Shielding of a Lipooligosaccharide IgM Epitope Allows Evasion of Neutrophil-Mediated Killing of an Invasive Strain of Nontypeable *Haemophilus influenzae*

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ABSTRACT **Nontypeable *Haemophilus influenzae* is a frequent cause of noninvasive mucosal inflammatory diseases but may also cause invasive diseases, such as sepsis and meningitis, especially in children and the elderly. Infection by nontypeable *Haemophilus influenzae* is characterized by recruitment of neutrophilic granulocytes. Despite the presence of a large number of neutrophils, infections with nontypeable *Haemophilus influenzae* are often not cleared effectively by the antimicrobial activity of these immune cells. Herein, we examined how nontypeable *Haemophilus influenzae* evades neutrophil-mediated killing. Transposon sequencing (Tn-seq) was used on an isolate resistant to neutrophil-mediated killing to identify genes required for its survival in the presence of human neutrophils and serum, which provided a source of complement and antibodies. Results show that nontypeable *Haemophilus influenzae* prevents complement-dependent neutrophil-mediated killing by expression of surface galactose-containing oligosaccharide structures. These outer-core structures block recognition of an inner-core lipooligosaccharide epitope containing glucose attached to heptose HepIII-β1,2-Glc by replacement with galactose attached to HepIII or through shielding HepIII-β1,2-Glc by phase-variable attachment of oligosaccharide chain extensions. When the HepIII-β1,2-Glc-containing epitope is expressed and exposed, nontypeable *Haemophilus influenzae* is opsonized by naturally acquired IgM generally present in human serum and subsequently phagocytosed and killed by human neutrophils. Clinical nontypeable *Haemophilus influenzae* isolates containing galactose attached to HepIII that are not recognized by this IgM are more often found to cause invasive infections.**

IMPORTANCE **Neutrophils are white blood cells that specialize in killing pathogens and are recruited to sites of inflammation. However, despite the presence of large numbers of neutrophils in the middle ear cavity and lungs of patients with otitis media or chronic obstructive pulmonary disease, respectively, the bacterium nontypeable *Haemophilus influenzae* is often not effectively cleared from these locations by these immune cells. In order to understand how nontypeable *Haemophilus influenzae* is able to cause inflammatory diseases in the presence of neutrophils, we determined the mechanism that underlies resistance to neutrophil-mediated killing. We have shown that nontypeable *Haemophilus influenzae* prevents binding of antibodies of the IgM subtype through changes in their surface lipooligosaccharide structure, thereby preventing complement activation and clearance by human neutrophils.**

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Invasive disease caused by *Haemophilus influenzae* has decreased dramatically with the introductions of the *H. influenzae* type B conjugate vaccine (1). Currently, nontypeable *H. influenzae* (NTHi) is the most commonly isolated form of *H. influenzae* causing invasive infections in Europe (2). This group of *H. influenzae* strains lacks a capsule, which renders them more sensitive to antibacterial actions of the innate immune system. As such, NTHi is often found as a cause of mucosal inflammatory diseases, including otitis media (OM) (3), sinusitis (4), and exacerbations in patients with chronic obstructive pulmonary disease (COPD) (5). However, NTHi can also cause more invasive diseases, such as sepsis and meningitis (6). In all these situations, NTHi encounters the antimicrobial activity of the host's immune system and therefore has developed mechanisms to survive and multiply during infection.

The immune response to NTHi includes secretion of cytokines and chemokines by the respiratory epithelium and resident immune cells, which attracts various types of nonresident immune cells, including large numbers of neutrophils, to the site of inflammation (7). Neutrophils possess multiple antimicrobial activities contributing to the clearance of bacterial pathogens, including the generation of reactive oxygen species (ROS), release of antimicrobial peptides and proteases from their granules, formation of extracellular traps (neutrophil extracellular traps [NETs]), and potent phagocytic capacity (8). Even though neutrophils are specialized killers, these cells are often not able to clear NTHi

TABLE 1 R2866 mutant library challenged with or without neutrophils with active complement for 2 h[a]

Total no. of pseudoreads, NHS	Total no. of pseudoreads, NHS + neutrophils	Fold change	Adjusted *P* value	Gene locus	Gene name	Product
379	5	−71.7	3.3E−17	R2866_0222	*galE*	UDP-glucose 4-epimerase
260	6	−41.0	1.1E−09	R2866_1061	*rfaE*	Fused heptose 7-phosphate kinase/heptose 1-phosphate adenyltransferase
113	4	−26.4	2.2E−06	R2866_1286	*rfaD*	ADP-L-glycero-D-manno-heptose-6-epimerase
125	5	−23.6	1.0E−06	R2866_1295	*rfaF*	ADP-heptose-LOS heptosyltransferase II
293	13	−21.7	3.6E−14	R2866_0033	*lic2A*	Lipooligosaccharide biosynthesis protein Lic2A
359	37	−9.5	2.9E−08	R2866_1581	*galU*	Glucose-1-phosphate uridylyltransferase
168	21	−7.8	1.9E−04	R2866_0055	*waaQ*	ADP-heptose-LPS heptosyltransferase III
1,056	193	−5.5	5.1E−06	R2866_0326	*lgtC*	1,4-Alpha-galactosyltransferase (LgtC)

[a] Genes related to LOS biosynthesis with a >5-fold decrease in total pseudoreads for NHS plus neutrophils compared to pseudoreads for NHS, $P < 0.05$, were selected.

infections. Neutrophil recruitment has even been shown to be an advantage for NTHi survival by eliminating sensitive competitors like *Streptococcus pneumoniae* (9). In addition, NTHi actively induces NET formation *in vitro*, which may promote its survival (10, 11).

Bacteria need to be opsonized by antibodies and/or complement factors, such as C3b and C5b, to be efficiently phagocytosed by neutrophils (12). Unencapsulated bacteria, like NTHi, are particularly susceptible to complement-mediated killing and subsequent phagocytosis by neutrophils due to the lack of a protective surface structure. In response to this threat, NTHi has developed multiple strategies to evade the bactericidal activity of the human complement system. For instance, NTHi binds the complement control proteins C4b binding protein (C4BP) and factor H (13, 14). In addition, variation and modifications in its lipooligosaccharide (LOS) composition have been shown to prevent complement activation (15–18). The LOS structure of NTHi consists of three parts: (i) lipid A, (ii) an inner core comprised of a single 3-deoxy-D-manno-octulosonic acid (Kdo) linked to three conserved heptoses (Hep), and (iii) an outer core containing variable oligosaccharide extensions (19). Specific LOS structures have been shown to prevent binding of antibody and complement factor C4b, which increases their resistance to complement-mediated lysis (15, 16). In addition, NTHi prevents recognition by antibodies through incorporation of phosphorylcholine (PCho) and other "self-antigens" into the LOS structure (17, 18). However, mechanisms that prevent neutrophil-mediated killing are incompletely understood.

