flow cytometry as previously described (282). Briefly, 200 μl reactions containing mid-logarithmic phase bacterial cells in Hank's buffer without Ca2+ or Mg2+ (Gibco, San Diego, CA) supplemented with 5% fetal calf serum (HyClone, Logan, UT) were incubated with primary antibody for 60 min at 37°C. Primary antibody sources included naïve BALB/c serum (1:200 dilution), NHS (1:200 dilution), IgG purified from NHS (Sigma, 4.8 μg), IgM purified from NHS (Sigma, 3.7 μg), normal human nasal secretions (1:200 dilution), mAb 6E4 (1:100 dilution for H729, 1:500 dilution for Rd), and BRS (1:50 dilution). Serum collected from BALB/c mice that had been intranasally inoculated at day 0, 7, and 14 with either PBS (naïve) or $10^7$ CFU/mL of constitutive ChoP- type b strain H445 (immune) was also used as a source of primary antibody (1:20 dilution). Reactions mixtures were then washed and re-suspended in 1:200 dilutions of secondary antibody, followed by incubation at 4°C for 60 min. Secondary antibodies included goat anti-mouse IgG-FITC, goat anti-human IgG-FITC, goat anti-human IgA-FITC, goat anti-human IgM-FITC (Sigma), and goat anti-rabbit polyclonal C3-FITC (MP Biomedical Chappel, Irvine, CA). Reaction mixtures were then washed and re-suspended in PBS with 1% bovine serum albumin and 0.5% paraformaldehyde prior to flow cytometric analysis on a BD FACS Calibur flow cytometer (Becton Dickinson Biosciences). A total of 50,000 cells were collected from each reaction mixture, and the MFI of

antibody binding was determined using FlowJo software (Tree Star, Ashland, OR).

*Bactericidal assays*

Assays were conducted in 200 μl reaction mixtures containing 20 μl of mid-logarithmic phase bacterial cells ($OD_{620}$ 0.5) diluted to $10^5$ CFU/mL in Hank's buffer with $Ca^{2+}$ and $Mg^{2+}$ (Gibco). Following the addition of serum, reaction mixtures were incubated for 45 min at 37°C. One serum source was CRP-depleted NHS, used at a 1:10 dilution for H233 and Eagan, a 1:5-1:10 dilution for other NTHi strains, and a 1:50 dilution for Rd. CRP was depleted from NHS using immobilized *p*-aminophenyl phosphoryl choline gel, which also results in depletion of anti-ChoP antibodies, according to the manufacturer's protocol (Thermo Scientific, Rockford, IL). Another serum source was a 1:20 dilution of BRS, to which purified IgG from NHS was added (Sigma, 4.8 μg). IgG depletion of NHS was performed using a Protein G column (GE Healthcare Bio-Sciences, Uppsala, Sweden). IgG eluted from the Protein G column according to manufacturer's instructions was also used as a source of purified IgG (0.25 μg/mL) for bactericidal assays. Survival due to alternative pathway mediated killing alone was determined by comparing survival in BRS alone (no *H. influenzae* antibodies) and by chelating NHS with gelatin veronal buffer containing MgEGTA (Boston Bioproducts, Worcester, MA) (122). Serum pre-absorption of anti-LPS antibodies was conducted by incubation of NHS (1:50

dilution) with 1 μg LPS overnight at 4°C. Baby rabbit serum was used as a source of complement without antibody at a 1:20 dilution for H233, a 1:10 dilution for H729, and a 1:25 dilution for Rd. Bactericidal assays with baby rabbit serum were supplemented with IgG purified from NHS (4.8 μg) for H233, or mAb 6E4 at a 1:10 dilution for H729 and a 1:100 dilution for Rd. Percent survival was determined relative to complement-inactivated serum, which was incubated for 30 min at 56°C prior to use. Assays to determine EDTA sensitivity were performed by addition of EDTA (1-4 mM) to bacterial cells in sBHI diluted in Hank's buffer with $Ca^{2+}$ and $Mg^{2+}$, followed by incubation for four hours at 37°C. Percent survival was determined relative to no-EDTA controls.

*LPS extraction*

LPS extractions were performed using the phenol-chloroform-petroleum ether method as previously described (112), with modifications (338). Briefly, bacterial pellets were washed sequentially in ethanol, acetone, and petroleum ether prior to lyophilization. The lyophilized samples were re-suspended and mixed overnight in a 2:5:8 extraction mixture of phenol: chloroform: petroleum ether. Following filtration and evaporation of chloroform and petroleum ether, LPS was precipitated from phenol in 5:1 mixture of acetone: diethyl ether. Ultracentrifugation was used to further purify LPS re-suspended in water, followed by lyophilization.

*Trypsin digestion and Cy5 detection of surface proteins*

Partial digestion of outer membrane proteins with trypsin was performed as previously described (262). Briefly, 200 μl of mid-logarithmic phase bacterial cells ($OD_{620}$ 0.5) were washed and re-suspended in 10 mM Tris-HCL, pH 7.5. Following addition of 1mg/mL trypsin, bacteria were incubated for 2hrs at 37°C. The cells were then washed and re-suspended in 10 mM carbonate buffer prior to staining with Cy5 according to manufacturer's instructions (GE Biosciences Amersham, Buckinghamshire, UK). Surface proteins with exposed lysines were labeled with 10 μl of Cy5 (40pmol) in the dark for 20 min, and reactions were stopped with 20 μl of 10 mM lysine. Cells were washed with 10 mM carbonate buffer and re-suspended in PBS with 1% bovine serum albumin prior to flow cytometric analysis on a BD FACS Calibur flow cytometer (BD Biosciences). A total of 50,000 cells were collected from each reaction mixture, and the MFI of antibody binding was determined using FlowJo software (Tree Star).

*EtBr uptake assay*

Ethidium bromide was used as a measure of outer membrane permeability as previously described (270). Bacterial cells grown to stationary phase ($OD_{620}$ 0.8) were re-suspended in PBS, with 15 μg/mL polymyxin B and 6 μM EtBr added directly prior to each measurement. Fluorescence was measured at an excitation

wavelength of 544 nm, an emission wavelength of 610 nm using FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany). EtBr uptake was expressed by RFU/s.

*DSC analysis of LPS phase transition temperature*

Purified LPS samples for each strain were diluted to 2 mg/mL in PBS, sonicated, and subjected to three cycles of incubation at 56°C for five min, vortexing 1 min, and cooling to 4°C. Where specified, $MgCl_2$ was added to the PBS at 1:1 and 5:1 [$MgCl_2$]: [LPS] molar ratios. Following preparation, samples were stored at 4°C for several hours before running on the DSC instrument. Heat capacity profiles were determined at a scan rate of 60°C/hr over a temperature range of 10-60°C in a high-resolution differential scanning calorimeter (MCS, MicroCal, Amherst, MA). Three consecutive heating and cooling scans were measured per sample. PBS buffer-buffer references were subtracted from sample data, and concentration was normalized based on sample concentration. The Microcal ORIGIN software package was used to progress baselines. The gel-to-liquid crystalline phase transition temperature, T*m*, was determined by integration from baseline to calculate the midpoint of the transition.

*MS analysis*

Purified LPS samples were subjected to mild acid hydrolysis and electrospray ionization-mass spectrometry (ESI-MS) was performed as previously described (226).

*Statistical analysis*

Differences between groups were assessed for statistical significance using an unpaired Student's *t*-test (GraphPad PRISM4, GraphPad Software, La Jolla, CA).

*Gene reference numbers*

The reference numbers for genes mentioned in the text include: *opsX* (0261), *lpsA* (0765), *orfH* (0523) and *lpt6* (0258, 0259) from the Rd database for the published genome sequence (102), as well as *lic1A* (950399) and *lic1D* (950403) GeneID from the NCBI GenBank database.

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Table 1. List of strains used in this study

| Strain | Description | Reference |
|---|---|---|
| H233 | NTHi chronic bronchitis clinical isolate A860516 | (394) |
| H632 | NTHi otitis media clinical isolate SR7332.P1, Sm[a] | (243) |
| H707 | NTHi COPD[b] (non-exacerbation) clinical isolate | (282) |
| H708 | NTHi COPD (non-exacerbation) clinical isolate | (282) |
| H725 | NTHi COPD (exacerbation) clinical isolate | (282) |
| H729 | NTHi COPD (exacerbation) clinical isolate | (282) |
| Rd | Type d, unencapsulated isolate | (5) |
| H446 | Rd with *lic1D*::Km[c], constitutive ChoP- | (228) |
| H457 (H3)[d] | Rd with *lic1D* (Eagan) | (228) |
| H491 (H1)[e] | Rd with *lic1A*Δ$(CAAT)_n$, constitutive ChoP+ | (228) |
| *lpsA* | Rd *lpsA*::Km | (170) |
| *lpt6* | Rd *lpt6*::Km | (428) |
| *opsX* | Rd *opsX*::Km | (170) |
| *orfH* | Rd *orfH*::Km | (170) |
| Eagan | Type b clinical isolate | (413) |
| H625 | Eagan, unencapsulated isolate[f] | (165) |
| H445 | Eagan with *lic1D*:Km, constitutive ChoP- | (228) |

[a]Sm, spontaneous streptomycin resistant mutant, [b]COPD, chronic obstructive pulmonary disease, [c]Km, contains kanamycin resistance cassette, [d]H3, ChoP attached to hexose extension from $Heptose_{III}$, [e]H1, ChoP in natural position (for Rd) attached to hexose extension from $Heptose_{I}$, [f]type b- spontaneous mutant lacking both copies of the *cap* locus

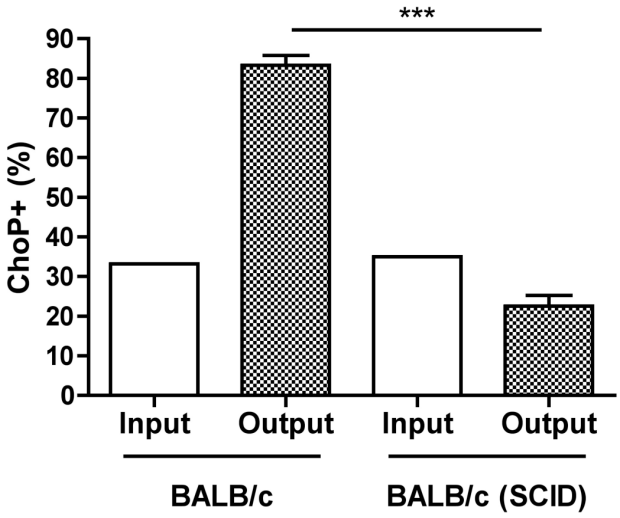


Figure 1. Adaptive immunity is required for selection of ChoP+ phase variants during colonization. BALB/c or BALB/c (severe combined immune deficiency, or SCID) mice were intranasally inoculated with $10^7$ CFU/mL of a 3:1 mixture of ChoP-:ChoP+ phase variants of NTHi strain H632. The percentage of ChoP+ phase variants was determined by colony immunoblotting of the inoculum (input, white bars) and the nasal lavage fluid following three days of colonization (output, stippled bars). Values are derived from five mice per group ± SD, ***p<.0001.

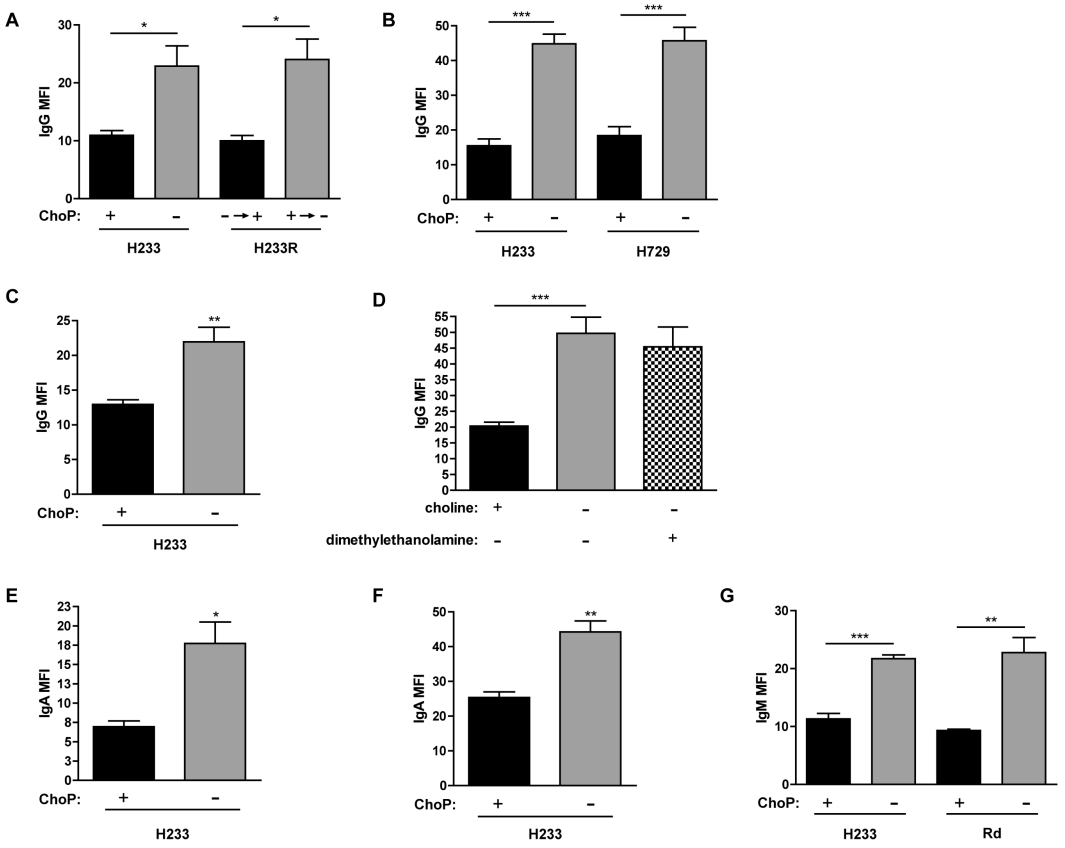


Figure 2. ChoP expression decreases antibody binding to the bacterial surface. Flow cytometric analysis was used to quantify antibody binding to bacterial cells. Representative histogram of BALB/c serum IgG binding to phase variants of NTHi strain H233, and the mean fluorescence intensity (MFI) for binding to H233 phase variants and revertants of the originally selected phase variants (A). MFI of IgG binding from the serum of naïve (intranasally inoculated with PBS) or immune (intranasally inoculated with constitutive ChoP- type b strain H445) BALB/c mice to phase variants of type b (unencapsulated) strain H625 (B). The MFI of normal human serum (NHS) IgG binding to phase variants of NTHi strains H233 and H729 (C) and purified IgG for strain H233 (D). The MFI of NHS IgG binding for Rd constitutive ChoP+ strain H491 grown in CDM alone (grey bar) or supplemented with choline ($(CH_3)_3N^+CH_2CH_2$, black bar) or dimethylethanolamine ($(CH_3)_2NCH_2CH_2$, stippled bar), (E). The MFI of IgA binding for phase variants of H233 using NHS (F) or pooled human nasal secretion IgA binding (G). The MFI of IgM purified from NHS binding for phase variants of H233 and Rd (H). The MFI of C3 binding from BRS to phase variants of H233 in the presence or absence of purified IgG (from NHS) (I). ChoP+ variants are shown in black bars, ChoP- variants are shown in grey bars. Values are derived from at least three independent experiments ± SD, *p<.05, **p<.01, ***p<.001.

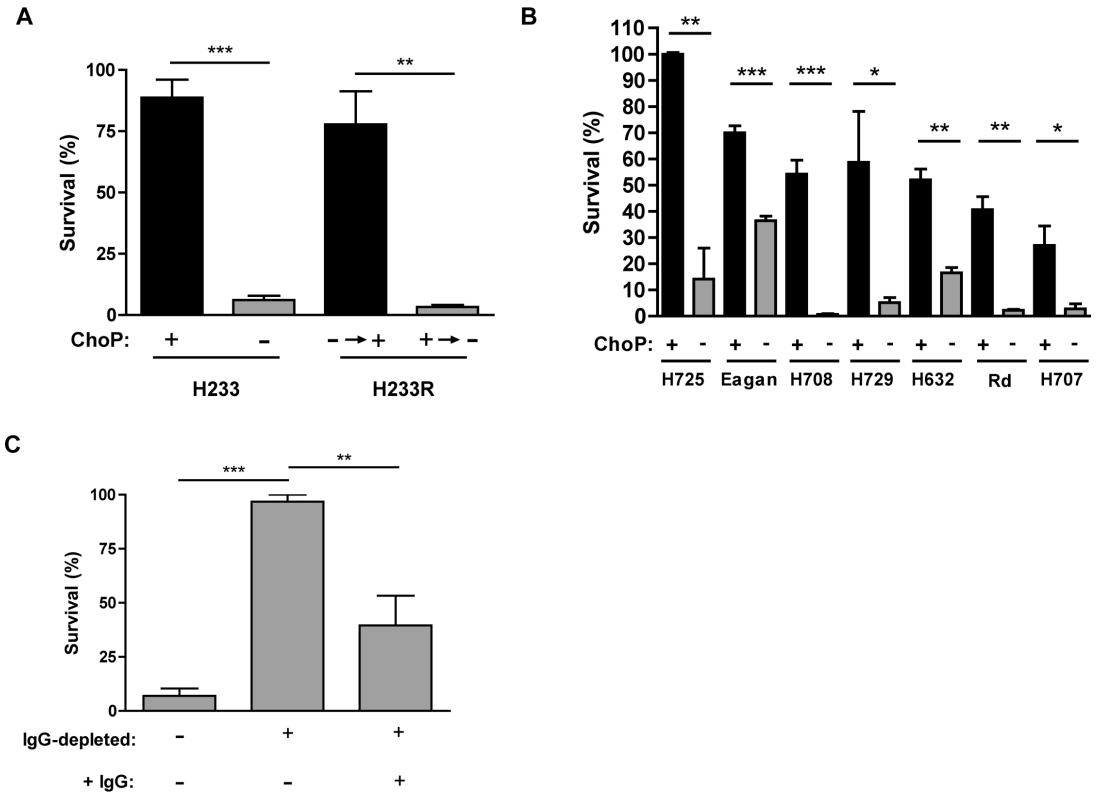


Figure 3. ChoP expression protects against bactericidal antibody. Percent survival in C-reactive protein (CRP)-depleted NHS (relative to complement-inactivated control) is shown for phase variants of NTHi strain H233 and revertants of the originally selected phase variants (A). Percent survival in purified IgG from NHS with BRS (complement source) for phase variants of H233 (B). Percent survival in CRP-depleted NHS for constitutive ChoP+ and ChoP- mutants in Rd, phase variants of type b strain Eagan, and multiple NTHi strains (C). Percent survival of NTHi strain H233 ChoP- phase variant in NHS, IgG-depleted NHS, or in IgG-depleted NHS supplemented with purified IgG, is shown as indicated (D). ChoP+ variants are shown in black bars, ChoP- variants are shown in grey bars. Values are derived from at least three independent experiments ± SD, *p<.05, **p<.01, ***p<.001.

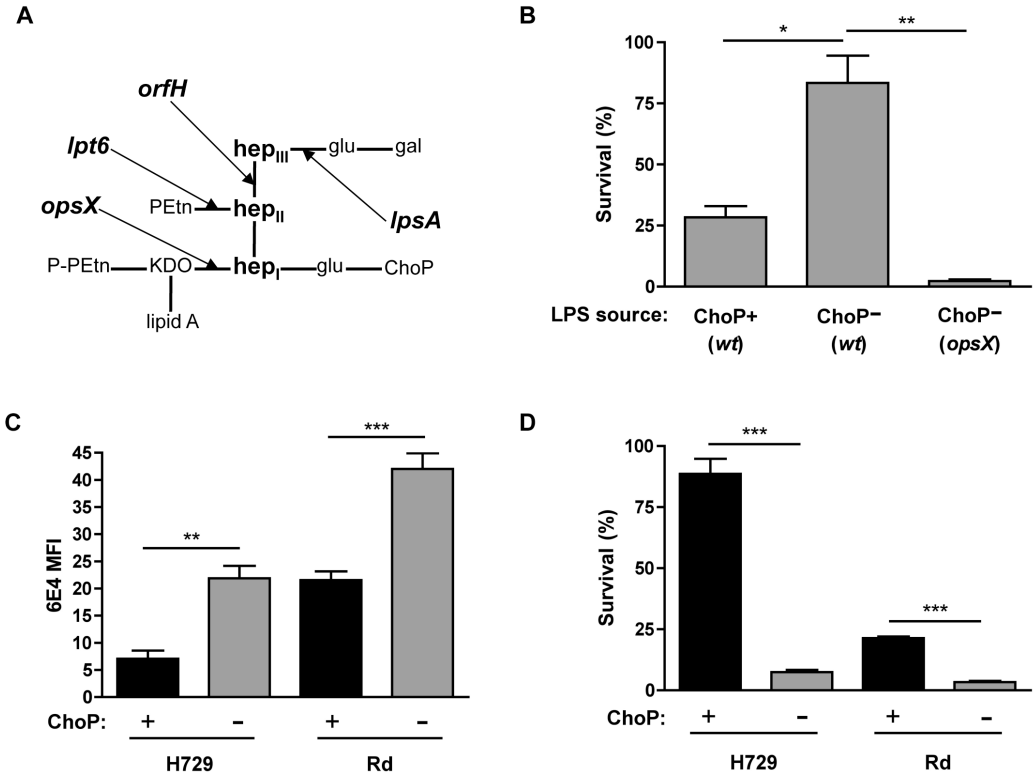


Figure 4. ChoP expression reduces binding of LPS bactericidal antibody. Rd LPS structure with arrows indicating sites of truncations for LPS biosynthesis mutants (A). Percent survival (relative to complement-inactivated control) for Rd constitutive ChoP- strain H446 in NHS that was absorbed with: purified Rd LPS from the constitutive ChoP+ mutant, the constitutive ChoP- mutant, or an *opsX* mutant (B). Flow cytometric analysis of the MFI of mAb 6E4 binding to phase variants of NTHi strain H729 and constitutive ChoP+ and ChoP- mutants in Rd (C). Percent survival in BRS (complement source) and mAb 6E4 for phase variants of NTHi strain H729 and constitutive ChoP+ and ChoP- mutants in Rd (D). ChoP+ variants are shown in black bars, ChoP- variants are shown in grey bars. Values are derived from at least three independent experiments ± SD, *p<.05, **p<.01, ***p<.001.

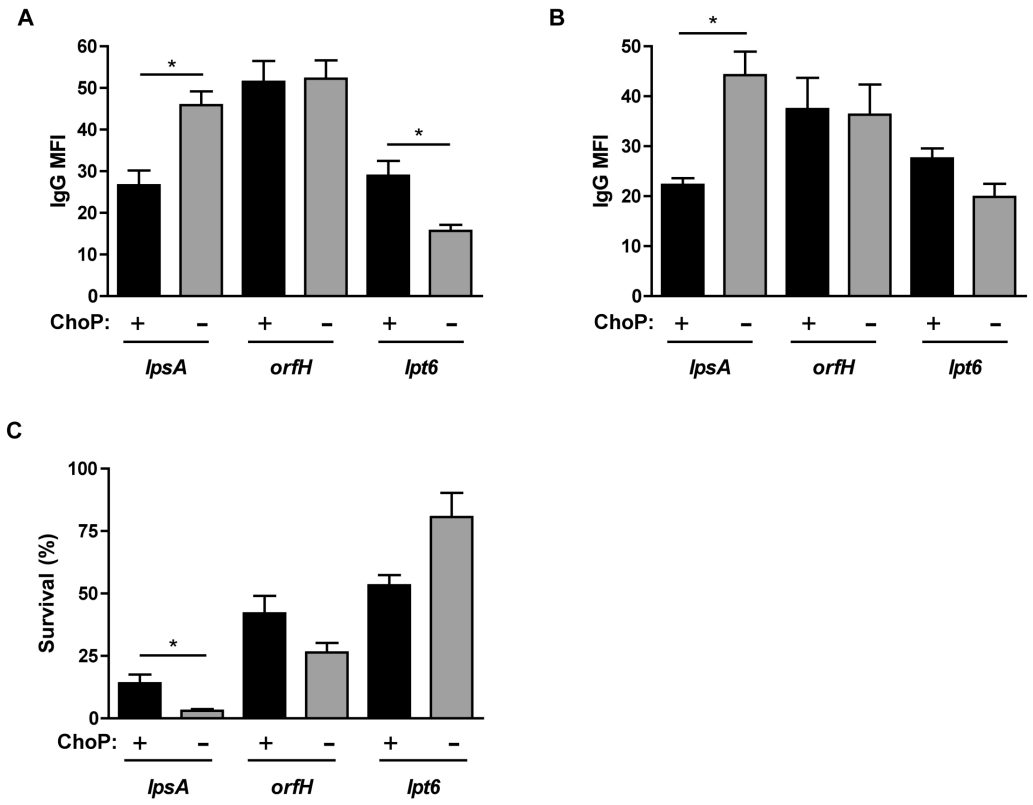


Figure 5. Core LPS structures are required for ChoP-mediated protection against antibody binding. Flow cytometric analysis of the MFI of IgG binding to phase variants of Rd LPS mutant strains *lpsA, orfH,* and *lpt6* following incubation in BALB/c serum (A) or NHS (B). Percent survival in CRP-depleted NHS (relative to complement-inactivated control) for phase variants of Rd mutant strains (C). ChoP+ variants are shown in black bars, ChoP- variants are shown in grey bars. Values are derived from at least three independent experiments ± SD, *p<.05, **p<.01.

