HHS Public Access

Author manuscript

Mol Microbiol. Author manuscript; available in PMC 2015 August 26.

Published in final edited form as:

Mol Microbiol. 2013 May ; 88(3): 603–618. doi:10.1111/mmi.12214.

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

Sarah E. Clark, **Kara R. Eichelberger**, and **Jeffrey N. Weiser***

Department of Microbiology, University of Pennsylvania, Philadelphia, PA, USA

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 galα1–4gal 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), (Murphy and Sethi, 1992; Harabuchi *et al*., 1994; Murphy *et al*., 2009; Mackenzie *et al*., 2010). 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 (Ulanova and Tsang, 2009; Agrawal and Murphy, 2011). 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

*For correspondence: weiser@mail.med.upenn.edu; Tel. (+1) 215 573 3511, Fax (+1) 215 573 4856.

The authors have no conflict of interest to declare.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

of this pathway including complement and antibody are present on the mucosal surface (Zola *et al*., 2009). The lipopolysaccharide, or LPS, is a major source of both intra- and interstrain surface variation in *H. influenzae* (Campagnari *et al*., 1987; Swords *et al*., 2003).

Several of the genes required for the expression of LPS structures in *H. influenzae* are phase variable due to the presence of tetranucleotide repeats (High *et al*., 1993; Hood *et al*., 1996b; Griffin *et al*., 2003; Fox *et al*., 2005). 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) (Weiser *et al*., 1989). 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 (Weiser *et al*., 1997). 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 (Weiser *et al*., 1998). 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 (Tong *et al*., 2000; Clark *et al*., 2012). ChoP and several other LPS phase variable structures increase bacterial survival in the presence of human antibody and complement, 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 with 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 with bacteria with outer core oligosaccharides (Fig. 1A and 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 (Clark *et al*., 2012), 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 with 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 and 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 with 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 (Weiser *et al*., 1998; Tong *et al*., 2000). 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 and 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 (Power *et al*., 2009) 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 of 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 and 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 with *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, galα1–4gal. 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 galα1–4gal structure is also present in humans in the form of P blood group antigens and, therefore, is not a target of human antibody (Virji *et al*., 1990; Bitzan *et al*., 1994). 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 (Clark *et al*., 2012). 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 (Fig. 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 survivors 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 (Noel *et al*., 1996). In both strains, serial passage in human serum resulted in selection for a resistant population (Fig. 3C and D). The serum resistant population of 2019 was *lic2A* phase-on, compared with the *lic2A* phase-off original population (Table 2). In Eagan, the serum resistant population was both *lic2A* and *lgtC* phase-on, compared with 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 galα1–4gal, 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 and 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

Haemophilus 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 (Clark *et al*., 2012). 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 (Forsgren and Quie, 1974). 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 and D; Fig. S1C and 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 (Spinola *et al*., 1990), 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 (Clark *et al*., 2012). 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 with 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 with 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 with 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 with 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* (Figueira *et al*., 2007). *H. influenzae* must acquire sialic acid from the environment (Severi *et al*., 2005), 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 (Severi *et al*., 2005). 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 with 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 and 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 (Zola *et al*., 2009). 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 (Weiser and Pan, 1998; Hood *et al*., 2001a; Griffin *et al*., 2005), 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 (Poole *et al*., 2013). 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 (Weiser *et al*., 1998; Tong *et al*., 2000; Marti-Lliteras *et al*., 2011). IgA1 protease expression is phase variable due to the presence of a seven base pair repeat in *igaB* (Fernaays *et al*., 2006). 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 6 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 (Weiser and Pan, 1998). 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 galα1–4βgal is found in human P blood group antigens (Naiki and Marcus, 1975), this structure is not 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 (Weiser and Pan, 1998; Erwin *et al*., 2006a).

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 (Deadman *et al*., 2009). 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 (Griffin *et al*., 2005). 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 favourable 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* tetra-nucleotide 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 (Fox *et al*., 2005; Erwin *et al*., 2006b), 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 (Nakamura *et al*., 2011). 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 suggest 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 (Clark *et al*., 2012). 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 (Clark *et al*., 2012). 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 with *lgtC*– mutants (Ho *et al*., 2007). 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 (Figs 5 and 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 with 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 with those without sialic acid (Figueira *et al*., 2007). 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 favourable for survival. For ChoP, it has been shown that CRP can recognize ChoP-modified LPS and initiate classical pathway complement-mediated killing (Weiser *et al*., 1998). In the case of di-galactoside and sialic acid, however, the disadvantages during infection are less clear. Galectins, which bind several galactose-associated LPS residues, have the potential to recognize these structures (Sato *et al*., 2009; Vasta, 2009). 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 galα1–4gal di-galactoside. Galectin binding can initiate immune responses resulting in bacterial clearance (Sato *et al*., 2009). Also, it has been shown that *lic2A* expression increases susceptibility of *H. influenzae* to infection with the bacteriophage HP1c1 (Zaleski *et al*., 2005). 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 galα1–4gal 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 (Schauer, 1985; Virji *et al*., 1990; Fan *et al*., 2001). 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 favours 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 μg ml^{-1} β-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 (Table S1) 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 (Table S1), introducing an XmaI site. The spectinomycin gene *aad9* was inserted at the XmaI site following amplification with the primer pair *aad9* (Table S1). The resulting plasmid used to transform R2846. The *siaP*– mutant was constructed as previously described (Severi *et al*., 2005). 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 (Weiser *et al*., 1997). 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 (Nakamura *et al*., 2011). 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 mean fluorescence intensity (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 (Clark *et al*., 2012). 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 h 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 (Clark *et al*., 2012). 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 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 4 h 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*, octanucleotide) 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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).

