Modified Lipooligosaccharide Structure Protects Nontypeable *Haemophilus influenzae* from IgM-Mediated Complement Killing in Experimental Otitis Media

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ABSTRACT **Nontypeable *Haemophilus influenzae* (NTHi) is a Gram-negative, human-restricted pathogen. Although this bacterium typically colonizes the nasopharynx in the absence of clinical symptoms, it is also one of the major pathogens causing otitis media (OM) in children. Complement represents an important aspect of the host defense against NTHi. In general, NTHi is efficiently killed by complement-mediated killing; however, various resistance mechanisms have also evolved. We measured the complement resistance of NTHi isolates isolated from the nasopharynx and the middle ear fluids of OM patients. Furthermore, we determined the molecular mechanism of NTHi complement resistance. Complement resistance was strongly increased in isolates from the middle ear, which correlated with decreased binding of IgM. We identified a crucial role for the R2866_0112 gene in complement resistance. Deletion of this gene altered the lipooligosaccharide (LOS) composition of the bacterium, which increased IgM binding and complement-mediated lysis. In a novel mouse model of coinfection with influenza virus, we demonstrate decreased virulence for the R2866_0112 deletion mutant. These findings identify a mechanism by which NTHi modifies its LOS structure to prevent recognition by IgM and activation of complement. Importantly, this mechanism plays a crucial role in the ability of NTHi to cause OM.**

IMPORTANCE **Nontypeable *Haemophilus influenzae* (NTHi) colonizes the nasopharynx of especially young children without any obvious symptoms. However, NTHi is also a major pathogen in otitis media (OM), one of the most common childhood infections. Although this pathogen is often associated with OM, the mechanism by which this bacterium is able to cause OM is largely unknown. Our study addresses a key biological question that is highly relevant for child health: what is the molecular mechanism that enables NTHi to cause OM? We show that isolates collected from the middle ear fluid exhibit increased complement resistance and that the lipooligosaccharide (LOS) structure determines IgM binding and complement activation. Modification of the LOS structure decreased NTHi virulence in a novel NTHi-influenza A virus coinfection OM mouse model. Our findings may also have important implications for other Gram-negative pathogens harboring LOS, such as *Neisseria meningitidis*, *Moraxella catarrhalis*, and *Bordetella pertussis*.**

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Nontypeable *Haemophilus influenzae* (NTHi) is a Gram-negative bacterial pathogen that colonizes the upper respiratory tract of humans, generally in the absence of clinical symptoms. However, NTHi is also able to ascend the Eustachian tube to the middle ear and cause inflammation, resulting in otitis media (OM) (1). As such, NTHi accounts for almost 50% of all bacterial OM infections (2). Although acute OM is typically self-limiting, it can also lead to important sequelae such as meningitis and permanent hearing loss (3). Despite the fact that OM is one of the most common childhood diseases, the molecular processes underlying the migration of NTHi from the nasopharynx to the middle ear are poorly understood.

An important part of the innate immune system intended to clear pathogenic bacteria is the complement system. Activation of complement leads to a cascade of protein activation and deposition of complement factor C3b on the surface of bacteria, including NTHi (4), which may result in direct killing through the formation of the membrane-attack complex. NTHi strains are generally considered to be sensitive to complement-mediated lysis; however, studies have also shown that NTHi possesses complement resistance mechanisms, including variation and modifications in its lipooligosaccharide (LOS) composition (4). The LOS structure of NTHi consists of three parts: lipid A, an inner core comprised of a single 3-deoxy-d-manno-octulosonic acid (Kdo) linked to three heptoses, and an outer core containing a heteropolymer of mainly glucose and galactose moieties. Additional modifications have also been reported, including sialic acid, *N*-acetylgalactosamine, and phosphorylcholine (5, 6). Variations

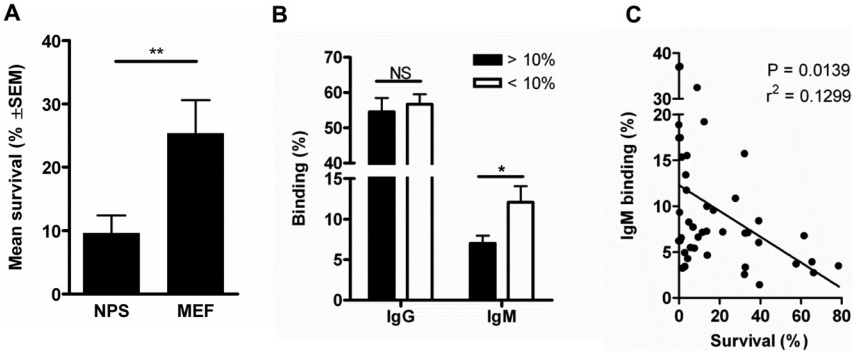


FIG 1 Determination of the complement resistance and IgM binding of clinical NTHi isolates. (A) Survival of MEF ($n = 22$) and NPS ($n = 24$) isolates was determined in 5% normal human serum and expressed as percent survival compared to that in 5% heat-inactivated human serum for 60 min. (B) Serum IgG and IgM binding on 25 serum-sensitive and 21 complement-resistant NTHi isolates was determined by flow cytometry. Statistical significance was determined with an unpaired *t* test with Welch's correction. *, $P < 0.05$; **, $P < 0.01$. (C) Correlation between complement resistance and IgM binding.

in the composition of the LOS structure have previously been associated with decreased binding of antibodies and reduced complement deposition and have been suggested to contribute to the development of disease (7-11). Recently an important link between the presence of NTHi-specific IgM antibodies and colonization of the host was demonstrated (12). Although IgM binding and complement resistance were shown to play an important role in the lower respiratory tract during exacerbation of chronic obstructive pulmonary disease (COPD) by NTHi (10), it remains unclear whether similar immune evasion mechanisms are important during OM.

In this study, we investigated the contributions of IgM binding and complement-mediated killing of NTHi during OM. We show that NTHi strains isolated from the middle ear of children with OM are more complement resistant than are strains isolated from the nasopharynx. This decreased susceptibility correlated with decreased binding of IgM to the bacterium. Expression of the R2866_0112 gene is essential for modifying the LOS structure, which prevents binding of IgM and confers bacterial resistance to complement-mediated killing, similar to our observation in clinical isolates. Finally, using a novel NTHi OM mouse model, we show that the R2866_0112 gene plays a crucial role in virulence.