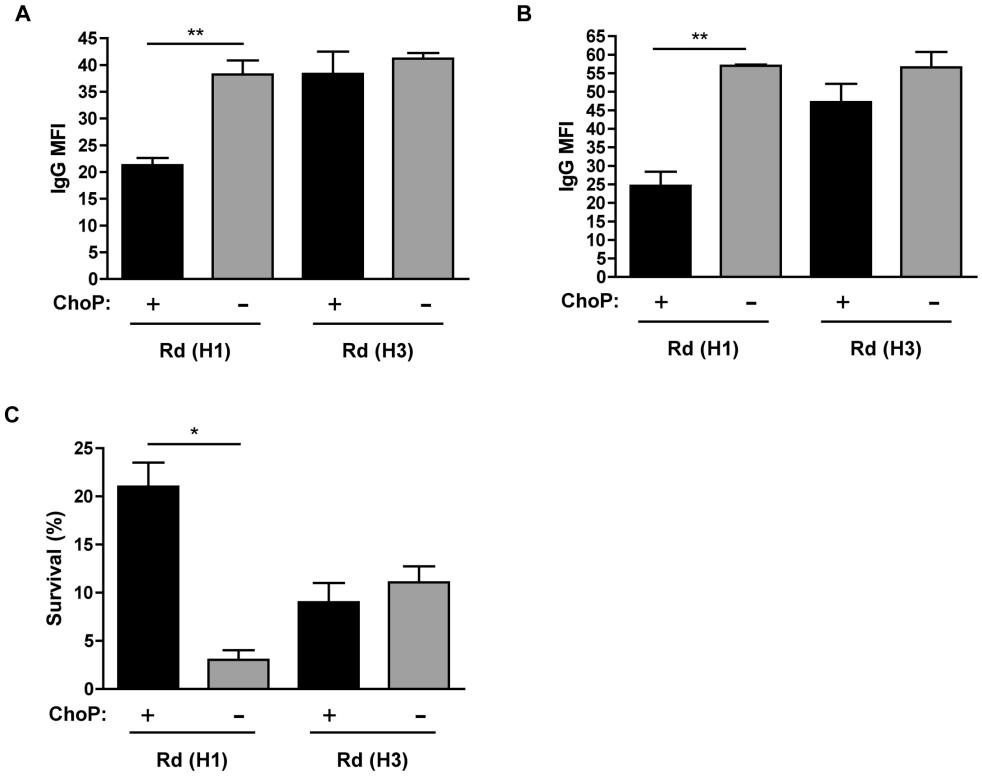


Figure 6. Position of ChoP affects ChoP-mediated protection against antibody binding. Flow cytometric analysis of the MFI of IgG binding to phase variants of Rd *lic1D* exchange mutants; H491 with Rd *lic1D* ($Heptose_{I}$, or H1), and H457 with Eagan *lic1D* ($Heptose_{III}$, or H3), from BALB/c serum (A) or NHS (B). Percent survival (relative to complement-inactivated control) was determined following incubation in CRP-depleted NHS (C). ChoP+ variants are shown in black bars, ChoP- variants are shown in grey bars. Values are derived from at least three independent experiments ± SD, *p<.05, **p<.01.

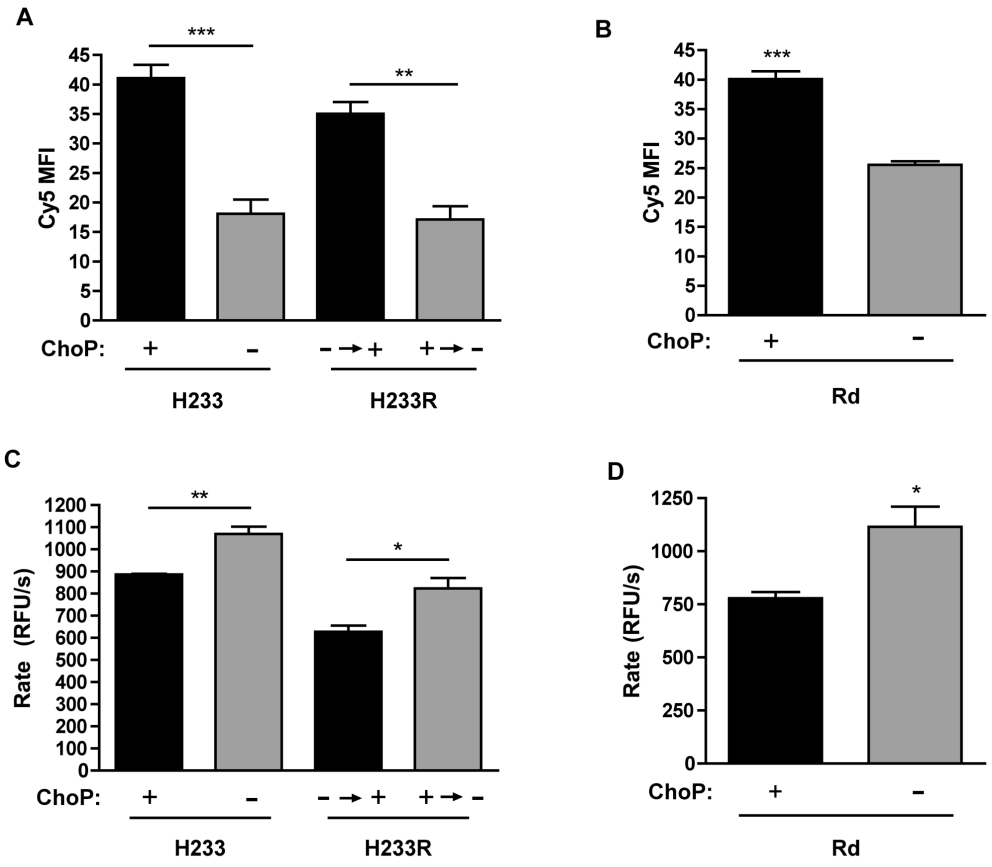


Figure 7. ChoP expression affects outer membrane accessibility and barrier function. Flow cytometric analysis of the MFI of Cy5 binding to outer membrane surface proteins following trypsin digestion for phase variants of NTHi strain H233 and revertants of the originally selected phase variants and constitutive ChoP+ and ChoP- mutants in Rd (A). Rate of ethidium bromide (EtBr) uptake, expressed in relative fluorescent units per second (RFU/s), in the presence of polymyxin B for phase variants of NTHi strain H233 and revertants and constitutive ChoP+ and ChoP- mutants in Rd (B). ChoP+ variants are shown in black bars, ChoP- variants are shown in grey bars. Values are derived from at least three independent experiments ± SD, *p<.05, **p<.01, ***p<.001.

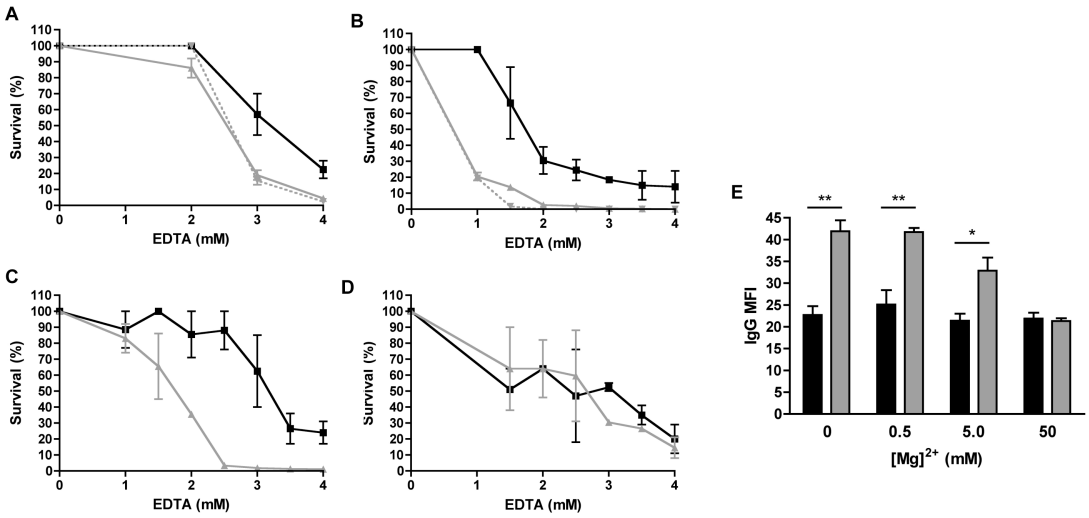


Figure 8. ChoP expression increases outer membrane integrity. Percent survival following incubation in EDTA was determined for ChoP+ (black solid), ChoP- (grey solid), and ChoP-,Gal$\alpha$1-4Gal+ (grey dashed) phase variants of NTHi strain H233 (A). Percent survival in EDTA for Rd constitutive ChoP+ strain H491 grown in CDM alone (grey solid), or supplemented with choline (black solid) or with dimethylethanolamine (grey dashed) (B). Percent survival in EDTA for Rd *lic1D* exchange mutants, Rd *lic1D* (H1) (C) and Eagan *lic1D* (H3) (D). Flow cytometric analysis of the MFI of NHS IgG binding in the presence of 0-50 mM $Mg^{2+}$ for ChoP+ (black bars) and ChoP- (grey bars) phase variants of NTHi strain H233 (E). Values are representative of three independent experiments performed in duplicate ± SD (A-D), or are derived from three independent experiments ± SD *p<.05, **p<.01 (E).

## CHAPTER 4

# Molecular basis of increased serum resistance among pulmonary isolates of non-typeable *Haemophilus influenzae*

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Abstract

Non-typeable *Haemophilus influenzae* (NTHi), a common commensal of the human pharynx, is also an opportunistic pathogen if it becomes established in the lower respiratory tract (LRT). In comparison to colonizing isolates from the upper airway, LRT isolates, especially those associated with exacerbations of chronic obstructive pulmonary disease, have increased resistance to the complement- and antibody-dependent, bactericidal effect of serum. To define the molecular basis of this resistance, mutants constructed in a serum resistant strain using the *mariner* transposon were screened for loss of survival in normal human serum. The loci required for serum resistance contribute to the structure of the exposed surface of the bacterial outer membrane. These included loci involved in biosynthesis of the oligosaccharide component of lipooligosaccharide (LOS), and *vacJ*, which functions with an ABC transporter encoded by *yrb* genes in retrograde trafficking of phospholipids from the outer to inner leaflet of the cell envelope. Mutations in *vacJ* and *yrb* genes reduced the stability of the outer membrane and were associated with increased cell surface hyrophobicity and phospholipid content. Loss of serum resistance in *vacJ* and *yrb* mutants correlated with increased binding of natural immunoglobulin M in serum as well as anti-oligosaccharide mAbs. Expression of *vacJ* and the *yrb* genes was positively correlated with serum resistance among clinical isolates. Our findings suggest that NTHi adapts to inflammation encountered during infection of the LRT by modulation of its outer leaflet through increased expression of *vacJ* and *yrb* genes to minimize recognition by bactericidal anti-oligosaccharide antibodies.

Author Summary

*Haemophilus influenzae* generally colonizes the human upper respiratory tract. When isolated from the lower respiratory tract, this opportunistic pathogen is associated with inflammatory conditions such as pneumonia and exacerbations of chronic obstructive pulmonary disease (COPD). Here we show that one of the adaptations made by *H. influenzae* isolated from the lower respiratory tract is increased resistance to the bactericidal effect of antibody and complement. To define the mechanism for increased resistance, mutants were screened to identify the complete set of genes required to inhibit killing by antibody and complement. These included multiple genes that all contribute to biosynthesis of the organism's surface oligosaccharide (lipooligosaccharide), which is targeted by bactericidal antibody. Our results also revealed a novel function for additional genes that maintain the lipid asymmetry of the surface membrane and thereby limit recognition of the pathogen by anti-oligosaccharide antibodies.

## Introduction

The mucosal surface of the human nasopharynx is serially colonized by different strains of *Haemophilus influenzae* (268). When host factors allow this opportunistic pathogen to gain access to the normally sterile parts of the respiratory tract, inflammatory diseases such as otitis media, sinusitis or

pneumonia may result (272). Widespread immunization against encapsulated strains with the type b polysaccharide has greatly reduced the incidence of invasive disease by *H. influenzae* in children. However, non-typeable strains (NTHi), which do not express a capsule, remain amongst the most common etiologic agents of localized infectious diseases of the airway in all age groups (389). The damaged airways in adults with chronic obstructive pulmonary disease (COPD) are especially susceptible, and identification of a newly acquired NTHi isolate in sputum is temporally associated with exacerbations of disease symptoms and decline in pulmonary function (14, 344). COPD ranks as the fourth leading cause of death in the US and is rapidly becoming recognized as a public health problem of similar proportions in other parts of the world (279, 297).

Characteristics of the organism that allow it to transition from its commensal state in the upper airway and survive the inflammatory milieu of the lower respiratory tract (LRT) and elsewhere are poorly understood. In particular, during early infection this predominantly extracellular pathogen will be exposed to increasing levels of natural (i.e. pre-existing) antibody and complement produced locally or extravasated from serum. For encapsulated *H. influenzae*, the thick polysaccharide coat protects the organism from recognition by immunoglobulin, the activation of complement and complement-dependent bactericidal activity. For gram-negative bacteria, the exposed surface is its outer membrane, an asymmetric lipid bilayer consisting of an outer leaflet of lipid A attached to a polysaccharide (LPS) and an inner leaflet of phospholipid (183, 189, 285). For *H. influenzae*, LPS is referred to as a lipooligosaccharide (LOS) because of its more

limited number of attached sugars. There is marked strain to strain heterogeneity in the presence and linkages of these sugars and oligosaccharide epitopes these residues generate indicating that antigenic variation may contribute to immune evasion by NTHi (339, 375). Structural features of the surface oligosaccharide that inhibit complement-dependent killing have been analyzed extensively (83, 95, 164, 415). The expression of many of these oligosaccharide components is controlled by highly repetitive DNA sequences and, as a consequence of slipped stranded mispairing, the expression of oligosaccharide structures is turned on and off at high frequency (413, 415). While this would predict that the presence of bactericidal antibody and complement would select for variants with increased resistance, many of these structures decorating the surface oligosaccharide are present on both serum sensitive and resistant isolates. Therefore, our current understanding does not fully account for why only some NTHi are serum resistant and how this phenotype correlates with the pathogenicity of the species.

In this study, we addressed whether increased resistance to the complement-mediated bactericidal activity of normal human serum is a characteristic of isolates from the LRT. We then used a whole genomic approach to identify the genes required for the expression of serum resistance among these isolates. We describe an important role for genes involved in trafficking of phospholipids in evading natural antibody and the expression of serum resistance by NTHi.

## Results

### *Lung isolates have increased serum resistance and decreased binding of natural IgM*

Collections of recent clinical isolates maintained with minimal *in vitro* passage were compared for their ability to survive following a 60 min incubation in 5% normal human serum (NHS). Bactericidal activity was complement-dependent, since killing was not observed in controls using heat-inactivated serum. Sputum isolates from the lower respiratory tract (LRT) (n=22) were significantly more serum resistant than colonizing strains (n=25) cultured from the upper respiratory tract (Fig. 1A). Among the LRT isolates, those obtained at the time of a COPD exacerbation were the most serum resistant. Next, we examined whether differences in serum resistance correlated with the binding of immunoglobulin present in normal human serum as measured by flow cytometry. There was no difference between serum resistant and serum sensitive isolates in binding of IgG (Fig. 1B1). In contrast, the serum sensitive strains bound significantly more IgM than serum resistant strains (Fig. 1B2). There was no difference between serum resistant and serum sensitive strains in killing by baby rabbit serum (2.5%), which lacks natural antibody to *H. influenzae*, as a source of complement (Fig. 1C). Addition of IgM, but not IgG, purified from NHS to baby rabbit serum significantly enhanced killing of serum sensitive, but not serum resistant isolates (Fig. 1C). Together these results demonstrate 1) an association between serum resistance

and the ability of NTHi to infect the LRT and 2) that resistant isolates bind less natural, bactericidal IgM.

*Genetic basis of increased serum resistance*

In order to identify the complete set of genes required for serum resistance in NTHi, we screened *mariner* transposon mutants generated in strain R2866, a previously described serum resistant isolate for which the whole genome sequence was available, for increased susceptibility to NHS (423). A total of 6912 mutants were individually screened to provide ~4-fold representation of open reading frames. Genomic DNA from candidates showing <10% survival was back transformed into the parent, R2866, and these back transformants were tested to confirm that the insertion mutation conferred a serum sensitive phenotype. Sixty serum sensitive mutants (representing 0.87% of the total strains screened) were identified and for these the *mariner* insertion site was defined. We focused on the genes (13 total and 12 of 'known' function) for which there was more than a single 'hit' (Table 1). Eight loci, including *lgtC, galE, waaQ, lic2A, lex2B, lpsA, yhxB* and *galU*, function in the biosynthesis of the surface oligosaccharide and these were not considered further (170). The most striking effect on serum resistance (Fig. 2A) was observed with mutations in HI0718 (encoding VacJ, a putative lipoprotein), and in a separate operon with 'hits' in HI1083, HI1085, and HI1086 (encoding orthologs of other gram-negative species; YrbB, a putative NTP binding protein; YrbD, an ABC transporter periplasmic protein; and YrbE, an ABC transporter permease, respectively).

VacJ, YrbD and YrbE each share homology (>60% sequence identity) with members of the *E. coli* Mla transport system, which have been proposed to function in preventing phospholipid accumulation in the outer leaflet and thereby maintain the lipid asymmetry and the barrier function of the gram-negative outer membrane (234). A double mutant in *vacJ* and the ABC transporter gene *yrbE* had a similar serum sensitive phenotype to that of each mutant, which confirmed that these may act in the same pathway. The effect on *vacJ* is unlikely to be caused by a polar effect of the insertion mutation, since a mutant in the immediate downstream gene, HI0719, maintained serum resistance (data not shown). There was no effect of *vacJ* or *yrb* genes on LOS or outer membrane protein profiles as assessed following separation using tricine gel electrophoresis followed by silver staining (data not shown).

*vacJ and yrb ABC transporter genes contribute to serum resistance and IgM binding*

After incubation in baby rabbit serum, no significant difference was observed in the binding of rabbit complement factor 3 between wild type and *vacJ* mutant (data not shown) indicating a requirement for antibody in the differential susceptibility of the mutants. To determine whether *vacJ* and *yrbE, B* and *D* mediate serum resistance by affecting antibody binding, we compared the deposition of IgG and IgM purified from NHS by flow cytometry. No detectible effect of these mutations on the binding of IgG was observed (data not shown). In contrast, there was a significant increase in the binding of IgM to the mutants

(Fig. 2B and 2C). To determine if increased binding of IgM was sufficient to account for loss of serum resistance of the mutants, IgM purified from human serum was used with 2.5% baby rabbit serum as a complement source. Under these conditions each of the mutants was more susceptible to IgM dependent killing (data not shown). To define the complement pathway affected, the survival of the mutants was studied in the presence of Mg-EGTA buffer, which inhibits the classical pathway. When the classical pathway was inhibited, a significant increase in the survival of each mutant was observed (Fig. 2D). The requirement for the classical pathway of complement showed that the anti-bacterial effect of IgM was not caused by agglutination. Together these results demonstrated that *vacJ* and *yrbE, B* and *D* are needed for serum resistance in R2866 by limiting the binding of natural IgM that promotes killing via the classical pathway of complement activation.

*vacJ and yrb ABC transporter genes affect binding of anti-LOS antibody*

Next, we considered the target of bactericidal, natural IgM affected by mutations in *vacJ* and *yrb* ABC transporter genes. We performed FACS analysis to compare the binding of murine mAbs 4C4 and TEPC-15, which bind specifically to LOS components, Galα1-4Gal and phosphorylcholine, respectively. Since these are both phase variable LOS epitopes, we first enriched for mAb 4C4 or TEPC-15 positive cells by colony immunoblotting. Mutations in *vacJ* and the *yrb* ABC transporter genes significantly increased the binding of mAbs 4C4 and

TEPC-15 (Fig. 3A and 3B). Furthermore, mAb 4C4 was bactericidal in the presence of normal mouse serum as a source of complement and each mutant was significantly more sensitive compared to the parent strain. (Fig. 3C). We then investigated whether *vacJ* affects the antibody binding to cell surface proteins by flow cytometry in two different ways. First, we compared binding of the mAb 7B11 to an exposed epitope on outer membrane protein P2 on strain H782 (143). However, the mutation in *vacJ* in strain H782 did not alter the binding of mAb 7B11 (data not shown). Second, we labeled exposed lysine residues on cell surface proteins by treating whole bacteria with the fluorescent dye Cy5. There was no difference in the levels of bound Cy5 between wild type and *vacJ* mutant (data not shown). Our data suggested that *vacJ* affects binding of antibody to exposed LOS but not outer membrane protein epitopes. Since mAb 4C4 is IgG and TEPC-15 is IgA, the effect on antibody binding and killing was not specific to IgM. This suggests that our observations about natural antibody in NHS could be because the bactericidal antibody targeting LOS is predominantly IgM.

*vacJ and yrb ABC transporter genes affect outer membrane stability*

To test whether vacJ and yrb ABC transporter genes affect the integrity of the outer membrane, we analyzed the sensitivity of the mutants to small antimicrobial compounds, including vancomycin (MW 1449), novobiocin (MW 613), bacitracin (MW 1423), and polymyxin-B (MW 1302). There was no difference in sensitivity to these compounds compared to the parent strain suggesting that the outer

membrane barrier of the mutants is largely intact (data not shown). We then addressed the stability of the outer membrane by the addition of EDTA, which chelates divalent cations and compromises the outer leaflet by interrupting intermolecular associations between LOS phosphate groups. A concentration of 25 mM EDTA had no effect on the parent strain, but resulted in a >3-log decrease in viability for each of the mutants (Fig. 4A). Similarly, the mutants were sensitive to the detergent deoxycholate at a concentration 4-fold lower than that required to inhibit growth of the parent strain (data not shown). Our observations of increased sensitivity to EDTA and deoxycholate demonstrated that the stability of the outer membrane was impaired in the mutants. Next, we compared the physical properties of the outer leaflet of the mutants by measuring the rate of uptake of 1-N-phenylnaphthylamine (NPN), a probe that changes fluorescence upon transfer from a hydrophilic to hydrophobic environment. We predicted that higher phospholipid content would increase the hydrophobic character of the cell surface of the mutants. As shown in Fig. 4B, vacJ and yrbE mutants had more rapid NPN uptake than the wild type strain, demonstrating that both mutants have increased surface hydrophobicity. In addition, we directly compared the content of surface exposed phospholipids by treating whole cells with phospholipase C and then detecting released diacylglycerol using thin layer chromatography (Fig. 4C). In comparison to the parent strain, amounts of released diacylglycerol were increased in vacJ (spot density increased 219%) and yrbE (spot density increased 143%) mutants, showing that the amount of surface phospholipid accessible to phospholipase C treatment was increased in

the mutants. The similar results for each mutant provided further evidence that vacJ and yrb genes act in the same pathway and were consistent with their previously proposed function in E. coli in excluding phospholipids from the outer leaflet described (234). Together our findings suggested that maintaining the asymmetry of the outer leaflet by the exclusion of phospholipids is important in limiting recognition of surface oligosaccharide epitopes by antibody.

