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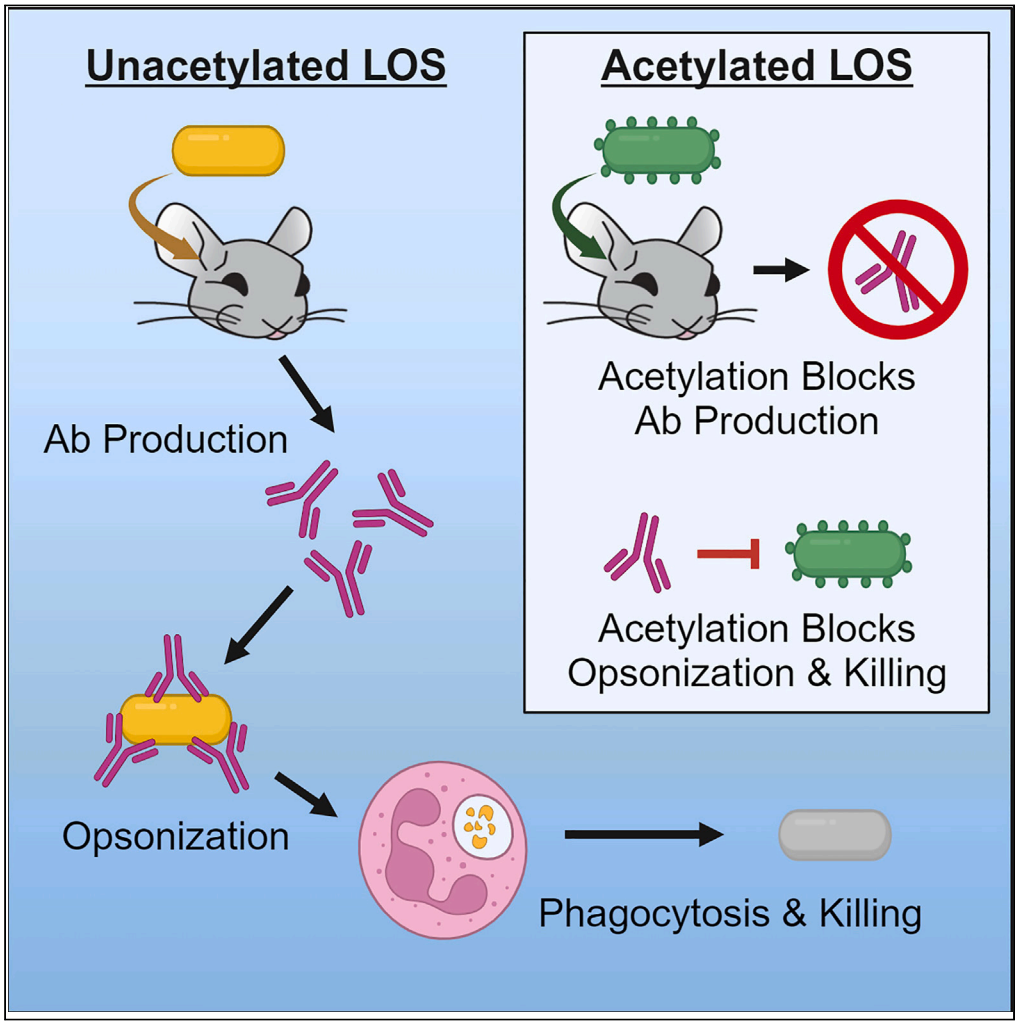

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Highlights

OafA phase variably acetylates NTHi lipooligosaccharide

Acetylation inhibits production of anti-LOS antibodies during infection

Acetylation of LOS blocks opsonization and phagocytic killing

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Phase variable acetylation of lipooligosaccharide modifies antibody production and opsonophagocytic killing of non-typeable *Haemophilus influenzae*

Brandon M. Wills,[1] Preeti Garai,[1] Quinn Dickinson,[2] Jesse G. Meyer,[2] and Kenneth L. Brockman[1,3,*]

SUMMARY

Non-typeable *Haemophilus influenzae* (NTHi) causes millions of infections each year. Though it is primarily known to cause otitis media, recent studies have shown NTHi is emerging as a primary pathogen for invasive infection, prompting the need for new vaccines and treatments. Lipooligosaccharide (LOS) has been identified as a potential vaccine candidate due to its immunogenic nature and outer membrane localization. Yet, phase variable expression of genes involved in LOS synthesis has complicated vaccine development. In this study, we used a chinchilla model of otitis media to investigate how phase variation of *oafA*, a gene involved in LOS biosynthesis, affects antibody production in response to infection. We found that acetylation of LOS by OafA inhibited production of LOS-specific antibodies during infection and that NTHi expressing acetylated LOS were subsequently better protected against opsonophagocytic killing. These findings highlight the importance of understanding how phase variable modifications might affect vaccine efficacy and success.

INTRODUCTION

Non-typeable *Haemophilus influenzae* (NTHi) is a Gram-negative bacterium that is a primary causative agent of acute otitis media (AOM) as well as several other respiratory infections. NTHi typically colonizes the human nasopharynx asymptomatically but can also cause infections throughout the upper and lower respiratory tracts.[1] Children (<5 years old) and the elderly (>65 years old) are most at risk for developing these infections.[2] AOM accounts for ~709 million infections worldwide annually and complications from OM-related infections are estimated to cause 21,000 deaths per year globally.[3] Though most cases are not fatal, AOM infection can progress to more severe middle ear (ME) diseases such as otitis media with effusion (OME) or chronic suppurative otitis media (CSOM), both of which can lead to hearing impairment.[4,5] This can be especially detrimental to young children, as OME infections have been associated with reduced cognition, impaired verbal skill development, and decreased performance in school.[6] In addition to OM, NTHi can cause other respiratory diseases including sinusitis, bronchitis, pneumonia, and exacerbations in the lungs of patients with chronic obstructive pulmonary disease (COPD). Exacerbations triggered by NTHi lead to lung inflammation that can damage the lung tissues and lead to complications that result in death. More recently, NTHi has emerged as the primary agent of invasive *Haemophilus* infections. A recent survey on invasive *Haemophilus* infections in Europe found NTHi to be the predominant pathogen (78%) among other *Haemophilus* serotypes in patients of all ages. NTHi was particularly dominant in invasive *Haemophilus* infections among children <1 month old, causing 97% of the cases.[7] Taken together, infections caused by NTHi, whether acute or invasive, are highly prevalent across the world and infection rates continue to increase each year.[7] Rising invasive NTHi infection rates may be due to a combination of several factors including introduction of the Hib vaccine, enhanced NTHi serotyping protocols, and increased NTHi virulence.[8] As there are no vaccines available against NTHi, antibiotics are the standard for treating NTHi infections. However, it is estimated that 30–40% of NTHi produce β-lactamases that cf. resistance against penicillin-based antibiotics.[9] Additionally, increases in ampicillin-resistant NTHi that lack β-lactamase have also been observed.[10] Unless new advances are made, our ability to successfully treat patients suffering from NTHi-induced illness will decline and the incidence of NTHi-related diseases and deaths will only increase. Thus, there is a critical need to develop an effective vaccine against NTHi.