RESULTS

NTHi isolates from MEF display increased complement resistance. NTHi isolates were obtained from middle ear fluid (MEF) and nasopharyngeal swab (NPS) samples collected from children with OM. Isolates collected from MEF ($n = 22$) were significantly more resistant to complement-mediated killing than were isolates collected from the nasopharynx ($n = 24$) (Fig. 1A). To rule out potential attenuation by *in vitro* culture, we passaged complement-resistant isolates for 5 generations in the absence of serum. Subsequent analysis of complement resistance showed no significant changes, suggesting that the phenotype of these minimally passaged strains reflects their *in vivo* phenotype (data not shown).

To investigate the mechanism by which NTHi was killed by serum, we measured surface binding of IgM and IgG. We found that serum-sensitive isolates (<10% survival) showed increased IgM binding compared to complement-resistant isolates (>10% survival) (Fig. 1B). This difference was not observed for the binding of IgG (Fig. 1B). The amount of IgM binding correlated with the ability of serum to kill the NTHi isolates (Fig. 1C), implying an important role for IgM in activating the classical complement pathway.

TABLE 1 Top list of genes identified in the serum resistance GAF screen at 30 min

Fold difference	Bayes, *P*	R2866 gene name	R2866 locus tag	R2866 annotation
−194.5	<1.00E−16		R2866_0112	Conserved hypothetical protein
−83.2	<1.00E−16	*lpsA2*	R2866_1629	Lipooligosaccharide glucosyltransferase LpsA
−16.0	<1.00E−16	*lic2A*	R2866_0033	Lipooligosaccharide biosynthesis protein Lic2A
−12.3	<1.00E−16	*galE*	R2866_0222	UDP-glucose 4-epimerase
–10.6	1.11E−16		R2866_0369	Conserved hypothetical protein
−9.6	3.35E−11	*bolA*	R2866_0424	Morphology-related protein BolA
			R2866_0425	Lipoprotein, putative
−9.4	<1.00E−16	*lgtF*	R2866_1822	UDP-glucose-lipooligosaccharide beta 1-4 glucosyltransferase
−9.2	<1.00E−16	*lgtc*	R2866_0326	1,4-Alpha-galactosyltransferase (LgtC)
−8.5	2.83E−06		R2866_1530	Hypothetical protein
−6.2	7.52E−07	*rfbB*	R2866_1509	dTDP-glucose 4,6-dehydratase
−4.9	<1.00E−16	*galU*	R2866_1581	Glucose-1-phosphate uridylyltransferase
−4.3	3.14E−09	*rfaD*	R2866_1286	ADP-L-glycero-D-mannoheptose-6-epimerase
−4.0	3.04E−13	*tex*	R2866_0016	Probable transcription accessory protein Tex
−3.9	2.44E−15	*lpt6*	R2866_0303	PE-tn-6–lipooligosaccharide phosphorylethanolamine transferase
−3.9	5.78E−07	*waaQ*	R2866_0055	ADP-heptose–lipooligosaccharide heptosyltransferase III
−3.9	9.28E−09	*hgpB*	R2866_1813	Hemoglobin and hemoglobin-haptoglobin binding protein B
−3.9	1.60E−07	*licA*	R2866_1070	Phosphorylcholine kinase LicA
−3.8	<1.00E−16	*ICE_orf31*	R2866_0596	Conserved hypothetical protein p31
−3.6	1.78E−15		R2866_1296	Conserved hypothetical protein
−3.6	1.11E−16	*accA*	R2866_0167	Acetyl coenzyme A carboxylase, subunit alpha

TABLE 2 Database for annotation, visualization, and integrated discovery (DAVID) analysis

Functional annotation	*P* value	Fold enrichment
Lipopolysaccharide biosynthesis	2.40E−05	27.4
Cell outer membrane	7.09E−04	11.9
Glycosyltransferase	1.00E−05	10.1
Signal	9.74E−05	5.9
Cell membrane	1.96E−04	2.8
Membrane	0.001	2.5
Transferase	0.007	2.2

The R2866_0112 gene affects NTHi complement resistance. In order to identify genes affecting IgM binding and complement resistance, the negative genome-wide screen genomic array footprinting (GAF) was performed (13, 14). For this screen, the sequenced complement-resistant strain R2866 was used (15). In total, 57 transposon mutants showed an attenuated phenotype (see Table S2 in the supplemental material), of which the top 20 most attenuated mutants are listed in Table 1. Functional class enrichment analysis showed that the majority of the identified genes were involved in LOS biosynthesis (Table 2). The R2866_0112 gene, coding for a conserved hypothetical protein, showed the most prominent phenotype, and transcriptional analysis indicated that expression of this gene was increased in complement-resistant isolates (Fig. 2A).

To validate a role for R2866_0112 gene expression in complement resistance, we determined survival of the R2866 wild type, the R2866Δ0112 mutant, and the R2866Δ*lgtC* strain as a control because the *lgtC* gene has previously been shown to confer complement resistance (8). Furthermore, we included the R2866Δ*licA* mutant because it showed a minor phenotype in the GAF screen (see Table S2 in the supplemental material). The Δ*0112* mutant was extremely sensitive to complement-mediated killing compared to the wild-type, Δ*licA* mutant, or Δ*lgtC* mutant strain (Fig. 2B). Heat inactivation of serum abrogated killing of the Δ*0112* mutant, which showed that the bactericidal activity was dependent on the action of the complement pathway (Fig. 2B). A polar effect of the R2866_0112 gene deletion was excluded by microarray data analysis of the R2866 wild type and the Δ*0112* mutant (Table 3). Besides the expression of R2866_0112 (251-fold decrease), a minor decrease in expression was observed for the hemoglobin and hemoglobin-haptoglobin binding protein B gene (*hgpB*), as was an increase in expression of gene locus R2866_1095—R2866_1101. However, the altered expression of *hgpB* and gene locus R2866_1095—R2866_1101 was not involved in complement resistance because deletion did not alter the complement-sensitive phenotype of the Δ*0112* mutant (Fig. 2C). To confirm the importance of this gene for complement resistance in other NTHi strains, we also deleted the R2866_0112-homologous gene from NTHi Rd, 3655, 86-028NP, and 1521062. All mutant strains showed strongly reduced complement resistance compared to their respective parental strain, albeit complement resistance levels varied extensively (Fig. 2D to G).