*Expression of vacJ and yrb ABC transporter genes correlates with serum resistance in clinical isolates*

To investigate the relationship between outer leaflet stability and serum resistance in NTHi, we compared the sensitivity of serum resistant and serum sensitive clinical isolates to EDTA. As shown in Fig. 5A, serum resistant isolates were significantly more resistant to EDTA than serum sensitive isolates. We then determined whether differences in serum resistance and resistance to EDTA correlated with the expression of *vacJ* and *yrb* genes by qRT-PCR. As shown in Fig. 5B, *vacJ* expression was ~5-fold higher in serum resistant strains compared to serum sensitive isolates. In addition, *yrbE* and *yrbD* expression was ~3-fold higher in serum resistant compared to serum sensitive isolates (Fig. 5C and 5D). To further investigate the relationship between serum resistance and *vacJ* expression, we serially passaged strain H725, a serum sensitive clinical isolate, in 2.5% NHS to select for variants with an increasing capacity to resist the bactericidal effect of serum. With each passage H725 became more serum resistant and this adaptation was associated with a stepwise increase in *vacJ*

expression and resistance to EDTA (Fig. 6A–C). Our data suggest that among clinical isolates differences in serum resistance correlate with the level of expression of *vacJ* and its effect on stability of the outer leaflet of NTHi.

## Discussion

Although generally co-existing in a commensal relationship with its host, NTHi is able to survive the robust inflammatory response it induces in normally sterile sites in the respiratory tract such as the lung. Since humans are serially colonized beginning early in childhood, prior exposure to NTHi, as well as to other microbial species that induce cross reactivity, provides an abundant source of natural antibody (89, 292). Individuals with defects in generating antibody are particularly susceptible to recurrent respiratory tract infection with NTHi (293). To survive the inflammatory response in the LRT, this pathogen has to evade the effects of the host's pool of pre-existing antibody, which when bound to the bacterial surface activates complement and induces lytic killing. In this report we used a serum killing assay to show that survival of NTHi isolated from the LRT is associated with increased resistance to the complement-dependent bactericidal effect of antibody. Additional evidence in support of this conclusion is the finding that serum resistance was highest among the isolates obtained at the time of COPD exacerbations when clinical signs of inflammation, such as increased production of sputum, are more pronounced. Although antibodies of other isotypes can be bactericidal, we found that most of the natural bactericidal

antibody present in NHS is IgM. Our findings demonstrate that serum killing correlates with binding of natural IgM followed by activation of the classical pathway of complement. Observations on the prominence of anti-LOS IgM in the bactericidal activity of NHS correlate with previous reports looking at natural bactericidal antibody present in animal sera (85). Findings in the current study are also consistent with prior reports from this laboratory using a mouse model of airway infection showing that complement and natural antibody protect the host from NTHi (440).

Previous analysis of serum resistance in NTHi has been limited by the marked heterogeneity within and between strains. This is largely caused by the rapid variation in the expression of surface antigens targeted by natural antibody and demonstrates the ability of the organism to escape antibody-dependent, complement-mediated killing. Several specific oligosaccharide structures that contribute to serum resistance have been described. Some strains express siayltransferases that use serum CMP-NANA to cap the oligosacchaide with sialic acid, which inhibits the activation of complement through the alternative pathway (95). The disaccharide Galα1-4Gal, which mimics the human Pk blood group antigen, blocks recognition by anti-LOS antibodies (415). The oligosaccharide structures requiring the galactosyltransferase LgtC affects expression of Galα1-4Gal modulating deposition of C4b and activation of the classical pathway (164). These oligosaccharide decorations are each variably expressed within and between strains. Our finding that 8 of the 13 genes necessary for the expression of serum resistance in a highly serum resistant

NTHi strain function in biosynthesis of the surface oligosaccharide highlights its central role in this phenotype and the pathogenicity of the organism. In addition, we demonstrate that the oligosaccharide is the major target of bactericidal human antibody. This may explain the predominance of natural IgM in targeting the NTHi oligosaccharide, since antibody to LPS antigens is dominated by IgM generated by B-1 cells (314). For example, a large proportion of B-1 cells generate IgM reactive with phosphorylcholine (31).

The selection for a more serum resistant phenotype in the LRT correlated with increased expression of *vacJ* and *yrb* genes, which alter cell surface characteristics and thereby limit the binding of bactericidal antibody. The identification of VacJ and an ABC transporter with related function as necessary for the expression of serum resistance shows that surface characteristics other than structural components of the oligosaccharide contribute to serum resistance of NTHi. The lipoprotein VacJ was previously identified as a virulence determinant contributing to intracellular survival by *Shigella flexneri* (372). The group of Silhavy proposed that VacJ acts with the Mla ABC transporter to maintain the lipid asymmetry of the outer membrane by recycling phospholipids from the outer leaflet back to the inner leaflet (234). In *E. coli*, mutants lacking these genes were more sensitive to the presence of SDS plus EDTA added to solid media, but not to antibiotics that need to access targets in the periplasmic space. These observations indicated that 1) stability of the outer leaflet generated through intermolecular bridging of LPS by divalent cations was compromised by the accumulation of phospholipids and 2) the permeability

barrier of the outer leaflet remains largely intact in the mutants. We observed similar characteristics of mutants in *vacJ* and Mla ABC transporter homologs in NTHi. In addition, we provided direct evidence that *vacJ* and *yrb* genes function in determining key characteristics of the cell surface (i.e. its hydrophobicity) and that these genes affect amounts of surface exposed phosholipid. The increase in antibody binding and killing seen in these mutants provides a new insight into how these physical characteristics of the outer membrane contribute to serum resistance. Our results suggest that the intramolecular forces bridging LOS molecules also serve to limit binding of antibodies to the oligosaccharide. Thus, when phospholipids are more thoroughly excluded (high expression of *vacJ* and *yrb* genes), LOS molecules are more tightly packed and accessibility of LOS epitopes is restricted (serum resistance). Whereas when phospholipids accumulate in the outer leaflet (low expression of *vacJ* and *yrb* genes), intermolecular associations of LOS molecules are interrupted allowing for increased access of oligosaccharide epitopes recognized by bactericidal antibodies (serum sensitivity).

Interestingly, the expression of *vacJ* and *yrb* genes, which are required for serum resistance, is variable within and between NTHi strains. Serum resistant isolates demonstrated increased levels of transcription compared to serum sensitive isolates and serial passage of a sensitive isolate in serum selected for increased resistance and resulted in a higher level of *vacJ* expression. Thus, differences in transcription of *vacJ* are another factor accounting for the marked differences in serum resistance that characterizes this species. It is somewhat

surprising that for many clinical isolates levels of *vacJ* expression is low enough to affect the stability of the outer membrane. This suggests that some level of phospholipid accumulation in the outer leaflet is tolerated by NTHi and implies that there must be an advantage to a less stable, more hydrophobic outer membrane, particularly during colonization when restricting recognition by antibody may be less critical for survival. It appears, however, that this fitness advantage is lost during infection of the lung when increased *vacJ* and *yrb* gene expression is selected for. Because NTHi resides in the respiratory tract where it is not exposed to the detergent effect of bile in the intestine, the physical requirements of its outer membrane may be different from the paradigm described in the classic studies based on enteric bacteria (286).

In conclusion, we show that resistance to the bactericidal effect of immunoglobulin together with complement correlates with the ability of NTHi to infect the human LRT. Analysis of a serum resistant isolate revealed that genes contributing to the biosynthesis of its surface oligosaccharide are required for this phenotype. In addition, we describe a novel mechanism for serum resistance whereby NTHi limits the binding of bactericidal anti-LOS antibody by increasing the exclusion of surface phospholipids.

## Materials and Methods

### *Bacterial strains and growth conditions*

Strains used in this study are listed in Table 2. *COPD strains.* Strains were isolated from expectorated sputum samples as part of a prospective study at the Buffalo VA Medical Center. COPD exacerbation strains fulfilled the following criteria: 1) First isolation of the strain in an adult with COPD based on molecular typing of isolates recovered from monthly sputum cultures; 2) NTHi is the only potential pulmonary pathogen isolated in the sputum sample; 3) simultaneous onset of clinical symptoms of an exacerbation (increased sputum volume, increased sputum purulence and increased shortness of breath compared to baseline symptoms). Non-exacerbation strains were from patients with COPD during clinically stable periods who fulfilled criteria 1 and 2 but symptoms unchanged from baseline upon acquisition of the strain. *Upper respiratory tract strains*. 25 NTHi strains were isolated from throat cultures of 25 healthy children attending 17 different day care centers (92, 363). Each isolate had a different pulse-field gel electrophoresis pattern and was confirmed to be *H. influenzae* (and not non-hemolytic *H. haemolyticus*) based on previous criteria (247, 268). The absence of *bexA* and *bexB* confirmed that the isolates were not capsule-negative variants.

Strains were routinely grown at 37°C in brain heart infusion broth (Becton Dickinson) supplemented with 2% Fildes enrichment (Remel) and 20 μg/ml β-NAD hydrate (NAD; Sigma). H782 was created from strain Rd by transformation with a PCR product from outer membrane protein P2 locus of strain 2019 followed by screening for expression of the surface epitope recognized by mAb 7B11 epitope (143).

*Ethics statement*

The strains from adults with COPD were obtained from subjects enrolled in a study at the Buffalo VA Medical Center that was approved by the IRB of the VA Western NY Healthcare System. All subjects provided written informed consent. Strains collected from healthy children in day care, under protocols reviewed by IRB Health at the University of Michigan, were deemed EXEMPT on the basis of: EXEMPTION #4 (45 CFR 46.101(b)(4)), because the data were collected and analyzed without personal identifiers attached to the bacterial isolates.

*In vitro transposon mutagenesis, DNA transformation and transposon library construction*

*Mariner* mutants of strain R2866 were created by *in vitro* transposon mutagenesis as previously described (210). Briefly, *in vitro* transposition reactions were carried out on NTHi genomic DNA treated with purified MarC9 transposase and *pEMspec* (240). To repair gaps, reactions were ethanol-precipitated and resuspended in the gap repair buffer [50 mM Tris (pH 7.8)], 10 mM MgCl2, 1 mM DTT, 100 nM dNTP, and 50 ng of BSA] and then treated with T4 DNA polymerase (Invitrogen) and *E. coli* DNA ligase (Invitrogen) (3). DNA was transformed into competent R2866 by the method of Herriott et al (161) and transformants where selected for on sBHI agar (1%) plates containing spectinomycin (100 µg/ml).

*Identification of serum sensitive transposon mutants*

To identify NTHi genes essential for serum resistance, *mariner* transposon mutants were screened in a 96-well serum bactericidal assay. Following growth from single colonies in 200 µl sBHI, mutants were diluted to 105 CFU/ml and 10 µl of the culture solution was added to 90 µl consisting of 55 µl PBS, 30µl Hank's buffer (Ca2+, Mg2+) and 5 µl of normal human serum (NHS). Serum was obtained from a single donor for the initial screen to minimize variability in screening large numbers of mutants. Bacteria were incubated at 37°C for 1 h with shaking before the reaction was stopped at 4°C. Controls included serum from the same donor treated at 56°C for 30 min to inactivate complement. Mutants showing >90% killing in the primary screen were further tested to minimize false positives. Genomic DNA was used to back transform competent R2866 and three spectinomycin resistant colonies were picked and rescreened in the bactericidal assay. The site of the transposon insertion was determined for mutants in which 3/3 back transformants were serum sensitive.

*Arbitrary primed PCR and nucleotide sequence*

The primers used for inverse PCR are listed in Table 2. The first round of PCR was performed in a final volume of 50 µl. Primers (0.5 µmol/reaction mixture) ARB1 paired with mag2F3 (transposon) were used in PCR under the following conditions: 1 cycle of 95°C for 8 min; 6 cycles of 95°C for 30 s, 30°C for 30 s, and

72°C for 1.5 min; 30 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 2 min; and 72°C for 5 min. A second round of PCR was performed with primers (0.5 μmol/reaction) ARB2 paired with the internal mag2F4 (transposon) primer under the following conditions: 1 cycle of 95°C for 8 min, 30 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 1.5 min, followed by 72°C for 10 min. PCR products were purified with the QIAGEN PCR cleanup kit and then used for nucleotide sequence analysis.

*Construction of ΔvacJ::KmR mutant*

The *vacJ* gene and flanking regions were amplified from strain R2866 using primers Hi_vacJ_H3_F2 and Hi_vacJ_R1 and cloned into pCR2.1TOPO vector. The entire *vacJ* gene was deleted by inverse PCR using Hi_vacJ_B1_F1 and Hi_vacJ_B1_R1 primers introducing a BamHI site. The resulting Δ*vacJ* fragment was subcloned into pUC19 and the kanamycin-resistance cassette from pUC4K was then inserted using BamHI creating pUCΔ*vacJ*::KmR. This plasmid was used to transform strains R2866 and 69G3 strains creating single and double Δ*vacJ*::KmR mutants. Disruption of *vacJ* was confirmed by PCR using primers vacJ_ F9 and Hi_vacJ_R7 located outside of the originally cloned region.

*Serum bactericidal assays*

To test clinical isolates in bactericidal assays, serum was collected, pooled and

stored at -80°C from 5 healthy adult volunteers. Assays were performed with 20 µl of a suspension of midlog phase organisms (OD620 0.3–0.4) diluted to 105 CFU/ml in Hank's buffer with Ca2+ and Mg2+ (GIBCO, Auckland, New Zealand), 10 µl of NHS, 110 µl of PBS and 60 µl of Hank's buffer with Ca2+ and Mg2+. After incubation for 60 min at 37°C with rotation, the assay was stopped by cooling to 4°C and dilutions were made for quantitative culture. To inhibit the classical pathway of complement, veronal buffer (pH 7.4) containing 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM MgCl2, and 10 mM Mg-EGTA was substituted for Hank's buffer (246). To calculate the percent survival, viable counts were compared to controls in which complement activity had been eliminated by heat-inactivation at 56°C for 30 min. IgG was removed from NHS using a protein G column according to the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). The flow-through of IgG column was used for purification of IgM. Purified IgM was obtained by using IgM purification column according to the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). The concentration of purified IgG and IgM were determined by the Micro BCA protein assay (Pierce Chemical Co.) and a titrated enzyme-linked immunosorbent assay (ELISA). Where indicated, three-four week old baby rabbit serum was used as a complement source (Pel-Freez Biologicals, Rogers, AR).

*Colony immunoblotting*

Colonies lifted onto nitrocellulose were immunoblotted to separate phase variants

as previously described (417). Briefly, the LOS and ChoP epitope on colonies lifted onto nitrocellulose were detected using mAbs 4C4 against Galα1-4Gal (141) and TEPC-15 (Sigma) against phosphorylcholine followed by alkaline phosphatase–conjugated anti– mouse IgG and IgA, respectively. Single colonies of a uniform phenotype were selected and confirmed by repeated immunoblotting.

*Labeling of surface proteins*

200 µl of a suspension of midlog phase organisms, R2866 and *vacJ* mutant, (OD620 0.5) was pelleted, washed twice with 1 ml of 10 mM carbonate buffer (pH 8.5), and then resuspended in 200 µl of buffer (10 mM carbonate, 1 M urea). Surface-exposed lysine residues were labeled with 10 µl of Cy5 (400 pmol, GE Healthcare) on ice in the dark for 20 min (196). Reactions were stopped by addition of 20 µl of 10 mM lysine. The resulting pellets were washed twice with 500 µl of carbonate buffer, centrifuged, and resuspended in 200µl 1% BSA. Cy5 binding on bacteria was analyzed by flow cytometry with 10,000 events analyzed per sample.

*Deposition of complement factor 3*

R2866 and *vacJ* mutant were grown to an OD620 ~0.5. The bacterial suspension (200 µl) was pelleted and resuspended in Hank's buffer with Ca2+ and Mg2+

(GIBCO, Auckland, New Zealand) with 5% fetal calf serum (HyClone) and incubated with 5µl of baby rabbit sera for 1°h. Bacteria were pelleted and resuspended in Hanks' buffer plus 5% fetal calf serum containing a 1:100 dilution of a FITC-conjugated polyclonal goat anti-rabbit C3 antibody (MP Biomedical Cappel, Irvine, CA) for 60 min at 4°C in dark and analyzed by flow cytometry.

*Quantitative real-time RT-PCR*

Total cellular RNA was extracted from mid-log phase grown NTHi clinical isolates by using the RNeasy mini Kit (QIAGEN). To eliminate genomic DNA, samples were incubated with 20 U of RNase-free DNase (QIAGEN) for 20 min at 25°C using the RNeasy columns, according to the manufacturer's instructions. 1 to 1.5 µg RNA was used for reverse transcription in a 20 µl reaction with the high-capacity cDNA reverse transcription kit (Applied Biosystems) together with random primers and 20 U RNase inhibitor (Promega). 1 µl of cDNA from this reaction was used as template with 0.5 µM primers (*vacJ*-F: *5′-TCCGTGGGCATTAGTGAAAT-3′*; *vacJ*-R:5′-AATTCTGCATTATTGAGATTTTTCG-3′; *yrbD*-F:*5′-TACTGTGACGGCAACTTTCG-3′*; *yrbD*-R:*5′-AATCGCGATGCTTACTTTCG-3′*; *yrbE*-F:*5′-TCGTGTTAATCGATTTTTCTGC-3′*; *yrbE*-R:*5′-CAGGGCCTAATTCTCGTAAAAG-3′*) and SYBR Green PCR Master Mix in a 20-µl reaction (Applied Biosystems). Standard runs of the reactions on fast optical 96-well reaction plates (Applied Biosystems) were carried out using the

StepOnePlus Real-Time PCR system (Applied Biosystems). The *gyrA* gene (primers *gyr*-F: *5′-GCGTGTTGTGGGTGATGTAA-3′*; and *gyr*-R: *5′-GTTGTGCCATACGAACGATG-3′*) was used as the internal standard gene for RNA quantity normalization (124). Quantitative comparison was obtained through the ΔΔ*C*T method as described at http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041435.pdf.

*Antibody binding assays*

200 µl of mid-logarithmic-phase bacterial cells (OD620 0.5) were pelleted and resuspended in 200 µl of Hanks' buffer without Ca2+ and Mg2+ (Gibco) supplemented with 5% fetal calf serum (HyClone). Primary antibodies (1:200 for heat inactivated NHS, 1:50 for mAb 4C4, 1:1000 for mAb TEPC-15, and 1:100 for mAb 7B11) were added to reaction and then incubated at 37°C for 60 min. Bacteria were pelleted and incubated with Hanks' buffer without Ca2+ and Mg2+ plus 5% fetal calf serum containing a 1:200 dilution of the appropriate secondary antibody; goat anti-human IgG-FITC conjugate (Sigma), goat anti-human IgM FITC conjugate (Sigma), anti-mouse IgG-FITC conjugate (Sigma), anti-mouse IgA-FITC conjugate (Sigma) for 60 min at 4°C in the dark. Reaction mixtures were then washed and resuspended in 200 µl of PBS containing 1% bovine serum albumin and 0.5% paraformaldehyde. A total of 50,000 cells were collected for each sample. All samples were subjected in full volume to flow

cytometry analysis on a BD FACS Calibur flow cytometer (BD Biosciences), and groups were compared using FlowJo software (Tree Star).

*Growth inhibition assays*

Ethylenediaminetetraacetate (EDTA) was added to 500 µl of an overnight culture in sBHI broth (25 mM final concentration) and incubated at 37°C for 4 h under aeration prior to obtaining viable counts. The minimum inhibitory concentration of vancomycin, novobiocin, bacitracin, polymyxin-B and deoxycholate was determined by broth microdilution method. Briefly, 96-well plates containing 2-fold dilution of chemical were prepared in sBHI and wells were inoculated with ~5×105 cfu/ml of overnight bacterial cultures. Viable counts were determined after incubation for 18–24 h at 37°C.

*NPN uptake assay*

1-*N*-phenylnaphthylamine (NPN) (Sigma) uptake assay was performed as described previously (241). Briefly, bacteria were grown to OD620 0.5 in sBHI broth. Pellets were washed and resuspended in 5 mmol/L HEPES buffer (pH 7.2). NPN was dissolved in acetone for a 500 µmol/L stock solution and diluted to 40 µmol/L in HEPES buffer. The NPN solution (50 µl) was added after 15 s to the bacterial suspension (150 µl) for a final NPN concentration of 10 µmol/L. Fluorescence was monitored for a total of 90 s using the PTi fluorescence

system (Photon Technology International), with excitation at 350 nm, emission at 420 nm, and slit width of 2 nm.

*Phospholipase C treatment*

Phospholipase C treatment and thin layer chromatography (TLC) were performed as described previously (128). 1 L of each bacterial culture were grown in sBHI broth to OD620 0.5 and centrifuged ×5000 g for 20 min. Pellets were washed once and resuspended in a sucrose-PBS-MgCl2 buffer [0.4M sucrose, 1×PBS, 15 mM MgCl2, (pH 7.5)] to a final volume of 1.8 ml. 200 µl aliquots of bacterial suspension were treated with 5u of phospholipase C from *Bacillus cereus* (Sigma) for 20 min at 37°C. Reactions were stopped by adding 600 µl CHCl3-methanol 1:2 (v/v). The lipids were extracted by the method of Bligh and Dyer (30). Lipid samples were normalized to protein content and separated by TLC on glass backed silica gel 60 plates (Merck) with CHCl3-Acetone 94:4 (v/v) solvent system and were stained by iodine. The standard was 10 µg of 1,2-diglycerides from pig liver (Serdary Research Lab).

*Statistical analysis*

All data were analyzed using StatView software (Abacus Concepts, Cary, NC). The significance of differences between or among groups was examined using ANOVA followed by Tukey or Dunnett post-tests.

*Acknowledgments*

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*Footnotes*

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Table 1. List of sites with multiple transposon insertions affecting serum resistance in strain R2866.

| HI no.* | Gene name | Protein_id# | Function |
|---|---|---|---|
| 0258 | *lgtC* | NP_438427* | UDP-galactose-LOS-galactosyltransferase |
| 0351 | *galE* | ZP_00156190.2 | UDP-glucose 4-epimerase |
| 0461 | | ZP_00156296.2 | Hypothetical protein |
| 0523 | *waaQ* | ZP_00349685.1 | ADP-heptose-lipooligosaccharide heptosyltransferase III |
| 0550 | *lic2A* | ZP_00156370.1 | UDP-galactose-LOS-galactosyltransferase |
| 0653 | *lex2B* | ZP_00156456.1 | UDP-glucose-LOS-glucosyltransferase |
| 0718 | *vacJ* | ZP_00156519.2 | Lipoprotein (associated to retrograde PLs trafficking) |
| 0740 | *yhxB* | ZP_00156601.1 | phosphomannomutase |
| 0765 | *lpsA* | ZP_00203076.1 | LOS-glycosyltransferase |
| 0812 | *galU* | ZP_00156667.2 | UTP-glucose-1-phosphate uridylyltransferase |
| 1083 | *yrbB* | ZP_00156925.2 | Putative NTP binding protein |
| 1085 | *yrbD* | ZP_00156927.2 | ABC transporter periplasmic protein |
| 1086 | *yrbE* | ZP_00203123.1 | ABC transporter permease |

*NCBI Reference Sequence: NC_000907.1 (*Haemophilus influenzae* Rd KW20, complete genome).
#NCBI Reference Sequence: NZ_AADP01000001 and NZ_AADP01000002 (*Haemophilus influenzae* R2866 whole genome).