We used transposon sequencing (Tn-seq) technology to identify genes required for NTHi to survive in the presence of human neutrophils. We identified an important role for phase-variable incorporation of oligosaccharides into the LOS outer core, shielding an epitope that is recognized by naturally acquired IgM, thereby preventing complement activation, opsonization, and killing by human neutrophils.

RESULTS

Identification of genes important for survival in the presence of human neutrophils. To identify genes that were important for resistance to neutrophil-mediated killing, we used Tn-seq to compare survival of transposon mutants of the invasive clinical isolate R2866. Mutants were screened in the presence of 10% pooled normal human serum (NHS) containing opsonizing antibodies and active complement only as a control and NHS in the presence of freshly isolated human neutrophils from peripheral blood as a challenge condition (see Fig. S1A in the supplemental material). Viable counts of the mutant library exposed to NHS or NHS with neutrophils did not differ at the start of the experiment, after 2 h of incubation, or after the 3 h of growth in sBHI (brain heart infusion [BHI] broth [Becton, Dickinson] supplemented with 2% Fildes enrichment [Thermo Scientific] and 2 μg/ml β-NAD [Sigma-Aldrich]) needed to obtain sufficient DNA for genomic DNA isolation (see Fig. S1B).

Tn-seq analysis identified a total of 20,759 TA insertion sites with at least 10 reads for the control condition. These insertion sites included 17,507 insertions into 1,498 out of the 1,876 predicted genes in the R2866 genome. Among others, we found that transposon insertions in multiple genes known to be involved in lipooligosaccharide (LOS) biosynthesis conferred a significant decrease in survival in the presence of neutrophils (Table 1), with the largest effects seen for a strain carrying transposon mutations in the *galE* gene, encoding a UDP-galactose 4-epimerase needed for reversible conversion of UDP-glucose to UDP-galactose (20).

Neutrophil-mediated killing of R2866Δ*lic1/galE* is dependent on phagocytosis and serine-protease activity. To study the effects of the *galE* gene on resistance to neutrophil-mediated killing, we replaced the entire *galE* gene with a spectinomycin resistance cassette. In preliminary experiments, we observed increased neutrophil-mediated killing of the R2866Δ*galE* mutant (data not shown) but also selection for phase variants incorporating PCho into the LOS of the R2866Δ*galE* mutant, as determined by TEPC-15 binding assessed by flow cytometry (see Fig. S2A in the supplemental material). PCho is known to bind C-reactive protein and germline-encoded IgM (21, 22) and modulates resistance to complement through decreased binding of IgG (17). Therefore, we inactivated PCho incorporation by deletion of either *lic1A* or *lic1D* (further referred to as *lic1* mutants) to evaluate the effect of *galE* or other gene deletions on resistance to neutrophil-mediated killing in the absence of this structure. Growth of R2866Δ*lic1* and R2866Δ*lic1/galE* in sBHI were not significantly different (see Fig. S2B).

Neutrophil-mediated killing of R2866Δ*lic1/galE* in 5% NHS was dose dependent, with an NTHi/neutrophil ratio of 1:100, resulting in ~80% killing (Fig. 1A), which was used in all the following experiments. Neutrophil-mediated killing was completely dependent on the presence of active complement, since neutrophils

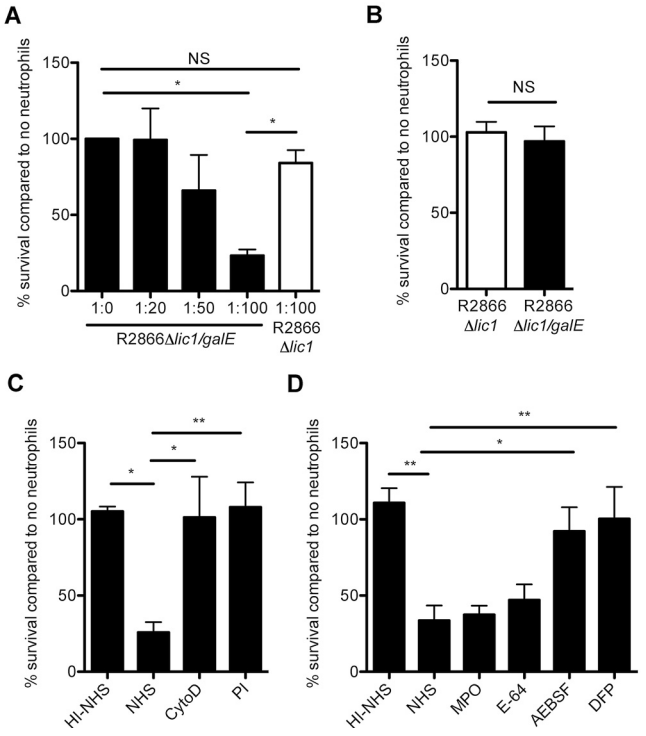

FIG 1 Neutrophil-mediated killing of R2866Δ*lic1/galE* is dependent on phagocytosis and serine protease activity. R2866Δ*lic1* and R2866Δ*lic1/galE* were incubated with 5% pooled normal human serum (NHS) (A) or 5% heat-inactivated (HI)-NHS (B) with or without neutrophils at different target-to-effector ratios, and survival was determined after incubation at 37°C for 30 min. (C and D) Neutrophils were preincubated with 20 μM cytochalasin D (CytoD) or 1× protease inhibitor (PI) cocktail (C) or with 1 mM MPO inhibitor, 10 μM E-64, 500 μM AEBSF, or 200 μM DFP (D) for 15 min at 37°C. R2866Δ*lic1/galE* was incubated with 5% NHS with or without neutrophils, and survival was determined after incubation at 37°C for 30 min. Statistical significance was determined using an unpaired Student *t* test (B) or one-way analysis of variance and the Tukey *post hoc* test (A and C to D). NS, not significant; *, $P < 0.05$; **, $P < 0.01$.

