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*Trends In Microbiology (review)*

## ***Haemophilus influenzae* and the complement system**

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## **Abstract**

The respiratory tract pathogen *Haemophilus influenzae* is responsible for a variety of infections in humans including septicemia, bronchitis, pneumonia, and acute otitis media. The pathogenesis of *H. influenzae* relies on its capacity to resist the human host defenses including the complement system, and thus *H. influenzae* has developed several efficient strategies to circumvent the complement attack. In addition to attracting specific host complement regulators directly to the bacterial surface, the capsule, lipooligosaccharides, and several outer membrane proteins contribute to resistance against the complement-mediated attacks and hence increased bacterial survival. Insights into the mechanisms of complement evasion of *H. influenzae* are very important for understanding the pathogenesis, for development of vaccines and for new therapies aimed for patients with, for example, chronic obstructive pulmonary disease. This review gives an overview of our current knowledge on the different mechanisms by which *H. influenzae* conquers the host complement attack.

## **The respiratory pathogen *Haemophilus influenzae***

*H. influenzae* is a Gram-negative human specific pathogen responsible for a variety of diseases, and can be divided into encapsulated strains and unencapsulated strains according to the presence of a polysaccharide capsule. The capsule is the major virulence factor of invasive *H. influenzae* strains, and the encapsulated strains belong to one of six serotypes (a to f), where type b (Hib; see Glossary) is the most virulent one. Invasive disease caused by Hib mainly affects infants and children, causing potentially life-threatening conditions such as meningitis, epiglottitis, and severe sepsis [1]. After introduction of the capsular polysaccharide conjugate vaccine against Hib in the early 1990s, the incidence of invasive disease caused by Hib has decreased substantially in the Western hemisphere [2]. Since the

Hib vaccines do not protect against other capsule types or unencapsulated *H. influenzae* strains, invasive infections caused by non-type b strains have increased in frequency recent years [3-6]. Another important issue is that the Hib vaccine to date is not commonly used in developing countries, making large populations susceptible to all types of *H. influenzae* infections.

In contrast to encapsulated *H. influenzae*, non-typeable *Haemophilus influenzae* (NTHi) is a commensal of the respiratory tract particularly in children but also among adults and is rarely associated with invasive disease [7, 8]. NTHi mainly causes local disease in the upper and lower respiratory tract, *e.g.* acute otitis media (AOM), sinusitis, and bronchitis. However, NTHi is one of the most common causes of exacerbations in patients that suffer from chronic obstructive pulmonary disease (COPD) [9]. Moreover, NTHi is a frequent cause of AOM in children [8, 10]. NTHi are only sometimes invasive, but since the introduction of the Hib vaccine, there has been a serotype replacement with non-type b encapsulated *H. influenzae* and the incidence of invasive disease caused by non-type b *Haemophilus*, including NTHi has been suggested to increase in some countries and indigenous populations [6, 11-13]. Thus, there may be a shift in invasive *H. influenzae* disease from children to adults and the elderly over 65 years [12, 13].

Almost all pathogens possess ways to circumvent the complement system, and recent years a growing body of knowledge on interactions with this important part of the immune system has been compiled. Similar to most Gram-negative bacteria, *H. influenzae* is sensitive to the cytolytic activity of the complement system. To be able to infect the human host, *H. influenzae* has to survive this potentially lethal attack. *H. influenzae* is generally considered as a relatively serum sensitive pathogen, but it has also developed sophisticated strategies to

inhibit complement attack. The goal of this review is to give an overview of different ways *H. influenzae* can conquer innate immunity of its host by interfering with the complement system.

## **The complement system**

The complement system is the first line of defense and an essential part of the innate immune system. Activation of complement leads to a cascade of protein activation and deposition on the surface of the pathogen, resulting in formation of the membrane attack complex (MAC) and opsonization of the pathogen followed by phagocytosis [14]. Invading pathogens activate complement either spontaneously due to differences in envelope or membrane composition compared to host (alternative pathway (AP) and lectin pathway (LP)) or through antibody binding (classical pathway (CP)) (Figure 1). All three pathways lead to the formation of C3 convertase and thereafter they follow the same terminal pathway.

The CP is initiated when the Fc region of the antigen-bound immunoglobulin G (IgG) or IgM binds and activates the C1 complex consisting of the pattern recognition molecule C1q and two C1r and C1s proteases each (C1qr2s2). The binding results in a conformational change of C1q, leading to proteolytic activation of C1r. C1r then cleaves C1s, producing an active enzyme capable of interacting with and cleaving complement component 4 (C4) and C2 to form the C3 convertase (C4bC2b<sup>1</sup>) of the CP.