References

Agrawal A, Murphy TF. *Haemophilus influenzae* infections in the *H. influenzae* type b conjugate vaccine era. J Clin Microbiol. 2011; 49:3728–3732. [PubMed: 21900515]

Bitzan M, Richardson S, Huang C, Boyd B, Petric M, Karmali MA. Evidence that verotoxins (Shiga-like toxins) from *Escherichia coli* bind to P blood group antigens of human erythrocytes in vitro. Infect Immun. 1994; 62:3337–3347. [PubMed: 8039905]

Campagnari AA, Gupta MR, Dudas KC, Murphy TF, Apicella MA. Antigenic diversity of lipooligosaccharides of nontypable *Haemophilus influenzae*. Infect Immun. 1987; 55:882–887. [PubMed: 3549563]

Clark SE, Snow J, Li J, Zola TA, Weiser JN. Phosphorylcholine allows for evasion of bactericidal antibody by *Haemophilus influenzae*. PLoS Pathog. 2012; 8:e1002521. [PubMed: 22396641]

Deadman ME, Hermant P, Engskog M, Makepeace K, Moxon ER, Schweda EK, Hood DW. Lex2B, a phase-variable glycosyltransferase, adds either a glucose or a galactose to *Haemophilus influenzae* lipopolysaccharide. Infect Immun. 2009; 77:2376–2384. [PubMed: 19289512]

Erwin AL, Allen S, Ho DK, Bonthuis PJ, Jarisch J, Nelson KL, et al. Role of *lgtC* in resistance of nontypeable *Haemophilus influenzae* strain R2866 to human serum. Infect Immun. 2006a; 74:6226–6235. [PubMed: 16966407]

Erwin AL, Bonthuis PJ, Geelhood JL, Nelson KL, McCrea KW, Gilsdorf JR, Smith AL. Heterogeneity in tandem octanucleotides within *Haemophilus influenzae* lipopolysaccharide biosynthetic gene *losA* affects serum resistance. Infect Immun. 2006b; 74:3408–3414. [PubMed: 16714571]

Fan X, Goldfine H, Lysenko E, Weiser JN. The transfer of choline from the host to the bacterial cell surface requires *glpQ* in *Haemophilus influenzae*. Mol Microbiol. 2001; 41:1029–1036. [PubMed: 11555284]

Fernaays MM, Lesse AJ, Cai X, Murphy TF. Characterization of *igaB*, a second immunoglobulin A1 pro-tease gene in nontypeable *Haemophilus influenzae*. Infect Immun. 2006; 74:5860–5870. [PubMed: 16988265]

Figueira MA, Ram S, Goldstein R, Hood DW, Moxon ER, Pelton SI. Role of complement in defense of the middle ear revealed by restoring the virulence of nontypeable *Haemophilus influenzae siaB* mutants. Infect Immun. 2007; 75:325–333. [PubMed: 17088344]

Forsgren A, Quie PG. Influence of the alternate complement pathway in opsonization of several bacterial species. Infect Immun. 1974; 10:402–404. [PubMed: 4152919]

Fox KL, Yildirim HH, Deadman ME, Schweda EK, Moxon ER, Hood DW. Novel lipopolysaccharide biosynthetic genes containing tetranucleotide repeats in *Haemophilus influenzae*, identification of a gene for adding O-acetyl groups. Mol Microbiol. 2005; 58:207–216. [PubMed: 16164559]

Griffin R, Cox AD, Makepeace K, Richards JC, Moxon ER, Hood DW. The role of *lex2* in lipopolysaccharide biosynthesis in *Haemophilus influenzae* strains RM7004 and RM153. Microbiology. 2003; 149:3165–3175. [PubMed: 14600228]

Griffin R, Bayliss CD, Herbert MA, Cox AD, Makepeace K, Richards JC, et al. Digalactoside expression in the lipopolysaccharide of *Haemophilus influenzae* and its role in intravascular survival. Infect Immun. 2005; 73:7022–7026. [PubMed: 16177385]

Harabuchi Y, Faden H, Yamanaka N, Duffy L, Wolf J, Krystofik D. Nasopharyngeal colonization with nontypeable *Haemophilus influenzae* and recurrent otitis media. Tonawanda/Williamsville Pediatrics. J Infect Dis. 1994; 170:862–866. [PubMed: 7930728]