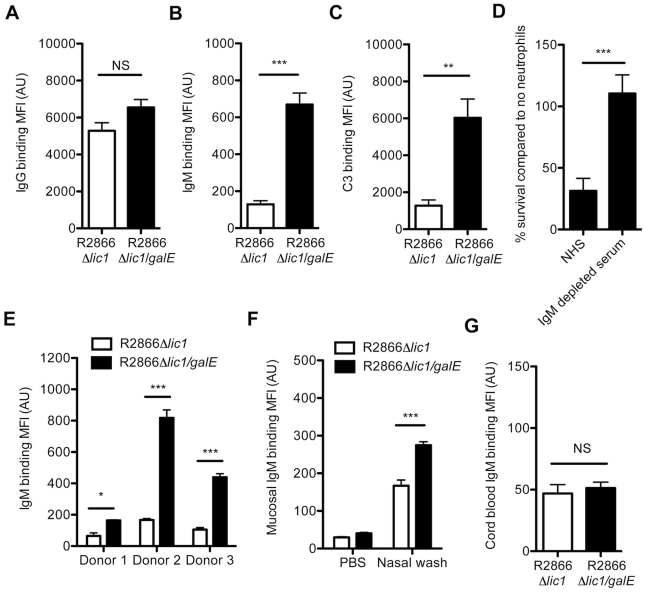

FIG 2 Increased IgM and C3 binding to the bacterial surfaces of R2866Δ*lic1/galE*. R2866Δ*lic1* and R2866Δ*lic1/galE* were incubated for 15 min with 5% NHS at 37°C, and binding of human IgG (A), IgM (B), or complement factor C3 (C) to the bacterial surface was determined by flow cytometry. (D) R2866Δ*lic1/galE* was incubated for 15 min with 5% NHS or 5% IgM-depleted serum at 37°C, and binding of human IgM to the bacterial surface was determined by flow cytometry. R2866Δ*lic1* and R2866Δ*lic1/galE* were incubated for 15 min with 5% NHS from individual donors (E), human nasal airway surface fluid (F), or 10% cord blood plasma at 37°C (G), and binding of IgM to the bacterial surface was determined by flow cytometry. Statistical significance was determined using an unpaired student *t* test. NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

with heat-inactivated NHS were not able to kill R2866Δ*lic1* or R2866Δ*lic1/galE* (Fig. 1B).

Next, we determined by which mechanisms R2866Δ*lic1/galE* was killed by human neutrophils. Cytochalasin D and protease inhibitor (PI) cocktail showed complete inhibition of neutrophil-mediated killing of R2866Δ*lic1/galE* (Fig. 1C). Of note, we were not able to determine the contribution of reactive oxygen species (ROS) in neutrophil-mediated killing, since diphenylene iodonium (DPI) affected survival of NTHi at concentrations needed to inhibit ROS production by neutrophils (data not shown). We used specific inhibitors to elucidate which type of protease was responsible for neutrophil-mediated killing of NTHi. Whereas myeloperoxidase (MPO) inhibitor and the cysteine protease inhibitor E-64 showed no effect on bactericidal activity of neutrophils, the serine protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and diisopropylfluorophosphate (DFP) showed complete inhibition of neutrophil-mediated killing of R2866Δ*lic1/galE* (Fig. 1D). Therefore, we conclude that neutrophil-mediated killing of R2866Δ*lic1/galE* is dependent on phagocytosis that involves complement-dependent uptake of the bacteria and activity of serine proteases.

The R2866Δ*lic1/galE* mutant shows increased IgM and C3 binding. Since neutrophil-mediated killing of the R2866Δ*lic1/galE* mutant was dependent on phagocytosis, we compared IgG, IgM, and C3 deposition on the bacterial surface of R2866Δ*lic1*and R2866Δ*lic1/galE* by flow cytometry. Whereas binding of IgG was not different (Fig. 2A), a marked increase in IgM binding (Fig. 2B) and C3 deposition (Fig. 2C) was found on the surface of the R2866Δ*lic1/galE* mutant compared to findings for the R2866Δ*lic1* control. Binding of IgM was needed for neutrophil-mediated killing of the R2866Δ*lic1/galE* mutant, since depletion of IgM (see Fig. 2C in the supplemental material) abrogated neutrophil-mediated killing completely (Fig. 2D). Significant increased IgM binding was observed not only with pooled NHS but also when donor specific NHS (Fig. 2E) or human nasal airway surface fluid (Fig. 2F) was used.

Serum IgM can be divided into germ line-encoded IgM and naturally acquired IgM synthesized after immune stimulation (21). IgM is not able to pass the placental barrier; therefore, IgM present in cord blood plasma is exclusively germ line encoded. No difference in IgM binding (Fig. 2G) or IgG binding (see Fig. S2D in the supplemental material) between R2866Δ*lic1* and R2866Δ*lic1/galE* was observed with cord blood plasma. Altogether, we conclude that IgM that recognizes an epitope(s) on the R2866Δ*lic1/galE* mutant was acquired from previous exposure and is generally present in human serum.

IgM binds to HepIII-β1,2-Glc in the R2866Δ*lic1/galE* LOS structure. Next, we set out to identify the specific LOS epitope(s)

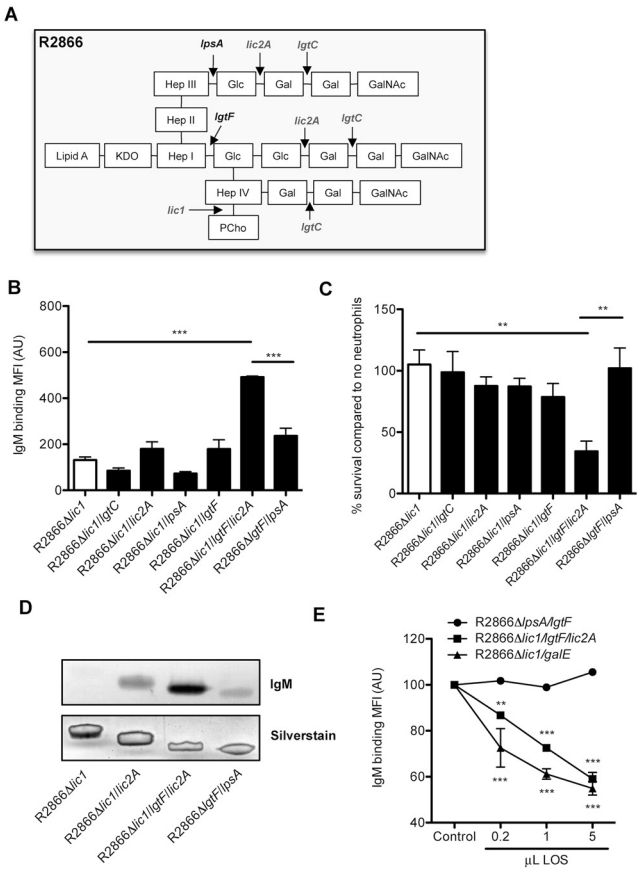

FIG 3 Binding of IgM to HepIII-β1,2-Glc increases neutrophil-mediated killing. (A) Predicted LOS structure of NTHi strain R2866 (23; Elke Schweda, personal communication) with phase-variable LOS synthesis genes in gray. (B) R2866 LOS mutants were incubated for 15 min with 5% NHS at 37°C, and binding of IgM to the bacterial surface was determined by flow cytometry. (C) R2866LOS mutants were incubated with 3% NHS with or without neutrophils, and survival was determined after incubation at 37°C for 30 min. (D) LOS was isolated from R2866 LOS mutants, separated by Tris Tricine SDS-PAGE, and visualized by silver staining, or IgM binding was detected by Western blot analysis. (E) R2866Δ*lic1/lgtF/lic2A* was incubated for 15 min with 5% NHS that was preincubated with LOS isolated from R2866Δ*lic1/lgtF/lic2A*, R2866Δ*lic1/galE*, or R2866Δ*lpsA/lgtF* for 15 min at 37°C, and binding of IgM to the bacterial surface was determined by flow cytometry. Statistical significance was determined with a one-way analysis of variance and the Tukey *post hoc* test (B and C) or two-way analysis of variance and the Bonferroni *post hoc* test (E). **, $P < 0.01$; ***, $P < 0.001$.

on the R2866Δ*lic1/galE* mutant that is recognized by IgM present in human serum. The predicted LOS structure of R2866 has oligosaccharide chains extending from both HepI and HepIII and the presence of a HepIV containing additional oligosaccharides (Fig. 3A) (23; Elke Schweda, personal communication).