Development of an effective vaccine requires both identification of a suitable target(s), as well as a comprehensive understanding of the associated immune responses. Lipooligosaccharide (LOS) has been considered as a potential vaccine target due to its immunogenic nature. LOS is a virulence factor expressed on the outer membrane of some Gram-negative bacteria, including NTHi. Similar to LPS found in other Gram-negative bacteria, LOS of NTHi consists of a lipid A-KDO inner core and a saccharide-rich outer core but lacks the O-antigen chain found on LPS. The outer core of LOS can undergo several modifications, including sialylation and acetylation, which are thought to protect the bacterium from host defenses. Several animal studies have shown the effectiveness of various LOS conjugates to induce a protective

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immune response in mice, rabbits, and chinchillas.[11–13] These results suggest that development of human-compatible LOS conjugate vaccines may reduce NTHi infection rates and disease severity. A problem hindering the development of a human-compatible LOS-conjugate vaccine is that several of the genes involved in LOS biosynthesis are phase variable and can randomly switch expression status. This phase variability is due to tetranucleotide repeats found within the LOS biosynthesis genes. The long tracts of simple nucleotide repeats can cause slipped-strand mispairing during DNA replication and result in frameshift mutations that cause a protein that is normally expressed to no longer be expressed, or vise-versa.[14] Such changes may alter LOS structure and antigenicity, allowing NTHi to escape the vaccine-induced immune response. At least 7 of the genes involved in NTHi LOS biosynthesis are known to be phase variable. These include *lic1A*, *lic2A*, *lic3A*, *lic3B*, *lex2A*, *lgtC*, and *oafA*.[15,16] The work presented here focuses on one of these genes, *oafA*.

The *oafA* gene was first identified as a phase variable gene when DNA-probing experiments revealed the presence of GCAA tetranucleotide repeat tracts in the *oafA* sequences of 10 different NTHi strains.[15] As a phase variable gene, *oafA* can either exist in the ON state (expressed) or the OFF state (not expressed). Studies have shown that OafA expression status is selected for depending on the microenvironment encountered by the bacterium.[16–18] When NTHi that expressed *oafA* was repetitively passaged in normal human serum (NHS), expression of OafA was maintained and never shifted from ON to OFF.[17] OafA expression has also been studied in NTHi isolated from both healthy individuals and those suffering from NTHi-related diseases. NTHi isolated from healthy patients were found to be majority *oafA* OFF whereas NTHi isolated from the ME and sterile sites, such as blood, were found to be majority *oafA* ON.[16,18] Taken together, these data suggest that in some instances, expression of *oafA*, or lack thereof, may provide a survival advantage for NTHi depending on the types of microenvironments the pathogen encounters inside of the host. While previous studies have shown that acetylation of LOS by OafA is protective against serum-mediated killing (Fox et al., 2005), the natural immunologic response to LOS during infection is unknown. Here we use a chinchilla model of otitis media to show that acetylation of LOS by OafA affects the adaptive immune response by altering both production and specificity of antibodies against LOS isolated from NTHi strain 723. These results are biologically significant, as NTHi with acetylated LOS are better protected from opsonophagocytic killing than NTHi that express unacetylated LOS.

RESULTS

OafA alters antibody production during NTHi-induced otitis media

In vitro studies have found that expression of *oafA* affects survival of NTHi in the presence of NHS.[15] Yet, *in vivo* studies on the relevance of these findings are lacking. NTHi strain 723 has 6 known LOS biosynthesis genes capable of phase variation (Table 1). Individual laboratory variants of NTHi strain 723 were sequenced to determine the ON/OFF status of each gene. Sequencing revealed that the *oafA* gene was out of frame in one of the variants (*oafA* OFF) and in frame in the other (*oafA* ON). The other 5 phase variable LOS biosynthesis genes had the same number of repeats and thus identical statuses in both of these variants, with *lic1A* (*lex1_1*), *lic2A* (*lex1_3*), *lic3A* (*lex1_4*), and *lic3B* (*NTHI_00744*) in the ON state and *lgtC* in the OFF state (Table 1). Adult chinchillas were challenged with either the *oafA* ON variant or the *oafA* OFF variant via transbullar inoculation directly into the ME space to induce AOM. All animals developed similar signs of experimental disease, which included ME fluids, mild erythema, and bulging of the tympanic membrane. Fourteen days after challenge, serum and any fluids present within the ME were collected (Figure 1A). The bacterial burden within the MEs of animals challenged with *oafA* OFF was greater than that for animals challenged with *oafA* ON (Figure S1). Western blots were performed to determine antibody response toward outer membrane preparations (OMPs) isolated from these two NTHi variants. Due to the outbred nature of these animals, overall antibody response varied among animals. However, antibodies against a ~10 kDa molecule were present in post-serum and ME fluids from animals challenged with *oafA* OFF (Figure 1B, arrowheads) but not in the post-serum or ME fluids from animals challenged with *oafA* ON (Figure 1C). These antibodies were not present in serum collected prior to NTHi challenge (Figures 1B and 1C, pre-serum), indicating that these particular antibodies were produced during NTHi infection. Furthermore, the antibodies appeared to be specific for OMPs isolated from the *oafA* OFF variant but not those isolated from the *oafA* ON variant (Figure 1B, compare OFF OMPs to ON OMPs). A total of 5 chinchillas across 2 independent studies were challenged with each variant. Four of the five animals (80%) infected with *oafA* OFF developed antibodies against the 10 kDa molecule, whereas none of the animals infected with *oafA* ON developed antibodies against this molecule (Table 2).