High NJ, Deadman ME, Moxon ER. The role of a repetitive DNA motif (5′-CAAT-3′) in the variable expression of the *Haemophilus influenzae* lipopolysaccharide epitope alpha Gal(1-4)beta Gal. Mol Microbiol. 1993; 9:1275–1282. [PubMed: 7523834]

Ho DK, Ram S, Nelson KL, Bonthuis PJ, Smith AL. *lgtC* expression modulates resistance to C4b deposition on an invasive nontypeable *Haemophilus influenzae*. J Immunol. 2007; 178:1002–1012. [PubMed: 17202363]

Hood DW, Deadman ME, Allen T, Masoud H, Martin A, Brisson JR, et al. Use of the complete genome sequence information of *Haemophilus influenzae* strain Rd to investigate lipopolysaccharide biosynthesis. Mol Microbiol. 1996a; 22:951–965. [PubMed: 8971716]

Hood DW, Deadman ME, Jennings M, Bisercic M, Fleischmann RD, Venter JC, Moxon ER. DNA repeats identify novel virulence genes in *Haemophilus influenzae*. Proc Natl Acad Sci USA. 1996b; 93:11121–11125. [PubMed: 8855319]

Hood DW, Cox AD, Gilbert M, Makepeace K, Walsh S, Deadman ME, et al. Identification of a lipopolysaccharide alpha-2,3-sialyltransferase from *Haemophilus influenzae*. Mol Microbiol. 2001a; 39:341–350. [PubMed: 11136455]

Hood DW, Cox AD, Wakarchuk WW, Schur M, Schweda EK, Walsh SL, et al. Genetic basis for expression of the major globotetraose-containing lipopolysaccharide from *H. influenzae* strain Rd (RM118). Glycobiology. 2001b; 11:957–967. [PubMed: 11744630]

Lundstrom SL, Li J, Deadman ME, Hood DW, Moxon ER, Schweda EK. Structural analysis of the lipopolysaccharide from nontypeable *Haemophilus influenzae* strain R2846. Biochemistry. 2008; 47:6025–6038. [PubMed: 18465844]

Lysenko E, Richards JC, Cox AD, Stewart A, Martin A, Kapoor M, Weiser JN. The position of phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* affects binding and sensitivity to C-reactive protein-mediated killing. Mol Microbiol. 2000; 35:234–245. [PubMed: 10632893]

Mackenzie GA, Leach AJ, Carapetis JR, Fisher J, Morris PS. Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. BMC Infect Dis. 2010; 10:304. [PubMed: 20969800]

Marti-Lliteras P, Lopez-Gomez A, Mauro S, Hood DW, Viadas C, Calatayud L, et al. Nontypable *Haemophilus influenzae* displays a prevalent surface structure molecular pattern in clinical isolates. PLoS ONE. 2011; 6:e21133. [PubMed: 21698169]

Murphy TF, Sethi S. Bacterial infection in chronic obstructive pulmonary disease. Am Rev Respir Dis. 1992; 146:1067–1083. [PubMed: 1416398]

Murphy TF, Faden H, Bakaletz LO, Kyd JM, Forsgren A, Campos J, et al. Nontypeable *Haemophilus influenzae* as a pathogen in children. Pediatr Infect Dis J. 2009; 28:43–48. [PubMed: 19057458]

Naiki M, Marcus DM. An immunochemical study of the human blood group P1, P, and PK glycosphingolipid antigens. Biochemistry. 1975; 14:4837–4841. [PubMed: 1182122]

Nakamura S, Shchepetov M, Dalia AB, Clark SE, Murphy TF, Sethi S, et al. Molecular basis of increased serum resistance among pulmonary isolates of non-typeable *Haemophilus influenzae*. PLoS Pathog. 2011; 7:e1001247. [PubMed: 21253576]

Noel GJ, Brittingham A, Granato AA, Mosser DM. Effect of amplification of the Cap b locus on complement-mediated bacteriolysis and opsonization of type b *Haemophilus influenzae*. Infect Immun. 1996; 64:4769–4775. [PubMed: 8890238]

Poole JJ, Foster ED, Chaloner K, Hunt JR, Jennings MP, Bair T, et al. Analysis of nontypeable *Haemophilus influenzae* phase variable genes during experimental human nasopharyngeal colonization. J Infect Dis. 2013 (in press).

Power PM, Sweetman WA, Gallacher NJ, Woodhall MR, Kumar GA, Moxon ER, Hood DW. Simple sequence repeats in *Haemophilus influenzae*. Infect Genet Evol. 2009; 9:216–228. [PubMed: 19095084]

Sato S, St-Pierre C, Bhaumik P, Nieminen J. Galectins in innate immunity: dual functions of host soluble beta-galactoside-binding lectins as damage-associated molecular patterns (DAMPs) and as receptors for pathogen-associated molecular patterns (PAMPs). Immunol Rev. 2009; 230:172–187. [PubMed: 19594636]

Schauer R. Sialic acids and their role as biological masks. Trends Biochem Sci. 1985; 10:357–360.