Altered oligosaccharide chain lengths of HepI, HepIII, and HepIV for the R2866Δ*lic1/lgtC* and R2866Δ*lic1/lic2A* mutants or complete deletion of oligosaccharide chains on HepI (which includes HepIV) or HepIII for the R2866Δ*lic1/lgtF* and R2866Δ*lic1/lpsA* mutants, respectively, did not increase binding of IgM (Fig. 3B) or IgG (see Fig. S2E in the supplemental material) significantly, as determined by flow cytometry. However, deletion of the oligosaccharide chain of HepI in combination with truncation of oligosaccharide chain length on HepIII for the R2866Δ*lic1/lgtF/lic2A* mutant increased IgM binding significantly (Fig. 3B). The presence of glucose (Glc) attached to HepIII in a β1,2 linkage was needed for recognition by IgM, because the R2866Δ*lgtF/lpsA* mutant lacking any oligosaccharide chains on HepI and HepIII showed a significant decrease in IgM binding compared to the R2866Δ*lic1/lgtF/lic2A* mutant (Fig. 3B). In accordance with the findings for R2866Δ*lic1/galE*, an increase in IgM binding to the R2866Δ*lic1/lgtF/lic2A* mutant but not the other LOS mutants increased neutrophil-mediated killing significantly (Fig. 3C).

Increased binding of IgM to the bacterial surface of the R2866Δ*lic1/lgtF/lic2A* mutant was due to an altered LOS structure, because marked binding of IgM to the LOS of the R2866Δ*lic1/lgtFilic2A* mutant was detected, whereas binding of IgM was nearly undetectable for the R2866Δ*lic1* mutant and increased slightly for the R2866Δ*lic1/lic2A* mutant (Fig. 3D). From these data, we conclude that IgM binds an epitope that contains HepIII-β1,2-Glc on the R2866Δ*lic1/lgtF/lic2A* mutant.

To determine whether IgM recognized a similar epitope in the LOS of the R2866Δ*lic1/galE* and R2866Δ*lic1/lgtF/lic2A* mutants, we preincubated NHS with LOS from the R2866Δ*lic1/galE*, R2866Δ*lic1/lgtF/lic2A*, or R2866Δ*lpsA/lgtF* mutant as a control, to compete with IgM binding to the bacterial surface of the R2866Δ*lic1/lgtF/lic2A* mutant. Preincubation of NHS with LOS from the R2866Δ*lpsA/lgtF* mutant did not decrease IgM binding to the R2866Δ*lic1/lgtF/lic2A* mutant. However, addition of LOS from either R2866Δ*lic1/lgtF/lic2A* or R2866Δ*lic1/galE* decreased IgM binding to the R2866Δ*lic1/lgtF/lic2A* mutant in a dose-dependent manner (Fig. 3E), which confirmed that IgM from human serum bound a similar epitope on the LOS of the R2866Δ*lic1/galE* and R2866Δ*lic1/lgtF/lic2A* mutants. From these data, we conclude that human serum contains naturally acquired IgM recognizing an epitope that includes HepIII-β1,2-Glc and that an oligosaccharide chain extension on HepIII-β1,2-Glc, as well as on oligosaccharides on HepI and HepIV, block binding of IgM to the R2866Δ*lic1* control strain.

Phase-variable incorporation of galactose on HepIII-β1,2-Glc prevents neutrophil-mediated killing. The glucose linked to HepIII can be substituted with galactose (Gal) by the phase-variable gene *lic2A* (24, 25) (Fig. 4A). While we conducted experiments with strain Rd, we observed increased IgM binding (Fig. 4B) and neutrophil-mediated killing (Fig. 4C) for both RdΔ*lic1/lgtF* and RdΔ*lic1/lgtF/lic2A*, whereas binding of IgG was similar (see Fig. S2F in the supplemental material). The lack of difference for IgM binding and neutrophil-mediated killing between RdΔ*lic1/lgtF* and RdΔ*lic1/lgtF/lic2A* would suggest that *lic2A* was "phase off" in strain RdΔ*lic1/lgtF*, which we confirmed by sequencing (see Fig. S3). Accordingly, we observed that sequential exposure (3 rounds) of RdΔ*lic1/lgtF* to NHS and neutrophils selected for RdΔ*lic1/lgtF* with *lic2A* "phase on" (a shift from 23 to 22 tandem repeats of CAAT within the *lic2A* gene) (see Fig. S3). Phase variation of *lic2A* coincided with an increased LOS size (Fig. 4D), decreased IgM binding (Fig. 4E), and increased resistance to neutrophil-mediated killing (Fig. 4F), whereas no difference in IgG binding was observed (see Fig. S2G). We confirmed that *lic2A* phase-on selection was essential for decreased IgM binding and neutrophil-mediated killing because RdΔ*lic1/lgtF/lic2A* remained sensitive to neutrophil-mediated killing after serial treatment with NHS and neutrophils (3 rounds) (Fig. 4G). Therefore, these data show that phase-variable incorporation of