Antibodies induced by *oafA* OFF target LOS

Based on its size and outer membrane localization, we hypothesized that the molecule these antibodies were detecting around 10 kDa was LOS. To test this, serum antibody response to proteinase K-treated OMPs isolated from both NTHi variants was assessed via Western blot. As expected, reactivity against multiple OMP components was observed in the untreated control samples (Figure 2A) and matched the findings shown in Figure 1. Following proteinase K treatment, reactivity to only a single band at 10 kDa was observed in the treated *oafA* OFF OMPs whereas no reactivity was observed in the treated *oafA* ON OMPs (Figure 2B). This result suggested that the 10 kDa molecule was a non-protein outer membrane component. To confirm that the 10 kDa molecule of interest was indeed LOS, pure LOS was isolated from *oafA* OFF and *oafA* ON via phenol extraction.[19] The isolated LOS was similar in size to LPS from *E. coli* but lacked the characteristic ladder-like appearance that differentiates LPS from LOS (Figure 2C). Antibody reactivity against pure LOS was assessed via Western blot and reactivity to a single band at 10 kDa was observed for LOS produced by *oafA* OFF but not for LOS produced by *oafA* ON (Figure 2D). The pattern was identical to that observed with the proteinase K-treated samples, confirming that the 10 kDa molecule in the OMPs was indeed LOS.

Table 1. Sequencing of LOS genes in NTHi strains

		723 oafA ON		723 oafA OFF		723 ΔoafA		86-028NP		C486 #1		C486 #1 ΔoafA		C486 #2		C486 #2 ΔoafA		R2866		477		1209		
Target gene	Alternate name	# of repeats	Status	# of repeats	Status	# of repeats	Status	# of repeats	Status	# of repeats	Status	# of repeats	Status	# of repeats	Status	# of repeats	Status	# of repeats	Status	# of repeats	Status	# of repeats	Status	Repeat Seq
oafA	*oafA*	14	ON	16	OFF	N/A	Δ	8	OFF	17	ON	N/A	Δ	17	ON	N/A	Δ	9	OFF	11	ON	8	ON	AGCA
lex1_1	*licA*	21	ON	21	ON	21	ON	15	ON	31	OFF	31	OFF	31	OFF	31	OFF	36	ON	21	ON	39	ON	CAAT
lex1_3	*lic2A*	23	ON	23	ON	23	ON	14	ON	32	ON	32	ON	31	OFF	31	OFF	4	ON	19	OFF	22	ON	ATCA
lex1_4	*lic3A*	34	ON	34	ON	34	ON	18	ON	26	ON	26	ON	28	OFF	28	OFF	19	OFF	21	ON	35	OFF	TCAA
NTHI_00744	*lic3B*	16	ON	16	ON	16	ON	18	ON	No gene	N/A	No gene	N/A	No gene	N/A	No gene	N/A	No gene	N/A	19	OFF	No gene	N/A	CAAT
lgtC	*lgtC*	15	OFF	15	OFF	15	OFF	10	ON	16	OFF	16	OFF	16	OFF	16	OFF	26	ON	16	OFF	43	OFF	GACA

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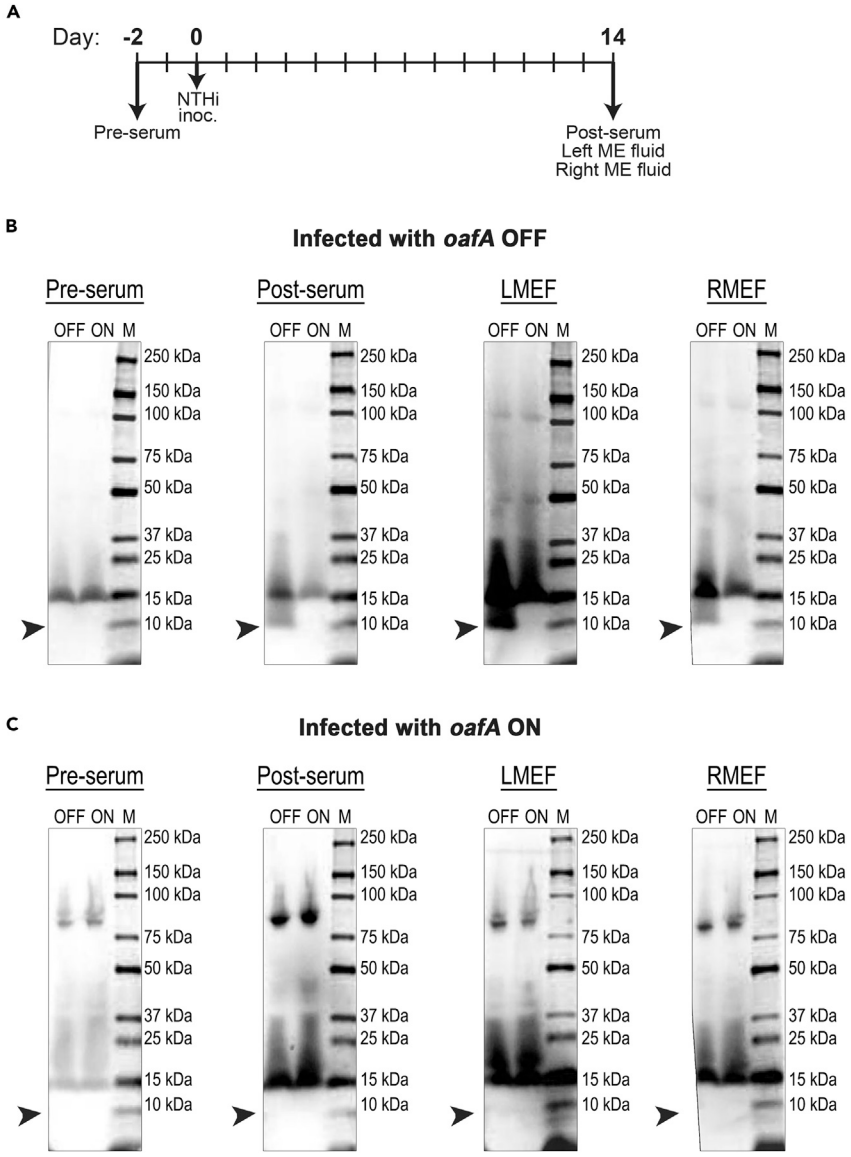

Figure 1. Antibodies in serum and middle ear fluids (MEFs) collected from animals challenged with NTHi

(A) Chinchillas were challenged with either *oafA* OFF or *oafA* ON via direct inoculation into the middle ear. 14 days after challenge, serum as well as MEFs were collected.