Severi E, Randle G, Kivlin P, Whitfield K, Young R, Moxon R, et al. Sialic acid transport in *Haemophilus influenzae* is essential for lipopolysaccharide sialylation and serum resistance and is dependent on a novel tripartite ATP-independent periplasmic transporter. Mol Microbiol. 2005; 58:1173–1185. [PubMed: 16262798]

Spinola SM, Kwaik YA, Lesse AJ, Campagnari AA, Apicella MA. Cloning and expression in *Escherichia coli* of a *Haemophilus influenzae* type b lipooligosaccharide synthesis gene(s) that encodes a 2-keto-3-deoxyoctulosonic acid epitope. Infect Immun. 1990; 58:1558–1564. [PubMed: 1692806]

Swords WE, Jones PA, Apicella MA. The lipooligosaccharides of *Haemophilus influenzae*: an interesting array of characters. J Endotoxin Res. 2003; 9:131–144. [PubMed: 12831454]

Tong HH, Blue LE, James MA, Chen Y, DeMaria TF. Evaluation of phase variation of nontypeable *Haemophilus influenzae* lipooligosaccharide during nasopharyngeal colonization and development of otitis media in the chinchilla model. Infect Immun. 2000; 68:4593–4597. [PubMed: 10899860]

Ulanova M, Tsang RS. Invasive *Haemophilus influenzae* disease: changing epidemiology and host-parasite interactions in the 21st century. Infect Genet Evol. 2009; 9:594–605. [PubMed: 19460326]

Vasta GR. Roles of galectins in infection. Nat Rev Microbiol. 2009; 7:424–438. [PubMed: 19444247]

Virji M, Weiser JN, Lindberg AA, Moxon ER. Antigenic similarities in lipopolysaccharides of Haemophilus and Neisseria and expression of a digalactoside structure also present on human cells. Microb Pathog. 1990; 9:441–450. [PubMed: 1711142]

Weiser JN, Pan N. Adaptation of *Haemophilus influenzae* to acquired and innate humoral immunity based on phase variation of lipopolysaccharide. Mol Microbiol. 1998; 30:767–775. [PubMed: 10094625]

Weiser JN, Love JM, Moxon ER. The molecular mechanism of phase variation of *H. influenzae* lipopolysaccharide. Cell. 1989; 59:657–665. [PubMed: 2479481]

Weiser JN, Shchepetov M, Chong ST. Decoration of lipopolysaccharide with phosphorylcholine: a phase-variable characteristic of *Haemophilus influenzae*. Infect Immun. 1997; 65:943–950. [PubMed: 9038301]

Weiser JN, Pan N, McGowan KL, Musher D, Martin A, Richards J. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. J Exp Med. 1998; 187:631–640. [PubMed: 9463413]

Zaleski P, Wojciechowski M, Piekarowicz A. The role of Dam methylation in phase variation of *Haemophilus influenzae* genes involved in defence against phage infection. Microbiology. 2005; 151:3361–3369. [PubMed: 16207918]

Zola TA, Lysenko ES, Weiser JN. Natural antibody to conserved targets of *Haemophilus influenzae* limits colonization of the murine nasopharynx. Infect Immun. 2009; 77:3458–3465. [PubMed: 19451240]