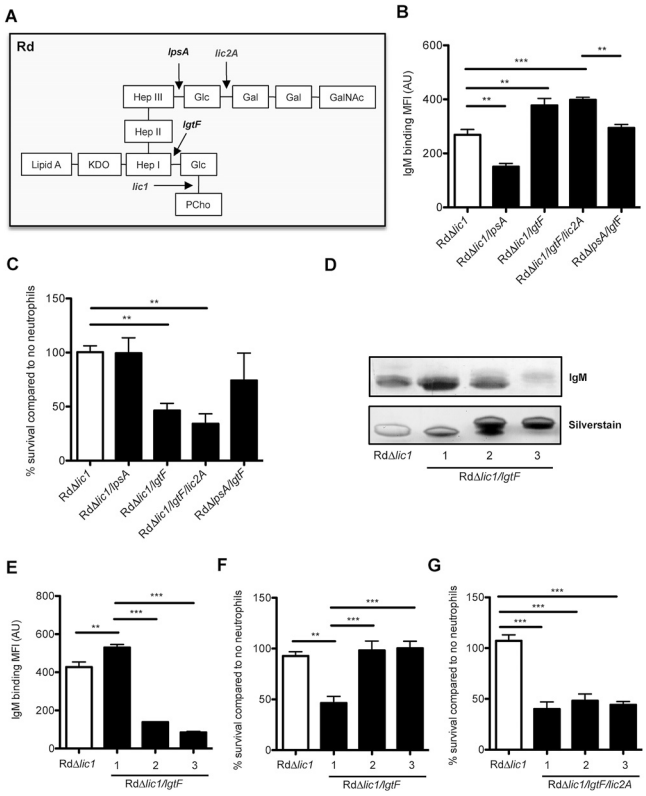

FIG 4 Phase-variable incorporation of galactose on HepIII-β1,2-Glc blocks binding of IgM and neutrophil-mediated killing. (A) Predicted LOS structure of Rd (25) with phase-variable LOS synthesis genes in gray. (B) Rd LOS mutants were incubated for 15 min with 5% NHS at 37°C, and binding of IgM to the bacterial surface was determined by flow cytometry. (C) RdLOS mutants were incubated with 1% NHS with or without neutrophils, and survival was determined after incubation at 37°C for 30 min. (D) LOS was isolated from Rd LOS mutants, separated by Tris Tricine SDS-PAGE, and visualized by silver staining, or IgM binding was detected by Western blot analysis. (E) RdΔ*lic1/lgtF* mutants exposed to 1% NHS with neutrophils for three rounds were incubated for 15 min with 5% NHS at 37°C, and binding of IgM to the bacterial surface was determined by flow cytometry. (F) RdΔ*lic1/lgtF* mutants exposed to 1% NHS with neutrophils were incubated with 1% NHS with or without neutrophils for three rounds, and survival was determined after incubation at 37°C for 30 min. (G) RdΔ*lic1/lgtF/lic2A* mutants exposed to 1% NHS with neutrophils were incubated with 1% NHS with or without neutrophils for three rounds, and survival was determined after incubation at 37°C for 30 min. Statistical significance was determined with a one-way analysis of variance and the Tukey *post hoc* test. **, $P < 0.01$; ***, $P < 0.001$.

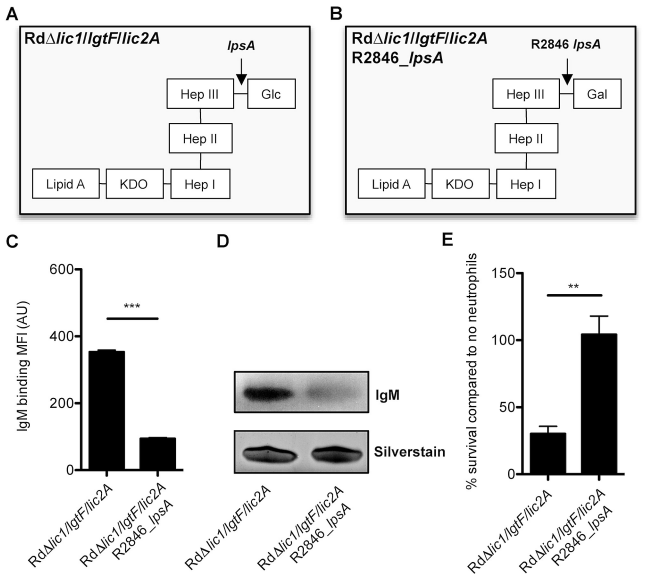

FIG 5 Binding of IgM is specific for an epitope that includes HepIII-β1,2-Glc, but not HepIII-β1,2-Gal. (A) Predicted LOS structures of RdΔ*lic1/lgtF/lic2A*and (B) RdΔ*lic1/lgtF/lic2A* with *lpsA* gene from R2846. (C) RdΔ*lic1/lgtF/lic2A* and RdΔ*lic1/lgtF/lic2A* R2846_*lpsA* mutants were incubated for 15 min with 5% NHS at 37°C and binding of IgM to the bacterial surface was determined by flow cytometry. (D) LOS was isolated from RdΔ*lic1/lgtF/lic2A* and RdΔ*lic1/lgtF/lic2A* R2846_*lpsA* mutants, separated by Tris-Tricine SDS-PAGE, and visualized by silver staining or IgM binding was detected by Western blotting. (H) RdΔ*lic1/lgtF/lic2A* and RdΔ*lic1/lgtF/lic2A* R2846_*lpsA* mutants were incubated with 1% NHS with or without neutrophils, and survival was determined after incubation at 37°C for 30 min. Statistical significance was determined using an unpaired Student *t* test. **, $P < 0.01$; ***, $P < 0.001$.

galactose onto HepIII-β1,2-Glc by Lic2A prevents binding of IgM and increases resistance to neutrophil-mediated killing.

Binding of IgM to HepIII-β1,2-Glc but not HepIII-β1,2-Gal contributes to neutrophil-mediated killing of NTHi. Incorporation of glucose onto HepIII is dependent on the *lpsA* gene. Besides incorporation of glucose onto HepIII, LpsA can also incorporate galactose, which is strain specific and determined by a single amino acid difference at position 151 (26) (see Fig. S4A in the supplemental material). In order to determine the specificity for IgM binding for either a glucose or galactose attached to HepIII, we constructed an RdΔ*lic1/lgtF/lic2A* strain that has a HepIII-β1,2-Glc epitope (25) (Fig. 5A) and an RdΔ*lic1/lgtF/lic2A* strain with the *lpsA* gene from strain R2846 that has a HepIII-β1,2-Gal epitope (Fig. 5B), and *lpsA* gene replacement was confirmed by sequencing (see Fig. S4B). A change from glucose to galactose attached to HepIII decreased IgM binding (Fig. 5C) but not IgG binding (see Fig. S2H), as measured by flow cytometry and Western blot analysis (Fig. 5D). More importantly, neutrophil-mediated killing of the RdΔ*lic1/lgtF/lic2A* mutant was significantly decreased with incorporation of galactose on HepIII for the RdΔ*lic1/lgtF/lic2A* R2846_*lpsA* mutant (Fig. 5E).

Invasive NTHi isolates incorporate galactose onto HepIII more frequently. The presence of Thr at position 151 in the LpsA protein results in specificity for galactose, whereas the presence of Cys, Ala, or Met results in the incorporation of glucose. From 82 recently published NTHi genomes (27), 13 publically available NTHi genomes, and 10 additional clinical NTHi strains, 103 (98%) contained an *lpsA* gene, indicating that ~2% of NTHi strains do not incorporate any oligosaccharides on HepIII. ClustalW2 alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/) of the LpsA proteins showed the presence of Thr in 26/105 (25%), Cys in 61/105 (58%), Met in 15/105 (14%), or Ala in 1/105 (1%) (see Fig. S4A in the supplemental material) on position 151, predicting incorporation of glucose or galactose in 73% or 25% of the strains, respectively.