Binding of mannose binding lectin (MBL) or ficolins to carbohydrates on the surface of a microbe initiates the LP [15]. MBL undergoes a conformational change when it binds mannose residues on the microbe, leading to the activation of the MBL associated proteases

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<sup>1</sup> Sometimes referred to as C4bC2a [14].

(MASPs), which cleave C4 and C2 resulting in C4bC2b (C3 convertase), and the activation of this pathway follows the same route as the CP [16]. The C3 convertases have the ability to cleave C3 into C3a and C3b [14]. C3a is an anaphylatoxin that induces pro-inflammatory activities. Thereafter, C3b can bind covalently to the complement activating surface and C4bC2b, forming the CP and the LP C5 convertase, C4bC2bC3b. C3b binds factor B (FB), which allows factor D (FD) to cleave FB into Bb and Ba, forming the C3 convertase of the AP (C3bBb). This convertase is relatively unstable and decays easily, but an increased stability is provided when properdin binds. The C3 convertase will cleave more C3 in an amplification loop. C3b also functions as an opsonin and mediates uptake of C3b-coated pathogens via complement receptor 1 (CR1) by phagocytes [17]. When additional C3b is attached to C3bBb, the C5 convertase of the AP is formed [14]. The C5 convertases bind and cleave C5 to C5b and the pro-inflammatory anaphylatoxin C5a. C5b initiates the assembly of the late complement components (C5b, C6, C7, C8 and C9) forming a pore in the membrane. These channels, designated MAC, reduce osmotic pressure and cause lysis of the target cell. Opsonization, lysis, and pro-inflammatory signaling contribute to the host antimicrobial defense.

The complement system is tightly regulated, otherwise it would cause extensive host tissue damage. Host cells are protected from unwanted complement damage by membrane bound regulators as well as soluble regulators, which are attracted by the chemical composition of the cell surfaces (e.g., sialic acid and glycosaminoglycans). In addition, the soluble regulators also control complement activation in solution. Decay accelerating factor, CR1, membrane cofactor protein, and protectin are regulators present on host cell membranes and these are involved in inhibiting C3 and/or C5 convertases or MAC formation [17]. C4b-binding protein (C4BP), factor H (FH), and FH like protein-1 (FHL-1) are all soluble regulators that inhibits

complement by reducing the production of the C3 convertases both in fluid phase and on surfaces [18, 19]. Vitronectin and clusterin are regulators of the terminal pathway and they inhibit the insertion of C5b-7 into the membrane and also the polymerisation of C9 [17]. In addition, complement FH related protein 1 (FHR1) inhibits C5 convertase activity and MAC assembly [20]. Taken together, the complement system maintains a balance between activation and inhibition to protect self-cells and to allow activation on invading microbes.

### **Complement in the respiratory tract**

For many microorganisms, including *H. influenzae*, the primary interaction with the human host is through colonization of the mucosal surface of the respiratory tract. Therefore, it is of highest interest for the host to exploit an efficient defense at these surfaces. The complement system is classified as a part of serum, but there are several studies demonstrating the presence of complement in various sites of the body [21, 22]. However, reports of the presence of complement components in the respiratory tract of healthy individuals are scarce. In contrast, there are several studies indicating the importance of complement in the respiratory tract during infections [23-26]. Upon inflammation, the permeability of the mucosa increases, and plasma, including complement proteins, Igs and components of the coagulation and fibrinolysis systems, enters the airway lumen [21, 22]. This process is as designated plasma exudation and has been suggested to be the first line of the mucosal defense. Complement components, *i.e.*, C3a and C5a, have been found in the mucosa of the nose and lower airways in various respiratory tract challenges, including allergy and infection with influenza virus and in patients with nasal polyps [26-28]. FH, FHL-1 and FHR-1/2/3/4/5 are complement components found in middle ear effusions of patients with chronic otitis media with effusion [24]. Highly elevated levels of C3a have been observed in middle ear

effusions, indicating ongoing complement activation [25]. Furthermore, Marc and coworkers found increased concentrations of C5a in patients with COPD, suggesting the involvement of complement in the pathogenesis of COPD [23]. The presence of complement proteins and regulators in the respiratory tract indicates the importance of the use of efficient complement evasion strategies by respiratory pathogens.