R2866_0112 gene expression alters the LOS structure. As our results (Table 2), as well as previous reports, suggested an important role for LOS in complement resistance, we determined whether deletion of the R2866_0112 gene affected the LOS composition. As a control, the Δ*lgtC* mutant was included because previous reports showed an altered LOS structure for this mutant in the R2866 strain (8, 16). The Δ*0112* mutant showed a different LOS migration pattern than did the R2866 wild type and Δ*lgtC* mutant (Fig. 3A), suggesting that the R2866_0112 gene affects LOS biosynthesis. Deletion of the R2866_0112-homologous gene from NTHi Rd, 3655, 86-028NP, and 1521062 also altered the LOS migration pattern (see Fig. S1 in the supplemental material), which confirms a conserved function for this gene.

Although changes in LOS structure may directly affect complement resistance by changing membrane stability, this was not the case for the Δ*0112* mutant because the mutant and wild-type strains showed similar sensitivities to EDTA (Fig. 3B), which interrupts intermolecular associations between LOS phosphate groups (10). Also, sensitivity to polymyxin B, which increases membrane permeability, was equal for the R2866 wild-type and Δ*0112* mutant strains (Fig. 3C). Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis of the LOS showed that both the R2866 wild-type and Δ*0112* mutant strains contained a conserved inner-core triheptosyl moiety and belong to a group of NTHi strains expressing a heptose (HepIV) in the outer core (5). The R2866 wild-type LOS showed major ions at *m/z* 2124 and 1876, whereas the spectrum of the Δ*0112* mutant showed smaller major ions at *m/z* 1521 and 1265 (Table 4). In conclusion, the LOS structure of the Δ*0112* mutant strain showed various truncations, a finding which corresponds to the altered migration pattern (Fig. 3A).

Complement activation is dependent on direct binding of IgM to NTHi LOS. Because our primary observation in clinical isolates showed a correlation between IgM binding and complement resistance, we also determined IgG and IgM binding to the

TABLE 3 R2866 and R2866Δ*0112* mutant gene expression array results[a]

R2866 locus tag	R2866 annotation	R2866 wild-type array signal	R2866Δ*0112* array signal	Bayes, *P*	Fold difference
R2866_0112	Conserved hypothetical protein	5,345	21	<1.00E−16	−251.3
R2866_1813	Hemoglobin and hemoglobin-haptoglobin binding protein B	5,312	1,249	<1.00E−16	−4.3
R2866_1095	Putative TPR[b] protein	302	4,685	<1.00E−16	15.3
R2866_1096	Hypothetical protein	302	6,706	<1.00E−16	21.9
R2866_1097	Putative TPR protein	604	10,290	<1.00E−16	16.7
R2866_1098	Hypothetical protein	308	6,610	<1.00E−16	21.3
R2866_1099	Putative TPR protein	200	9,008	<1.00E−16	44.4
R2866_1100	Hypothetical protein	309	6,792	<1.00E−16	21.7
R2866_1101	Putative TPR protein	297	9,918	<1.00E−16	32.7

[a] Genes regulated 2.5-fold with *P* values of <0.001 are included in the table.
[b] TPR, tetratricopeptide repeat.

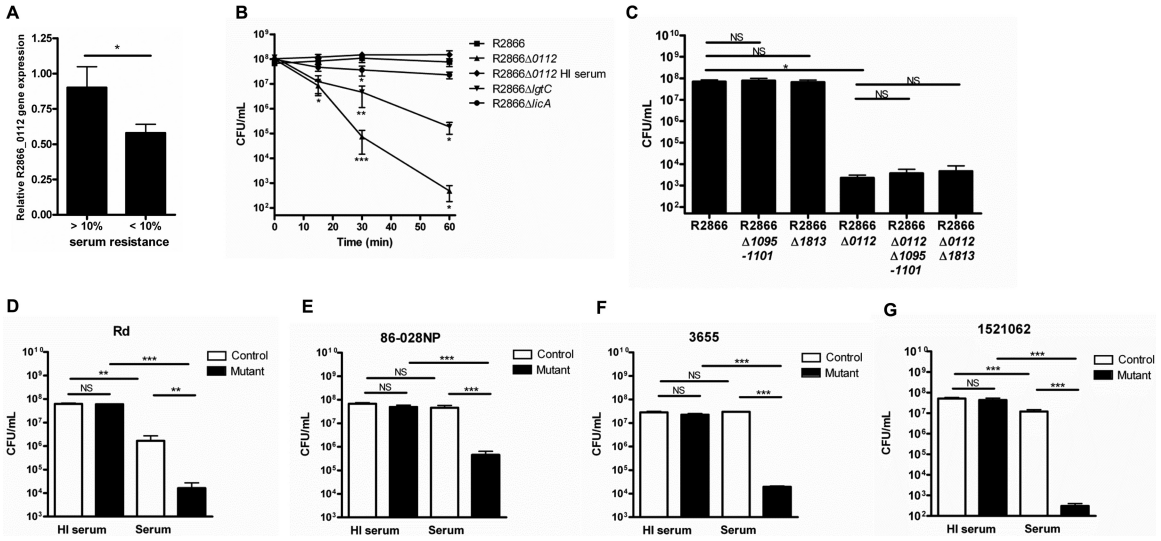


FIG 2 The R2866_0112 gene deletion mutant exhibits decreased complement resistance. (A) Relative expression of R2866_112 mRNA was analyzed by qRT-PCR in 25 serum-sensitive and 21 complement-resistant clinical isolates Statistical significance was determined with an unpaired *t* test with Welch's correction. (B) Complement resistance of R2866, Δ*0112* mutant, and Δ*lgtC* mutant ($n = 4$) was determined with 40% NHS or 40% heat-inactivated NHS. Statistical significance was determined with a two-way analysis of variance and the Bonferroni *post hoc* test. (C) Complement resistance of R2866, Δ*0112* mutant, Δ*1813* mutant, Δ*1095-1101* mutant, Δ*0112*/Δ*1813* mutant, and Δ*0112*/Δ*1095-1101* mutant was determined in 40% NHS ($n = 4$). Statistical significance was determined with a one-way analysis of variance and the Tukey *post hoc* test. (D to G) Complement resistance of Rd (HI0461) (D), 86-028NP (NTHI0592) (E), 3655 (CGSHi3655_02894) (F), and 1521062 (G) was determined with 10% (Rd), 20% (3655), or 40% (86-028NP and 1521062) serum, respectively ($n = 3$). Statistical significance was determined on $\log_{10}$-transformed data with a one-way analysis of variance and the Tukey *post hoc* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