Table 2. The list of strains, plasmids and primers used in this study.

| Strains, plasmids, primers | Description | Reference |
|---|---|---|
| **Strains** | | |
| Rd | Rough type d isolate, genome sequence reference strain | (102) |
| 2019 | Clinical isolates of nontypeable *H. influenzae* | (302) |
| R2866 | NTHi clinical isolate from the bloodstream | (423) |
| 32F2 | R2866 *vacJ* disrupted by *mariner* Tn | This study |
| 49E3 | R2866 *yrbD* ABC transporter periplasmic protein disrupted by *mariner* Tn | This study |
| 66B5 | R2866 *yrbB* NTP binding protein disrupted by *mariner* Tn | This study |
| 69G3 | R2866 *yrbE* ABC transporter perimease disrupted by *mariner* Tn | This study |
| H782 | Rd transformed with DNA of 2019 to express m7B11 epitope on OMP P2 | This study |
| H725 | Clinical isolate of NTHi from lower respiratory tract | This study |
| H816 | H782 transformed with DNA of 32F2 to disrupt *vacJ* | This study |
| *vacJ*::Km | R2866 transformed with pUCΔ*vacJ*KmR to disrupt *vacJ* | This study |
| *vacJ*::Km,Δ*yrbE* | 69G3 transformed with pUCΔ*vacJ*KmR to disrupt *vacJ* | This study |
| **Plasmids** | | |
| pEMspec | Contains *mariner* Tn carrying SpecR cassette | (240) |
| pUCΔ*vacJ*KmR | *vacJ* replaced with KmR cassette from pUC4K | This study |
| **Primers** | | |
| ARB1 | *GGCCACGCGTGCACTAGTAC* (N)10 TACNG | (250) |
| ARB2 | *GGCCACGCGTGCACTAGTAC* | (250) |
| MAG2F3 | *GGAATCATTTGAAGGTTGGTA* | (155) |
| MAG2F4 | *ACTAGCGACGCCATCTATGTG* | (155) |
| Hi_vacJ_H3_F2 | *AAGCTTAAAATGTAGCAGGTAAACGTCG* | This study |
| Hi_vacJ_R1 | *ACGTAATGCCATCGTTTTAGAC* | This study |

| | | |
|---|---|---|
| Hi_vacJ_B1_F1 | *CGGGATCCTAAACAGAAAAGTGCGGTAAAAATT* | This study |
| Hi_vacJ_B1_R1 | *CGGGATCCTTTTAATCCTTACATAAATATGGGATTAT TC* | This study |
| Hi_vacJ_F9 | *CGTGCGTTCTTTAATGACTCTCG* | This study |
| Hi_vacJ_R7 | *CAATGTGAAGTGGAAAAGCCCC* | This study |

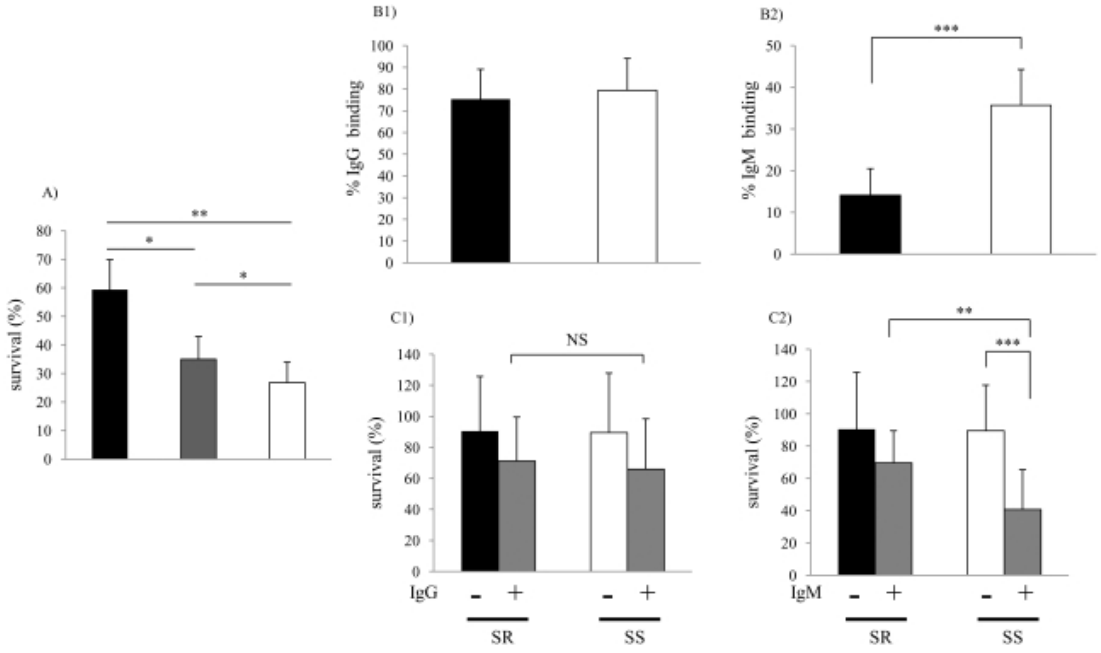


Figure 1. Characterization of clinical isolates. (**A**) Comparison of serum sensitivity between lower and upper respiratory tract isolates. Survival was determined over 60 min in 5% normal human serum and expressed relative to controls in which complement was inactivated. Groups included lower respiratory tract isolates from patients with chronic obstructive pulmonary disease (COPD) at the time of clinical exacerbation (black bars; n=11); isolates from patients with COPD during clinically stable periods (grey bars; n=11); and upper respiratory tract colonizing strains (white bars; n=25). Values are the mean of three determinations in triplicate ± SEM. (**B**) Comparison of antibody binding in serum resistant (>50% survival in pooled NHS, black bars; n=10) and serum sensitive (<50% survival in pooled NHS, white bars; n=10) isolates. B1 and B2 show percent of IgG and IgM bound following incubation in 5% heat-inactivated NHS as determined by flow cytometry, respectively. (**C**) Serum IgM contributes to bactericidal killing of clinical isolates of NTHi. Strains were incubated with or without IgG (C1, 0.25 µg/ml) or IgM (C2, 0.07µg/ml) purified from NHS for 60 min in 2.5% baby rabbit serum as a complement source. Percent survival was calculated by viable counts with and without antibody. The mean values of two independent experiments in triplicate are shown ± SD, **P*<0.05, ***P*<0.01, ****P*<0.001. Serum sensitive (SS), Serum resistant (SR).

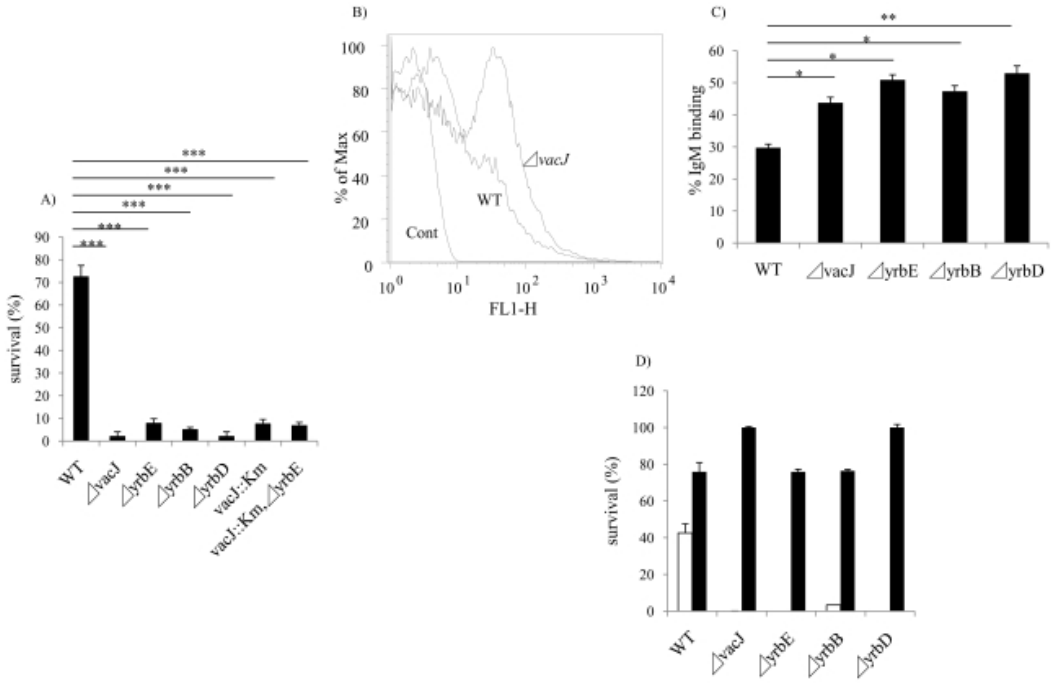


Figure 2. Characterization of *vacJ* and *yrb* mutants. (**A**) Effect of mutations in *vacJ* and genes of the *yrb* ABC transporter on serum resistance of strain R2866. Survival was determined over 60 min in 5% normal human serum and expressed relative to controls in which complement was inactivated. (**B**) Representative histogram comparing the binding, as measured by fluorescence intensity (x-axis), of total IgM purified from normal human serum to parent strain (WT) or *vacJ* by flow cytometry. Control performed without IgM (**C**) Percent IgM binding for each mutant was determined by calculating the percentage of 50,000 events with an increase in mean fluorescence intensity following incubation in 5% heat-inactivated normal human serum compared to no serum controls. (**D**) Survival of mutants in 10% normal human serum in the presence (black bars) or absence (white bars) of Mg-EGTA to inhibit the classical pathway of complement activation. Values represent two independent experiments in triplicate ± SD. **P*<0.05, ***P*<0.01, ****P*<0.001.

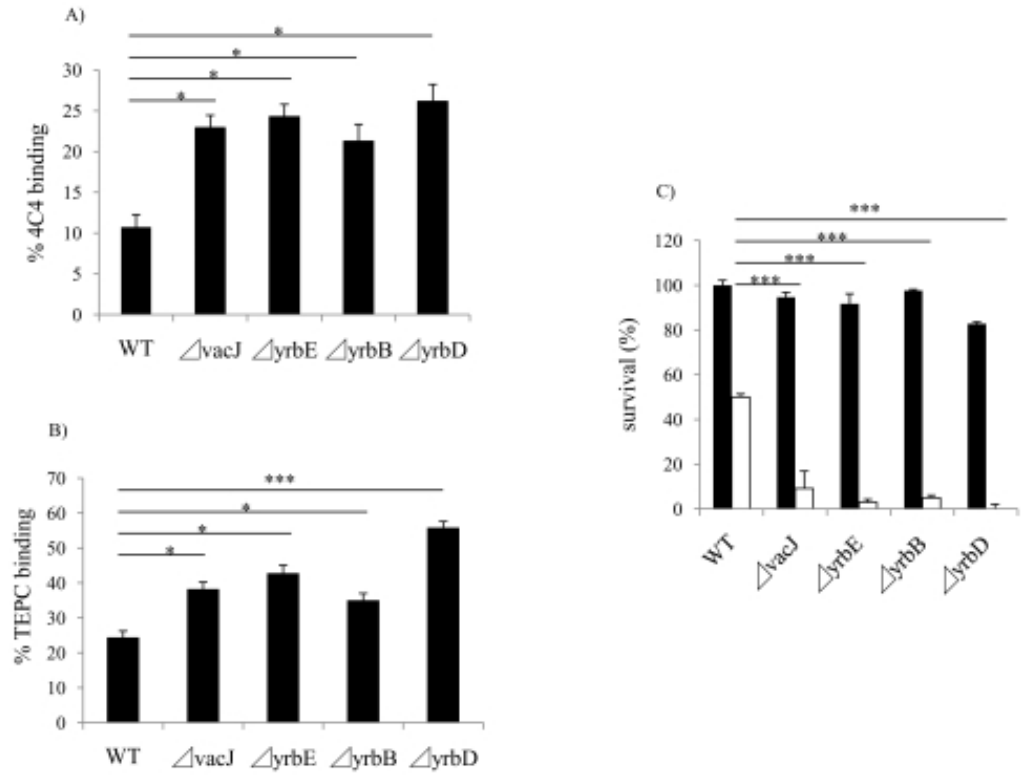


Figure 3. Effect of *vacJ* and *yrb* mutants on antibody binding and bactericidal activity. Binding of (**A**) mAb 4C4 to the LOS structure Galα1-4Gal or (**B**) mAb TEPC-15 to the LOS structure phosphorylcholine was compared by flow cytometry for the mutants indicated. Percent mAb binding for each mutant was determined by calculating the percentage of 50,000 events with an increase in mean fluorescence intensity compared to no primary antibody controls. (**C**) Effect of mutations in *vacJ* and genes of the *yrb* ABC transporter on the bactericidal effect of mAb 4C4. Bactericidal assays were performed with (white bars) or without (black bars) mAb with 5.0% normal mouse serum as a complement source. Values represent two independent experiments in triplicate ± SD. **P*<0.05, ****P*<0.001.

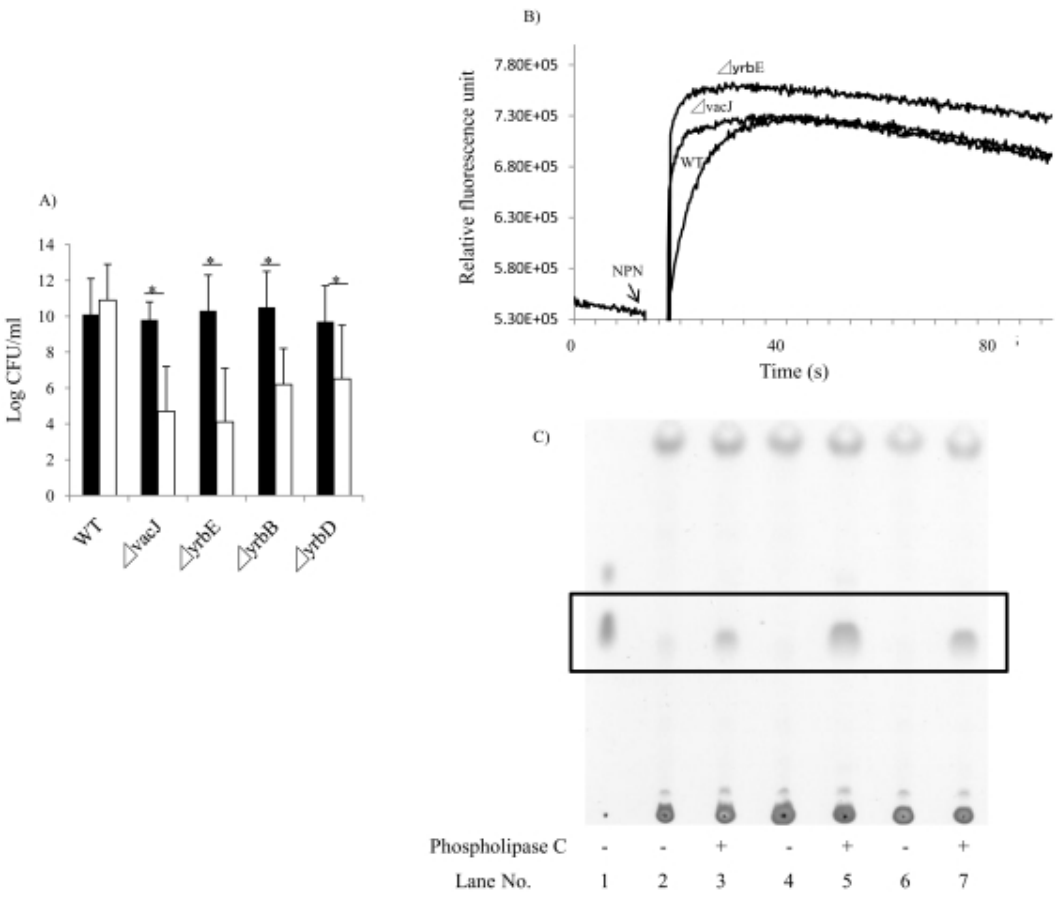


Figure 4. Effect of mutations in *vacJ* and genes of the *yrb* ABC transporter on outer membrane characteristics. (**A**) To compare outer membrane stability, following overnight culture of the strain indicated, viable counts were obtained after incubation at 37°C for 4 h in the presence (white bars) or absence (black bars) of 25 mM EDTA. Values represent two independent experiments in triplicate ± SD. **P*<0.05. (**B**) To compare surface hydrophobicity, the rate of uptake of membrane permeant 1-*N*-phenylnaphthylamine (NPN) was monitored by fluorescence. NPN was added at the time indicated and a representative experiment shown. (**C**) To compare amounts of surface phospholipids, diacylglycerol (boxed area) released by phospholipase C treatment of whole bacteria was detected by thin layer chromatography. Lane 1; diacylglycerol (standard), Lane 2 and 3; R2866 (wild type), Lane 4 and 5; 32F2 (*vacJ* mutant), Lane 6 and 7; 69G3 (*yrbE* mutant).

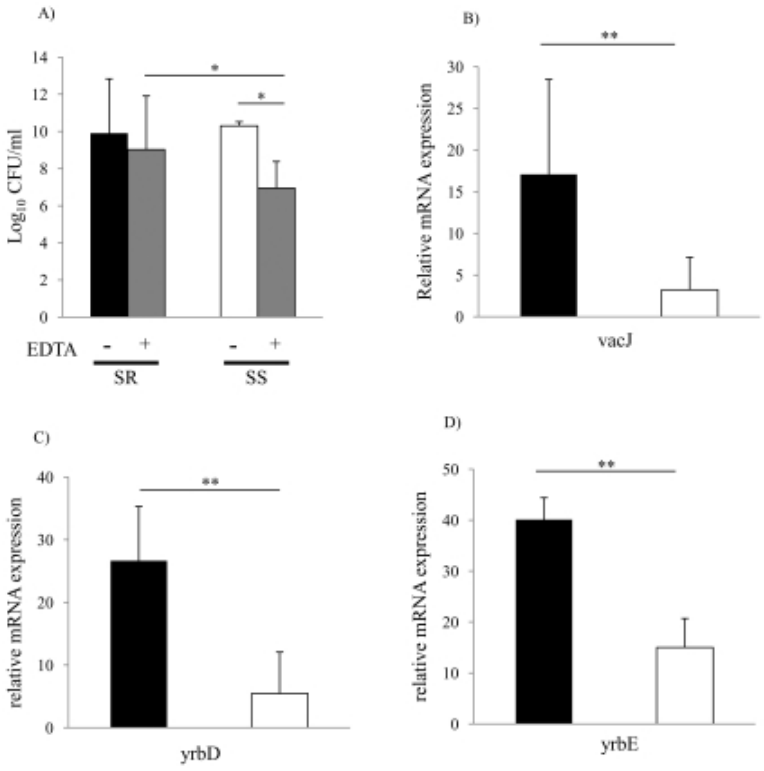


Figure 5. Membrane stability and *vacJ* and *yrb* expression among clinical isolates. (**A**) Outer membrane stability of serum sensitive (n=31) compared to serum resistant (n=16) clinical isolates. Following overnight culture, viable counts were obtained after incubation at 37°C for 4 h with or without 25 mM EDTA. Values represent two independent experiments in triplicate ± SD. **P*<0.05, Serum sensitive (SS), Serum resistant (SR). (**B**) Relative expression of *vacJ* mRNA, (C) *yrbD* and (D) *yrbE* by qRT-PCR in serum sensitive (white bar, n=7) and serum resistant isolates (black bar, n=7). Error bars indicate SD, ***P*<0.01.

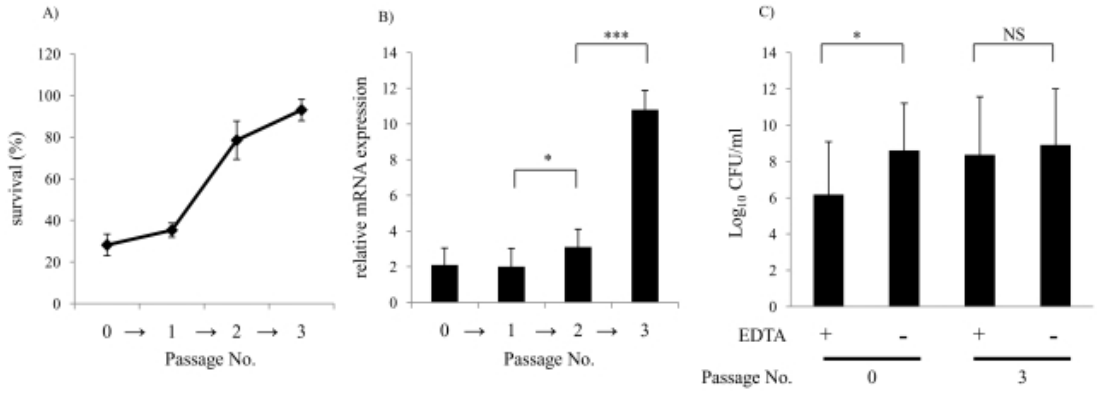


Figure 6. Repeated serum treatment selects for serum resistance, increased *vacJ* expression, and increased outer membrane stability. (**A**) Serum sensitive clinical isolate H725 was treated three times in 2.5% NHS and survival quantified after the passage indicated. (**B**) Following each passage in the bactericidal assay survivors were tested for relative expression of *vacJ* mRNA by qRT-PCR and (**C**) survival in the presence of 25 mM EDTA. Values represent two independent experiments in triplicate ± SD.*$P$<0.05, ***$P$<0.001.

# CHAPTER 5

## Evasion of killing by human antibody and complement through multiple variations in the surface oligosaccharide of *Haemophilus influenzae*

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Summary

The lipopolysaccharide (LPS) of *H. influenzae* is highly variable. Much of the structural diversity is derived from phase variation, or high frequency on-off switching, of molecules attached during LPS biosynthesis. In this study, we examined the dynamics of LPS phase variation following exposure to human serum as a source of antibody and complement in multiple *H. influenzae* isolates. We show that *lic2A*, *lgtC*, and *lex2A* switch from phase-off to phase-on following serial passage in human serum. These genes, which control attachment of a gal$\alpha$1-4gal di-galactoside structure (*lic2A* and *lgtC* phase-on) or an alternative glucose extension (*lex2A* phase-on) from the same hexose moiety, reduce binding of bactericidal antibody to conserved inner core LPS structures. The effects of the di-galactoside and alternative glucose extension were also examined in the context of the additional LPS phase variable structures phosphorylcholine (ChoP) and sialic acid. We found that di-galactoside, the alternative glucose extension, ChoP, and sialic acid each contribute independently to bacterial survival in the presence of human complement, and have an additive effect in combination. We propose that LPS phase variable extensions serve to shield conserved inner core structures from recognition by host immune components encountered during infection.

Introduction

The gram-negative bacterium *Haemophilus influenzae* has established the human nasopharynx as its niche. Between 20-60% of the population is colonized asymptomatically by *H. influenzae*, and colonization is thought to be a pre-requisite for respiratory tract diseases including pneumonia, otitis media, and exacerbations of chronic obstructive pulmonary disease (COPD), (148, 231, 275, 277). While the Hib vaccine protects against type-b *H. influenzae*, there is still a significant burden of disease caused by non-typeable *H. influenzae* (NTHi) strains (2, 391). Evasion of the host immune system is critical to the persistence of *H. influenzae* in the nasopharynx. *H. influenzae* is susceptible to classical pathway complement-mediated lysis, and components of this pathway including complement and antibody are present on the mucosal surface (440). The lipopolysaccharide, or LPS, is a major source of both intra- and interstrain surface variation in *H. influenzae* (47, 375).

Several of the genes required for the expression of LPS structures in *H. influenzae* are phase variable due to the presence of tetranucleotide repeats (107, 137, 163, 171). Slipped-strand mispairing, which occurs with these tandem repeats, creates stochastic on-off switching of gene expression. The result is high frequency ($10^2$-$10^3$ times per generation) phase variation of LPS modifications, as changes in the number of tetranucleotide repeats shifts the reading frame in and out of frame (translational switch), (413). As there are several phase variable genes controlling attachment of LPS structures, the

population of a given *H. influenzae* strain contains many different phase variants with distinct LPS structural configurations. In addition, the distribution of these genes varies among isolates. The selective pressure of host immune components can enrich for phase variants that are resistant to recognition and clearance. This has been demonstrated previously in the case of the phase variable structure ChoP, where attachment to the LPS is dependent on the *lic1* locus, of which *lic1A* contains the tetranucleotide repeats determining phase variant status (417). Bacteria with ChoP-modified LPS (*lic1A* phase-on variants) are targets of C-reactive protein (CRP), which initiates complement-mediated killing of ChoP expressing bacteria (416). In environments with high CRP levels, such as in the blood, there is selection against bacteria with ChoP-modified LPS. However, *lic1A* phase-on variants are enriched during colonization, as ChoP expression reduces antibody binding and complement-mediated killing (384, 416). ChoP and several other LPS phase variable structures increase bacterial survival in the presence of human serum, although the dynamics of these effects and how they relate to each other has not previously been explored. We sought to conduct a comprehensive analysis of the LPS phase variable structures that contribute to the evasion of complement-mediated killing in *H. influenzae*. Our findings highlight the independent and additive effects of several phase variable LPS structures on bacterial evasion of recognition by host immune components present at the mucosal surface.