(B) Outer membrane preparations (OMPs) isolated from *oafA* OFF and *oafA* ON were probed with either pre-serum (collected prior to infection), post-serum (day 14), or MEFs (LMEF, left ear; RMEF, right ear) from a single animal infected with *oafA* OFF. Serum from animals infected with *oafA* OFF had a specific response to a molecule around 10 kDa present only in OMPs isolated from *oafA* OFF (OFF) and was absent in OMPs isolated from *oafA* ON (ON).

(C) No reactivity at 10 kDa was observed when serum collected from animals infected with *oafA* ON were used to probe the same sets of OMPs. Representative blots are shown. Full results are presented in Table 2.

OafA acetylates at the proximal hexose of LOS

To determine differences in LOS composition that may lead to differential recognition by these antibodies, LOS isolated from *oafA* OFF and *oafA* ON was analyzed with ESI-MS in positive mode. Several ions, including the ions with the greatest intensity at m/z 685.700 and 847.749, were shared between *oafA* OFF and *oafA* ON, representing LOS with the same composition between the variants (Figures 2E and 2F). In addition to the shared peaks, the *oafA* ON variant contained additional peaks, notably those at m/z 706.701 and 868.754, which have an approximately m/z 21 difference between these and the shared peaks (Figure 2F). As the single acetylation of LOS would add a mass of 42.011 and these are doubly charged ions, it is likely that these additional peaks demonstrate the acetylation of LOS in the *oafA* ON variant. In addition to the peaks labeled here, ten additional peaks with differences of m/z 21 between the two strains were determined using ESI-MS in negative mode (Figures S2A and S2B).

Table 2. Summary of Western blotting results

Serum from animals infected with:	OafA status	*oafA* OFF OMPs	*oafA* ON OMPs	Δ*oafA* OMPs
723 oafA OFF	–	**80% (4/5)**	0% (0/5)	**100% (5/5)**
723 oafA ON	+	0% (0/5)	0% (0/5)	0% (0/5)
723 Δ*oafA*	Δ	**66% (2/3)**	0% (0/3)	**66% (2/3)**

Bolded numbers indicate OMPs that were detected by >50% of the sera tested.

To determine the location of acetylation in LOS isolated from NTHi strain 723, tandem mass spectrometry (MS2 or MS/MS) in positive mode was performed on each sample on the peaks at 685.700 and 706.701. The precursor at 685.700 produced several notable peaks (Figure 2G) including those at m/z 328.116 and 1043.274, the sum of which equals the mass of the precursor LOS (328.116 + 706.701 = 1371.39 vs. 685.700*2 = 1371.4). The precursor at m/z 706.701 produced a similar peak at m/z 1043.274 but no peak at m/z 328.116 (Figure 2H). Instead, it contained a peak at m/z 370.127, approximately the 42 m/z difference expected by acetylation. Similar patterns can be seen for the precursor ions at m/z 847.749 and m/z 868.754 (Figures S2C and S2D), suggesting that this pattern of acetylation is consistent among LOS species.

Given the masses of the produced ions and the expected masses of each of the moieties within LOS, we were able to propose the composition and structure of each of the analyzed LOS (Figures 2I and 2J). The shared ion between the two MS2 spectra has an m/z of 1043.274, which is approximately the monoisotopic mass of three heptose (192.063*3 = 576.189), a ketodeoxyoctonic acid (KDO) (220.058), two phosphoethanolamines (PEtn) (123.009*2 = 246.018) and a proton (1.008) (1043.273 vs. 1043.274). For the other two peaks, the masses of phosphocholine (166.063) and hexose (162.053) add to the m/z of the first ion (328.116) and the second ion by addition of acetate (42.011) (370.127 vs. 370.127). A similar pattern is observed for the precursor ions at m/z 847.749 and m/z 868.754 (Figures S2E and S2F). Based on these data, we conclude that LOS isolated from the *oafA* OFF variant is not acetylated whereas LOS isolated from the *oafA* ON variant is acetylated at the hexose attached to the proximal heptose of the outer core.

Antibody specificity is dependent on LOS acetylation status

To confirm that acetylation by OafA inhibited the production and specificity of LOS specific antibodies, we generated an isogenic *oafA* knockout mutant (*ΔoafA*) in the *oafA* ON variant background. There were no differences in the status of any of the other phase variable LOS biosynthesis genes, aside from *oafA* (Table 1). At 14 days post-challenge, bacterial burden of NTHi within the middle ears of chinchillas challenged with Δ*oafA* was similar to that of animals challenged with *oafA* OFF and was greater than that of animals challenged with *oafA* ON (Figure S1). Serum collected from all animals was then used to probe OMPs isolated from the *oafA* OFF, *oafA* ON, and Δ*oafA* variants. As expected, antibodies in serum from animals infected with *oafA* OFF recognized the 10 kDa LOS band in OMPs isolated from NTHi that lacked OafA (*oafA* OFF and Δ*oafA*) but did not recognize this band in OMPs isolated from *oafA* ON (Figure 3A). Antibodies in serum from animals infected with *oafA* ON did not react to LOS from any of the OMPs (Figure 3B). These results matched the initial findings that animals challenged with an *oafA*-expressing NTHi variant do not produce significant antibodies against LOS. To determine if the loss of acetylation due to OafA was sufficient to induce the production of antibodies during disease, serum from animals infected with the *ΔoafA* mutant was used to probe the same set of OMPs. A majority of the animals infected with the Δ*oafA* mutant (2 of 3) produced antibodies specific for unacetylated LOS (Figure 3C; Table 2). These findings are consistent with the results observed for the *oafA* OFF variant, indicating that acetylation by OafA impacts the development of antibodies specific to unacetylated LOS.