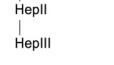
R2866 wild type and the Δ*0112* mutant. No significant change in IgG binding was observed. However, the Δ*0112* mutant showed a ~2-fold increase in IgM binding (Fig. 4A). Binding of IgM to the bacterial surface was essential for killing the Δ*0112* mutant, as depletion of IgM from serum completely prevented killing (Fig. 4B). The reduced killing of the Δ*0112* mutant with IgM-depleted serum was not due to decreased bactericidal activity, because an NTHi strain dependent on C-reactive protein (CRP)-mediated complement activation was killed equally as well with normal serum as with IgM-depleted serum (data not shown). Sera with different levels of IgM showed a strong correlation between IgM binding and killing of the bacteria, confirming the essentiality of IgM in complement-mediated killing of the Δ*0112* mutant (Fig. 4C).

To determine whether IgM directly recognizes the LOS of NTHi, Western blot experiments were performed. While IgM predominately binds to the lower band of the wild-type LOS, IgM also binds strongly to the truncated LOS band of the Δ*0112* mutant (Fig. 4D). The Δ*0112* mutant LOS bound ~2-fold-more IgM than did the R2866 wild-type LOS (Fig. 4E), which corresponds to the flow cytometry results (Fig. 4A). Also, the Δ*lgtC* mutant showed an increase in IgM binding, although not significant (Fig. 4E).

TABLE 4 Positive-ion ESI-MS data and proposed compositions for glycoforms in dephosphorylated and permethylated oligosaccharide derived from NTHi strains R2866 and R2866Δ*0112*[a]

	Relative abundance (%)		
$[M + Na]^+$	R2866	R2866Δ*0112*	Proposed composition
2,736	Tr[b]		Hex7 · Hep4 · AnKdo-ol
2,369	9		HexNAc · Hex4 · Hep4 · AnKdo-ol
2,166		8	HexNAc · Hex3 · Hep4 · AnKdo-ol
2,124	**19**		Hex4 · Hep4 · AnKdo-ol
2,080	10	9	Hex5 · Hep3 · AnKdo-ol
1,920	13	5	Hex3 · Hep4 · AnKdo-ol
1,876	**21**	7	Hex4 · Hep3 · AnKdo-ol
1,716	9		Hex2 · Hep4 · AnKdo-ol
1,672	6	6	Hex3 · Hep3 · AnKdo-ol
1,521	8	**27**	Hex1 · Hep4 · AnKdo-ol
1,468		5	Hex2 · Hep3 · AnKdo-ol
1,265	5	**35**	Hex1 · Hep3 · AnKdo-ol

[a] The major ions are depicted in bold. All glycoforms contain Hep3 · AnKdo-ol. Points of elongation appear from HexI and/or HepIII in the following structure:



[b] Tr, traces.

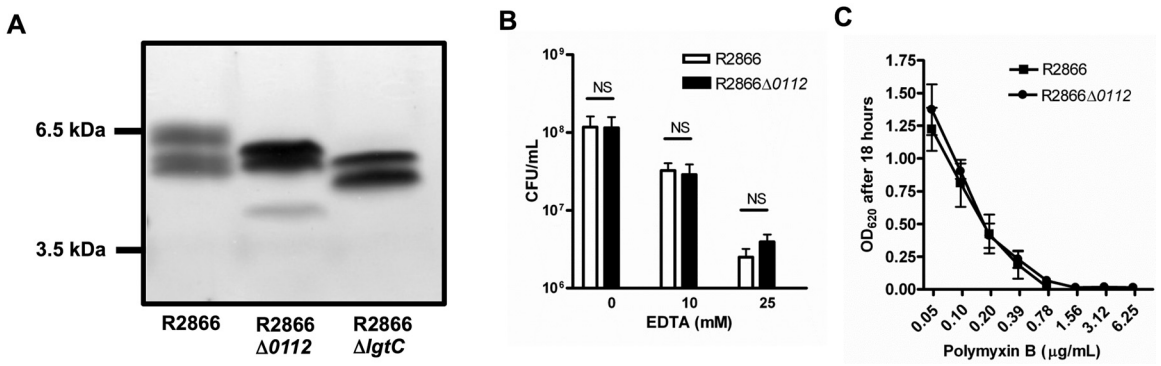


FIG 3 The R2866_0112 gene deletion mutant expresses an altered LOS structure. (A) LOS analysis of R2866, Δ*0112* mutant, and Δ*lgtC* mutant strains by Tris-Tricine SDS-PAGE and silver staining. (B and C) Outer membrane stability of R2866 wild type and mutant as determined by sensitivity to EDTA ($n = 5$) (B) or polymyxin B ($n = 8$) (C). Statistical significance was determined with a one-way analysis of variance and the Tukey *post hoc* test or a with a two-way analysis of variance and the Bonferroni *post hoc* test, respectively. OD_{620}, optical density at 620 nm; NS, not significant.

Furthermore, we determined IgM binding to 4 complement-resistant and 4 complement-sensitive isolates. In accordance with the data for the complement-sensitive Δ*0112* mutant, LOS from complement-sensitive isolates bound significantly more IgM than did LOS from complement-resistant isolates (Fig. 4F and G).