Of the NTHi isolates, 22 were carriage strains, 41 were obtained from patients with otitis media (OM), 25 were from patients with COPD, and 17 were invasive isolates. The percentage of strains predicted to incorporated galactose or no saccharide (no *lpsA* gene) onto HepIII was 14% for carriage strains, whereas this was 29% and 24% for strains isolated from patients with OM or COPD, respectively (Table 2). Incorporation of galactose or no saccharide onto HepIII was predicted for 41% of the strains col-

TABLE 2 Predicted distribution of glucose or galactose (Gal) incorporation with HepIII by LpsA in 105 clinical NTHi isolates

Incorporation	No. (%) of isolates				
	Carriage	Otitis media	COPD	Invasive	Total
Glc	19 (86.4)	29 (70.7)	19 (76.0)	10 (58.8)	77
Gal or no saccharide	3 (13.6)	12 (29.3)	6 (24.0)	7 (41.2)	28
Total	22	41	25	17	105

lected from patients with invasive disease (Table 2), which was ~3-fold more frequent than for carriage strains, indicating a relationship between incorporation of galactose or no saccharide onto HepIII and the ability to cause invasive disease (Pearson chi-square, 3.815; $P = 0.05$). Collectively, these data support a role for incorporation of galactose or no saccharide onto HepIII, thereby preventing binding of IgM, which increases resistance to neutrophil-mediated killing, although the presence and activity of other glycosyltransferases such as *lic2A*, as shown in Fig. 4, also contribute to the overall resistance phenotype.

DISCUSSION

In this study, we used Tn-seq to identify genes important for survival in the presence of pooled NHS with or without freshly isolated human neutrophils. Multiple genes involved in LOS biosynthesis were identified (Table 1), including the *rfaD*, *rfaE*, *rfaF*, and *waaQ* genes, important for synthesis of the LOS inner core (28, 29). The *rfaD* gene mutant showed decreased virulence in a chinchilla model for OM (30) and increased killing by neutrophil extracellular traps (NETs) (11), emphasizing a critical role for the LOS inner core in virulence of NTHi; however, the mechanisms remained unexplored.

Multiple genes involved in synthesis of the variable LOS outer core, including *galE*, *galU*, *lic2A*, and *lgtC*, were shown in our work to be important in resisting killing by human neutrophils. Many of these genes have previously been shown to contribute to resistance to complement-mediated killing in the absence of neutrophils. Erwin et al., for example, described an important role for *lgtC* in resistance to complement-mediated killing of strain R2866 (31), and each of these genes has been identified in recently published transposon mutagenesis screens for genes involved in resistance to the complement-dependent bactericidal effect of serum (15, 32). The activation of complement on the bacterial surface has both direct antimicrobial activity (formation of the membrane attack complex) and indirect effects (opsonization). Throughout our study, we have compared NHS containing active complement with and without neutrophils, controlling for the effect of complement alone and allowing us to focus on the additional contribution of neutrophils to NTHi killing.

Truncations of the R2866 LOS outer core resulted in increased binding of IgM from pooled NHS. Even though detectable levels of IgG binding were observed for NTHi with or without an intact LOS outer core, increased binding of IgM was required to initiate sufficient complement activation to promote neutrophil-mediated uptake and killing. In contrast, the amount of complement deposition resulting from IgG binding did not appear to be as effective at opsonizing the bacteria.

The contribution of IgM binding to the bacterial surface of NTHi to efficient complement-mediated killing has been demonstrated in various studies. We have previously shown that the level of IgM binding correlated with complement-mediated killing for NTHi strains collected from children with OM (15) and complement-resistant NTHi isolates from patients with COPD showed decreased IgM binding (32). Decreased binding of IgM to complement-resistant isolates is likely to be multifactorial, because changes in the LOS, as well as exclusion of phospholipids from the outer leaflet of the bacterial membrane, decreased IgM binding and increased resistance to complement-mediated killing (15, 32). Therefore, it appears that IgM recognition of NTHi plays an important role in controlling infections with NTHi. This is corroborated by clinical data from patients with hyper-IgM syndrome, who have a significantly lower risk of NTHi carriage than children with panhypogammaglobulinemia, suggesting that IgM antibodies, even in the absence of IgG or IgA, are protective and contribute to the prevention of respiratory tract colonization and infection (33).

Most vaccine studies for NTHi are focused on the use of immunogenic membrane-exposed proteins (34). However, antigenic variation and the absence of certain proteins in selected NTHi isolates make it difficult to formulate a broadly protective protein-based vaccine. Therefore, LOS might be an interesting alternative, and this concept is supported by a vaccination study with protein-conjugated LOS (35). Although the LOS of NTHi is heterogeneous as well, immunization against specific LOS epitopes masking immune-dominant structures might be an interesting alternative, and several studies demonstrate the potential of opsonizing LOS antibodies in opsonophagocytosis and protection in mouse models. Mouse monoclonal antibodies recognizing NTHi LOS after intraperitoneal immunization were shown to be mainly IgM, and selected clones conferred bactericidal activity and increased phagocytosis by human neutrophils (36). A mouse immunization study with *M. catarrhalis* was shown to induce cross-reactive IgM recognizing *H. influenzae* LOS, which increased bactericidal activity in the presence of complement, demonstrating that antibodies to single LOS epitopes could be protective (37).

We identified an important role for binding of IgM to LOS in neutrophil-mediated killing. In order to determine the exact epitope recognized by IgM, we generated a series of mutant strains that either had truncated oligosaccharide extensions or a complete absence of oligosaccharides on HepI or HepIII. Although we have not confirmed the LOS structures by mass spectrometry, altered size as confirmed by Tris-Tricine gel electrophoresis and consistent results for strains Rd, R2846 (data not shown), and R2866 present strong evidence that IgM from human serum recognizes an epitope that contains HepIII-β1,2-Glc but not HepIII-β1,2-Gal. Both galactose and glucose can be attached to HepIII in a β1,2 or β1,3 linkage (26), but we have not addressed whether the linkage has effect on binding of IgM.

Although NTHi can incorporate a galactose on HepIII, thereby increasing its resistance to neutrophil-mediated killing, in 73% of NTHi strains incorporation of a glucose at this site is predicted (Table 2). Therefore, it is tempting to speculate that the presence of glucose on the HepIII might have advantages for NTHi under specific conditions, like colonization of the upper respiratory tract. Such apparent discrepancy is also found for incorporation of PCho into the LOS structure. Although PCho is recognized by C-reactive protein (CRP) and germ line-encoded IgM (21, 22), thereby activating the classical complement pathway, the presence of PCho contributes in colonization in animal models (17) and in

a human colonization model (38). Similar factors might be selecting for incorporation of glucose onto HepIII, but to our knowledge this not been investigated.