## Results

### *LPS structural requirements for evasion of complement-mediated lysis*

We sought to determine the minimal LPS structural components required for bacterial resistance to human antibody and complement-mediated lysis. Initial analysis was conducted with mutants in the *H. influenzae* strain Rd, which is a type-d, unencapsulated strain with a minimally complex LPS structure compared to most NTHi isolates (see Table 1 for a full list of strains and mutants used in this study). We first examined the targets of human antibody binding by flow cytometry. We found that an *opsX*- mutant in Rd, which has a severely truncated inner core oligosaccharide, has increased binding of naturally acquired human IgG compared to bacteria with outer core oligosaccharides (Fig. 1A,B). This was demonstrated in a *lic1D*- background, as the *lic1D*-dependent attachment of ChoP to the LPS has previously been shown to reduce antibody binding (53), and we sought to determine whether additional LPS structures play a similar role. The phenotype of the *lic1D*- mutant is similar to that of a wild-type, ChoP phase-off variant. The difference in antibody binding between *opsX*- and *lic1D*- strains was maintained in the presence or absence of digestion with trypsin, suggesting that antibody binds to non-proteinaceous structures, and this is blocked by the presence of LPS outer core extensions (aside from ChoP).

Next, human serum was used as a source of antibody and complement for bactericidal assays, and survival was determined relative to complement-inactivated controls. Serum was depleted of CRP to prevent killing of ChoP

phase-on bacteria. The Rd *opsX*- mutant was sensitive to human serum compared to the *lic1D*- strain. Surviving colonies from these bactericidal assays were then grown and re-exposed to human serum, under the same conditions as in the original bactericidal assays. This was repeated several times, to determine if the level of serum resistance changed following serial passage in human serum. The *opsX*- mutant remained constitutively serum sensitive (Fig. 1C). Selective truncations of Rd oligosaccharide structures were used to establish the requirements for evasion of complement-mediated lysis. Serial passage of bacteria in human serum results in selection for increased resistance in an *lpsA*- mutant, which lacks hexose extensions from the third inner core heptose ($Hep_{III}$) of Rd (Fig. 1A,D). In Rd, ChoP is attached to the hexose extension from $Hep_{I}$, leading us to hypothesize that increased bacterial survival was due to the enrichment of *lic1A* phase-on variants in the resistant population, compared to the original population. To determine phase variant status, the 5' end of the *lic1A* gene was sequenced from genomic DNA isolated from the original and resistant bacterial populations, and the tetranucleotide repeats within these sequences were enumerated. We found that while the original population was *lic1A* phase-off, the more resistant population was predominantly *lic1A* phase-on (Table 2). This result was corroborated by colony immunoblotting using the ChoP-specific antibody TEPC-15 to distinguish between *lic1A* phase-on and *lic1A* phase-off colonies. By colony immunoblotting, the original population was phenotypically <2% *lic1A* phase-on, while the serum resistant population was >94% *lic1A* phase-on (data not shown). Previous work has documented the selection for

*lic1A* phase-on variants *in vivo* following colonization in animal models and humans, validating this *in vitro* approach for phase variant analysis (384, 416). The Rd *lpsA*-, *lic1D*- mutant, in contrast, remained constitutively sensitive to human serum (Fig. 1E).

Serial passage in human serum results in selection for increased serum resistance in the *lgtF*- mutant, which lacks the hexose extensions from the first inner core heptose ($Hep_I$) of Rd (Fig. 1A,F). As there is no possibility for ChoP attachment in the absence of *lgtF*, this resistant population potentially contains changes in phase variable genes other than *lic1A* that affect resistance to antibody and complement. A screen of all ten known genes with tetranucleotide repeats (not including *lic1A*) in Rd (305) was performed to compare phase variants in the original and serum resistant bacterial populations of the *lgtF*- mutant (see Table 3 for all primers used for tetranucleotide repeat sequence analysis). The only gene with altered phase variant status was *lic2A*, which was phase-off in the original population, and phase-on in the serum resistant population (Table 2). The expression of *lic2A* results in the attachment of a galactose residue to the LPS, which enables further hexose extensions in the presence of other phase-on LPS biosynthesis genes (Fig. 1A). Therefore, the *lic1D*-, *lic2A*- mutant contained the minimal LPS truncations required for constitutive sensitivity to human serum in Rd (Fig. 1G). These data established that selection for ChoP or galactose phase-on variants is necessary for evasion of complement-mediated killing in this strain.

In order to extend these observations to a *H. influenzae* strain with a more complex LPS structure, survival in human serum was examined for selective LPS truncation mutants in the NTHi clinical isolate R2846 (Fig. 2A). Serial passage of the R2846 *lic1D*-, *lpsA*- mutant in human serum results in selection for a population with increased resistance, while the *lic1D*-, *lgtF*- mutant is constitutively serum sensitive (Fig. 2B,C). Sequence analysis of the LPS phase variable genes present in the *lic1D-, lpsA*- mutant determined that the serum resistant population of this strain was *lic2A* phase-on, compared to *lic2A* phase-off in the original population, as with Rd (Table 2). However, unlike in the Rd strain, both the original and serum resistant populations of R2846 were also *lgtC* phase-on. The expression of *lic2A* and *lgtC* results in attachment of a di-galactoside structure, gal$\alpha$1-4gal. The proximal galactose of this di-galactoside is added in *lic2A* phase-on variants, and the distal galactose is added in *lic2A* and *lgtC* phase-on variants. The gal$\alpha$1-4gal structure is also present in humans in the form of P blood group antigens, and, therefore, is not a target of human antibody (28, 401). Interestingly, selection for *lic2A* phase-on variants was observed even though in R2846 galactose is attached to a glucose extension from $Hep_I$, rather than $Hep_{III}$ as in Rd (Fig. 2A). Therefore, the R2846 *lic1D*-, *lic2A*- mutant (also *lex2A*-; see below) contains the minimal LPS truncations required for constitutive sensitivity to human serum in this strain (Fig. 2D). In the absence of functional *lic2A*, there is no possibility for attachment of either galactose, regardless of *lgtC* phase variant status. Our analysis of LPS structures contributing to evasion of complement-mediated killing in two separate *H. influenzae* strains revealed that

selection for either ChoP or galactose phase-on variants aids bacterial survival in the presence of human serum.

*Variable LPS hexose extensions increase bacterial survival in human serum*

The contribution of hexose oligosaccharide extensions to serum resistance was examined in a *lic1D*- background of multiple strains, as the role of ChoP expression has previously been described for several *H. influenzae* isolates (53). Experiments with *lic1D*- mutants were conducted in serum without CRP depletion, as the presence of CRP does not affect survival of these strains. Serial passage in human serum results in selection for *lgtC* phase-on variants in the Rd *lic1D*- strain (Figure 3A, Table 2). As the original and serum resistant populations in this strain background were also *lic2A* phase-on, the serum resistant population expressed the di-galactoside structure. Similarly, serial passage in human serum resulted in selection for variants expressing the di-galactoside structure in the R2846 *lic1D*- strain (Fig. 3B, Table 2). Of note, sequence analysis of the original and serum resistant populations of R2846 included all LPS phase variable genes used in the first Rd screen as well as three additional LPS phase variable genes found in R2846 that are not present in Rd; *lex2A, oafA,* and *losA1* (which has a eight base pair repeat). We also performed sequence analysis on bacterial populations from each passage in between the original and serum resistant populations of Rd and R2846 *lic1D*- strains to determine the timing of the change in phase variant status. We confirmed that

either *lgtC* (Rd) or *lic2A* (R2846) switched to phase-on in the round of passage in human serum corresponding to the shift in resistance (data not shown).

To extend these observations to other relevant *H. influenzae* strains, *lic1D*- mutants of the NTHi clinical isolate 2019 and a capsule- variant of the type-b strain Eagan were also exposed to human serum. Without capsule, Eagan is significantly less resistant to complement-mediated lysis (287). In both strains, serial passage in human serum resulted in selection for a resistant population (Fig. 3C,D). The serum resistant population of 2019 was *lic2A* phase-on, compared to the *lic2A* phase-off original population (Table 2). In Eagan, the serum resistant population was both *lic2A* and *lgtC* phase-on, compared to the *lic2A* and *lgtC* phase-off original population (Table 2). Thus, passage in human serum results in selection for expression of the components of the same di-galactoside structure in four distinct *H. influenzae* strains.

LPS mutants in *lic2A* and *lgtC* were used to confirm the contribution of the di-galactoside to survival in the presence of human serum. The mAb 4C4 specifically recognizes gal$\alpha$1-4gal, and was used for phenotypic confirmation of *lic2A*- mutants by Western blotting (data not shown). In the Rd *lic1D*- background, the *lic2A* and *lgtC* phase-on strain was the most serum resistant, the *lic2A* phase-on, *lgtC*- mutant had an intermediate level of serum resistance, and the *lic2A*- mutant was the least serum resistant (Fig. 4A). These data show that both *lic2A* and *lgtC* contribute individually to bacterial survival. In the R2846 *lic1D*- background, the *lic2A* and *lgtC* phase-on strain was also more serum

resistant than the *lic2A*- mutant (Fig. 4B). We constructed the Rd and R2846 *lic1D-, lic2A*- mutants using two separate background strains; the serum resistant population (*lic2A* and *lgtC* phase-on) and the original population (*lic2A* and *lgtC* phase-off). The *lic2A*- mutants had the same phenotype in both background strains, supporting the proposition that phase variation of *lic2A* and *lgtC* accounts for the only difference between the original and serum resistant populations. Similar results were also observed for Eagan and 2019 (Fig. 4C,D). In 2019, the *lic2A* and *lgtC* phase-on variant population was isolated using the mAb 4C4, as the resistant population isolated after passage in human serum was *lic2A* phase-on, *lgtC* phase-off. Similar to Rd, in 2019 both *lic2A* and *lgtC* contribute individually to bacterial survival in the presence of human serum. These results demonstrate that each galactose extension on LPS in *lic2A* and *lgtC* phase-on variants contributes independently to the evasion of complement-mediated killing in multiple *H. influenzae* strains.

In a minority of cases, serial passage of the R2846 *lic1D*- strain in human serum resulted in selection for a resistant population that was *lex2A* phase-on instead of *lic2A* phase-on (Table 2). The expression of *lex2A* in R2846 results in the attachment of glucose to the same hexose moiety as galactose is attached to when *lic2A* is phase-on (Fig. 2A). The R2846 *lex2A* phase-on strain was more serum resistant than a *lex2A*- mutant (Fig. 4B). In order to determine whether the presence of ChoP affects selection for di-galactoside or the alternative glucose extension controlled by *lex2A* expression, we also exposed *lic1A* phase-on *H. influenzae* strains to serial passage in human serum. In R2846, the *lic1A* phase-

on serum resistant population was *lic2A* phase-on in one experiment, and *lex2A* phase-on in another (Table 2). In Rd, serial passage of the *lic1A* phase-on strain in human serum resulted in selection for a *lic2A* phase-on serum resistant population (Table 2). These results demonstrate that LPS hexose extensions contribute to evasion of complement-mediated killing independent of ChoP expression.

*Di-galactoside expression reduces antibody binding and classical pathway complement-mediated killing*

*H. influenzae* is susceptible to alternative and classical pathway complement-mediated lysis. We have previously shown that ChoP attachment to the LPS reduces antibody binding to the bacterial surface, increasing survival in the presence of complement (53). To determine whether modification of the LPS with di-galactoside or the alternative glucose extension affects antibody binding, flow cytometry was used to measure binding of human serum IgG to the bacterial surface. The Rd *lic2A* and *lgtC* phase-on variant population had the lowest amount of IgG binding, the *lic2A* phase-on, *lgtC*- strain had an intermediate amount of IgG binding, and the *lic2A*- mutant had the most IgG binding (Fig. 5A). Bactericidal assays in human serum with or without IgG depletion were used to determine whether the difference in antibody binding correlated with a difference in classical pathway complement-mediated killing. The Rd *lic2A*- mutant was killed in the presence, but not the absence, of human IgG, while the *lic2A* and

*lgtC* phase-on variant was resistant in either condition (Fig. 5B). Similar results were observed for bactericidal assays conducted with purified human IgG either added or not added to a complement source that lacks antibody to *H. influenzae*, baby rabbit serum (BRS), (Fig. S1A). In contrast, there was no difference in IgM binding or bactericidal activity between *lic2A* and *lgtC* phase-on variants and *lic2A*- mutants (data not shown). A further comparison between alternative and classical pathway complement-mediated killing was performed using Mg-EGTA treated human serum. Mg-EGTA treatment inhibits classical and lectin, but not alternative pathway complement-mediated lysis (104). The Rd *lic2A*- mutant was sensitive to normal human serum, but not Mg-EGTA-treated human serum, confirming that the classical pathway, rather than the alternative pathway, is responsible for the sensitivity of the *lic2A*- mutant (Fig. S1B). Similar results were observed for the R2846 *lic2A* and *lgtC* phase-on strain versus the *lic2A*- mutant (Fig. 5C,D and Fig. S1C,D). Collectively, these data show that di-galactoside expression reduces IgG binding and bactericidal activity in multiple strains of *H. influenzae*.

The LPS mAb 6E4, which binds to an inner core LPS epitope that includes the KDO structure (360), was used to determine the effect of di-galactoside expression on antibody binding to LPS. Previously, we showed that ChoP expression reduces mAb 6E4 binding and killing in the presence of complement (53). Titration of mAb 6E4 was used to determine an antibody concentration where binding between modified and un-modified LPS could be differentiated by flow cytometry. R2846 retains mAb 6E4 binding in an *lgtF*- mutant (data not

shown), which is missing all $Hep_I$ extensions (Fig. 2A), suggesting that the di-galactoside itself is not required for mAb 6E4 recognition of LPS. Serial passage of the R2846 *lic1D*- strain in mAb 6E4 and a complement source (BRS) resulted in selection for a resistant population (Fig. 6A). Sequence analysis determined that the mAb 6E4 resistant population was *lic2A* phase-on (Table 2). These results are consistent with the changes observed following serial passage of R2846 in human serum. Bactericidal assays with LPS mutants were conducted to confirm the role of di-galactoside in evasion of mAb 6E4-mediated killing. The R2846 *lic2A* and *lgtC* phase-on strain is the most resistant to killing by mAb 6E4 and complement, the *lic2A* phase-on, *lgtC*- mutant has an intermediate level of resistance, and the *lic2A*- mutant is the least resistant (Fig. 6B). These data show that both *lic2A* and *lgtC* contribute to survival in the presence of the mAb 6E4 and complement. Next, flow cytometry was used to examine the amount of mAb 6E4 binding to the bacterial surface. Consistent with the bactericidal assay data, there was evidence for an individual contribution of both *lic2A* and *lgtC* in the reduction of mAb 6E4 binding (Fig. 6C). These results show that di-galactoside expression reduces the binding and bactericidal activity of an anti-LPS antibody that requires an inner core LPS structure for bacterial recognition.

Serial passage in both human serum and mAb 6E4 with complement resulted in selection for di-galactoside expression. We next compared the *lic2A* and *lgtC* phase-on variants isolated following serial passage in human serum to those isolated after serial passage in mAb 6E4 with complement. First, the R2846 *lic2A* and *lgtC* phase-on population isolated after serial passage in human

serum had reduced binding of mAb 6E4 compared to the *lic2A*- mutant (Fig. S2C). Similar results were also observed for Rd (Fig. S2D). Second, the R2846 *lic2A* and *lgtC* phase-on population isolated after serial passage in mAb 6E4 and complement had increased survival in the presence of human serum, compared to the *lic2A*- mutant (Fig. S2E). These results demonstrate that di-galactoside expressing variants isolated following exposure to different antibody sources recognizing inner core LPS epitopes have the same resistance profile in the presence of complement.

We also found that *lex2A* contributes to evasion of antibody binding to the bacterial surface. In the R2846 *lic1D*- strain, the *lex2A* phase-on population had reduced binding of human IgG compared to the *lex2A*- mutant (Fig. S2A). Upon repetition of the experiment where the R2846 *lic1D*- strain was serially passaged in mAb 6E4 and complement, one of the mAb 6E4 resistant populations was *lex2A* phase-on instead of *lic2A* phase-on (Table 2). Flow cytometry confirmed that the *lex2A* phase-on population had reduced mAb 6E4 binding compared to the *lex2A*- mutant (Fig. S2B). These data show that in addition to contributing to evasion of killing in human serum, both di-galactoside and the alternative glucose extension from the same hexose moiety reduce antibody binding to the bacterial surface. The expression of these phase variable hexose extensions protects *H. influenzae* against antibody binding conserved inner core LPS structures.

*LPS phase variable structures have independent and additive effects on bacterial survival in the presence of complement*

LPS phase variable structures including ChoP and di-galactoside contribute to evasion of antibody-dependent, complement-mediated lysis. We wanted to determine next whether the effects of these structures on bacterial survival are independent, additive, or both. Selection for di-galactoside expressing variants in the presence or absence of ChoP (Table 2) suggests that these two modifications contribute to survival independently, but may have an additive effect in combination. Included in the subsequent analysis was another phase variable LPS structure, sialic acid (Neu5Ac). The modification of LPS with sialic acid increases resistance to alternative pathway complement-mediated killing in *H. influenzae* (95). *H. influenzae* must acquire sialic acid from the environment (346), so the presence or absence of sialic acid was controlled by supplementation of growth media with Neu5Ac. This approach was validated using a *siaP-* mutant, which inactivates the sialic acid transporter SiaPQM TRAP, preventing sialic acid uptake and LPS attachment (346). The Rd *lic1D-, siap-* mutant remained constitutively sensitive to human serum, regardless of the presence of Neu5Ac, while the Rd *lic1D-* strain had increased survival in human serum with Neu5Ac added to growth media (data not shown). We found that in the presence of human serum and Neu5Ac, there was selection for increased serum resistance in the Rd *lic1D-* strain, and sequence analysis determined that this population was *lgtC* phase-on (Table 2). As *lic2A* was phase-on in the original and serum resistant populations, serial passage in human serum with

sialic acid selected for di-galactoside expressing variants. These results suggest there is an independent, but additive effect of di-galactoside and sialic acid expression on bacterial resistance to human serum.

To examine the dynamics of ChoP, di-galactoside, and sialic acid expression on bacterial survival, mutants were compared with phase variant populations following exposure to various concentrations of human serum. Human serum was used in increasing concentrations to resolve additive contributions of separate phase variable structures to bacterial survival. For the Rd *lic1D*- strain, there was an independent and additive effect of sialic acid and di-galactoside attachment to the LPS on serum resistance (Fig. 7A). Repetition of this experiment in Rd *lic1A* phase-on variants showed that there is also an independent and additive contribution of sialic acid and di-galactoside in the presence of ChoP (Fig. 7B). This experiment indicates that there is an additive effect for all three LPS modifications; ChoP, di-galactoside, and sialic acid each contribute to evasion of complement-mediated lysis, and can do so in combination for maximum serum resistance. In the R2846 *lic1D*- background, di-galactoside, the alternative glucose extension, and sialic acid each had an independent and additive effect on bacterial survival in human serum, similar to results observed for Rd (Fig. 7C). In addition to the di-galactoside, we tested the effect of a single galactose on bacterial survival in the presence or absence of sialic acid. We found that Rd and R2846 *lic2A* phase-on, *lgtC*- mutants also had an additive effect on bacterial survival in human serum in combination with sialic acid (Fig. S3A). In R2846 *lic1A* phase-on variants, an independent and additive

contribution of sialic acid, the alternative glucose extension (*lex2A* phase-on) and ChoP was observed (Fig. 7D). We found that there was no difference in survival for *lex2A* and *lgtC* phase-on variants compared to a *lex2A* phase-on, *lgtC*-mutant, demonstrating there is no contribution of *lgtC* in combination with *lex2A* in R2846 (Fig. S3B). Evidence for an independent and additive effect of sialic acid and di-galactoside attachment to LPS was also observed in the *lic1D*-backgrounds of Eagan and 2019 (Fig. S3C,D). These experiments demonstrate, in four distinct *H. influenzae* isolates, that phase variable LPS structures can contribute individually to evasion of complement-mediated lysis, and have an additive effect on bacterial survival in combination.

## Discussion

During colonization and disease, *H. influenzae* must evade killing by host immune components including antibody and complement (440). In this study, normal human serum was used to provide a source of antibody and complement for bactericidal assays, intended to test the effect of these factors at the mucosal surface. While several studies have highlighted individual LPS phase variable genes that contribute to bacterial survival following exposure to human serum (136, 168, 415), a comprehensive analysis of their combined effects has not been described. We hypothesize that LPS phase variation allows bacteria to evade recognition and killing by host immune components *in vivo* through

selective enrichment of resistant bacteria from a mixed population. A recent study conducted by M. Apicella and colleagues at the University of Iowa explored this hypothesis using experimental human colonization with the NTHi clinical isolate 2019, which was also tested in the current study (303). They found that the population of bacteria isolated from healthy volunteers following six days of colonization was enriched for *lic1A* and *igaB* (an IgA1 protease-encoding gene) phase-on variants. The selection for *lic1A* phase-on variants during human colonization confirms previous data in animal models and the observation that clinical samples of *H. influenzae* are enriched for ChoP–expressing variants (239, 384, 416). IgA1 protease expression is phase variable due to the presence of a seven base pair repeat in *igaB* (93). Selection for bacteria expressing the IgA1 protease underlines the importance of antibody evasion at the mucosal surface.

In the human carriage study there was also an increase in the *lex2A* phase-on variant population, although this did not achieve statistical significance after six days of colonization. While the di-galactoside encoding genes *lic2A* and *lgtC* remained phase-off during the course of the carriage study, it may take longer periods of colonization to see enrichment in di-galactoside expressing bacteria. Alternatively, modification of LPS with di-galactoside or the alternative glucose extension may be more important during inflammation. In a study of phase variant populations from clinical samples, bacteria isolated from asymptomatic patients and patients with pneumonia were all *lic1A* phase-on variants (415). Interestingly, while *lic2A* and *lgtC* expression in asymptomatic

patients was variable, both genes were phase-on in every isolate obtained from patients with pneumonia. As we demonstrate in the current study that there is an additive effect for ChoP and di-galactoside expression, ChoP expression may be sufficient for successful colonization, while both ChoP and di-galactoside could be required for survival in a more inflammatory milieu. Human colonization studies are critical for the analysis of the *in vivo* contribution of LPS phase variable structures because the repertoire of antibodies differs between humans and animals used to model *H. influenzae* colonization and disease. For example, while the di-galactoside structure gal$\alpha$1-4$\beta$gal is found in human P blood group antigens (281), this structure isn't present in rodents. As a result, rodents are capable of making antibody specific for this di-galactoside, and infection selects against *lic2A* and *lgtC* phase-on bacteria (83, 415).

We demonstrate in the current study that both components of the di-galactoside and the alternative glucose extension from the same hexose moiety contribute to bacterial survival in the presence of complement (Fig. 4). In some strains, *lex2A* expression controls attachment of a galactose rather than glucose, which allows for subsequent di-galactoside attachment (73). In this way, all three phase variable structures controlled by *lic2A*, *lgtC,* and *lex2A* expression could have an additive effect on evasion of antibody binding and complement-mediated lysis (136). Of note, selection for *lex2A* phase-on variants was a more rare occurrence in R2846 than selection for *lic2A* phase-on variants (Table 2). Also, while *lex2A* is also present in Eagan, selection for *lex2A* phase-on variants was not observed. It is possible that due to the additive effect of *lic2A* and *lgtC*, which

does not occur for *lex2A* and *lgtC*, selection for di-galactoside expression is more favorable for bacterial evasion of complement-mediated killing.