To quantify the relative antibody avidity to LOS from each of the variants, ELISAs were performed using purified LOS. Results of the ELISAs were consistent with the OMP Western blot data. High levels of antibody binding were observed when serum from animals infected with *oafA* OFF was used to probe LOS isolated from *oafA* OFF or the Δ*oafA* mutant. Yet, this same serum elicited significantly less binding to LOS isolated from the *oafA* ON variant (Figure 3D). Low levels of antibody binding were observed when serum from animals infected with *oafA* ON was used to probe LOS isolated from any of the variants (Figure 3E). However, high levels of antibody binding occurred when serum from animals infected with the Δ*oafA* mutant were used to probe LOS (Figure 3F). Antibody binding was greater for unacetylated LOS compared to acetylated LOS. As NTHi strain 723 is also known to employ a phase variable DNA methyltransferase (ModA2) for global gene regulation, we assessed the role of OafA in *modA2* OFF and *modA2* ON backgrounds. ModA2 expression status did not affect LOS-specific antibody production or recognition, as assessed by Western blot and ELISA (Figure S3).

Antibodies cross-reactivity varies between NTHi strains with structurally diverse LOS

The *oafA* gene is highly conserved among NTHi species, appearing in all 105 complete NTHi genomes available from NCBI. A majority (61 of 105, 58.1%) of these genomes were found to contain an *oafA* gene with tetranucleotide repeats and were thus capable of undergoing phase variation (Table S2). To determine if antibodies produced against unacetylated LOS from NTHi strain 723 were cross reactive against other NTHi strains, we selected five other strains that encoded a phase variable *oafA* but had differences in the expression of the other phase variable LOS biosynthesis genes. The expression status of each phase variable LOS gene in all strains tested is shown in Table 1. OMPs were isolated from each strain and probed with serum collected from animals infected with the strain 723 Δ*oafA* mutant. As expected, antibodies in the serum detected LOS in the 723 *oafA* OFF OMPs. Serum antibodies also cross reacted with LOS in OMPs isolated from strain 477 but not

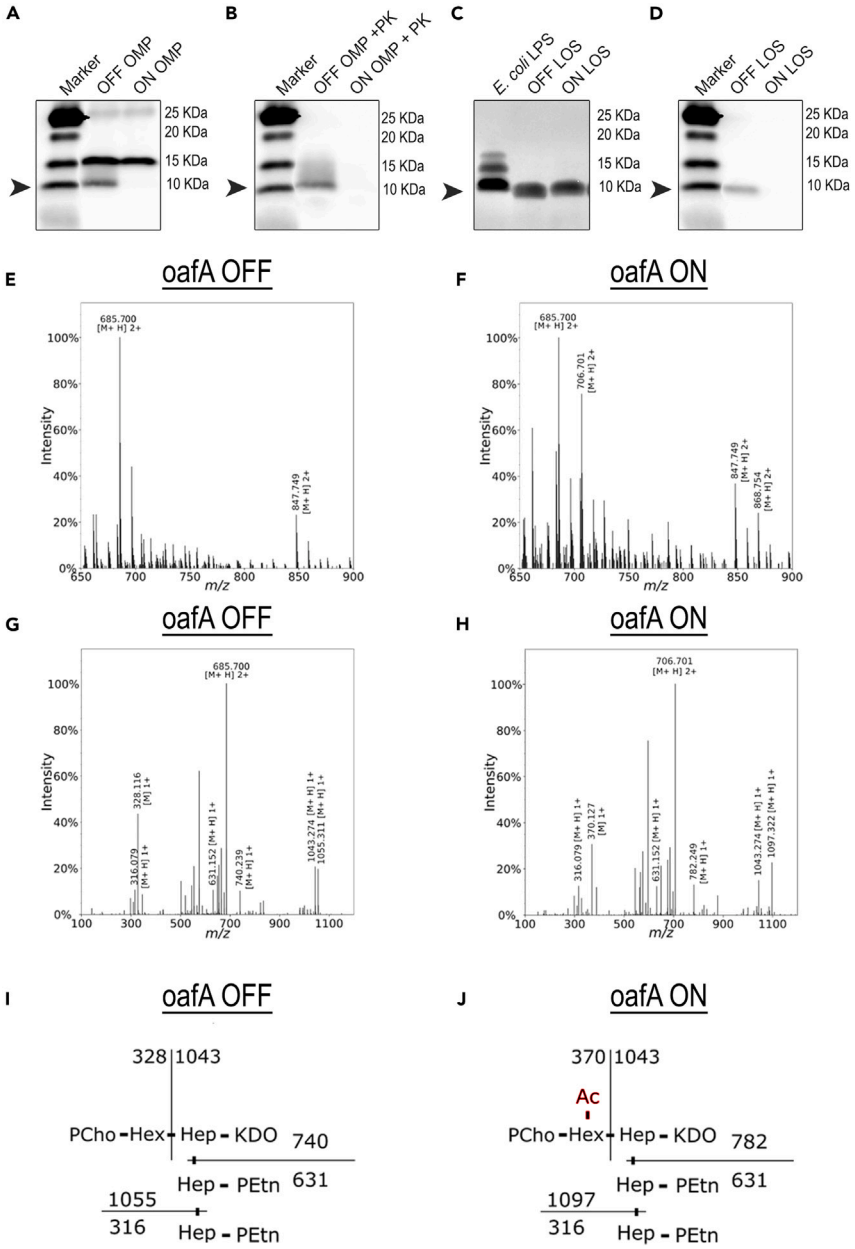

Figure 2. Antibodies induced during infection with *oafA* OFF are specific for unacetylated LOS

Untreated OMPs, proteinase K treated-OMPs, or purified LOS from each variant were probed with chinchillas serum that contained anti-LOS antibodies.

(A) Serum antibodies detected multiple bands in the untreated OMPs isolated from either variant. A band at 10 kDa was observed only with OMPs isolated from *oafA* OFF, indicated by arrowhead.