### ***H. influenzae* and the complement system**

The pathogenesis of many microorganisms relies on their capacity to avoid, resist or neutralize the host defense including the complement system [29, 30]. *H. influenzae* uses two major mechanisms for complement evasion; I) building physical barriers for the activation and deposition of complement proteins, and II) acquiring soluble complement regulators for preventing amplification and lysis (Box 1, Table 1). Encapsulated *H. influenzae* are often invasive, but NTHi can also cause invasive disease and are resistant to the actions of human serum [31]. A delayed C3 deposition on the cell surface was found with serum resistant invasive NTHi strains, resulting in prevention of MAC accumulation [31]. *H. influenzae* biogroup aegyptius is a group of NTHi strains that causes the pediatric disease Brazilian purpuric fever (BPF) [32]. Isolates from patients with BPF have been shown to be serum resistant compared to non-BPF isolates and the lysis of the non-BPF isolates require an intact classical pathway [32]. However, the exact complement resistance mechanism(s) used by BPF isolates are unknown. Another interesting finding is that some pathogens may collaborate to conquer the innate immune system. Tan *et al.* showed that outer membrane vesicles produced by *Moraxella catarrhalis* neutralize C3 and thus contribute to an increased survival of *H. influenzae* in human serum [33]. This observation may explain why these two pathogens often are found together in the human respiratory tract. Finally, patients with C2 and C3 deficiencies are associated with an increased susceptibility to several bacterial species



including *H. influenzae* [34]. Surviving the complement-mediated attack is an essential step in the pathogenesis of most pathogens, including *H. influenzae*.

### **Bacterial factors involved in evasion of complement-mediated lysis**

#### *The polysaccharide capsule*

Encapsulated *H. influenzae* are characterized by the presence of structurally and serologically distinct polysaccharide capsules [35]. The Hib capsule is composed of a polymer of ribose and ribitol-5-phosphate (polyribosylribitolphosphate-PRP). The concentration of polysaccharide among type b strains seems to be important for bacterial survival, since an increased production of polysaccharide is associated with increased resistance to lysis (Figure 2a) [36, 37]. The Cap b locus contains the genes responsible for type b capsule expression and in most Hib strains two copies are required for capsule expression [38]. Hib isolates from patients with invasive disease have been shown to frequently have three or more copies of the Cap b locus [39]. A study by Noel *et al.* demonstrated that Hib strains containing four copies of the Cap b locus were more resistant to complement-induced lysis, in addition to a decreased complement-mediated opsonization and a reduced C3 deposition [40]. These findings suggested that amplification of the Cap b locus increases their resistance to complement-mediated attacks. Moreover, the type b capsule confers resistance to phagocytosis by macrophages [41]. When compared to other capsule types, Hib has been shown to be more resistant to the bactericidal effect of complement [37]. To analyze whether there are differences in resistance to the actions of complement depending on the capsular serotype, Swift and coworkers used capsule deficient mutants that were identical with respect to outer membrane proteins, LPS and antibiotic susceptibility [42]. In this study the capsule types a, b and e transformants were shown to be equally resistant to complement activity as

compared to the Hib wild type. Thus, the polysaccharide capsule expressed by typeable *H. influenzae* is an important protective coat against the complement-mediated attack.

### *Lipooligosaccharides*

In contrast to some other Gram-negative bacteria, *H. influenzae* produces lipooligosaccharide (LOS), which lacks the O-chain. LOS is a major glycolipid in the outer membrane of NTHi, which is highly variable between strains [43]. The LOS biosynthetic phase-variable gene *lgtC* encodes a galactosyltransferase and the expression of LgtC is involved in serum resistance of the serum resistant isolate R2866 [44]. Furthermore, a recent study demonstrated that serum resistance is facilitated in an NTHi strain by a delay of C4b deposition (Figure 2a) [45]. When *lgtC* was inactivated, the C4b deposition increased and the survival in serum and blood was reduced. Furthermore, *losA* is a phase variable gene present in most NTHi isolates and its product is involved in the biosynthesis of LOS and serum resistance [46, 47]. *lex2* is another phase variable locus that is required for expression of an epitope in *H. influenzae* LOS [48]. The epitope was shown to be a digalactoside-containing tetrasaccharide that mimics human antigens found on several different human cell types. The ability to synthesize this epitope enhances *H. influenzae* resistance to complement-mediated killing.

### *Phosphorylcholine*

Phosphorylcholine (ChoP) is a phase variable molecule linked to different hexoses on LOS depending on the particular strain and is involved in serum resistance [49]. ChoP<sup>+</sup> strains are more susceptible to the bactericidal actions of human serum than the ChoP<sup>-</sup> counterpart and the C reactive protein (CRP) mediates this susceptibility to serum by binding directly to the ChoP<sup>+</sup> strains and thereby activating the CP. CRP is an acute phase reactant that activates the classical pathway by binding C1q [14]. The level of ChoP expressed on the surface of NTHi

correlates with the amount of CRP bound to NTHi, and CRP facilitates complement-mediated killing of the pathogen [50]. Furthermore, ChoP expression is associated with more efficient colonization of the nasopharynx in an experimental infant rat model [49]. Thus, the ability of *H. influenzae* to vary the expression of ChoP correlates with its ability to colonize and persist on mucosal surfaces, and to cause invasive infections by evading innate immunity.