Deletion of the R2866_0112 gene attenuates colonization and otitis media. Although the Δ*0112* mutant was significantly less resistant to complement-mediated lysis, the consequences with regard to host colonization and/or OM remain uncertain. Because NTHi by itself does not infect mice efficiently, we adapted our previously described murine model of influenzae A virus (IAV)-*Streptococcus pneumoniae* coinfection (17). In these experiments, mice are primed first with influenza virus or mock treated and subsequently challenged with NTHi. For these experiments, we used NTHi strain 1521062, which we had previously used in animal experiments. Infection of mice with IAV significantly increased the number of NTHi bacteria in the nose at both 48 h and 96 h postchallenge (Fig. 5A). Importantly, IAV infection also facilitated replication of NTHi in the middle ears (Fig. 5B).

Similar to R2866, deletion of the R2866_0112 gene from the NTHi 1521062 strain (designated the 1521062 mutant) attenuated complement resistance (see Fig. S2 in the supplemental material). IAV-NTHi coinfection experiments with a 1:1 mixture of the wild type and the mutant strain showed that the mutant was strongly outcompeted by the wild type. The mutant strain was attenuated in the nasopharynx as well as the middle ears, both at 48 h and at 96 h (Fig. 5C to H), suggesting that clearance of the mutant strain already occurs early during infection. Interestingly, although the CFU counts of the wild type in the nose decreased slightly from 48 h to 96 h, the CFU counts in the middle ears increased, suggesting that there is local bacterial replication in the middle ear cavity. In summary, these data demonstrate an important *in vivo* role for the R2866_0112 gene in complement resistance and bacterial pathogenesis.

DISCUSSION

Although NTHi is generally sensitive to complement-mediated killing, some strains have developed immune evasion mechanisms that aid in colonization and disease. Previous data from animal models suggest that the complement system comprises an important aspect of the host defense against NTHi. For instance, depletion of complement by cobra venom resulted in the development of otitis media in chinchillas by "avirulent" NTHi strains (18). Recently, it was shown that NTHi isolates from the lower respiratory tract exhibit increased complement resistance compared to colonizing strains (10). Here, we provide strong evidence that NTHi strains isolated from the middle ears of children suffering from otitis media are more resistant to complement-mediated killing than are nasopharyngeal isolates.

The observation that bacterial isolates from the middle ear cavity show increased complement resistance may be explained by at least two mechanisms. One explanation is that the local inflammatory environment determines the bacterial phenotype of OM strains. Evidence to support this mechanism is the observation that serial passage of a serum-sensitive NTHi strain in the presence of active complement increased its complement resistance (10). This is particularly relevant in the context of OM, since large quantities of complement factors are present in the middle ears during inflammation of the middle ear (19, 20). An alternative hypothesis is that only complement-resistant NTHi isolates are able to cause OM, which implies the existence of "colonizing" and "otitis media" genotypes. Despite a very high level of genetic heterogeneity among NTHi strains, especially for LOS synthesis-related genes (5), clinical isolates causing inflammatory diseases, including OM, display a distinct genetic profile that confers increased complement resistance (21). Future experiments that investigate whether these OM isolates are equally efficient at colonizing the mucosal surfaces of the nasopharynx, and whether this changes in the presence of inflammation (e.g., due to a viral infection), may shed more light into when and how NTHi requires complement resistance. Various modifications in the NTHi LOS structure have previously been described to contribute to complement resistance. Modification of LOS by the phase-variable LOS synthesis gene *lgtC* has been shown to delay C4b deposition on the bacterium, resulting in increased complement resistance (8). Another strategy used by NTHi is the incorporation of sialic acid in LOS, which also confers protection against complement attack (9) and leads to prolonged survival in the middle ear cavity in a chinchilla model (22). In this study, we identified a complement evasion mechanism, which is dependent on the R2866_0112 gene. This gene was also identified in a complement resistance screen by Nakamura et al. (10), and transposon mutants of Rd showed decreased growth or survival in the mouse lungs (23). The