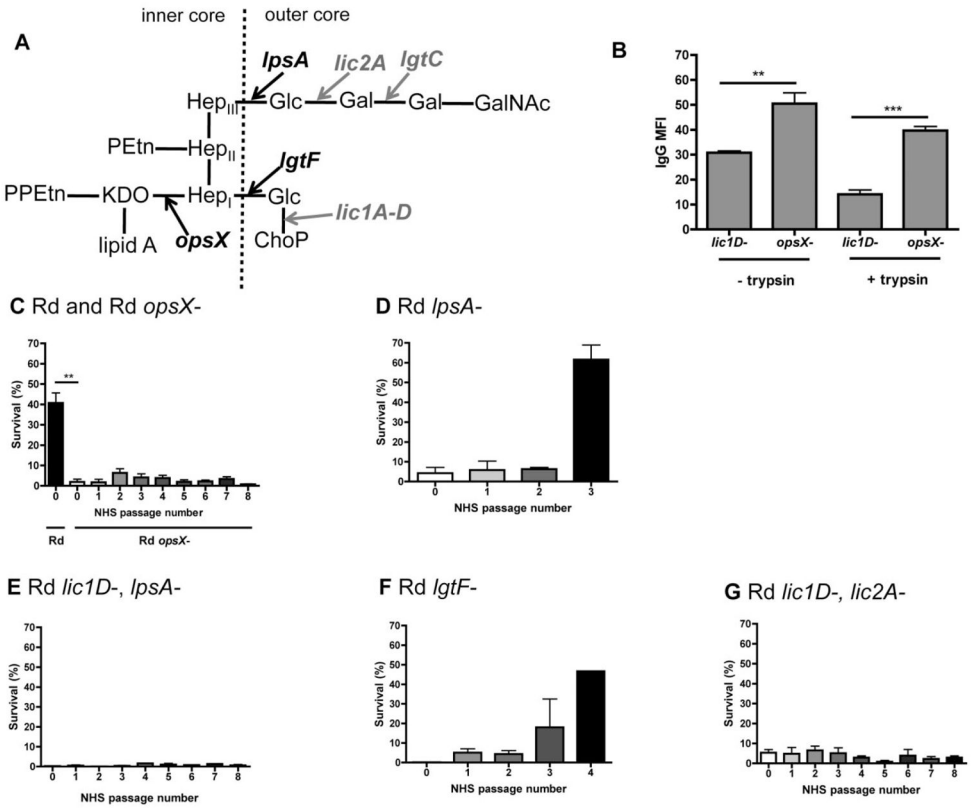

Fig. 1.

LPS structural requirements for resistance to human serum in the strain Rd. LPS structure proposed for Rd (Hood *et al.*, 2001b), 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 with the *opsX*– mutant, with or without trypsin digestion (B). Bactericidal assays, where survival in human serum is determined relative to 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; $^{**}P < 0.01$, $^{***}P < 0.001$.

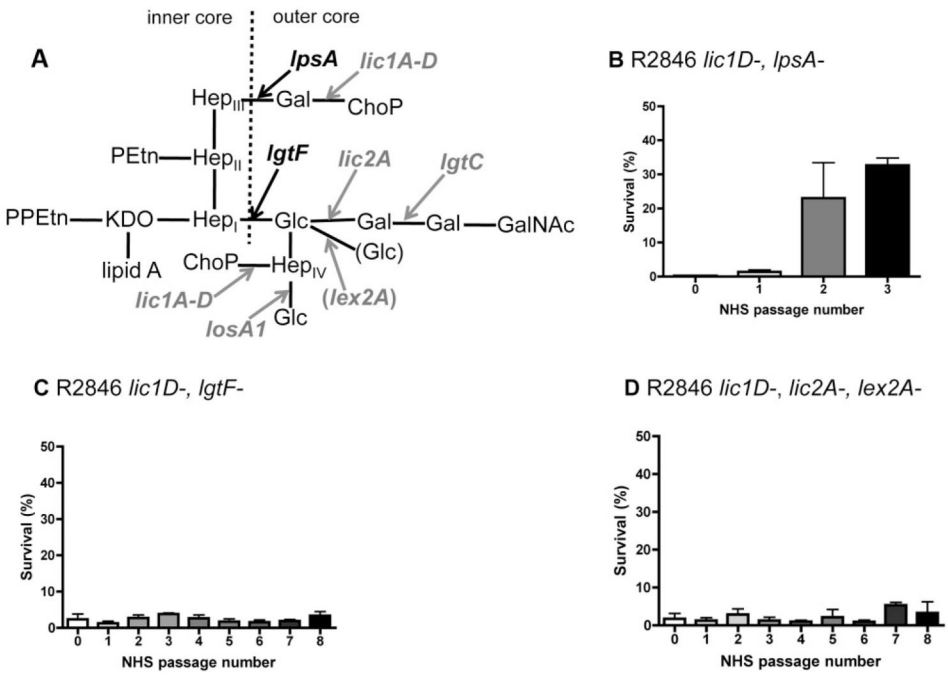

Fig. 2.

LPS structural requirements for resistance to human serum in the strain R2846. LPS structure proposed for NTHi clinical isolate R2846 (Lundstrom *et al*., 2008), 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 serial passage in 5% normal human serum (NHS), with survival following serial passage in human serum, for R2846 mutants (B–D). Data shown are means and SEM (representative experiments in triplicate).