### *Sialic acid*

Surface-exposed sialic acid on human cells increases the affinity for FH for surface deposited C3b, which keeps the activation of the AP under control on self surfaces [14]. By incorporating sialic acid into their cell surfaces, many pathogens mimic the host cells and thereby circumvent the immune response of the host [51]. *H. influenzae* is incapable of synthesizing sialic acid and therefore the majority of NTHi strains incorporate sialic acid from the host environment as a terminal non-reducing sugar in LOS [52, 53]. The uptake of sialic acid is mediated through a single transport system that is a member of the tripartite ATP-independent periplasmic (TRAP) transporter family and deletion of this system resulted in complete loss of sialic acid uptake [54, 55]. Interestingly, sialic acid was protective for *H. influenzae* against complement-mediated killing of human serum [55, 56]. Sialylation of LOS is also catalyzed by sialyltransferases, and several types have been characterized in *H. influenzae*. The main one is Lic3A, which is involved in serum resistance and also essential for bacterial survival in an animal model of otitis media [53, 57]. Furthermore, the complement system has a central role in the innate immune defense against NTHi in an experimental model of AOM [52]. When depleting complement in chinchillas, two otherwise avirulent *siaB* mutants, which were defective in their ability to sialylate LOS, caused severe otitis media that was similar to their wild-type counterparts.

### *Outer membrane proteins*

The immunogenic outer membrane protein P2 varies in length and sequence at both DNA and protein levels between *H. influenzae* isolates, and this variability allows the bacteria to evade protective antibodies and hence induction of the CP of complement activation [58]. However, mucosal immunization of mice with recombinant P2 appears to induce antibodies protective against multiple strains of NTHi [59]. Another interesting observation is that an *H. influenzae* strain devoid of the outer membrane protein P6 showed increased sensitivity to complement-mediated killing as compared to the wild type when exposed to human serum (Figure 2a) [60]. When P6 is mutated (deleted), the architecture of the outer membrane is altered and this was suggested to be a reason for why the P6-deficient mutant was more sensitive to the cytolytic activity of the MAC.

ArcA is another protein expressed by *H. influenzae* and this protein has also been shown to be involved in serum resistance (Figure 2a) [61]. An *arcA* mutant demonstrated a significantly reduced survival to human serum compared to the wild-type isogenic counterpart. Complementation of the *arcA* mutant fully restored serum resistance.

Fimbriae are major adhesins of Hib and attach to mucosal surfaces and cells. Interestingly, fimbriated strains are more susceptible to the bactericidal effects of human serum as compared to non-fimbriated strains [62]. An increased deposition of C3 has also been detected on fimbriated strains. However, *H. influenzae* can vary its fimbriae expression by classical phase variation. This phenomenon suggests that fimbriae expression correlates with its ability to colonize and persist on mucosal surfaces, and to cause invasive infections by evading the complement system by downregulating the fimbriae expression.

*H. influenzae* outer membrane proteins Hsf and PE [63, 64], which both bind vitronectin, are described in detail below.

### ***Haemophilus*-dependent utilization of complement regulators**

Serum resistance is crucial for bacterial species of *H. influenzae* for survival in the human host and binding of complement inhibitors such as C4BP, FH, FHL-1, and vitronectin are efficient survival strategies [63-66]. Recently described interactions are outlined below.

#### *The classical and lectin pathways*

C4BP inhibits the formation and accelerates the decay of the C3 convertase (C4bC2b) and serves as a cofactor for Factor I (FI) in the proteolytic degradation of C4b [18]. Interestingly, NTHi has been shown to specifically bind C4BP, the regulator of the CP and LPs [65] (Figure 2b). In contrast to the C4BP-binding NTHi strains, the majority of the typeable *H. influenzae* strains (a-f) tested showed no binding [65]. The main isoform of C4BP contains seven identical  $\alpha$ -chains and one  $\beta$ -chain linked together with disulfide bridges. Each  $\alpha$ -chain is composed of eight complement control protein (CCP) modules and NTHi did not interact with recombinant C4BP lacking CCP2 or CCP7, proving that these two CCPs are important for the binding to NTHi [65]. When exposed to human serum it was also demonstrated that a low C4BP binding isolate had an increased deposition of C3b followed by a reduced survival as compared to a high C4BP-binding isolate. A decrease in survival was seen with the C4BP-binding NTHi strain when it was incubated with serum depleted of C4BP, suggesting that C4BP at the surface indeed protected NTHi against the complement system. C4BP bound to the surface of *H. influenzae* also retained its cofactor activity as determined by analysis of C3b and C4b degradation. Consequently, such degradation prevents C4b and C3b from participating in the opsonization of the pathogen. The binding of C4BP renders the NTHi more resistant to serum-mediated killing and may consequently contribute to their virulence.