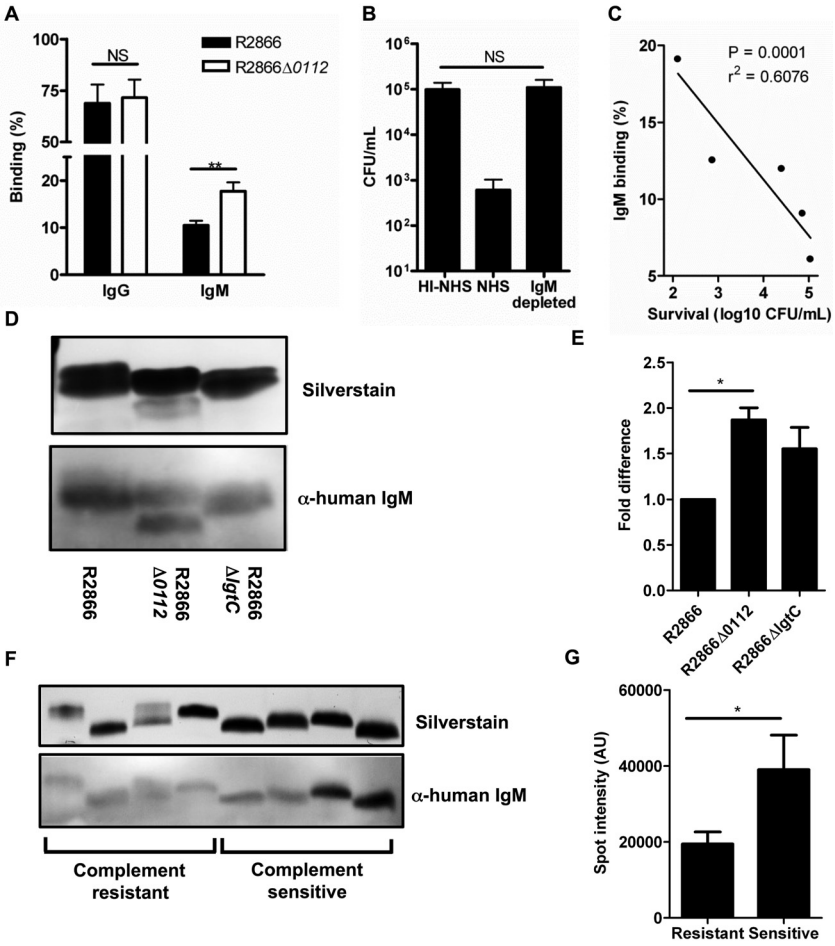


FIG 4 IgM binds R2866Δ*0112* mutant LOS directly. (A) Serum IgG and IgM binding on R2866 and Δ*0112* mutant as determined by flow cytometry ($n = 9$). Statistical significance was determined with an unpaired *t* test with Welch's correction. (B) Complement resistance of R2866 and Δ*0112* mutant strains was determined in heat-inactivated NHS (HI-NHS), NHS, or IgM-depleted serum ($n = 4$). Statistical significance was determined with a one-way analysis of variance and the Tukey *post hoc* test. (C) Correlation between Δ*0112* mutant serum survival and IgM binding. (D and F) Direct binding of IgM to LOS was analyzed by silver staining (loading control) and Western blotting. (E) Relative IgM binding to LOS of R2866, Δ*0112*, and Δ*lgtC* mutant was calculated ($n = 3$). (G) Signal intensities in arbitrary units (AU) of IgM binding to LOS of clinical isolates were calculated ($n = 4$). Statistical significance was determined with a one-way analysis of variance and the Tukey *post hoc* test or with an unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$; NS, not significant.

R2866_0112 gene is highly conserved in all NTHi strains sequenced to date, and deletion of the homologous genes from all NTHi strains that we tested resulted in a dramatic decrease in complement resistance. Here, we demonstrate a role for the R2866_0112 gene in LOS structure synthesis. The high number of truncated glycoforms in LOS of mutant strains lacking this gene, together with the absence of one dominant LOS glycoform, suggests that the R2866_0112 gene is not a transferase involved in LOS synthesis directly. Identifying the specific function of this gene remains the subject of ongoing investigation.

Interestingly, the R2866_0112 gene mutant showed an increase in IgM binding, which was essential for complement-mediated killing. This was also observed for the clinical isolates, in which IgM binding correlated with complement resistance. Another study focusing on COPD also reported decreased binding of IgM to complement-resistant NTHi isolates from the lower respiratory tract (10), and recently, Micol et al. showed that patients with hyper-IgM syndrome were protected from NTHi colonization but not from other respiratory pathogens (12). Our results and these studies both point to an essential function for IgM in the recognition of NTHi. Consequently, increasing the level of bactericidal IgM antibodies, either by therapeutic administration or by vaccination, may effectively reduce NTHi colonization as well as disease. Such a strategy may be highly effective, as an initial study using a detoxified NTHi LOS protein conjugate vaccine already showed protection in a chinchilla and mouse model of OM (24, 25).

To assess the importance of the R2866_0112 gene for *in vivo* virulence, we made use of a novel murine IAV-NTHi coinfection model. Here, we show that coinfection of mice with IAV and NTHi results in enhanced bacterial colonization and progression to OM, similar to *S. pneumoniae* (17, 26, 27). A competition experiment between a wild-type strain and a mutant strain lacking the R2866_0112 gene resulted in a strong attenuation of the mutant in both colonization and survival in the middle ears at 48 h and 96 h. The exact mechanism by which mice clear NTHi is currently unclear. Because we used naive mice, polyspecific natural IgM antibodies may play an important role in this bactericidal