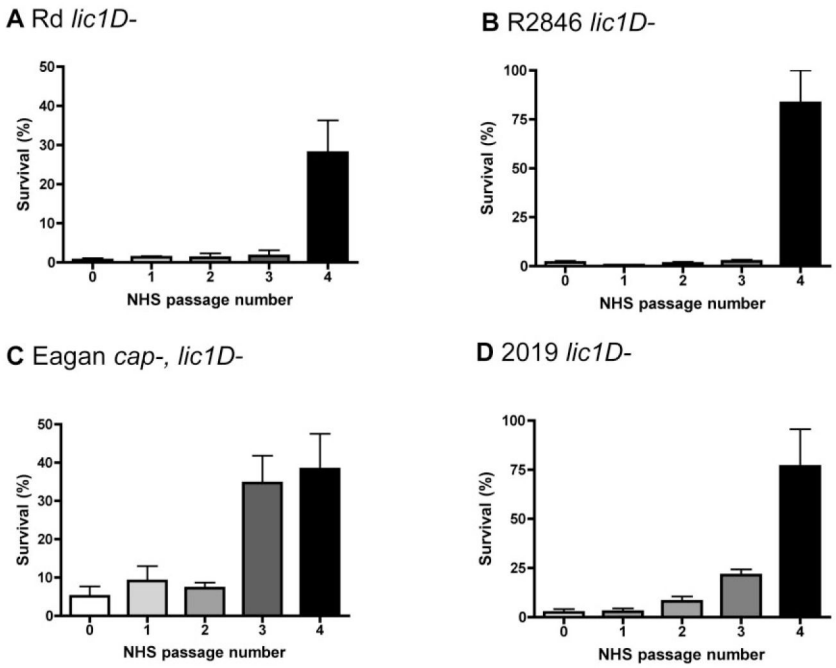

Fig. 3.

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*– (C, 2% NHS) and 2019 *lic1D*– (D, 4% NHS). Data shown are means and SEM (representative experiments in triplicate).

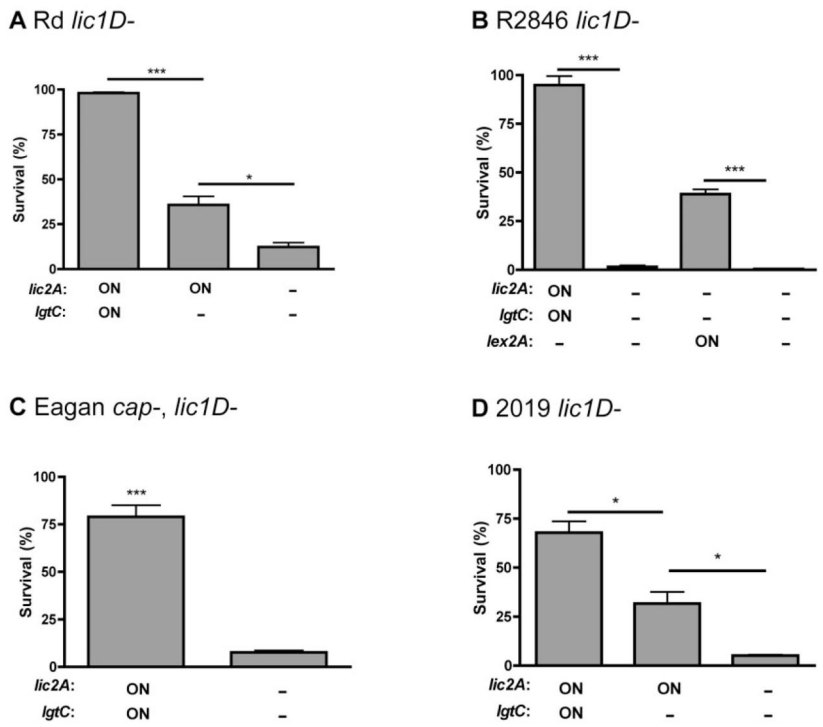

Fig. 4.

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; $*P < 0.05$, $***P < 0.001$.