(B) Reactivity against the 10 kDa band remained following proteinase K treatment. However, no other bands were observed.

(C) Silver stained SDS-PAGE of purified NTHi LOS was similar in size to *E. coli* LPS.

(D) Chinchilla serum that contained anti-LOS antibodies recognized LOS isolated from *oafA* OFF but not *oafA* ON.

(E and F) Positive mode ESI-MS spectrum of LOS purified from *oafA* OFF (E) and *oafA* ON (F). Labeled peaks represent selected LOS species with a difference of approximately 21 m/z from another peak, corresponding to the addition of an acetyl group on a doubly charged ion (acetyl mass ~42 Da). All ions with a difference of 21 are plotted in Figure S1.

(G and H) Positive mode ESI-MS/MS of m/z 685.70 (G) and m/z 706.70 (H). Labeled peaks represent the precursor ion and the ions important for the determination of the LOS structure.

(I and J) Proposed structure determined from fragment ions from *oafA* OFF (I) and *oafA* ON (J) samples. Labeled ion masses were calculated by the following masses: PCho = 166.063, Hex = 162.053, Ac = 42.011, Hep = 192.063, KDO = 220.058, PEtn = 123.009.

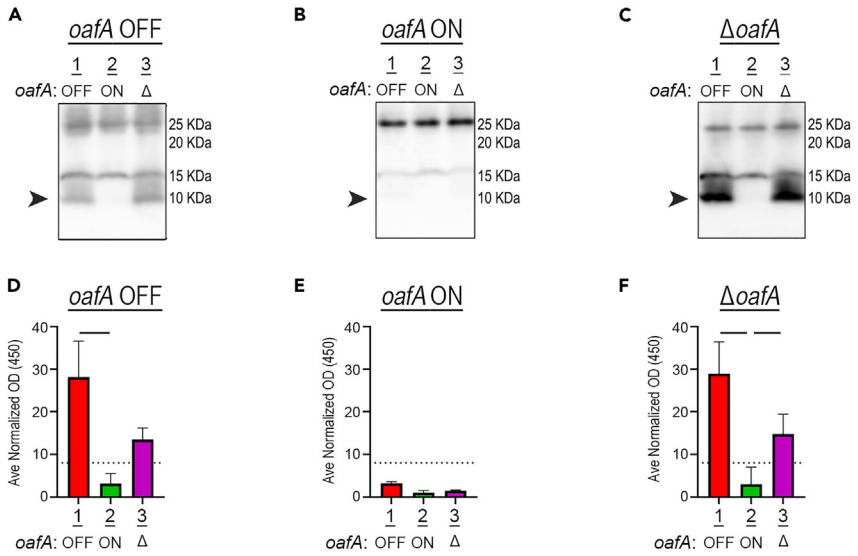

Figure 3. Infection with NTHi strains that lack OafA induces production of antibodies against unacetylated LOS

(A–C) OMPs isolated from *oafA* OFF, *oafA* ON, and Δ*oafA* were separated via SDS-PAGE and probed with chinchilla serum collected 14 days post-challenge. (A) Serum from animals infected with *oafA* OFF had a specific response to LOS in OMPs isolated from strains that also lacked OafA. (B) Serum from animals infected with *oafA* ON did not react with LOS from any of the variants. (C) Serum from animals infected with Δ*oafA* recognized LOS in OMPs isolated from strains that lacked OafA. Lane 1, *oafA* OFF OMPs; Lane 2, *oafA* ON OMPs; Lane 3, Δ*oafA* OMPs. Representative blots are shown. Compiled data for all blots are shown in Table 2.

(D–F) ELISAs to determine serum antibody response to purified LOS isolated from the 3 NTHi variants. (D) Sera collected from animals challenged with *oafA* OFF bound strongly to unacetylated LOS (1 and 3) but poorly to acetylated LOS (2). (E) Sera collected from animals challenged with *oafA* ON did not recognize LOS isolated from any of the 3 NTHi variants. (F) Serum from animals infected with Δ*oafA* contained antibodies that recognized unacetylated LOS (1 and 3) significantly more than acetylated LOS (2). Average absorbance values normalized to anti-lipid A control antibodies are shown. Dashed line indicates a baseline threshold value of 8. Bars over graph indicate $p < 0.05$; One-way ANOVA, Friedman test.

with LOS isolated from the other strains tested (Figure 4A). These results suggest that LOS isolated from 723 *oafA* OFF and 477 may share an epitope recognized by antibodies produced during infection with strain 723. Differences in the underlying LOS epitopes are likely responsible for the varied reactivity against LOS in the strains tested.

To test whether acetylation of LOS provided similar protection in a strain not recognized by 723-induced antibodies, we generated Δ*oafA* knockouts in two isolates of NTHi strain C486. Isolates C486 #1 and C486 #2 were selected as they differed in their expression of the phase variable genes *lic2A* (*lex1_3*) and *lic3A* (*lex1_4*) (Table 1). Chinchillas were challenged via transbullar inoculation and sera collected 14 days post-challenge were used to probe OMPs isolated from the parental isolates and Δ*oafA* mutants. Regardless of *oafA* status, none of the animals produced antibodies toward LOS isolated from any of the variants (Figure 4B, arrowheads). These results suggest that the immunogenic epitopes of LOS isolated from strain 723 may be missing or inaccessible on LOS of C486, thus preventing the production of LOS-specific antibodies during infection. As no LOS-specific antibodies were produced following infection with any of the C486 variants, it remains unclear how acetylation may affect antibody specificity with this strain.