Serial passage of bacteria in human serum resulted in the identification of changes in *lic2A*, *lex2A*, and *lgtC* tetranucleotide repeats in several *H. influenzae* strains (Table 2). Despite the LPS structural differences and varying levels of initial serum resistance of these strains, the same genes were found to contribute to bacterial resistance. However, there were no repeat shifts detected for *lic3A* (Rd or R2846), *oafA* (R2846), or *losA1* (R2846). Expression of *lic3A* results in sialic acid attachment to the LPS. As all bacterial populations tested were *lic3A* phase-on, we controlled sialic acid expression by selective addition of Neu5Ac to growth media. We found that sialic acid increased bacterial survival in human serum for all *H. influenzae* strains included in the present study. While previous work has demonstrated that *oafA* and *losA1* can contribute to bacterial survival in serum (84, 107), in this study we did not isolate any *oafA* or *losA1* phase-off variants, so these genes could not be fully accounted for in our analysis. In a previous study from this laboratory, a screen of genes contributing to serum resistance in the NTHi clinical isolate R2866 found that several LPS biosynthesis genes, including *lic2A*, *lgtC*, and *lex2A,* are important for bacterial survival in human serum (282). In addition to these, a role for *vacJ* and its associated genes was described, where increased *vacJ* transcription reduced IgM binding. In contrast, we did not observe a change in *vacJ* expression between serum sensitive and resistant populations of Rd (data not shown). While other spontaneous mutations between the original and resistant populations isolated in

this study cannot be ruled out without full genome sequence analysis, our data suggests the difference in survival is due solely to the change in repeat number of the identified LPS phase variable genes.

Previous work has shown that ChoP attachment to the LPS reduces antibody binding through an effect on the physical properties of the outer membrane, reducing membrane accessibility (53). For example, *lic1A* phase-on bacteria are more resistant to EDTA treatment, which is a measure of outer membrane stability. In contrast, di-galactoside expression does not affect EDTA sensitivity (53). Also, ChoP has a more global effect on antibody binding, while di-galactoside expression reduced binding of IgG, but not IgM, to the bacterial surface (Fig. 5), suggesting these two LPS modifications affect sensitivity to antibody binding through different mechanisms. The contribution of *lgtC* expression to bacterial survival has been previously explored in the NTHi strain R2866. It was found that *lgtC* phase-on bacteria have reduced deposition of the complement protein C4b, compared to *lgtC*- mutants (164). Reduction in C4b and IgG binding decreases sensitivity to classical pathway complement-mediated killing, and both could contribute to resistance. In the current study, we found that the di-galactoside and alternative glucose extension from the same hexose moiety each reduce binding of human IgG and the mAb 6E4, which binds an inner core LPS structure that includes KDO (Fig. 5,6, Fig. S2). We also showed that human IgG recognizes conserved inner core LPS structures, as there was increased antibody binding to the truncated *opsX*- mutant compared to bacteria with more complete oligosaccharide extensions (Fig. 1B). These data suggest

that LPS hexose structures affect antibody binding indirectly, possibly through steric hindrance. The attachment of sialic acid to the LPS, in contrast to ChoP and di-galactoside, affects the alternative pathway of complement-mediated lysis. It was shown previously that bacteria with sialylated LPS have delayed deposition of complement components on the outer membrane compared to those without sialic acid (95). While ChoP, di-galactoside, the alternative glucose extension, and sialic acid attachment to LPS affect host recognition in different ways, all contribute to survival in the presence of complement (Fig. 7). The use of divergent mechanisms may contribute to the ability of these phase variable molecules to have an additive effect on resistance to complement-mediated killing.

The LPS structural variations introduced by the attachment of ChoP, di-galactoside, the alternative glucose extension, and sialic acid may contribute to survival in other *in vivo* contexts that have yet to be explored. However, each of these structures is phase variable, which suggests there are also host environments for which expression is not favorable for survival. For ChoP, it has been shown that C-reactive protein (CRP) can recognize ChoP-modified LPS and initiate classical pathway complement-mediated killing (416). In the case of di-galactoside and sialic acid, however, the disadvantages during infection are less clear. Galactins, which bind several galactose-associated LPS residues, have the potential to recognize these structures (332, 399). In many *H. influenzae* strains, the residue GalNAc, which is recognized by some galectins, can be attached to the terminal end of hexose extensions including the gal$\alpha$1-

4gal di-galactoside. Galactin binding can initiate immune responses resulting in bacterial clearance (332). Also, it has been shown that *lic2A* expression increases susceptibility of *H. influenzae* to infection with the bacteriophage HP1c1 (435). The galactose that is attached to the LPS in *lic2A* phase-on variants is likely part of the receptor required for phage predation. Host immune and microbial factors that target *H. influenzae* LPS modifications can thereby also provide a negative selective pressure for phase variants in some host environments.

In summary, we show that exposure to human antibody and complement drives selection for bacteria expressing phase variable LPS structures including the gal$\alpha$1-4gal di-galactoside and the alternative glucose extension attached to the same hexose moiety. Selection for di-galactoside expression was observed in several distinct *H. influenzae* strains, and the attachment of di-galactoside or the alternative glucose extension reduces antibody binding to the LPS. Each of the phase variable structures including ChoP, sialic acid and di-galactoside are outer core modifications with reduced host recognition through molecular mimicry (90, 334, 401). While LPS modifications provide a source of surface variation in *H. influenzae*, there is a limited repertoire of structural conformations and epitopes achieved by phase variation alone. Instead, phase variation of LPS outer core structures may serve to shield conserved inner core structures from antibody binding and complement deposition. LPS modifications including ChoP, sialic acid, di-galactoside, and the alternative hexose extension each contribute to bacterial survival in the presence of antibody and complement, and have an

additive effect in combination. The additive effect of each of these LPS phase variable structures is supportive of the hypothesis that in the presence of human antibody and complement, survival favors bacteria with the most highly decorated LPS. Phase variation ensures that *H. influenzae* populations are staged for rapid enrichment of the most-fit variants for successful colonization.

## Experimental procedures

### *Bacterial strains and growth conditions*

See Table 1 for a full list of all *H. influenzae* strains and mutants of these strains included in the present study. Bacteria were grown in brain heart infusion media (Becton Dickinson Biosciences, Franklin Lake, NJ) supplemented with 2% Fildes enrichment (Remel, Lenexa, KS) and 20 $\mu$g ml$^{-1}$ $\beta$-Nicotinamide adenine dinucleotide hydrate (Sigma, St. Louis, MO). Strains with multiple mutations (ex. *lic1D*-, *lpsA*-) were created through transformation of one mutant strain with genomic DNA of the second mutant strain. All mutants constructed for the present study were back-transformed and confirmed by PCR analysis.

For the *lex2A* mutant, a spectinomycin resistance cassette was inserted to disrupt gene function. The *lex2A* gene and flanking regions was amplified from R2846 with the primer pair *lex2A* amplify (Supplementary Table 1) and cloned into the pCR™ 2.1-TOPO® vector, according to manufacturer's instructions (Invitrogen, Grand Island, NY). A region of *lex2A* was deleted by inverse PCR

with the primer pair *lex2A* XmaI (Supplementary Table 1), introducing an XmaI site. The spectinomycin gene *aad9* was inserted at the XmaI site following amplification with the primer pair *aad9* (Supplementary Table 1). The resulting plasmid used to transform R2846. The *siaP*- mutant was constructed as previously described (346). Briefly, the *siaP* gene (carried by the pUC19 plasmid) was disrupted by deletion of a BglII restriction fragment, and a kanamycin resistance cassette from the pUC4Kan plasmid was inserted. The resulting plasmid was used to transform the Rd *lic1D*- strain. In R2846, *lic1A* phase-on colonies were identified by colony immunoblotting with the mAb TEPC-15, as described previously (417). The TEPC-15 enriched *lic1A* phase-on population is > 98% *lic1A* phase-on. Similarly, the mAb 4C4 was used for detection and enrichment of *lic2A* and *lgtC* phase-on variants of 2019. Binding of mAb 4C4 was also used for phenotypic verification of *lic2A*- mutant strains, as was TEPC-15 binding for *lic1D*- strains.

*Flow cytometric analysis*

Flow cytometry was used to detect antibody binding to the bacterial surface as described previously (282). Briefly, 200 μl reactions were conducted in Hank's buffer without $Ca^{2+}$ or $Mg^{2+}$ (Gibco, San Diego, CA) supplemented with 5% fetal calf serum (HyClone, Logan, UT) with a 20 μl of mid-logarithmic phase bacterial cells ($OD_{620}$ 0.5) diluted to $10^5$ CFU $ml^{-1}$. Reactions were incubated with primary antibody for 60 min at 37°C. Primary antibody sources consisted of purified IgG

from NHS (4.8 μg) and mAb 6E4 (1:200 dilution). A Protein G column (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used to purify IgG from NHS. An alternative source of purified IgG from a mixed NHS pool was obtained from Sigma. Reactions were washed and re-suspended in 1:200 dilutions of secondary antibody, followed by incubation at 4°C for 60 min. Secondary antibody sources included goat anti-human IgG-FITC and goat anti-mouse IgG-FITC (Sigma). After secondary incubation, reactions were washed and re-suspended in PBS with 1% bovine serum albumin and 0.5% paraformaldehyde prior to analysis on a BD FACS Calibur flow cytometer (Becton Dicksinson Biosciences). A total of 50,000 cells were collected, and MFI was determined using FlowJo software (Tree Star, Ashland, OR). Partial digestion of outer membrane proteins with trypsin was performed, where specified, as described previously (53). Briefly, 200 μl reactions containing 20 μl of mid-logarithmic phase bacterial cells ($OD_{620}$ 0.5) diluted to $10^5$ CFU $ml^{-1}$ in 10 mM Tris-HCL, pH 7.5 were washed and re-suspended in 1 mg $ml^{-1}$ trypsin. Following incubation for 2 hrs at 37°C, cells were washed and re-suspended in Hank's buffer for flow cytometry as described above.

*Bactericidal assays*

Bactericidal assays were conducted as described previously (53). Briefly, 200 μl reaction mixtures in Hank's buffer were incubated for 45 min at 37°C following addition of serum. Serum sources included NHS, CRP-depleted NHS, and baby

rabbit serum (BRS) to which the mAb 6E4 (1:200 dilution) or purified IgG from NHS (4.8 μg/200 μl reaction) was added. NHS was obtained from a single donor to provide a consistent repertoire of human antibody, although representative experiments were repeated with similar results from other NHS donors. Where specified, Neu5Ac (Sigma) was added to growth media prior to bactericidal assays to a final concentration of 0.1 mg $ml^{-1}$. CRP was depleted from NHS for bactericidal assays conducted with *lic1A* phase-on variants with immobilized *p*-aminophenyl phosphoryl choline gel (Thermo Scientific, Rockford, IL). Percent survival was determined relative to complement-inactivated serum, which was obtained by incubation of NHS for 30 min at 56°C. For serial passage in NHS or mAb 6E4 and BRS, 5-25 surviving colonies were picked and grown for the subsequent bactericidal assay, performed under the same conditions. Inactivation of classical and lectin pathways of complement was accomplished by chelation of NHS with gelatin veronal buffer containing MgEGTA (50 μM final concentration in NHS), (Boston Bioproducts, Worcester, MA). EDTA sensitivity was compared by addition of EDTA (1-4 mM) to bacterial cells in Hank's buffer followed by incubation for four hrs at 37°C. Percent survival was determined relative to no-EDTA controls.

*Sequence analysis of bacterial populations*

Genomic DNA was isolated from multiple colonies of each population (original and post exposure to NHS or mAb 6E4 with BRS) for sequence analysis. Primers

listed in Table 3 were used to amplify a portion of the target gene and surrounding sequence that included the tetranucleotide (or in the case of *losA1,* octonucleotide) repeat. The number of repeats was counted from each sequence and used to determine reading frame status as in frame (ON) or out of frame (OFF), as listed in Table 2.

*Statistical analysis*

Differences between experimental groups were assessed for statistical significance with an unpaired Student's two-tailed *t*-test (GraphPad Prism 4, GraphPad Software, La Jolla, CA).

Acknowledgements

We thank Derek W. Hood (University of Oxford) for providing LPS structural mutants noted in Table 1. Also, we thank Michael A. Apicella (University of Iowa) for generously providing mAb 6E4. This work was supported by training grants awarded to the University of Pennsylvania from the US Public Health Service (GM007229-35 and AI060516). The authors have no conflict of interest to declare.

Table 1. *H. influenzae* strains used in this study

| Strain | Mutant (-) or phase-ON variant | Reference |
|---|---|---|
| [a]Rd | | |
| | *opsX*- | (170) |
| | *lpsA*- | (170) |
| | *lgtF*- | (282) |
| | *lic2A*- | (282) |
| | *lgtC*- | (282) |
| | *lic1D*- | (228) |
| | *lic1A*-ON | (53) |
| | *lic1D*-, *siaP*- | (346) |
| | *lic1D*-, *lpsA*- | This study |
| | *lic1D*-, *lic2A*- | This study |
| | *lic1D*-, *lic2A*-ON, *lgtC*- | This study |
| | *lic1D*-, *lic2A*-ON, *lgtC*-ON | This study |
| | *lic1A*-ON, *lic2A*-ON, *lgtC*-ON | This study |
| [b]R2846 | | |
| | *lic1D*- | This study |
| | *lic1A*-ON | This study |
| | *lic1D*-, *lpsA*- | This study |
| | *lic1D*-, *lgtF*- | This study |
| | *lic1D*-, *lic2A*-, *lex2A*- | This study |
| | *lic1D*-, *lic2A*-, *lex2A*-ON | This study |
| | *lic1D*-, *lex2A*-ON, *lgtC*-ON | This study |
| | *lic1D*-, *lic2A*-ON, *lgtC*-, *lex2A*- | This study |
| | *lic1D*-, *lic2A*-ON, *lgtC*-ON, *lex2A*- | This study |
| | *lic1A*-ON, *lex2A*-ON | This study |
| [c]2019 | | |

| | | |
|---|---|---|
| | *lic1D-* | This study |
| | *lic1D-, lic2A-* | This study |
| | *lic1D-, lic2A*-ON, *lgtC-* | This study |
| | *lic1D-, lic2A*-ON, *lgtC*-ON | This study |
| [d]Eagan *cap*- | | |
| | *lic1D-* | This study |
| | *lic1D-, lic2A-* | This study |
| | *lic1D-, lic2A*-ON, *lgtC*-ON | This study |

**a.** Type d, unencapsulated variant, avirulent strain

**b.** NTHi clinical isolate from patient with otitis media

**c.** NTHi clinical isolate from patient with chronic obstructive pulmonary disease

**d.** Type-b, spontaneous capsule- derivative of the clinical isolate Eagan

Table 2. Exposure to NHS[a] drives selection for LPS phase variants in *H. influenzae*

| Strain | Serial passage | Gene | No. Repeats (Reading frame) | Times observed |
|---|---|---|---|---|
| Rd | | | | |
| *lpsA*- | NHS | *lic1A* | 17 (OFF)[b] → 18 (ON) | 3/3 |
| *lgtF*- | NHS | *lic2A* | 23 (OFF) → 22 (ON) | 3/3 |
| *lic1D*- | NHS | *lgtC* | 21 (OFF) → 22 (ON) | 3/3 |
| *lic1D*- | Neu5Ac + NHS | *lgtC* | 21 (OFF) → 22 (ON) | 4/4 |
| *lic1A*-ON[c] | NHS | *lic2A* | 21 (OFF) → 22 (ON) | 3/3 |
| R2846 | | | | |
| *lpsA*- | NHS | *lic2A* | 24 (OFF) → 25 (ON) | 3/3 |
| *lic1D*- | NHS | *lic2A* | 24 (OFF) → 25 (ON) | 6/7 |
| | | *lex2A* | 16 (OFF) → 14 (ON) | 1/7 |
| *lic1D*- | mAb 6E4 + BRS[d] | *lic2A* | 24 (OFF) → 25 (ON) | 3/4 |
| | | *lex2A* | 16 (OFF) → 14 (ON) | 1/4 |
| *lic1A*-ON[c] | NHS | *lic2A* | 24 (OFF) → 25 (ON) | 1/2 |
| | | *lex2A* | 16 (OFF) → 14 (ON) | 1/2 |
| 2019 | | | | |
| *lic1D*- | NHS | *lic2A* | 17 (OFF) → 16 (ON) | 3/3 |
| Eagan (*cap*-) | | | | |
| *lic1D*- | NHS | *lic2A* | 17 (OFF) → 16 (ON) | 4/4 |
| | | *lgtC* | 23 (OFF) → 24 (ON) | 4/4 |

**a.** NHS, normal human serum

**b.** Refers to the reading frame of the indicated gene as in frame (ON) or out of frame (OFF) based on the number of tetranucleotide repeats

**c.** Bactericidal assays with *lic1A*-ON variants were conducted with CRP-deleted NHS

**d.** BRS, baby rabbit serum

Table 3. Primers used in this study

| Target gene (repeat)[a] | Direction | Sequence | Reference |
|---|---|---|---|
| *lic1A* (CAAT) | F | 5'-AGCTAACCGAGCTTGGGTAAAA-3' | This study |
| | R | 5'-AAATCATTGTGGCACGGACG-3' | |
| *lic2A* (CAAT) | F | 5'-CAAGTGATTTATCCCCACGCGCCA-3' | (415) |
| | R | 5'-CGTTCTTTTTCCAATCCGCTTGTT-3' | |
| *lgtC* (GACA) | F | 5'-TTTCATATCAAGAATATAAAAATT-3' | (415) |
| | R | 5'-GGTTTTGAAGAAAAAGGCGAA-3' | |
| *lex2A* (GCAA) | F | 5'-GGCGGAATTATGTTAATCAC-3' | (83) |
| | R | 5'-GCTTGCATATAAGCTTTTCG-3' | |
| *oafA* (GCAA) | F | 5'-TTCCAGAATTACTTGTAGGATCTTTG-3' | (83) |
| | R | 5'-CATTAAAAACAAGCAGGAAAATAATAG-3' | |
| *lic3A* (CAAT) | F | 5'-CTCAGCCTTTCGGCACCCCG-3' | This study |
| | R | 5'-GGCATCAAAGGCGGGTAGCTTGT-3' | |
| *losA1* (CGAGCATA) | F | 5'-TCGAGCATCCATTTTCCCACT-3' | This study |
| | R | 5'-TGCCCTCAAAGAGATCCAACG-3' | |
| *hgp* hemoglobin and hemoglobin-haptoglobin binding protein, *HI_0635* (CCAA) | F | 5'-TCATCAACCCCTCGAACTGC-3' | This study |
| | R | 5'-TCGTCAAGATCCTGTTGCCC-3' | |
| *hgp* hemoglobin and hemoglobin-haptoglobin binding protein, *HI_0661* (CCAA) | F | 5'-CTTTGCCCAAAACGTCCAGC-3' | This study |
| | R | 5'-ACGTGCTTGCCTATTCCGTT-3' | |
| *hgp* hemoglobin and hemoglobin-haptoglobin binding protein, *HI_1565* (CCAA) | F | 5'-TTATGCTTGGGCTAACGGCA-3' | This study |
| | R | 5'-CCGGTTTCATAGCGCACAAG-3' | |
| *hgp* hemoglobin and hemoglobin-haptoglobin binding protein, *HI_0712* | F | 5'-TTCAGCTTGACGAAGCCCAT-3' | This study |

| | | | |
|---|---|---|---|
| *hgp* hemoglobin and hemoglobin-haptoglobin binding protein*, HI_0712* (CCAA) | F | 5'-TTCAGCTTGACGAAGCCCAT-3' | This study |
| | R | 5'-TCCGCTGGGAAAGTCACATC-3' | |
| Drug/metabolite exporter, *HI_0687* (TTTA) | F | 5'-GCAGTTATTGGTTGGGCTGC-3' | This study |
| | R | 5'-GCATCCCATAAAAGCCAGCG-3' | |
| *mod* type III restriction/modification system methylase*, HI_1058* (TGAC) | F | 5'-TTTTGCGTCAAAAAGCCGGT-3' | This study |
| | R | 5'-TGTGTATTGAATGGCGGGCA-3' | |
| Putative glycosyltransferase, *HI_1386* (CCAA) | F | 5'-TTGGAGAAGATGGCAAAGGCT-3' | This study |

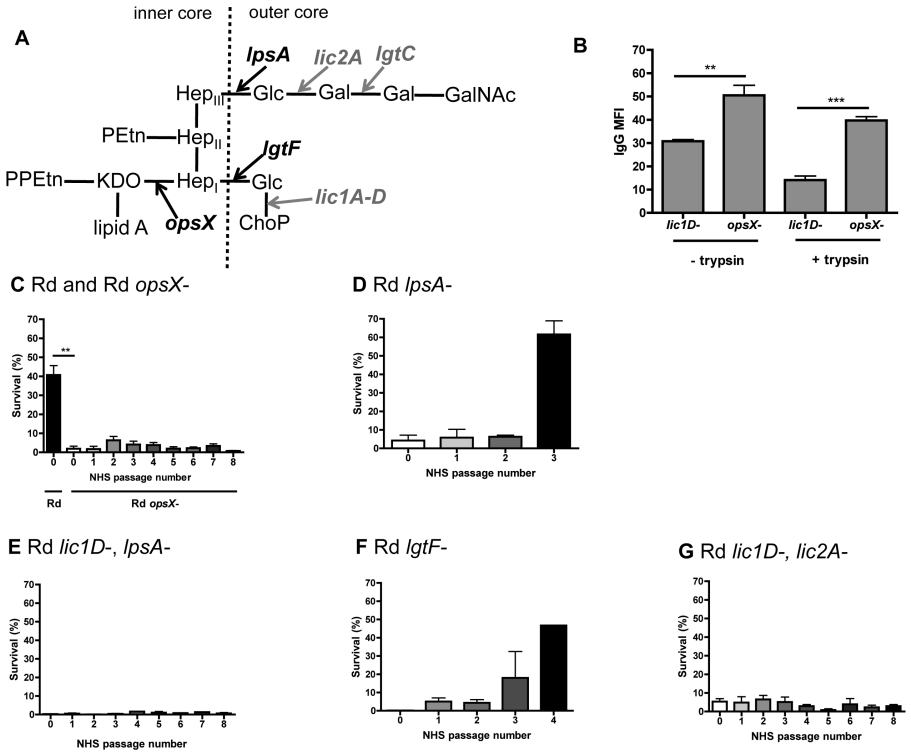



Figure 1. LPS structural requirements for resistance to human serum in the strain Rd. LPS structure proposed for Rd (169), with arrows indicating extensions dependent on LPS biosynthesis genes (black, bold), or phase variable genes (grey, bold), and dashed line indicating the border between inner and outer core LPS structures (A). Mean fluorescence intensity (MFI) for binding of purified human IgG to the surface of the Rd *lic1D*- strain compared to the *opsX*- mutant, with or without trypsin digestion (B). Bactericidal assays, where survival in human serum is determined relative controls with the same serum heat-inactivated to eliminate complement activity. Survival following serial passage in human serum is indicated for Rd and Rd mutants (C-G) in 3% normal human serum (NHS). Data shown are means and SEM (representative experiment in triplicate shown for D-G). Statistical analysis ($n \geq 3$) was performed by an unpaired *t*-test; $^{**}P<0.01$, $^{***}P<0.001$.