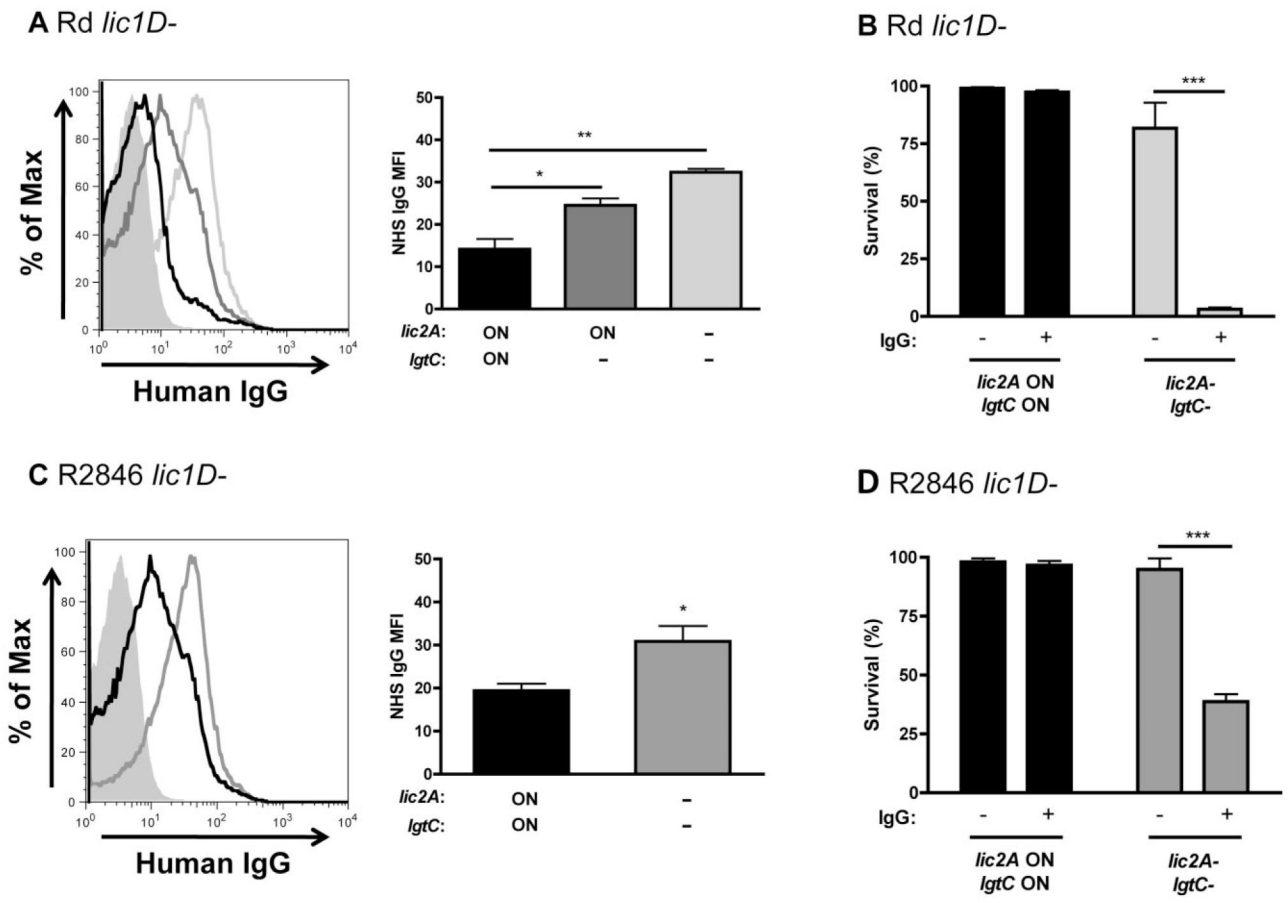

Fig. 5.

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.

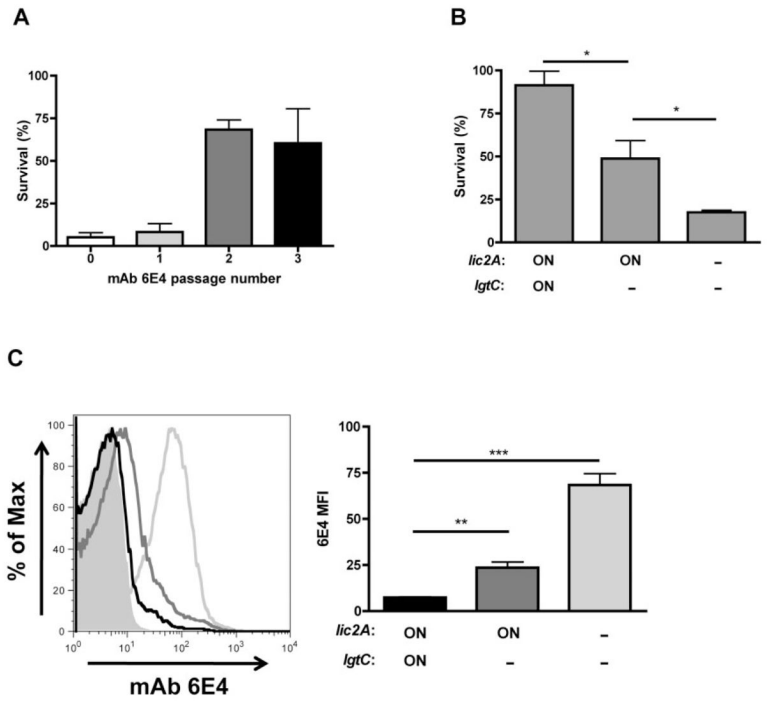

Fig. 6.

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 and 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 and mAb 6E4). Histogram and graphical summary for binding of mAb 6E4 to *lic2A* and *lgtC* phase-on variants (black), a *lic2A* phase-on, *lgtC*– mutant (dark grey) and a *lic2A*– mutant (light grey) 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.