### *The alternative pathway*

Both encapsulated *H. influenzae* and NTHi bind FH and FHL-1 (Figure 2b) [66]. FHL-1 is a product of alternative splicing of the FH gene [19]. These fluid phase proteins regulate the AP of the complement system via binding of C3b, accelerating the decay of the AP C3-convertase (C3bBb), and acting as a cofactor for the FI-mediated cleavage of C3b. FH is a glycoprotein in human plasma composed of 20 CCP domains. FHL-1 is composed of 7 CCPs identical to the N-terminal CCPs of FH and includes an extension of four amino acids at its C-terminal end [17]. There is a significant difference in the amount of bound FH between Hib strains [66], which may explain why a low FH-binding Hib strain was more sensitive to killing by human serum than a high FH-binding Hib strain. In contrast to incubation with normal human serum, Hib had a reduced survival in complement active FH-depleted human serum, thus demonstrating that FH mediates a protective role at the bacterial surface. Two Hib-binding domains were identified within FH; one binding site common to both FH and FHL-1 was located in CCPs 6-7, whereas the other, specific for FH, was located in the C-terminal CCPs 18-20. Importantly, both FH and FHL-1 when bound to the surface of Hib retained cofactor activity as determined by analysis of C3b degradation. Proteins responsible for interactions with the CP and AP are at present unknown. Utilization of the complement regulators FH and FHL-1 contributes to the protection of Hib and NTHi against the complement mediated attack.

### *The terminal pathway*

Hib binds vitronectin and this interaction is mediated by the surface exposed outer membrane protein *Haemophilus* surface fibril (Hsf) (Figure 2b) [64]. Flow cytometry analysis of an Hsf-deficient mutant revealed that Hsf is the major vitronectin binding protein in Hib. These findings are in contrast to another study, where no binding to soluble vitronectin could be

detected [67]. The reason for this discrepancy is presently unclear. Hsf-expressing Hib and *Escherichia coli* bound both soluble and immobilized vitronectin [64]. Moreover, *H. influenzae* devoid of Hsf had a markedly reduced survival as compared to the isogenic wild type when exposed to human serum. Since vitronectin is also a part of the extra cellular matrix, the Hsf molecule may consequently be able to bind two vitronectin molecules and stabilize adherence despite the physical forces in the respiratory tract, including the mucociliary escalator, sneezing, and coughing.

The ubiquitous adhesin protein E (PE) [68], which exists both in encapsulated and NTHi strains [69, 70], binds vitronectin (Figure 2b), and this interaction is important for survival of NTHi in human serum [63]. A PE-deficient mutant showed reduced binding to vitronectin at low concentrations as compared to the isogenic wild-type counterpart. Moreover, a PE-deficient NTHi mutant had a markedly reduced survival in serum as compared to the PE-expressing wild type. Vitronectin bound to the surface of NTHi maintained its inhibitory activity and reduced MAC induced hemolysis in a hemolytic assay. Consequently, this inactivation of the terminal pathway protects the pathogen. In addition, when bacteria were incubated in serum lacking vitronectin, a decreased bacterial survival was seen with NTHi. Thus, these experiments demonstrated that binding of vitronectin protects NTHi against the complement-mediated attacks.

In a recent study we showed that binding of complement regulators, including C4BP, FH and vitronectin in addition to serum resistance is equally important for NTHi strains isolated from blood (i.e., invasive isolates) as well as for nasopharyngeal isolates from patients with upper respiratory tract infection [71]. That study demonstrated thus that NTHi has adapted to evade the innate immunity in the upper respiratory tract. In addition, a correlation between disease

severity and serum resistance was identified in cases of NTHi invasive disease. The utilization of different human complement regulators is an efficient complement evasion strategy used by both typeable *H. influenzae* and NTHi strains. Typeable *H. influenzae* attracts FH, FHL-1 and vitronectin, while NTHi strains are able to bind C4BP as well. Whether *H. influenzae* is able to bind and utilize all human regulators simultaneously or at different stages of infection remains to be studied. However, the expression of different surface structures and binding of complement regulators contribute to the survival of *H. influenzae* and thus to the virulence and ability to infect the human host.