Whereas incorporation of a galactose on HepIII is considered to be a terminal moiety, glucose on HepIII can be substituted with galactose depending on *lic2A* gene activity (19), which is controlled by phase variation, a mechanism that switches translation of genes on or off (39). Phase-variable on-off translation of genes is a stochastic process dependent on slipped-strand mispairing of tandem repeats present in the gene, which occurs with a high frequency (10^{-2} to 10^{-3} times per generation) and leads to switching the coding region in or out of frame. We showed that selection for *lic2A* phase-on variants incorporating oligosaccharides onto HepIII-β1,2-Glc blocked binding of IgM, which prevented neutrophil-mediated killing. These experiments are in accordance with experiments performed by Clark et al., where serial exposure to NHS selected for *lic2A* phase-on variants and increased complement resistance through decreased binding of IgG (40). Since this is a phase-variable process, it raises the question of the advantage for NTHi in switching between these two LOS types. As for PCho, oligosaccharide extensions on HepIII-β1,2-Glc might be an advantage or disadvantage for NTHi depending on the location where it resides in the human host. Shielding of conserved immune-dominant epitopes with more-variable structures has been observed not only for NTHi but also for other pathogens, such as *S. pneumoniae*. For instance, pneumococcal surface protein A (PspA) binds to PCho and thereby blocks binding of CRP to the bacterial surface, preventing C3 deposition (41).

Because IgM recognizing an epitope containing HepIII-β1,2-Glc was not present in cord blood plasma, we concluded that it is not germ line encoded but is acquired in human serum from previous exposure. In contrast, we were unable to identify significant levels of IgG recognizing this epitope. There is evidence that a large proportion of memory B cells do not class switch but give rise to a population called IgM^+ $CD27^+$ memory B cells producing antigen-specific IgM. This population of memory B cells accumulates somatic mutations, although to a lower extent than IgG-producing B cells, but does not class switch (42, 43), and we propose that IgM recognizing an epitope that includes HepIII-β1,2-Glc is produced by this type of memory B cell. Although the origin and function of IgM^+ $CD27^+$ memory B cells are under debate (44), they appear to be an important part of the adaptive immune response to various pathogens. The percentages of IgM^+ $CD27^+$ memory B cells in the peripheral blood of children are low, increase to almost 20% in adults, and decline in the elderly (45), which corresponds with susceptibility to bacterial infections in children and the elderly. An important protective role for IgM^+ $CD27^+$ memory B cells was shown in patients with common variable immunodeficiency (CVID) or splenectomized or asplenic patients, where the presence of anticapsular IgM^+ $CD27^+$ memory B cells protected against bacterial pneumonia (46, 47). In addition, transient predisposition to infections caused by *S. pneumoniae* in very young children (<2 years) was associated with the lack of IgM^+ $CD27^+$ memory B cells and antipneumococcal capsular IgM (47). Altogether, IgM appears to play an important role in the adaptive immune response to pathogens, and exposing the immune system through vaccination might increase the ability of neutrophils to clear NTHi infections.

In conclusion, we have shown that IgM binding to the NTHi LOS facilitates efficient neutrophil-mediated killing of NTHi, but this pathogen has developed at least three mechanisms that prevent binding of IgM to HepIII-β1,2-Glc: (i) oligosaccharide extensions of HepI and possibly other heptose residues, (ii) phase-variable incorporation of galactose onto HepIII-β1,2-Glc, and (iii) incorporation of galactose onto HepIII. Our findings suggest that strategies to increase binding of IgM might enhance opsonization and phagocytic activity of human neutrophils and thereby limit infections by NTHi.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table S1 in the supplemental material (53–58). Bacteria were grown with shaking at 37°C in brain heart infusion (BHI) broth (Becton, Dickinson) supplemented with 2% Fildes enrichment (Thermo Scientific) and 2 μg/ml β-NAD (Sigma-Aldrich) (sBHI). Viable bacterial counts were determined by plating serial dilutions in phosphate-buffered saline (PBS) on sBHI agar plates grown at 37°C with 5% CO_2. Optical density was measured at 620 nm. For mutant library and gene mutants, 100 μg/ml spectinomycin (Calbiochem), 20 μg/ml kanamycin (Fisher Bioreagents), or 12.5 μg/ml tetracycline (Sigma-Aldrich) was used.

Human neutrophils, normal human serum, and human nasal airway surface fluid. Blood was drawn from healthy adult donors in sodium-heparin anticoagulation tubes (BD Vacutainer) and isolated using Ficoll-Paque (GE Healthcare) as described previously (48). Before use, neutrophils were taken up in Hanks' buffered salt solution (HBSS) without phenol red containing Ca^{2+} and Mg^{2+} plus 0.1% gelatin to the desired concentration. All experiments were performed with the same batch of pooled normal human serum (NHS) containing opsonizing antibodies and active complement obtained from GTI Diagnostics (catalogue no. PHS-N100). For IgM depletion, 2 ml of 20% NHS in PBS was incubated with 200 μl of PBS-washed Sepharose beads coupled to anti-human IgM antibody (Sigma). After 2 h of incubation while shaking at 4°C, Sepharose beads were removed by centrifugation, and sera were diluted to 10% with PBS and immediately stored at −80°C. Donor-specific NHS was obtained from healthy adult volunteers, using serum tubes (BD Vacutainer), centrifuged, and stored immediately at −80°C. Human nasal airway surface fluid was obtained from a healthy adult volunteer as described previously (49).

Identifying NTHi genes essential for survival with NHS and human neutrophils. Genomic DNA from NTHi strain R2866 was isolated from mid-log-phase cultures using the Qiagen Genomic-tip 20/G tips (Qiagen), following the manufacturer's protocol. The NTHi R2866 *marinerT7*-MmeI transposon mutant library was generated as described previously for NTHi 86-028NP (50).

The NTHI R2866 *marinerT7*-MmeI transposon mutant library was diluted to 2×10^6 CFU/ml in HEPES-buffered RPMI containing 1 μg/ml hemin and 2 μg/ml β-NAD and incubated for 2 h in the presence of 10% normal human serum (control) or 10% normal human serum with 2×10^7 neutrophils/ml (multiplicity of infection [MOI], 0.1) in quadruplicate at 37°C (end volume, 1 ml). After challenge, neutrophils were lysed by adding 100 μl 10% saponin in PBS and incubated for 10 min at room temperature. Subsequently, 4 ml sBHI was added, and the mutant library was grown to an optical density at 620 nm (OD_{620}) of ~0.2 (~4 h), and chromosomal DNA was isolated for Tn-seq analysis. Generation times were confirmed by viable bacterial counts (see Fig. S1B in the supplemental material). Genomic DNA was isolated using a DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's protocol. A readout of the mutant library and data analysis were performed as described previously (50). Analysis data can be found at http://bamics2.cmbi.ru.nl/websoftware/essentials/essentials_run.php?session=essentials%neutrophil.