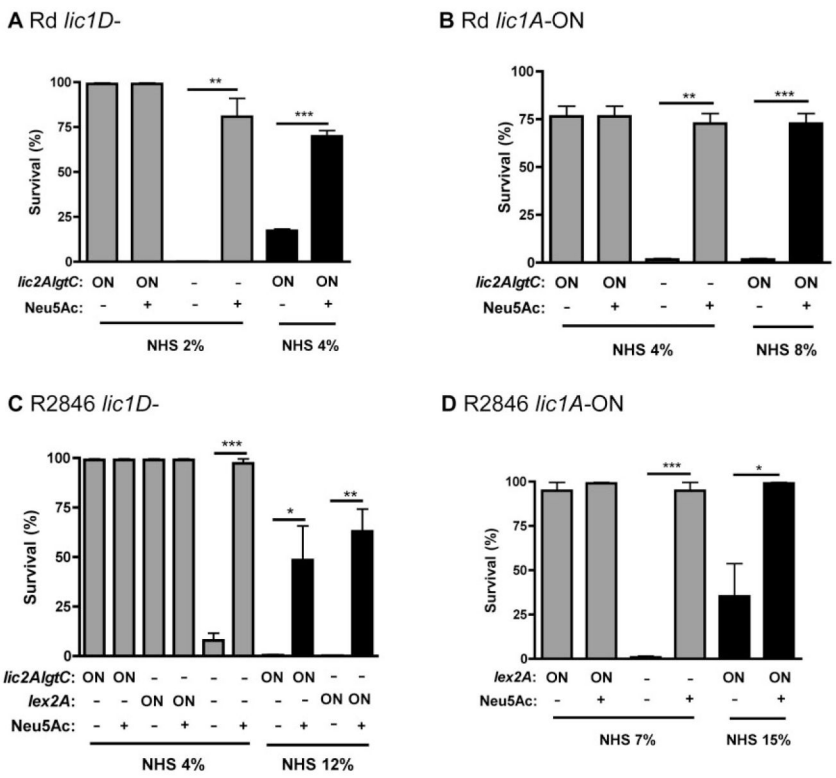

Fig. 7.

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; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 1

H. influenzae strains used in this study.

Strain	Mutant (−) or phase-on variant	Reference
Rd[a]	*opsX*–	Hood *et al*. (1996a)
	lpsA–	Hood *et al*. (1996a)
	lgtF–	Nakamura *et al*. (2011)
	lic2A–	Nakamura *et al*. (2011)
	lgtC–	Nakamura *et al*. (2011)
	lic1D–	Lysenko *et al*. (2000)
	lic1A-ON	Clark *et al*. (2012)
	lic1D–, *siaP*–	Severi *et al*. (2005)
	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
R2846[b]	*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
2019[c]	*lic1D*–	This study
	lic1D–, *lic2A*–	This study
	lic1D–, *lic2A*-ON, *lgtC*–	This study
	lic1D–, *lic2A*-ON, *lgtC*-ON	This study
Eagan *cap*-[d]	*lic1D*–	This study
	lic1D–, *lic2A*–	This study
	lic1D–, *lic2A*-ON, *lgtC*-ON	This study

[a] Type d, unencapsulated variant, avirulent strain (Hood *et al*., 1996a).

[b] NTHi clinical isolate from patient with otitis media (Lundstrom *et al*., 2008).

[c] NTHi clinical isolate from patient with chronic obstructive pulmonary disease (Tong *et al*., 2000).

[d] Type b, spontaneous capsule– derivative of the clinical isolate Eagan (Deadman *et al*., 2009).

Table 2

Exposure to NHS drives selection for LPS phase variants in *H. influenzae*.

Strain	Serial passage	Gene	No. repeats (reading frame)[a]	Times observed
Rd				
lpsA–	NHS	*lic1A*	17 (OFF) → 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[b]	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	*lic2A*	24 (OFF) → 25 (ON)	3/4
		lex2A	16 (OFF) → 14 (ON)	1/4
lic1A-ON[b]	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] 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.

[b] Bactericidal assays with *lic1A*-ON variants were conducted with CRP-deleted NHS.

NHS, normal human serum; 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′	Weiser and Pan (1998)
	R	5′-CGTTCTTTTTCCAATCCGCTTGTT-3′	
lgtC (GACA)	F	5′-TTTCATATCAAGAATATAAAAATT-3′	Weiser and Pan (1998)
	R	5′-GGTTTTGAAGAAAAAGGCGAA-3′	
lex2A (GCAA)	F	5′-GGCGGAATTATGTTAATCAC-3′	Erwin *et al*. (2006a)
	R	5′-GCTTGCATATAAGCTTTTCG-3′	
oafA (GCAA)	F	5′-TTCCAGAATTACTTGTAGGATCTTTG-3′	Erwin *et al*. (2006a)
	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 haemoglobin and haemoglobin-haptoglobin binding protein, *HI_0635* (CCAA)	F	5′-TCATCAACCCCTCGAACTGC-3′	This study
	R	5′-TCGTCAAGATCCTGTTGCCC-3′	
hgp haemoglobin and haemoglobin-haptoglobin binding protein, *HI_0661* (CCAA)	F	5′-CTTTGCCCAAAACGTCCAGC-3′	This study
	R	5′-ACGTGCTTGCCTATTCCGTT-3′	
hgp haemoglobin and haemoglobin-haptoglobin binding protein, *HI_1565* (CCAA)	F	5′-TTATGCTTGGGCTAACGGCA-3′	This study
	R	5′-CCGGTTTCATAGCGCACAAG-3′	
hgp haemoglobin and haemoglobin-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
	R	5′-TGAAGTCACTACCGCAACGG-3′	

[a]Sequence of the tandem repeat present within the sequence amplified for each target gene.