### **Concluding remarks and future perspectives**

The respiratory pathogen *H. influenzae* and its role in colonization in, for example, patients with COPD have gained much interest in recent years [72]. It is now well established that *H. influenzae* plays a major role in the chronic inflammation seen in this group of patients. NTHi also is an important pathogen causing AOM in children [8, 10], and due to the introduction and now widespread use of vaccines against pneumococci, a replacement phenomenon with a higher incidence of NTHi carriage and infections may be expected in the near future [73]. As has been shown with other bacterial species, outer membrane proteins binding complement regulators are interesting targets for vaccine development [74]. Characterization of host-cell interactions with focus on bacterial manipulation and/or inhibition of the complement system are a growing research area. However, little is known about the exact mechanisms of how *H. influenzae* avoids complement-mediated attacks. Several proteins, LOS and the capsule have been shown to be involved in the protection against the complement system but further investigations are required to fully clarify the exact mechanisms. Thus, expanding our knowledge of how *H. influenzae* interacts with the complement system is of utmost importance not only to increase our understanding of infection biology, but also for the



development of vaccines and new therapeutic options aimed at pre-school children, patients with respiratory diseases such as COPD, and the elderly. However, many questions still need to be answered (Box 2). The contribution of the different complement evasion mechanisms to the pathogenesis of *H. influenzae* need to be further studied and clarified. Development of *in vivo* models and additional research on the mechanisms to study the impact of the evasion strategies will provide a better understanding of the importance of complement in the pathogenesis of *H. influenzae*.

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**Table I. Bacterial factors involved in the protection of *H. influenzae* against the complement-mediated attack**

<i>H. influenzae</i>	Bacterial protein/structure	Host target	Resistance/mechanism	Reference
Hib	Capsular polysaccharide	Unknown	Serum resistance Reduced C3 deposition Decreased complement-mediated opsonization Resistance to phagocytosis	[36, 37, 40, 41]
Hib	ArcA	Unknown	Serum resistance	[61]
Hib	Unknown	Factor H, FHL-1	Serum resistance	[66]
Hib	Hsf	Vitronectin	Utilizing the inhibitory effect of vitronectin on the terminal pathway Serum resistance	[64]
Hib	Protein E	Vitronectin	Utilizing the inhibitory effect of vitronectin on the terminal pathway Serum resistance	[63]
NTHi	LOS/LosA	Unknown	Serum resistance	[46, 47]
NTHi	LOS/LgtC	Unknown	Serum resistance Delay of C4b deposition	[44, 45]
NTHi	LOS/Lex2B	Unknown	Mimics human antigens Serum resistance	[48]
NTHi	Sialic acid	Unknown	Serum resistance	[55, 56]
NTHi	P6	Unknown	Serum resistance	[60]
NTHi	Unknown	C4BP	Utilizing the inhibitory effect of C4BP on the CP/LP Serum resistance	[65]
NTHi	Unknown	Factor H, FHL-1	Reduced C3 deposition Utilizing the inhibitory effect of Factor H and FHL-1 on the AP Serum resistance	[66]
NTHi	Protein E	Vitronectin	Utilizing the inhibitory effect of vitronectin on the terminal pathway Serum resistance	[63]

## Glossary

**Acute otitis media (AOM):** is one of the most common bacterial infections in children and causes inflammation of the middle ear that may lead to hearing loss.

**Complement control protein (CCP):**, repetitive domains of 60 amino acids. Complement regulators, including C4BP, FH and FHL-1 are composed of CCPs.

**C4b-binding protein (C4BP):** is a regulator of the CP and LP of the complement system. It inhibits the formation and accelerates the decay of the C3 convertase. In addition, it acts as a cofactor for FI in the degradation of C4b.

**Chronic obstructive pulmonary disease (COPD):** a chronic lung disease that makes it difficult to breathe and consists of two conditions (*i.e.* chronic bronchitis and emphysema). Bronchitis causes large amounts of mucus and swelling in the main airways in the lungs and emphysema destroys the air sacs in the lungs.

**Factor H (FH):** a regulator of the AP of the complement system. FH inhibits the formation and accelerates the decay of the C3 convertase. In addition, it acts as a cofactor for FI in the degradation of C3b.

**Factor H like protein 1 (FHL-1):** is a product of alternative splicing of the *FH* gene and is composed of seven CCPs identical to the N-terminal CCPs of FH and four unique amino acids at the C-terminal end. It is a regulator of the AP of the complement system. FHL-1 inhibits the formation and accelerates the decay of the C3 convertase. Moreover, it acts as a cofactor for FI in the degradation of C3b.