Generation of NTHi-directed gene replacement mutants. Bacterial genomic DNA was isolated using a DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's protocol. Directed NTHi gene replacement mutants were generated by allelic exchange of donor genomic DNA or by allelic exchange of the target gene with an antibiotic resistance marker, as

described previously (50). Mutants were selected by plating on sBHI plates containing 100 μg/ml spectinomycin, 20 μg/ml kanamycin, or 12.5 μg/ml tetracycline and validated by PCR. All primers used in this study are listed in Table S1 in the supplemental material.

***lpsA* gene replacement in Rd*Δlic1/lgtF/lic2A*.** The R2846 *lpsA* gene was PCR amplified using the primers NTHi_lpsA_F_uptake and NTHi_lpsA_R_uptake (see Table S1 in the supplemental material), containing uptake sequences for increased transformation efficiency. The PCR product was purified using the QIAquick PCR purification kit (Qiagen), following the manufacturer's protocol, and 5 μg PCR product or water as a control was incubated 1 h with 500 μl M-IV competent (51) Rd*Δlic1/lgtF/lic2A* bacteria. Five hundred microliters sBHI was added to enable growth for 2 h at 37°C with shaking at 225 rpm. Cultures were washed with HBSS without phenol red containing Ca^{2+} and Mg^{2+} plus 0.1% gelatin and diluted to ~1×10^7 bacteria/ml. One hundred microliters culture (~1×10^6 bacteria) was incubated with 5% NHS for 30 min, plated on sBHI, and grown overnight at 37°C plus 5% CO_2 to select for complement-resistant transformants. Complement resistance assay was repeated with the surviving bacteria to enrich the selection. Single colonies were picked for growth and DNA isolation, and the *lpsA* gene was PCR amplified with the primers NTHi_lpsA_F2 and NTHi_lpsA_R1 (see Table S1). Replacement of the Rd *lpsA* gene (cysteine [C] at amino acid position 151) with the R2846 *lpsA* gene (threonine [T] at amino acid position 151) was confirmed by sequencing (see Fig. S4B).

Flow cytometric analysis. Bacteria were grown to an OD_{620} of ~0.6 in sBHI medium, washed with HBSS without phenol red containing Ca^{2+} and Mg^{2+}, and diluted to an OD_{620} of 0.2. Surface opsonization with serum was performed by incubating 50 μl bacteria with 50 μl 10% pooled NHS, 10% cord blood plasma, or 1:250-diluted TEPC-15 in HBSS without phenol red containing Ca^{2+} and Mg^{2+} or human nasal airway surface fluid for 15 min at 37°C with 5% CO_2. Opsonized bacteria were fixed for 20 min with 2% paraformaldehyde, washed, and incubated with 1:500-diluted fluorescein isothiocyanate (FITC)-labeled polyclonal goat anti-human C3 (MP Biomedicals), 1:100-diluted FITC-labeled Fc-specific goat anti-human IgG, 1:50-diluted FITC-labeled μ-chain-specific goat anti-human IgM (Sigma-Aldrich), or 1:100-diluted FITC-labeled α-chain-specific goat anti-mouse IgA for TEPC-15 in HBSS without phenol red containing Ca^{2+} and Mg^{2+} plus 5% FCS for 30 min at 4°C. Bacteria were washed and resuspended in PBS for flow cytometry. Binding was detected by flow cytometry using a FACS LSR II instrument (BD Biosciences). Data were analyzed using the software program FlowJo version 7.6.3. Measurement of geometric mean fluorescence intensity (MFI) was depicted in arbitrary units (AU).

Neutrophil killing assay. Bacteria were grown to an OD_{620} of ~0.6 in sBHI, washed with HBSS without phenol red containing Ca^{2+} and Mg^{2+}, and diluted to 2×10^5 CFU/ml in HBSS without phenol red containing Ca^{2+} and Mg^{2+} plus 0.1% gelatin. Neutrophils were resuspended in HBSS without phenol red containing Ca^{2+} and Mg^{2+} plus 0.1% gelatin to 4×10^6/ml (ratio, 1:20), 1×10^7/ml (ratio, 1:50), or 2×10^7/ml (ratio, 1:100). For inhibition experiments, neutrophils (2×10^7/ml) were preincubated with 20 μM cytochalasin D (Sigma), 1× protease inhibitor cocktail (Calbiochem), 1 mM myeloperoxidase (MPO) inhibitor, 10 μM E-64 (Sigma), 500 μM AEBSF (Calbiochem), or 200 μM DFP (Sigma) for 15 min at 37°C with 5% CO_2. Next, 10 μl heat-inactivated NHS (HI-NHS) or NHS of the desired concentration, 45 μl bacteria, and 45 μl neutrophils were mixed and incubated for 30 min at 37°C with 5% CO_2. CFU counts were determined by plating 10-fold dilutions on sBHI plates and incubated overnight at 37°C with 5% CO_2. For the Rd*Δlic1/lgtF* and Rd*Δlic1/lgtF/lic2A* mutants, surviving colonies from round 1 were subsequently incubated with 1% NHS with or without neutrophils, and survival was determined after a 30-min incubation (round 2), which was repeated for another round (round 3).

LOS analysis by Tris-Tricine SDS-PAGE. LOS was isolated as described previously (52). LOS samples were separated on a Tris-Tricine SDS-PAGE gel with a Protean II xi cell electrophoresis system (Bio-Rad) and visualized by silver staining or transferred to a polyvinylidene difluoride (PVDF) membrane for Western blotting. Membranes were blocked with 5% bovine serum albumin (BSA) in PBS for 1 h, incubated for 2 h with 5% NHS in PBS, washed 5 times for 5 min (each) with Tris-buffered saline plus 0.05% Tween 20, and incubated with 1:3,000-diluted alkaline phosphatase (AP)-labeled goat-anti-human IgM μ-chain (Sigma-Aldrich). Binding was detected with NBT (4-nitroblue tetrazolium chloride)-BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Roche), following the manufacturer's instructions.

Sequence analysis of *lic2A* phase-variable region. Genomic DNA was isolated from 1 ml culture (original and post-exposure to NHS and neutrophils) for sequence analysis. Primers lic2F and lic2R (see Table S1 in the supplemental material) were used to amplify the *lic2A* gene, which included the tetranucleotide 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) (see Fig. S3).

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software), where a P value of <0.05 was considered significant. The specific statistical tests that were used for the various experiments are specified in the figure legends. The program SPSS version 22 (IBM) was used to analyze the predicted galactose or glucose incorporation with HepIII with a chi-square test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01478-14/-/DCSupplemental.

Figure S1, PDF file, 0.2 MB.
Figure S2, PDF file, 0.3 MB.
Figure S3, PDF file, 0.2 MB.
Figure S4, PDF file, 1.1 MB.
Table S1, PDF file, 0.1 MB.

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