Anti-LOS antibodies enhance opsonophagocytic killing of NTHi

Previous studies have shown that acetylation by OafA protects NTHi against killing by NHS, but the role of LOS-specific antibodies has not been reported.[15] To determine if antibodies generated against unacetylated LOS provide protection against NTHi infection, opsonophagocytosis was assessed using a human HL-60 assay as described previously.[20,21] We hypothesized that NTHi that produce unacetylated LOS would be susceptible to opsonophagocytic killing in the presence of serum from animals infected with NTHi that lack OafA. Serum from a chinchilla challenged with *ΔoafA* that contained high titers of LOS-specific antibodies (See Figure 3C) was used to test opsonophagocytosis of the 3 NTHi variants. Serum collected from the same animal prior to bacterial challenge was used as a control to account for any pre-existing antibodies the animal may have had. No significant killing occurred for any of the variants when the pre-serum was used (Figure 5A). In contrast, killing was observed for all variants when post-serum was used, with the greatest killing occurring for variants that lacked OafA (Figure 5B). Percent killing during opsonophagocytosis was calculated at each concentration of chinchilla serum used. The variants that lacked OafA were completely killed at the highest serum concentrations as observed at the 1:10 and 1:20 dilutions, whereas the *oafA* ON variant was more resistant to killing, only 75% and 40% killed, respectively (Figure 5C). Although the killing of all variants decreased with decreasing concentrations of the serum, the *oafA* ON variant was killed the least at all serum concentrations. Based on the percent killing of each variant, area under each of the curves was calculated and used to assess overall killing across all serum dilutions tested. The *oafA* ON variant was killed

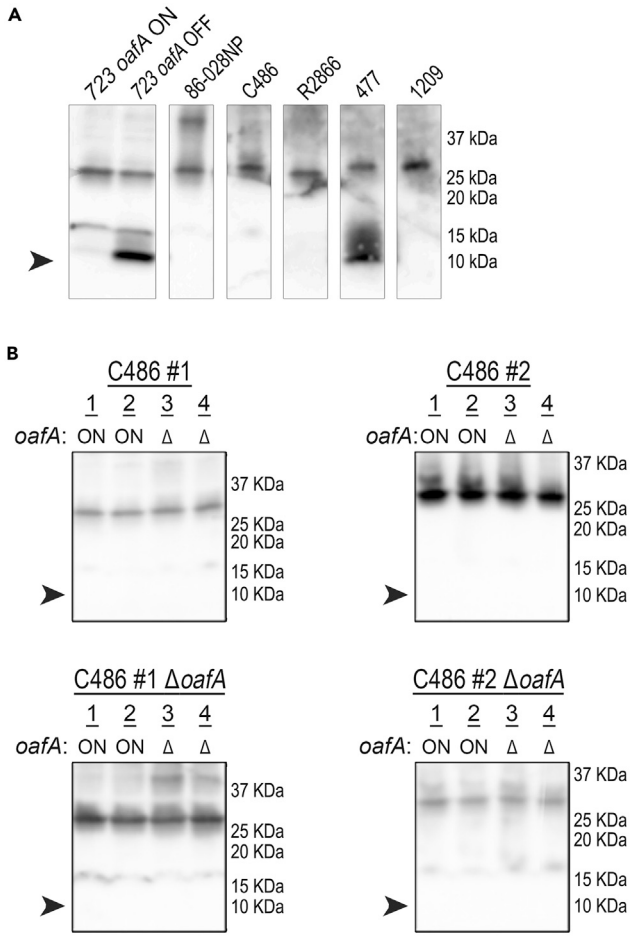

Figure 4. Specificity and production of anti-LOS antibodies with diverse strains of NTHi

(A) Sera from animals challenged with strain 723 were used to probe OMPs isolated from diverse NTHi strains. Antibodies bound to LOS in Δ*oafA* OFF OMPs and 477 OMPs but did not detect LOS in the other strains tested. Full gel and corresponding blot are shown in Figure S4.

(B) Chinchillas were challenged with two different variants of NTHi strain C486 and their respective Δ*oafA* mutants. Sera was collected 14 days post-challenge and used to probe OMPs isolated from the parental and Δ*oafA* mutant C486 variants. None of the sera contained antibodies against LOS, regardless of *oafA* status. Lane 1, C486 #2 OMPs; Lane 2, C486 #1 OMPs; Lane 3, C486 #2 Δ*oafA* OMPs; Lane 4, C486 #1 Δ*oafA* OMPs.

significantly less than the other two variants in which OafA was either not expressed or deleted (Figure 5D). Therefore, antibodies against LOS are able to induce more killing of the variants with unacetylated LOS compared to the variant with acetylated LOS. Taken together, these data suggest that expression of OafA, and subsequent acetylation of LOS, protects NTHi from opsonophagocytic killing. Furthermore, under the correct conditions, production of anti-LOS antibodies may protect the host from NTHi-infection via neutralization or opsonization-mediated bacterial clearance.

DISCUSSION

Several mucosal-associated Gram-negative bacteria express LOS on their cell surface. These include the respiratory pathogens *Haemophilus*, *Neisseria*, *Moraxella*, and *Bordetella*, as well as members of the *Campylobacter* genus.[22] LOS structures can vary greatly among bacteria and often undergo additional modification by bacterial or host factors. Common modifications include addition of sialic acids, phosphoenolamine, phosphorylcholne, and acetyl groups, all of which can further contribute to the protective nature of LOS.[23] Incorporation of sialic acid into the LOS structure has been shown to protect NTHi from bactericidal activity by blocking IgM binding to LOS.[24] Similarly, incorporation of phosphorylcholine into the LOS structure is known to protect NTHi from bactericidal activity by strengthening the integrity of the outer membrane.[25] Additionally, modifications that mimic host glycolipids are known to protect against immune recognition and clearance and can defend against complement or serum-mediated killing.[26] The use of LOS as a potential immunogen for vaccination has been of interest for some time and several LOS conjugate formulations have been tested in preclinical studies.[11–13] More recently, several of the genes involved in the biosynthesis of NTHi LOS have been shown to undergo phase variable expression. This phase variation is known to alter the