**Encapsulated *H. influenzae* type b (Hib):** is the most virulent type of the encapsulated *H. influenzae* causing severe and life-threatening diseases, including meningitis, septicaemia and epiglottitis.

**Lipooligosaccharides (LOS):** lack the O-chain (repetitive side chain) of lipopolysaccharides. LOS is a major glycolipid in the outer membrane of NTHi, which is highly variable between strains.

**Membrane attack complex (MAC):** the membrane attack complex (C5b-9) forms a major endpoint of the complement cascade. It is a pore that is inserted into a biological membrane and therefore reduces osmotic pressure and causes lysis of the target cell and consequently cell death.

#### **Glossary box, contd.**

**Nontypeable *H. influenzae* (NTHi):** lacks the capsule gene and thus expression of the polysaccharide capsule. NTHi causes upper and lower respiratory tract infections. NTHi is a significant cause of AOM in children and exacerbations as well as chronic carriage in patients with COPD.

**Opsonization:** a process through which a cell or microbe is tagged with marker proteins (*e.g.*, antibodies, C3b, and iC3b) to make it more susceptible to being engulfed by a phagocyte.

**Vitronectin:** a regulator of the terminal pathway of the complement system. Vitronectin binds the C5b-7 complex at its membrane-binding site and thereby inhibits the insertion of the MAC into the cell membrane. This regulator inhibits the polymerisation of C9 and consequently prevents lysis of the microbe.

#### **Box 1. *H. influenzae*-dependent interactions with the complement system**

*H. influenzae* is a Gram-negative human specific pathogen. Many Gram-negative bacterial species, including *H. influenzae*, are considered to be sensitive to complement-mediated killing, and the composition of the cell wall of Gram-negative bacteria does not make them resistant against MAC, the end product of the complement system [75]. The cell wall of the

Gram-negative bacteria consists of two lipid bilayers, the inner membrane (cytoplasmic membrane) and the outer membrane. The periplasmic space is located between the lipid bilayer and contains a thin peptidoglycan layer. The pore-forming MAC penetrates the bacterial envelope causing rupture of the membranes and consequently lysis of the cell. However, disruption of the outer membrane alone is unlikely to be lethal for the Gram-negative bacteria. Studies with *Escherichia coli* have shown that both outer and inner membranes are damaged by the MAC and this is lethal for the bacteria [76]. *H. influenzae* has developed several strategies to protect itself against the complement system. Encapsulated *H. influenzae* is surrounded by a layer of polysaccharides (*i.e.* the capsule), which protects the bacteria against the insertion of MAC and against phagocytosis, and this makes the encapsulated *H. influenzae* therefore more virulent than unencapsulated *H. influenzae* (NTHi) strains [39, 40]. In addition, the highly variable LOS, the outer membrane proteins ArcA and P6 are all involved in serum resistance of *H. influenzae* [44, 45, 60, 61]. Bacterial binding of complement regulators to the outer membrane surface in order to utilize their protective effect is another effective strategy to gain complement resistance. *H. influenzae* binds the regulators C4BP (CP and LP) and FH and FHL-1 (AP) [65, 66]. Moreover, encapsulated *H. influenzae* and NTHi bind vitronectin via Hsf and PE, respectively [63, 64]. The binding of these regulators results in increased survival in human serum. Taken together, *H. influenzae* has evolved different ways of protection against the different levels of the complement cascade in order to achieve maximal survival in the human host.

**Box 2. Future questions**

Which are the most important surface structures of *H. influenzae* involved in the protection against complement-mediated attacks?

Why are some capsular types of *H. influenzae* more resistant against the complement-mediated killing compared to other?

Which is the most important molecule associated with LOS that is involved in the protection against complement?

How do ArcA, P6 and sialic acid contribute to serum resistance of *H. influenzae*?