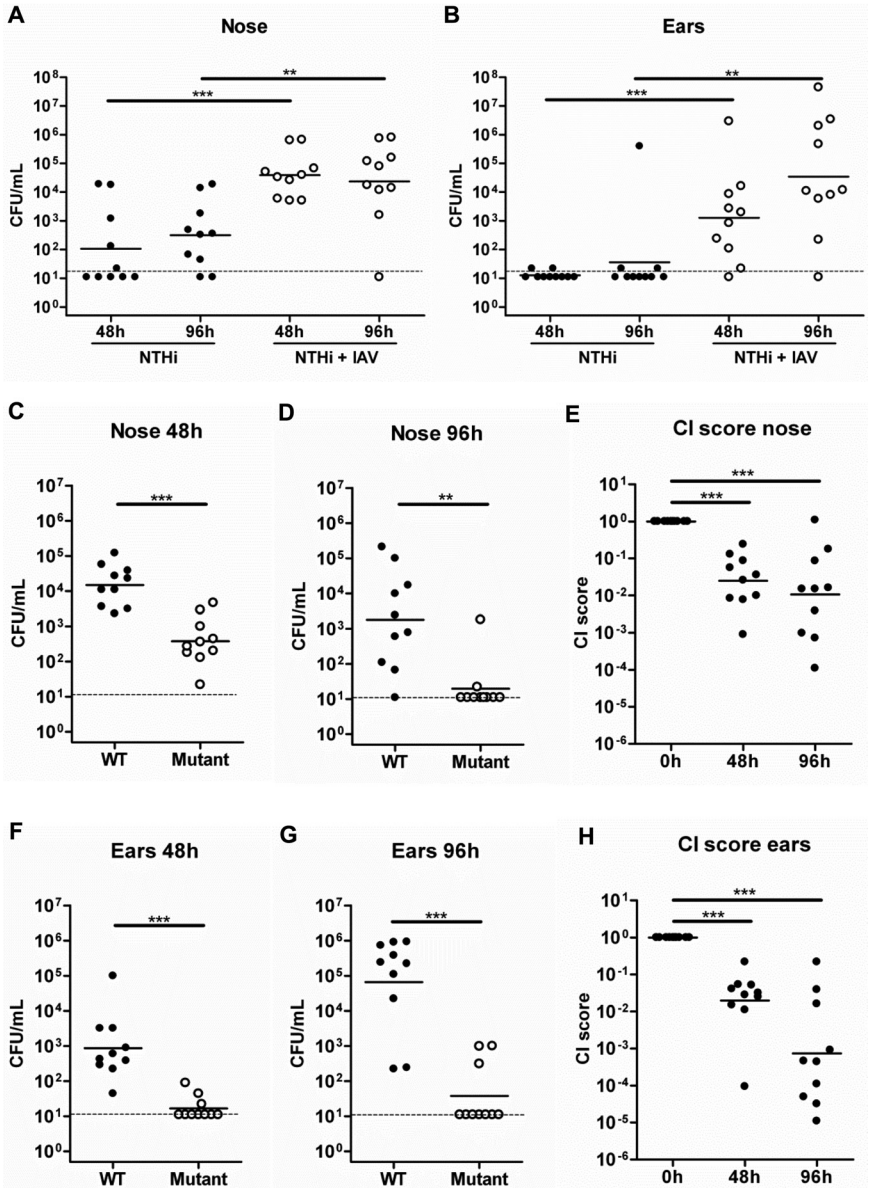


FIG 5 The R2866_0112 gene mutant shows decreased virulence in a murine coinfection otitis media model. Mice were inoculated with $10^{4.5}$ PFU of influenza A virus (IAV) or mock treated 3 days before intranasal infection with 5×10^7 CFU of NTHi. (A and B) CFU counts in the nose (A) or the middle ears (B) were determined 48 and 96 h postinfection ($n = 10$). (C, D, F, and G) Mice were infected with $10^{4.5}$ PFU of IAV 3 days before intranasal infection with a 1:1 ratio of NTHi 1521062 wild type (WT) and the R2866_0112 mutant (5×10^7 CFU total). CFU counts in the nose (C and D) or the middle ears (F and G) were determined 48 and 96 h postinfection ($n = 10$). Statistical significance was determined with a Mann-Whitney test. (E and H) CI scores were calculated. Statistical significance was determined with a one-way analysis of variance and the Tukey *post hoc* test. **, $P < 0.01$; ***, $P < 0.001$.

effect. A similar effect of natural IgM was observed by Zola et al. (28), who found a role for these antibodies in limiting NTHi colonization in mice. Interestingly, in our study, the mutant was attenuated not only in the middle ears but also in the nasopharynx, implying similar clearance mechanisms in the middle ears and the nasopharynx. One possibility is that the primary infection with IAV allows for abundant complement components to be present at the mucosal surface of the nasopharynx, thereby providing selective pressure. Although the exact mechanism by which IAV allows NTHi to replicate in either the nasopharynx or the middle ear cavity remains currently unclear, these data point to an important *in vivo* role for the R2866_0112 gene in complement resistance and the development of OM.

MATERIALS AND METHODS

Clinical isolates. Children up to 5 years of age who suffered from recurrent acute OM (rAOM) or chronic OM with effusion (COME) were enrolled in a retrospective clinical cohort study, which was approved by the Committee on Research Involving Human Subjects of the Radboud University Nijmegen Medical Centre (CMO 2007/239, international trial registration number NCT00847756). Legal guardians provided written informed consent. Middle ear fluid was collected during surgery using a middle ear fluid aspiration system (Kuijpers Instruments, Groesbeek,

Netherlands), and nasopharyngeal samples were obtained using a cotton wool swab (Copan, Brescia, Italy). Middle ear fluid was mixed with 2 ml saline prior to bacterial culture and stored at −80°C. Isolates were serotyped using slide agglutination (BD Biosciences). All clinical isolates were minimally passaged *in vitro* (<4 passages).

Bacterial strains. Strains used in this study are listed in Table S1 in the supplemental material. Strains were grown with shaking at 225 rpm in brain heart infusion (BHI; BD Biosciences) supplemented with 10 μg/ml hemin (Sigma) and 2 μg/ml β-NAD (Merck), at 37°C and 5% CO_2. Live bacterial counts were determined by plating serial dilutions in phosphate-buffered saline (PBS) on BHI plates. For mutant libraries and gene deletion mutants, 150 μg/ml spectinomycin (Calbiochem) was added.

Generation of *H. influenzae* R2866 transposon mutant library. Genomic DNA was isolated with Genomic-tip 20/G (Qiagen) as described previously (29). The *H. influenzae marinerT7* transposon mutant library was generated as described previously for *S. pneumoniae* (13) with plasmid pGSF8 as a donor of the *marinerT7* transposon conferring spectinomycin resistance. Mutagenized genomic DNA was introduced into the bacterium with the M-IV transformation method (30).

Identifying genes involved in complement resistance. Genes involved in resistance to complement-mediated killing were identified by GAF (13, 14). A volume of 0.1 ml (1×10^8 CFU/ml), containing approximately 30,000 unique mutants of the R2866 strain, was added to 0.4 ml of 50% normal human serum (NHS; GTI Diagnostics) or heat-inactivated (20 min, 56°C) NHS in phosphate-buffered saline (PBS) containing 0.1% gelatin (PBSG). Volumes of 100 μl were taken at 0, 30, and 60 min of incubation at 37°C; diluted directly in 5 ml of supplemented BHI (sBHI); and cultured for 4.5 h. The GAF experiment was performed on two independent days in duplicate.

GAF readout was performed essentially as described previously, with some minor modifications (13). Chromosomal DNA was digested with HpyCH4V (New England Biolabs). Two micrograms of Cy3-labeled cDNA was hybridized to custom-made *H. influenzae* R2866 GAF Nimblegen microarrays. Probe signals were normalized using Analysis of NimbleGen Arrays Interface Suite (ANAIS) (31). Probes with >2.0-fold probe signal differences and a Bayesian *P* value of <0.001 (http://cybert.microarray.ics.uci.edu) were set as underrepresented following the challenge.

Generation of NTHi directed gene mutants. Targeted gene deletion mutants of NTHi were generated by allelic exchange of the target gene with an antibiotic resistance marker, as described previously for *S. pneumoniae* (14). DNA was introduced into the bacterium with the M-IV transformation method (30). All primers (Biolegio, Nijmegen, Netherlands) used in this study are listed in Table S1 in the supplemental material.

qRT-PCR. RNA was extracted from mid-log-phase-grown NTHi clinical isolates by using the RNeasy minikit (Qiagen) and was DNase treated (Ambion). One microgram of cDNA was synthesized using the SuperScript III reverse transcriptase kit (Invitrogen). Quantitative reverse transcription-PCR (qRT-PCR) was performed in a 20-μl reaction mixture with SYBR green PCR Master Mix (Applied Biosystems) on a 7500 Fast Real-Time PCR system (Applied Biosystems). The *gyrA*, *rpoA*, and *frdB* genes were used as the internal standard genes for GeNorm normalization (32).