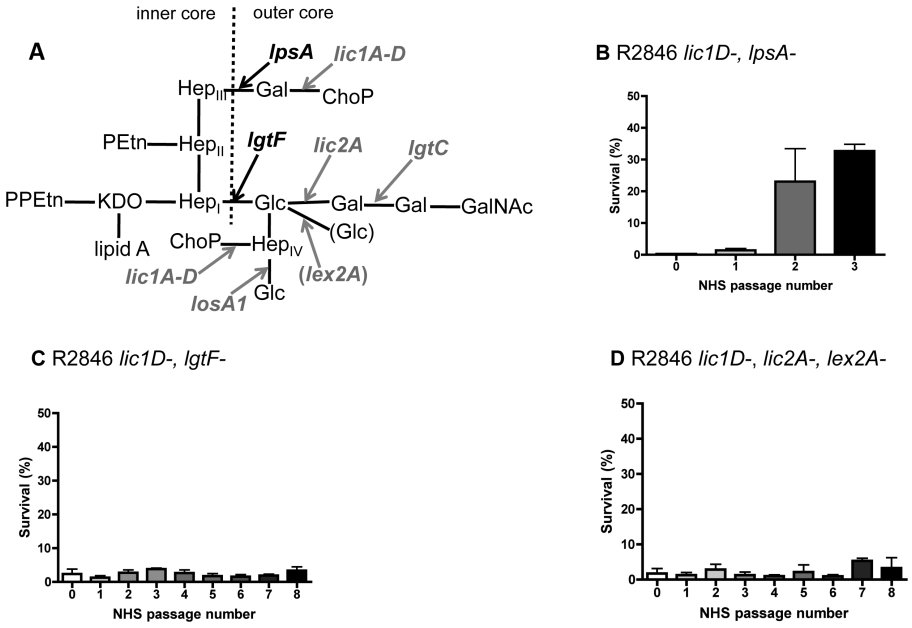



Figure 2. LPS structural requirements for resistance to human serum in the strain R2846. LPS structure proposed for NTHi clinical isolate R2846 (225), with arrows indicating extensions dependent on LPS biosynthesis genes (black, bold), or phase variable genes (grey, bold), and dashed line indicating the border between inner and outer core LPS structures (A). Also included in parentheses is the phase variable gene responsible for the proposed alternative glucose extension (grey, bold), which can be attached to the same hexose moiety as the di-galactoside. Bactericidal assays for the serial passage in in 5% normal human serum (NHS), with survival following the round of serum exposure indicated, for R2846 mutants (B-D). Data shown are means and SEM (representative experiments in triplicate).

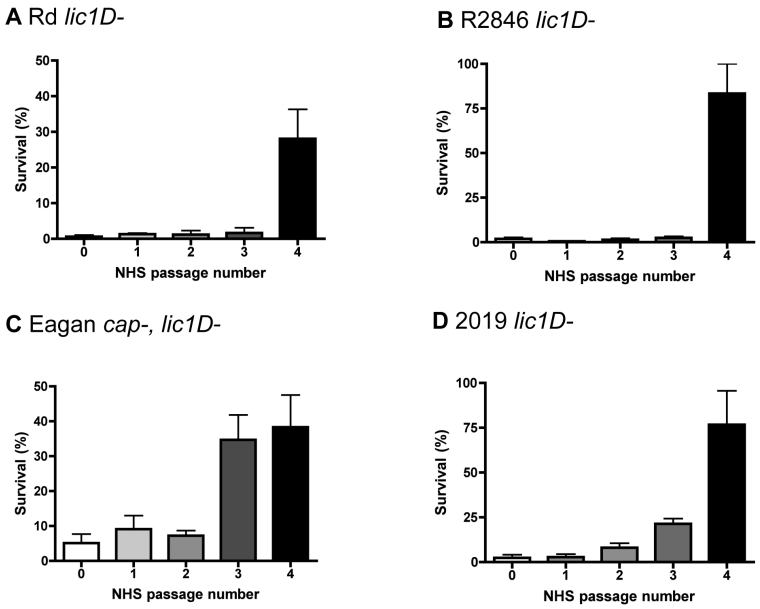



Figure 3. Exposure to human serum drives selection for resistant populations in multiple strains of *H. influenzae*. Bactericidal assays following serial passage of bacteria in human serum, with the round of serum exposure indicated, for Rd *lic1D*- (A, 3% normal human serum, NHS), R2846 *lic1D*- (B, 4% NHS), Eagan *cap-, lic1D*- (D, 2% NHS), and 2019 *lic1D*- (F, 4% NHS). Data shown are means and SEM (representative experiments in triplicate).

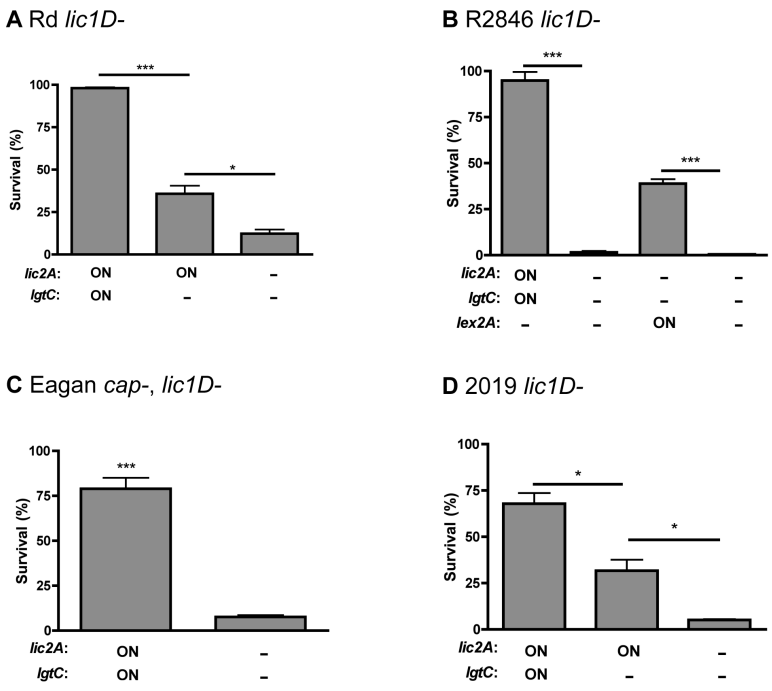



Figure 4. Contribution of LPS hexose extensions to survival in human serum. Bactericidal assays in human serum for *lic2A*, *lgtC*, and *lex2A* (R2846 only) phase-on variants (ON) and mutants (-) in Rd *lic1D*- (A, 2% normal human serum, NHS), R2846 *lic1D*- (B, 4% NHS), Eagan *cap-, lic1D*- (C, 1% NHS), and 2019 *lic1D*- (D, 4% NHS). Data shown are means and SEM. Statistical analysis ($n \geq 3$) was performed by an unpaired *t*-test; $^{*}P<0.05$, $^{***}P<0.001$.

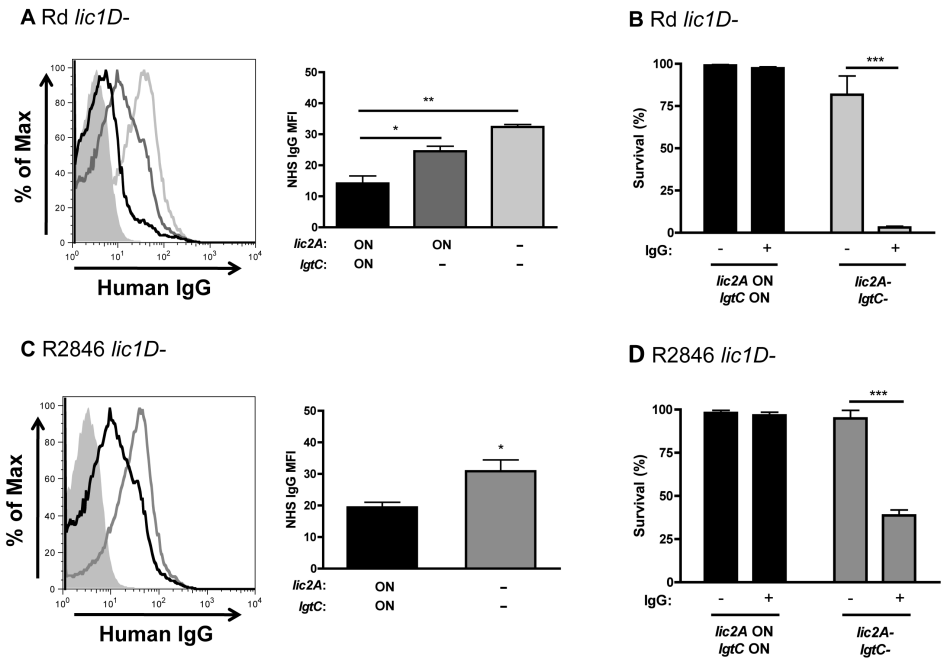



Figure 5. Di-galactoside expression protects against human IgG binding and bactericidal activity. Histogram of purified human IgG binding to *lic2A* and *lgtC* phase-on variants (black), a *lic2A* phase-on, *lgtC*- mutant (dark grey), and a *lic2A*- mutant (light grey) in Rd *lic1D*- (A). Graphical summary of IgG mean fluorescence intensity (MFI) is also shown. Bactericidal assays in IgG-depleted human serum with or without purified human IgG added back, as indicated, for *lic2A* and *lgtC* phase-on variants and *lic2A*- mutants in Rd *lic1D*- (B, 2% IgG-depleted normal human serum, NHS). Histogram of purified human IgG binding to *lic2A* and *lgtC* phase-on variants (black) and a *lic2A*- mutant (grey) of R2846 *lic1D*- (C), with a graphical summary of IgG MFI. Bactericidal assays in IgG-depleted human serum with or without purified IgG added back, as indicated, for *lic2A* and *lgtC* phase-on variants and *lic2A*- mutants in R2846 *lic1D*- (D, 4% IgG-depleted NHS). Data shown are means and SEM. Statistical analysis ($n \geq 3$) was performed by an unpaired *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

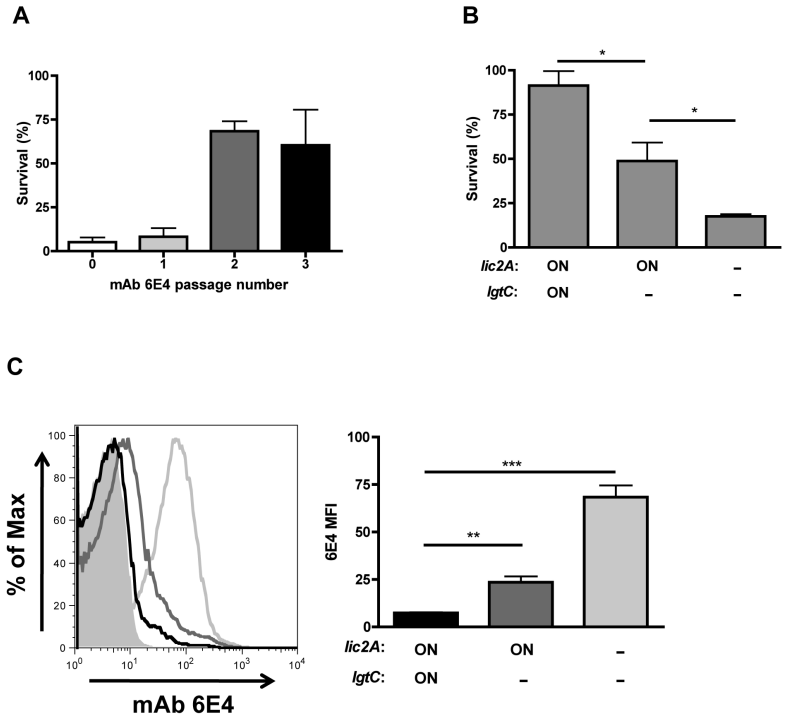



Figure 6. Di-galactoside expression protects against binding and bactericidal activity of the anti-LPS mAb 6E4. Bactericidal assays for the serial passage of R2846 *lic1D*- in mAb 6E4 and baby rabbit serum (BRS), with the round of exposure indicated (A, 15% BRS, 0.5% mAb 6E4; representative experiment in triplicate). Bactericidal assays in mAb 6E4 and BRS for *lic2A* and *lgtC* phase-on variants and mutants (B, 15% BRS, 0.5% mAb 6E4). Histogram and graphical summary for binding of mAb 6E4 to *lic2A* and *lgtC* phase-on variants and mutants in R2846 *lic1D*- (C). Data shown are means and SEM. Statistical analysis ($n \geq 3$) was performed by an unpaired *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

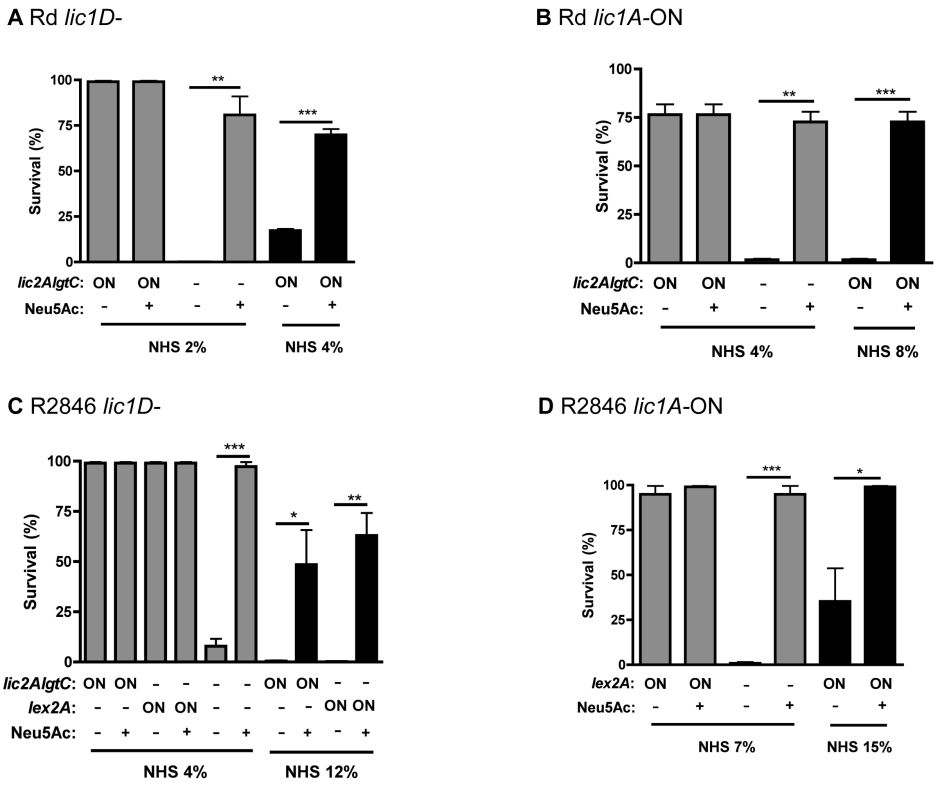
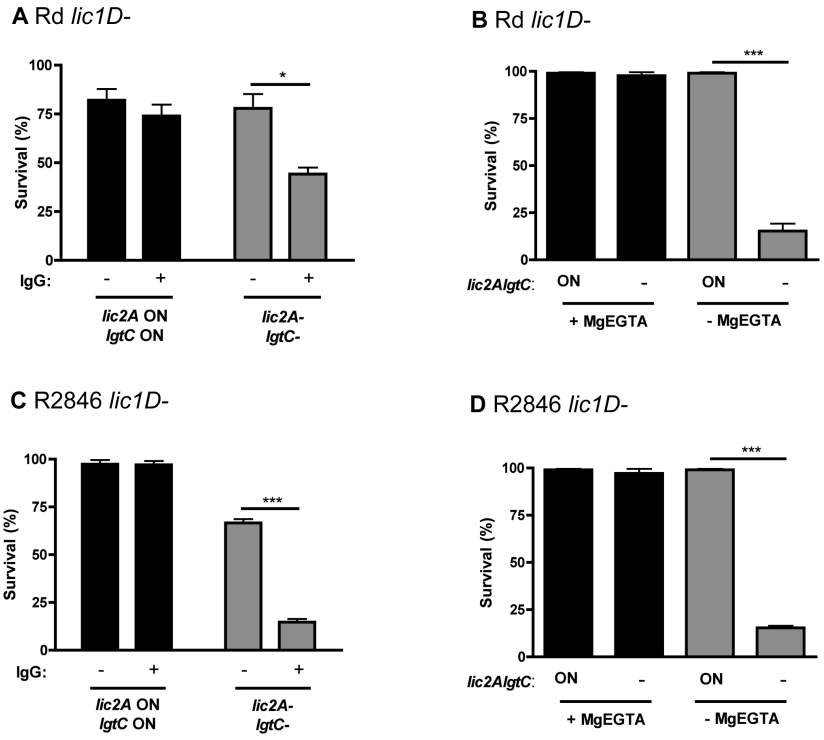



Figure 7. Di-galactoside, sialic acid, and ChoP have independent and additive effects on bacterial survival in human serum. Bactericidal assays in human serum for *lic2A*, *lgtC*, and *lex2A* (R2846 only) phase-on variants (ON) or mutants (-), with or without sialic acid (Neu5Ac) added to growth medium (+). Results are shown for Rd *lic1D-* (A), a *lic1A* phase-on variant population of Rd (B), R2846 *lic1D-* (C), and a *lic1A* phase-on variant population of R2846 (D). Bactericidal assays were performed at the concentration of normal human serum (NHS) indicated and in the case of *lic1A* phase-on variants, serum was first depleted of C-reactive protein. Data shown are means and SEM. Statistical analysis ($n \geq 3$) was performed by an unpaired *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

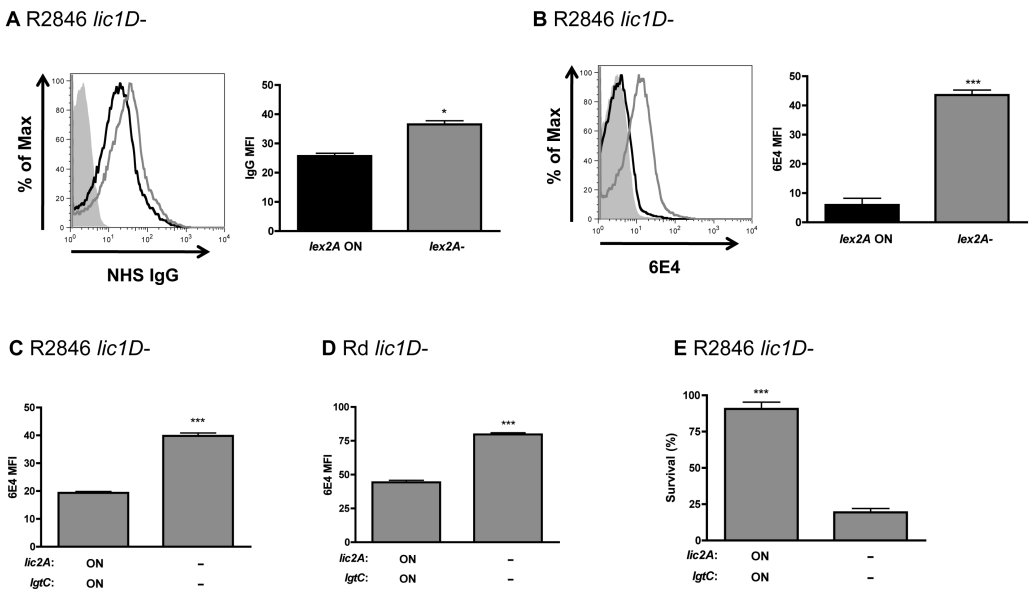
## Supplementary Table 1. Primers used for the construction of *H. influenzae* mutants

**Supplementary Table 1.** Primers used for the construction of *H. influenzae* mutants

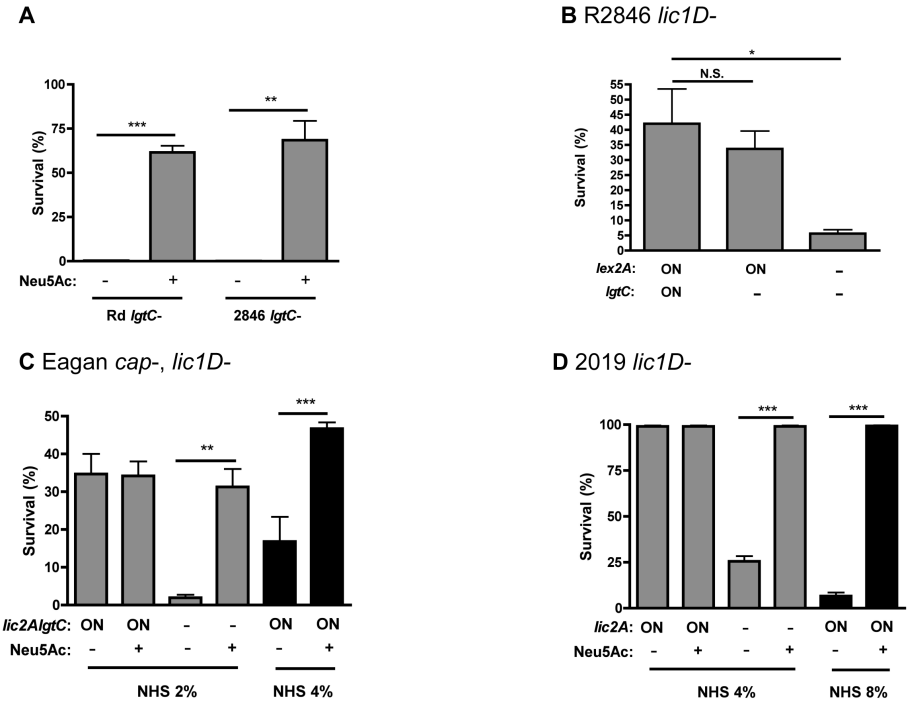
| Target gene | Direction | Sequence | Reference |
|---|---|---|---|
| *lex2A* amplify | F | 5'-ACCCCTTTGCATTCAACCGCT-3' | This study |
| | R | 5'-CCGAATGGTTCTGCGGAGGGC-3' | |
| *lex2A* XmaI | F | 5'-GCGGCGGGGCCCGCTTGCTTGCCAAGACTATC-3' | This study |
| | R | 5'-GCGGCGGGGCCCGCAAGCAAGCAAAGAGTGAC-3' | |
| *aad9* | F | 5'-GCGGCGGGGCCCATCGATTTTCGTTCGTGAATACATGTT-3' | This study |
| | R | 5'-GCGGCGGGGCCCTATGCAAGGGTTTATTGTTTTCTAAAATCTGA-3' | |
| *siaP* | F | 5'-GTTCACACAGGAGCGAAT-3' | (346) |
| | R | 5'-TACAGAGTATGCTGCTGC-3' | |




Supplemental Figure 1. Di-galactoside expression protects against classical pathway complement-mediated killing. Bactericidal assays in baby rabbit serum (BRS) with or without purified IgG for *lic2A* and *lgtC* phase-on variants and *lic2A*- mutants in Rd *lic1D*- (A, 4% BRS). Bactericidal assays in human serum with or without Mg-EGTA treatment for *lic2A* and *lgtC* phase-on variants and *lic2A*- mutants in Rd *lic1D*- (B, 2% normal human serum, NHS). Bactericidal assays in BRS with or without purified IgG in R2846 *lic1D*- (C, 15% BRS). Bactericidal assays in human serum with or without Mg-EGTA-treatment in R2846 *lic1D*- (D, 4% NHS). Data shown are means and SEM. Statistical analysis ($n \geq 3$) was performed by an unpaired *t*-test; $^{*}P<0.05$, $^{***}P<0.001$.




Supplemental Figure 2. Di-galactoside expressing variants have dual resistance to human IgG binding and mAb 6E4 binding and bactericidal activity. Histogram and graphical summary of human IgG (A) and mAb 6E4 (B) binding to *lex2A* phase-on variants (black) compared to a *lex2A*- mutant (grey) in R2846 *lic1D*-, with graphical MFI summaries. Summary of mean fluorescence intensity (MFI) for mAb 6E4 binding to *lic2A* and *lgtC* phase-on variants isolated following passage in normal human serum (NHS) compared to *lic2A*- mutants in R2846 *lic1D*- (C) and Rd *lic1D*- (D). Bactericidal assays in human serum for *lic2A* and *lgtC* phase-on variants isolated following passage in mAb 6E4 and BRS compared to *lic2A*- mutants in R2846 *lic1D*- (E, 4% NHS). Data shown are means and SEM. Statistical analysis ($n \geq 3$) was performed by an unpaired *t*-test; $^{*}P<0.05$, $^{***}P<0.001$.