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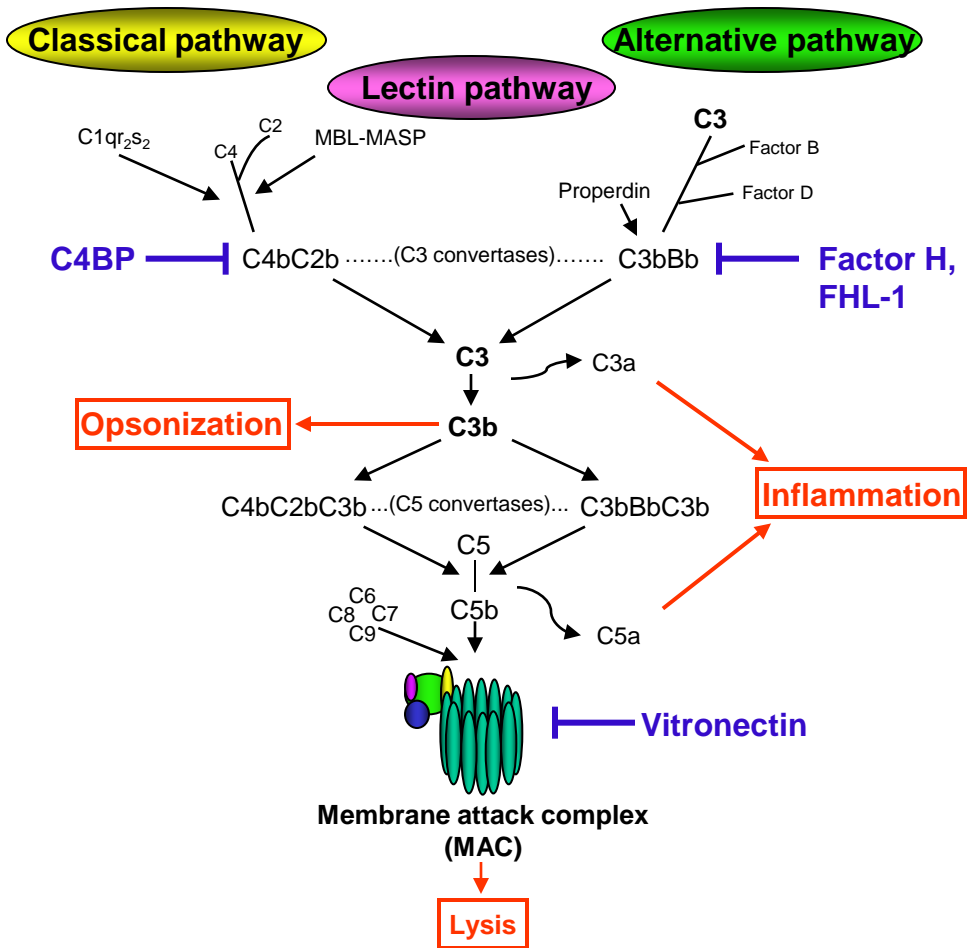
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## Figure legends

**Figure 1. The complement system.** The complement system is activated by pathogens either spontaneously due to differences in the envelope and membrane (AP and LP) or through antibody binding (CP). All three pathways lead to the formation of the C3 convertases, with subsequent cleavage of C3 to C3a (anaphylatoxin) and C3b (opsonin). Thereafter, the C5 convertases are formed, and all pathways follow the same terminal pathway resulting in the cytolytic MAC. The complement system is tightly regulated to avoid extensive host tissue damage. The complement regulators shown are utilized by *H. influenzae*.

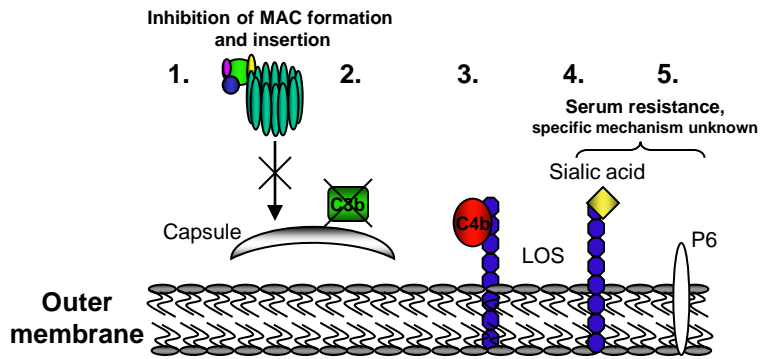
**Figure 2. Complement resistance mechanisms used by *H. influenzae*.** (a) The capsule protects *H. influenzae* against the cytolytic capacity of MAC (1) [36, 37] and against opsonization with C3 and hence phagocytosis (2) [41]. The expression of LOS (3) [44, 45], incorporation of sialic acid into LOS (4) [55, 57] and expression of P6 (5) [60] are also involved in serum resistance. LOS contributes to serum resistance by a delay of C4b deposition. However, the exact mechanisms by which sialic acid and P6 contribute to serum resistance of *H. influenzae* remain to be studied. (b) Binding of the complement regulators C4BP (1) [65], FH, FHL-1 (2) [66], and vitronectin (3) [63, 64] to the surface of *H. influenzae* protects against complement-mediated attacks and significantly contributes to the survival of *H. influenzae* in human serum. The ligands for C4BP, FH and FHL-1 are unknown.

Figure 1, Hallström et al





(a)



(b)