R2866 expression microarray analysis. RNA was extracted from mid-log-phase-grown NTHi R2866 wild-type or R2866Δ0112 mutant cells by using the RNeasy minikit (Qiagen) and was DNase treated (Ambion). Cy3-labeled cDNA was obtained according to the Nimblegen array user's guide (http://www.nimblegen.com/products/lit/NG_Expression_Guide_v5p1.pdf). Two micrograms of Cy3-labeled cDNA was hybridized to custom-made *H. influenzae* R2866 expression Nimblegen microarrays. Probe signals were normalized using Analysis of NimbleGen Arrays Interface Suite (ANAIS) (31). Genes with a >2.5-fold signal difference and a Bayesian *P* value of <0.001 (http://cybert.microarray.ics.uci.edu) were selected to be significantly regulated.

Flow cytometric analysis. Hanks' buffered salt solution (HBSS) without Ca^{2+}/Mg^{2+}, containing 5% (vol/vol) heat-inactivated fetal calf serum, was used for all dilutions and washes. Surface opsonization with serum was performed by incubating bacteria in 5% heat-inactivated pooled human serum for 1 h at 37°C with 5% CO_2. Bacteria were fixed in 2% paraformaldehyde, and surface-bound IgM or IgG was detected using anti-human IgG- or IgM-fluorescein isothiocyanate (FITC)-conjugated antibodies (Sigma) by flow cytometry using a FACSCalibur cytometer (BD Biosciences). Data were analyzed using FlowJo version 7.6.3.

Serum IgM depletion. Five milliliters of 20% NHS was incubated with 500 μl of PBS-washed Sepharose beads coupled to anti-human IgM antibody (Sigma). After 2 h of incubation on a rotating wheel at 4°C, Sepharose beads were removed by centrifugation, and sera were diluted to 10% with PBS and immediately stored at −80°C.

Complement resistance assays. All experiments were conducted with the same batch of pooled human serum obtained from GTI Diagnostics (catalogue no. PHS-N100). Complement resistance of the NPS and MEF isolates was determined with 5% NHS or heat-inactivated NHS as described previously (10). The complement resistance of NTHi R2866, 3655, 86-028NP, Rd, 1521062, Δ*lgtC* mutant, and Δ*0112* mutant strains was determined as described previously (15). To determine the contribution of IgM, complement resistance was determined in 5% IgM-depleted serum. For competition experiments, wild-type and mutant bacteria were mixed in a 1:1 ratio, and serum was added as described above. The competitive index (CI) score was calculated by dividing the output ratio of the CFU counts of the mutant to those of the wild type by the input ratio of the mutant to the wild-type bacteria.

LOS analysis by Tris-Tricine SDS-PAGE. LOS was prepared by the proteinase K-ethanol precipitation method as described previously (33). LOS samples were separated on a Tris-Tricine SDS-PAGE gel in a Protean II XI cell electrophoresis system (Bio-Rad) and visualized by silver staining (34) or transferred to nitrocellulose for Western blotting. Membranes were blocked with 5% bovine serum albumin (BSA) in PBS, incubated for 2 h with 2% NHS in PBS, and subsequently incubated with goat anti-human IgM coupled to horseradish peroxidase (HRP) in PBS (1:5,000). The intensity of IgM binding to LOS bands was calculated using ImageJ software (35).

Structural characterization of LOS glycoforms by mass spectrometry. LOS was extracted from lyophilized bacteria using phenol-chloroform-light petroleum as described previously (36). LOS preparations and LC-ESI-MS experiments were performed as described previously (37, 38) on a Waters 2690 high-pressure liquid chromatography (HPLC) system (Waters, Milford, MA) coupled to a Finnigan LCQ ion trap mass spectrometer (Finnigan-MAT, San Jose, CA). A microbore C_{18} column [Phenomenex Luna; 5-μm C18(2) column; 150 by 0.5 mm; Torrance, CA] was used with an eluent gradient consisting of 0.1 mM sodium acetate and 1% acetic acid in methanol as eluent A and 0.1 mM sodium acetate and 1% acetic acid in H_2O. Gradient elution was conducted as follows: 50% A at 0 min, 54% A at 15 min, 100% A at 35 min, 54% A at 55 min, and 50% A at 65 to 75 min. The flow rate was 0.018 ml/min. Average mass units were used for calculation of molecular weight values providing the basis for proposed compositions: hexose (Hex), 162.14; *N*-acetyl-hexosamine (HexNAc), 203.19; heptose (Hep), 192.17; reduced anhydro-Kdo (AnKdo-ol), 222.20; Me, 14.03; Na, 22.99. Relative abundance was estimated from the height of the ion peaks relative to the total (expressed as percent).

Influenza virus-NTHi coinfection mouse model. Six- to 8-week-old female, specific-pathogen-free (SPF) BALB/c mice (Harlan, Netherlands) were infected intranasally (i.n.) with $10^{4.5}$ PFU of egg-grown influenza virus strain A/Udorn/302/72 in a volume of 10 μl or a similar dilution of naive allantoic fluid (17). Three days later, mice were challenged with 5×10^7 CFU in 10 μl PBS of either the wild-type 1521062 strain alone or a 1:1 mixture of 1521062 wild-type and mutant strains. At 48 and 96 h following challenge with NTHi, mice were euthanized and perfused with PBS by intracardiac injection. The entire bulla from each ear was dissected, after

which a nasopharyngeal lavage was performed. Bullae were immediately homogenized (T10 basic Ultra-turrax; IKA), and serial dilutions of bulla homogenates and nasopharyngeal lavages were prepared in PBS and cultured on sBHI agar with or without 150 μg/ml of spectinomycin (Calbiochem) (39). The CI score was calculated as described above. All animal experiments were approved by the Animal Ethics Committee of the Radboud University Nijmegen Medical Centre (RU-DEC2-11-246).

Statistical analysis. All statistical analyses were performed in GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA), where $P < 0.05$ was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00079-12/-/DCSupplemental.

Figure S1, TIFF file, 1.7 MB.
Figure S2, TIFF file, 1.4 MB.
Table S1, TIFF file, 2.1 MB.
Table S2, TIFF file, 7.6 MB.

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