Supplemental Figure 3. Additive and independent effects on bacterial survival in human serum for LPS modifications in multiple *H. influenzae* strains. Bactericidal assays in human serum for a Rd *lic2A* phase-on, *lgtC*- mutant (2% normal human serum, NHS) and a R2846 *lic2A* phase-on, *lgtC*- mutant (4% NHS) with or without sialic acid (Neu5Ac) added (+) to growth medium (A). Also, bactericidal assays in human serum for *lex2A* and *lgtC* phase-on variants compared to a *lex2A* phase-on, *lgtC*- mutant and a *lex2A-, lgtC*- mutant in R2846 *lic1D*- (B, 4% NHS). Individual and collective contributions of *lic2A*, *lgtC* and sialic acid (Neu5Ac) modifications in Eagan *cap*-, *lic1D*- (C) and 2019 *lic1D*- (D). Bactericidal assays were performed at the concentration of normal human serum (NHS) indicated. Data shown are means and SEM. Statistical analysis ($n \geq 3$) was performed by an unpaired *t*-test; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

# CHAPTER 6

## Discussion and conclusions

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Model for the role of phase variation during *Haemophilus influenzae* colonization

*H. influenzae* must evade host recognition in order to successfully colonize the human respiratory tract. Antibody and complement are immune components present at the mucosal surface, and are important for the control of *H. influenzae* colonization and disease (78, 146, 202, 330). We have shown that the phase variable LPS structure phosphorylcholine (ChoP) is one of the bacterial factors that contributes to protection against host recognition (Chapter 3), (53). ChoP attachment to the LPS alters the physical characteristics of the outer membrane, resulting in reduced membrane accessibility. The altered membrane accessibility of ChoP phase-on variants limits antibody binding to the bacterial surface, and increases survival in the presence of complement. ChoP phase-on variants are also less susceptible to cationic antimicrobial peptides (229). Through a screen to identify NTHi genes required for complement resistance, we characterized an additional structure on the outer membrane that affects membrane accessibility in *H. influenzae*. The expression of *vacJ* and the associated *yrb* genes increase the expression of an ABC transporter that recycles phopsholipids from the outer leaflet of the outer membrane to the inner leaflet (Chapter 4) (282). In the absence of this activity, the outer membrane is less stable and there is increased antibody binding to LPS epitopes. ChoP and the *vacJ*/*yrb* associated transporter system are two examples of how alterations in membrane stability affect accessibility of host antibody to bacterial targets.

Aside from the *vacJ* and *yrb* genes, the remaining hits identified in the screen for complement resistance determinants were LPS biosynthesis genes, several of which were phase variable. We next examined the dynamics of phase variation in *H. influenzae*, and found that serial exposure to human antibody and complement drives selection for phase variants expressing ChoP, the gal$\alpha$1-4gal di-galactoside, and an alternative glucose extension (Chapter 5). Each of these structures, in addition to another LPS phase variable molecule, sialic acid, contributed independently to bacterial survival in the presence of complement. Finally, we observed an additive effect for ChoP, the di-galactoside, and sialic acid in combination. Phase variants with the most outer core LPS modifications had the highest level of complement resistance.

These data inform our model of how LPS phase variation in *H. influenzae* contributes to bacterial survival during human colonization and disease. We hypothesize that phase variation of LPS structures aids in the evasion of host recognition. Unlike most bacteria, *H. influenzae* has a limited number of two-component regulatory systems, where environmental triggers stimulate gene regulation; only four two-component systems have been identified and three of these have been characterized (72, 366, 369). Instead, *H. influenzae* has a number of phase variable genes, creating both intra- and interstrain surface diversity. Importantly, phase variation occurs in 'contingency' genes, not housekeeping genes, and affects bacterial survival during exposure to different host conditions (263). Phase variation of surface structures allows *H. influenzae* to spread survival risk among a heterogeneous population, enabling rapid

selection of the most-fit phase variants. This form of 'bet-hedging' is a specific evolutionary strategy, designed for survival in an environment where survival determinants are rapidly changing (309). Evolutionary theorists have modeled conditions for the evolution and maintenance of phase variation using *H. influenzae* as an example, and found that broad survival conditions select for the adoption of this strategy in bacteria (294).

The repertoire of host antibody present during colonization and disease development is a diverse and rapidly changing immune pressure on *H. influenzae*. In addition to antibody, complement levels increase dramatically during inflammation, which itself influences the type and amount of antibodies present on the mucosal surface (146). Over time, affinity maturation alters the repertoire of antibodies secreted (265). Phase variation of surface structures allows for the selective enrichment of bacteria with reduced antibody binding and complement deposition. However, any phase variant population resulting from a given selection event will still contain alternative phase variants, allowing rapid selection in the face of a different host immune response. We hypothesize that over the course of colonization and disease development, phase variants are constantly enriched for or selected against due to the immune pressures of antibody binding and complement-mediated lysis (see model in Figure 1).

Several of the phase variable LPS structures expressed by *H. influenzae* mimic host epitopes, and are not readily recognized by human antibody (90, 334, 401). We propose that, rather than create a source of antigenic variation, phase

variation of outer core LPS structures serves to shield conserved inner core structures from antibody binding. In this way, outer core LPS phase variants protect inner core structures from immune recognition, promoting bacterial survival without alteration of the conserved portion of the LPS. To summarize, in our model the phase variation of LPS structures allows *H. influenzae* to evade recognition by human antibody and complement, and this contributes to the successful colonization of this extracellular pathogen.

Multifunctional roles of phase variable LPS structures

Each of the phase variable LPS modifications discussed in this work reduces host recognition through a different mechanism. This divergence may contribute to the ability of each of these phase variable molecules to have an additive effect on bacterial survival in combination. For example, while sialic acid attachment to the LPS decreases alternative pathway complement-mediated lysis (95), the expression of the di-galactoside structure and the alternative glucose extension reduce antibody binding, contributing to survival in the context of classical pathway complement-mediated lysis (Chapter 5). Also, it was shown previously that the expression of *lgtC*, which is responsible for the attachment of the distal galactose residue of the di-galactoside structure, reduces binding of the complement protein C4b (164). The reduction of C4b binding and antibody

binding are two separate ways to affect classical pathway complement-mediated lysis, and both likely contribute to survival.

In contrast, ChoP has a more general effect on the physical properties of the outer membrane (53). In addition to reducing antibody binding and complement-mediated killing, ChoP expression increases bacterial survival in the presence of cationic antimicrobial peptides (229). Also, ChoP expression affects binding of IgG, IgM, and IgA, while di-galactoside expression affects binding of IgG, but not IgM. The mechanisms of the effect of *oafA* and other phase variable structures, such as *los1A*, have yet to be characterized. Some phase variable structures in *H. influenzae* are also phase variably expressed in other bacteria, including ChoP and the gal$\alpha$1-4gal di-galactoside structure (407, 431). However, the effects of these phase variable structures are not always equivalent. For example, sialylation in Neisseria increases factor H deposition, which helps to protect against complement-mediated lysis, but has no effect on factor H binding to *H. influenzae* (95, 310). Understanding the mechanisms of how each phase variable molecule contributes to bacterial survival is important for the design of strategies to minimize their effectiveness.

While we have shown that bacteria with the maximum number of outer core LPS extensions have the greatest resistance against antibody and complement-mediated killing, each of the structures we have highlighted is not constitutively expressed. A phase variable expression profile suggests there are also host environments for which these structures are detrimental to bacterial

survival. This concept has been explored in detail for the phase variation of ChoP. ChoP phase-on bacteria are recognized by C-reactive protein (CRP), which initiates complement-mediated killing (416). In environments with high levels of CRP, such as in the blood, ChoP phase-on variants are killed more readily than ChoP phase-off variants. In human serum, ChoP phase-off bacteria predominate, unless CRP is depleted from the serum (416). The conditions favoring fimbriae phase-on and phase-off variants have also been explored. *H. influenzae* isolated from the nasopharynx are highly fimbriated, while bacteria isolated from the bloodstream do not have fimbriae (254, 408). There may be immune components in the blood that recognize fimbriae and are less abundant on the mucosal surface, or their effect is not as detrimental as a lack of fimbriae for increased epithelial cell adherence on the mucosal surface. Another example of selection against phase-on variants is the increase in phase-off variants of the HMW1 and HMW2 adhesins over time in COPD patients, which correlates with increased anti-HMW1 and HMW2 titers in patients (51). In this case, antibody recognition of a phase variable structure is associated with selection against variants expressing that structure.

While the benefits and detriments of ChoP expression and some outer membrane proteins have been explored, the negative consequences of the other phase variable LPS structures remain unclear. One example of a potential disadvantage is an increase in susceptibility to phage infection. LPS structures are critical for phage recognition in several bacterial strains (181, 432). In *H. influenzae*, *lic2A* phase-on variants are more susceptible to infection with the

HP1c1 phage than *lic2A*- mutants (435). The *in vivo* consequences of phage predation are difficult to assess. Not all phages are lytic, and phage transformation results in exchange of genetic material that could be either advantageous or disadvantageous for survival. However, perhaps *lic2A* phase-on variants are selected against when there is an increase in lytic HP1c1 phage populations. Another potential consequence for the expression of LPS phase variable structures is recognition by host galectins. These molecules recognize structures found on the outer core of the LPS of *H. influenzae*, such as GalNAc (399). Galectin recognition, while not directly bactericidal, can increase immune responses resulting in bacterial clearance (332). In addition to phase variation, there may be consequences for the differential regulation of genes controlling the expression of other outer membrane structures. For example, *vacJ* expression is not always up-regulated, suggesting there may be advantages in some circumstances for reduced outer membrane stability, or at least less phospholipid turnover (282). In *Escherichia coli*, mutants in the Tat protein export system have increased membrane permeability and susceptibility to antimicrobial agents, but are also more resistant to phage infection (364). However, it is unclear what outer membrane protein or LPS structures were responsible for these effects in *tat*- mutants. Clearly, there is a need to further study the consequences of phase variable expression on bacterial survival in different host environments.

Bacterial targets of host antibody

Surface exposed structures on the outer membrane of *H. influenzae* are targets for recognition by host antibodies. Outer membrane proteins are susceptible to antibody binding, and immunization with purified outer membrane proteins can induce protein-specific antibodies (32, 175, 386). The only currently licensed vaccine with efficacy against NTHi disease contains the protein D outer membrane protein (307). However, it is unclear whether the efficacy against NTHi disease in protein D-immunized individuals is from direct protein D recognition and bacterial clearance, or from blocking its enzymatic activity. Protein D is a glycerophosphodiester phosphodiesterase, and is critical for uptake of choline from glycerophosphocholine, the major source of choline on the mucosal surface (90). Immunization against protein D prevents its enzymatic activity, and bacteria have reduced ChoP expression (185, 385). Therefore, individuals immunized against protein D may inhibit ChoP expression in colonizing bacteria, which reduces bacterial survival in the presence of natural host antibody and complement (53).

The LPS is also a major target of human antibody. In an experiment where purified LPS was used to deplete anti-LPS antibodies through incubation in serum (serum pre-absorption), it was found that IgM from rabbits binds primarily to LPS structures, rather than outer membrane proteins (85). Similar results were found in human serum, where the majority of the bactericidal activity was due to IgG recognition of LPS structures on *H. influenzae* (53). Studies where mice were immunized with heat-killed *H. influenzae* have led to the isolation of monoclonal antibodies capable of binding both LPS phase variable structures,

such as the gal$\alpha$1-4gal di-galactoside, and structures comprising the inner core region of the LPS (33, 34).

Our hypothesis, based on the evidence presented in this work, is that phase variation of LPS modifications protects conserved inner core structures from antibody recognition. We have shown two separate examples of phase variable structures that restrict recognition of inner core structures (Chapter 5), (53). These studies used LPS truncation mutants paired with defined monoclonal antibodies that had known epitope specificities. However, it is unclear whether LPS phase variation also affects outer membrane protein recognition. The most prominent candidate for this is ChoP, which has a general affect on membrane accessibility. While there may be a minor role for ChoP in reducing recognition of outer membrane proteins, we found that the majority of antibodies affected by ChoP expression targeted LPS structures.

Screens of *H. influenzae* strains have identified LPS biosynthesis genes as the major determinants of bacterial survival (427). For example, in the screen to identify genes necessary for complement resistance in NTHi (Chapter 4), all hits, other than the *vacJ* and associated *yrb* genes, affected LPS biosynthesis (282). Several LPS biosynthesis genes were also identified in a similar screen for complement resistance determinants in the same NTHi strain (211). One of the hits identified in both screens was a previously uncharacterized gene that encodes a hypothetical protein. This protein, R2866_0112, was found to be important for LPS outer core extensions, and mutants have increased IgM

binding to the bacterial surface as well as reduced virulence in a murine model of otitis media (211). A screen of *H. influenzae* genes required for short-term persistence in murine lungs determined that both metabolism genes and LPS biosynthesis genes were critical for bacterial survival (115). While these studies have identified many important LPS biosynthesis genes, some of which were hits in multiple screens, several of these genes cause severe LPS truncations when disrupted. The individual contributions to bacterial survival for each LPS structure affected by these mutations require further investigation.

It remains unclear if the list of genes affecting bacterial survival in the presence of host antibody and complement is complete. While we have shown a major role for LPS phase variable genes, there is still the possibility of minor contributions from other genes. For example, the outer membrane protein P6 affects outer membrane stability and increases survival in serum, and may therefore also affect antibody accessibility to the LPS or other outer membrane proteins (276). Modifications affecting the charge of the LPS may also be important for host recognition, as it has been shown that the sensitivity of *Salmonella typhimurium* mutants to antimicrobial peptides is related to the charge of the LPS in each mutant (311). In *H. influenzae*, mutation of *htrB*, which reduces lipid A acylation, results in increased sensitivity to the β-defensin HBD-2 (365). However, the potential effects of such mutations on antibody recognition remain largely undefined.

The study of the effect of phase variation on host recognition is reliant upon a source of antibodies that can bind *H. influenzae* surface structures. However, there is a major problem with the use of animal models for this purpose. While animal models of *H. influenzae* colonization and disease are useful for the investigation of many virulence factors *in vivo*, they are not ideal for the study of antibody evasion. The best example for why this is a problem is the differential recognition of the di-galactoside structure gal$\alpha$1-4gal in humans versus rodents. This di-galactoside mimics host glycoconjugates, specifically P blood group antigens, and humans do not make antibodies capable of recognizing these structures. However, the linkage of this structure is different in rodents, which make antibodies that bind the gal$\alpha$1-4gal di-galactoside on *H. influenzae* (33). The expression of the di-galactoside, therefore, is only advantageous in avoiding antibody recognition when exposed to human, not rodent, antibody (388). This example highlights the concern with a reliance on animal models for vaccination studies. It is clear that experiments incorporating human antibodies should be included in the design and testing of all *H. influenzae* vaccine candidates, as information from rodent models may be misleading. The group of M. Apicella and colleagues has developed a new experimental model of human NTHi colonization (303). This model will likely be useful for the study of bacterial factors that contribute to survival in the human nasopharynx. Importantly, a caveat of using human antibodies and the human carriage model in screening vaccination candidates is the lack of naïve hosts, as

it is estimated that all humans have been exposed to *H. influenzae* by adulthood, and colonization occurs early in life (113).

Vaccine design for protection against NTHi carriage and disease

While the Hib vaccine has been extremely successful in limiting invasive *H. influenzae* disease, there is still a large burden of respiratory tract disease associated with *H. influenzae* infection (2, 275). Acute otitis media infections and COPD exacerbations often are recurrent, as patients acquire new strains (148, 296). Otitis media-prone children produce less serum IgG against outer membrane proteins including protein D and P6, suggesting the frequency of otitis media events could be reduced through the stimulation of a more protective immune response (191). The primary objective for control of NTHi related disease is reducing colonization. Our model for the importance of phase variation in evasion of host recognition suggests that LPS phase variation should be considered during vaccine design. While there is currently no evidence that suggests LPS phase variation affects recognition of outer membrane proteins, several vaccine candidates include LPS structural targets (140, 167). Phase variation of the vaccine targets themselves, or other LPS structures that interfere with epitope recognition, should be considered.

Another issue related to the development of LPS-based vaccines for protection against NTHi disease is the stimulation of a protective immune

response. Similar to capsular antigens, LPS is a T cell independent epitope. Therefore, effective vaccination will likely require conjugation to a protein. In a murine model of otitis media, serum depleted of antibodies targeting outer membrane proteins was less protective following passive immunization than whole serum or serum without antibodies to LPS structures, suggesting that LPS antibodies are not protective (16). However, other studies have shown that protective anti-LPS antibodies can be generated using protein conjugate vaccines (167, 429). Our studies have identified a critical role for IgG and IgM in the recognition of LPS structures and killing in the presence of complement. While IgA is the most abundant natural antibody present on the mucosal surface, antigen-specific IgG and IgM generated following immunization can be isolated from the serum and respiratory tract (202, 379). The seven-valent *S. pneumoniae* conjugate vaccine, which reduces bacterial carriage, induces production of serum IgG as well as salivary IgA and IgG (291). It is likely that mucosal IgG and IgM will be required for protection against NTHi colonization.

Given our model for the importance of phase variation in shielding inner core epitopes, one strategy for future vaccination efforts is the inclusion of multiple LPS targets. For example, a vaccine could be designed to stimulate the production of antibodies that bind a phase variable structure as well as the inner core epitope it normally shields from recognition. In this way, phase-on and phase-off variants would both be vaccine targets. The initial promise of the protein D vaccine could also be expanded upon, perhaps by more direct targeting of ChoP. While natural anti-ChoP antibodies are low affinity,

conjugation to a protein carrier allows for stimulation of protective IgG anti-ChoP antibodies (379, 387).

Future directions

This work has focused on the impact of phase variation on bacterial survival in the presence of human antibody and complement. However, these are only some of the many immune components encountered by *H. influenzae* during colonization (Chapter 1). Moving forward, the role of phase variation should be explored in other contexts. This may reveal as yet uncharacterized advantages, and disadvantages, for the different phase variant molecules present in *H. influenzae*.

One area that requires further research is the effect of phase variation on recognition by immune cells, such as macrophages and neutrophils. Neutrophils are important for limiting *H. influenzae* colonization (440), and the effect of phase variation on neutrophil recognition has yet to be characterized. TLR4 stimulation is primarily lipid A-dependent, although it has been shown that core oligosaccharide is recognized by other pattern recognition receptors (66). While still largely unexplored, it is possible that phase variation affects cell-mediated, in addition to cell-free, immune responses.

Bacterial factors affecting transmission of *H. influenzae* to new hosts is another area requiring further investigation. For example, we hypothesize that

some phase variants have enhanced survival during the selective bottleneck of transmission. Unfortunately, there is no transmission model for *H. influenzae* to address such hypotheses. Transmission in humans would be even more difficult to track, due to the common carriage of *H. influenzae* (similar to the issue of a lack of naïve hosts). However, studies of infants and their caregivers or children in daycare settings are potential environments to address such questions through epidemiological data.

In addition to transmission to new hosts, phase variation could affect transmission to new anatomical sites within the same host. For example, some phase variants may be more successful during invasion of the middle ear, leading to otitis media, or the lung, which can cause bronchitis or COPD exacerbations (272). We have shown that upper respiratory tract isolates are more susceptible to IgM binding than lower respiratory tract isolates, and that this correlates with reduced *vacJ* expression (282). However, there may be other bacterial factors that contribute to survival in these different host environments. Even within the same host site, such as the nasopharynx, the repertoire of immune components is constantly changing. For example, there may be differences in survival conditions for bacteria on the un-inflamed versus inflamed mucosal surface. *H. influenzae* can induce inflammation in several ways (Chapter 1), and during inflammation there is increased leakage of serum components to the mucosal surface from the airway vasculature (135). There is also evidence that while ChoP is important for initial colonization, di-galactoside expression is perhaps more critical for long-term colonization or for survival

under inflammatory conditions (415). Bacterial factors contributing to survival during initial inflammation following bacterial exposure may also be important in the setting of chronic inflammatory diseases, such as COPD (409).

Finally, *H. influenzae* and its human host are not the only factors involved in the host-pathogen dynamic. In addition to phage predation of *H. influenzae*, discussed previously, there are also a number of bacteria in the respiratory tract flora that compete with *H. influenzae* for space and survival. For example, some *H. influenzae* strains are resistant to bacteriocins produced by competing bacteria (257). In a co-colonization study, neutrophil recruitment induced by *H. influenzae* colonization contributes to the clearance of *S. pneumoniae* (230). *H. influenzae* can also promote the survival of other bacteria. For example, quorum sensing molecules produced by *H. influenzae* promote the persistence of another prevalent nasopharyngeal bacterium, *Moraxella catarrhalis*, in a murine model of otitis media (8). The role of phase variation in evasion of host immunity may only be a starting point for how these important molecules contribute to bacterial survival.

## Concluding remarks

The evolution and maintenance of phase variable LPS biosynthesis genes in *H. influenzae* indicates the importance of these features in the promotion of bacterial survival. Phase variation of LPS structures allows bacteria to quickly

adapt to a range of different host conditions. In this work, we show the importance of LPS phase variation in bacterial evasion of antibody and complement-mediated lysis. We hypothesize phase variable outer core LPS structures shield conserved inner core structures from host recognition during colonization. While the mechanisms of how each phase variable LPS structure affects antibody and complement recognition are diverse, these modifications have an additive effect on bacterial survival in combination. The impact of phase variation on antibody and complement recognition of bacteria is important to consider in the efforts to design a vaccine to protect against both NTHi colonization and infection. While there is clearly a role for phase variation in evasion of host recognition, the impact of these phase variable structures on other aspects of the host immune response against *H. influenzae*, including the potential disadvantages of their expression, remain important areas for future investigation. We conclude that the phase variation of LPS structures is a critical strategy for the successful colonization of the extracellular bacterium *H. influenzae*.

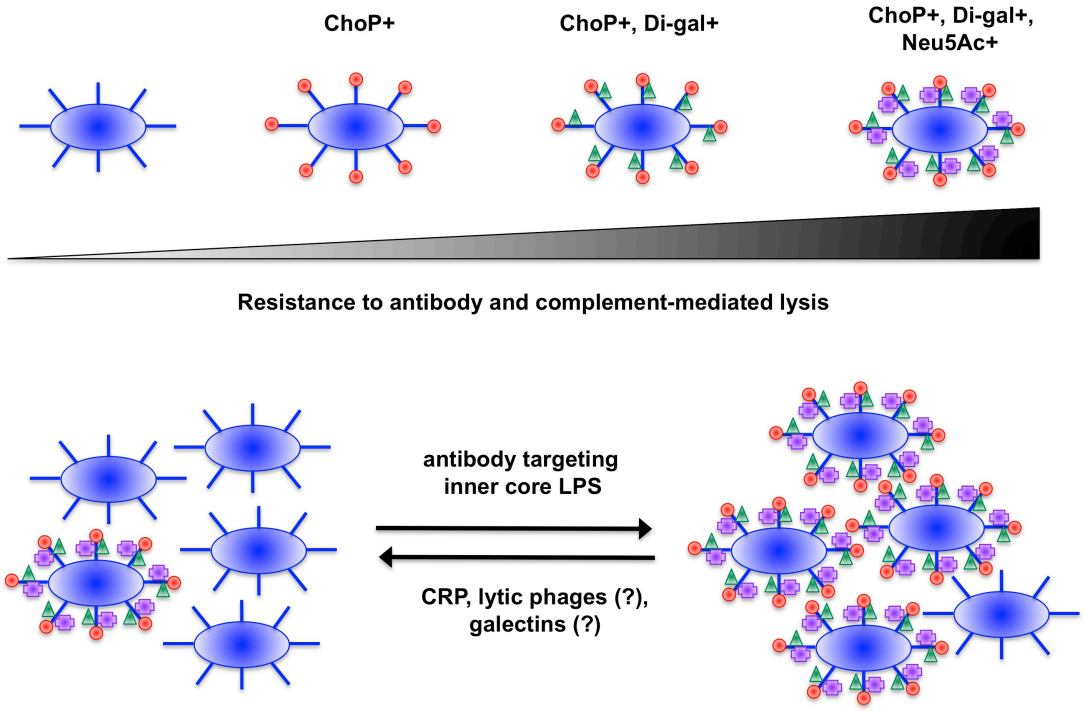


Figure 1. Model for the role of phase variation in evasion of antibody and complement-mediated lysis. Representation of bacteria with inner core LPS structures (blue) modified with phase variable structures including phosphorylcholine (ChoP, orange circles), the gal$\alpha$1-4gal di-galactoside (Di-gal, green triangles), and sialic acid (Neu5Ac, purple plus signs). The bacteria with the maximum number of outer core modifications have the greatest resistance to antibody and complement mediated lysis (top). Heterogeneous populations enriched for highly modified LPS (right) or minimally modified LPS (left) are selected depending on the host immune components present (bottom).

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