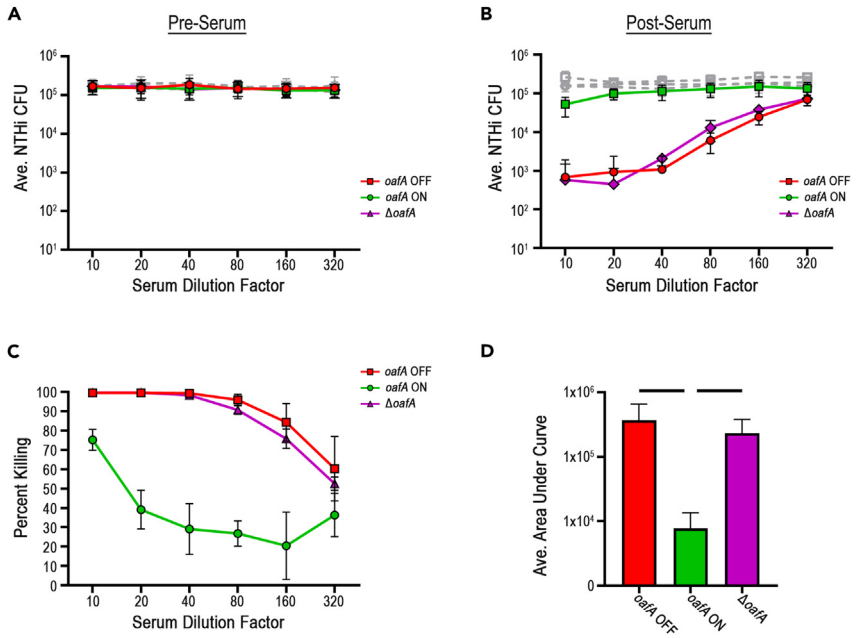

Figure 5. Antibodies that target unacetylated LOS promote oposonophogocitic killing

oafA OFF, *oafA* ON, and Δ*oafA* were incubated with HL-60 cells in the presence of pre- or post-serum from an animal challenged with the *ΔoafA* mutant and opsonophagocytic killing was measured.

(A) Opsonophagocytic killing in the presence of serum collected prior to infection (pre-serum).

(B) Opsonophagocytic killing with serum collected 14 days post-infection (post-serum) from an animal challenged with Δ*oafA*. For A and B, gray lines indicate corresponding results with a heat-inactivated control that lacked complement.

(C) Percent killing in the presence of post-serum was calculated with respect to a heat-inactivated control from each experiment. The highest percent killing for all 3 variants was observed at the highest concentration of serum. As the serum concentration decreased, percent killing also decreased for all 3 variants. The *oafA* ON variant was most resistant to killing at all serum concentrations tested.

(D) Area under the percent killing curves in panel C was calculated for each variant across all serum dilutions tested. NTHi variants with unacetylated LOS were killed significantly more than those with acetylated LOS. Bars over graph indicate $p < 0.0001$; One-way ANOVA, Uncorrected Fisher's LSD.

structure of LOS and subsequently modify resistance to complement mediated killing. However, little was known about how changes to LOS composition due to phase variation impact antibody production and response during infection. Here, we investigated the role of a phase variable *o*-acetyltransferase, OafA, on antibody development using a chinchilla model of otitis media.

We found that animals infected with NTHi that expressed OafA, and thus had LOS that was acetylated, did not develop significant antibodies against LOS. This suggests that acetylation of LOS by OafA inhibits LOS-specific antibody production. In contrast, antibodies were produced by animals infected with NTHi strain 723 that lacked OafA expression. Antibodies produced by these animals were specific to unacetylated LOS and did not recognize acetylated LOS, suggesting that acetylation may interfere with both antibody production and antibody binding to LOS. Due to the outbred nature of the chinchilla model used, it was possible for animals to have antibodies against a variety of bacterial targets, including OMPs and LOS, prior to the start of the study. To determine any preexisting antibodies, serum was collected from each animal prior to the study and used to probe OMPs isolated from the NTHi variants used in this study. The results of these pre-serum studies provided a baseline antibody reactivity to compare with post-infection serum to determine if any new antibodies were produced as a result of infection. As expected, pre-serum collected from every animal had reactivity against various outer membrane components of NTHi. Yet, none of the pre-serums showed reactivity to LOS in OMPs isolated from any of the variants, indicating that the animals developed antibodies toward LOS as a result of the experimental infection. Because of their heterogeneous nature, it was possible that some animals infected with the same NTHi variants would mount a varied adaptive immune response. Serum collected from 4 out of the 5 (80%) animals challenged with *oafA* OFF and serum collected from 2 out of the 3 (66%) animals challenged with *ΔoafA* produced LOS-specific antibodies as assessed by Western blot. Still, the majority of animals infected with NTHi that lack OafA elicited robust antibody production against unacetylated LOS.

Many NTHi strains, including strain 723, are known to encode a phase variable DNA methyltransferase, ModA, which can regulate expression of numerous genes via epigenetic modification to the chromosome, known as a phase variable regulon, or phasevarion.[27,28] To determine what role, if any, regulation by ModA played in the observed phenotypes, Δ*oafA* mutants were constructed in strain 723 *modA2* ON and *modA2* OFF backgrounds. We also tested strain 723 *modA2* ON and *modA2* OFF variants that expressed OafA at the time of challenge. ModA2 status, ON vs. OFF, did not affect the production of anti-LOS antibodies, or the specificity of antibodies toward LOS (see Figure S3). These results suggest that ModA2 does not regulate OafA expression and is not involved in LOS-specific antibody production or recognition.

This study reports a structural analysis of NTHi strain 723 LOS isolated from variants that either lack or express OafA. As *oafA* ON has a functional OafA, we expected that LOS isolated from this variant would be acetylated and that LOS isolated from the *oafA* OFF variant would not be acetylated. Previous studies have shown that LOS isolated from various NTHi strains can be acetylated in at least 4 different positions.[29–32] Of these 4 positions, *oafA* in NTHi strain 285 is responsible for acetylating the distal heptose of the inner core of LOS.[15] Based on structural similarities between LOS isolated from strain 723 and strain 285, we hypothesized that acetylation may occur at the same location in both strains. In contrast, our analysis found that OafA of NTHi strain 723 acetylates at the hexose attached to the proximal heptose of the outer core. It is unclear how these strain specific differences in the site of acetylation may affect immune response, antibody development, or protection against complement. To try to address this question, we challenged chinchillas with two NTHi strain C486 isolates that expressed OafA or their corresponding Δ*oafA* mutants. None of the chinchillas infected with these isolates produced any LOS-specific antibodies. Both of the C486 isolates tested lacked the *lic3B* (*NTHI_00744*) gene present in strain 723, and C486 #2 also lacked expression of lic2A (lex1_3) and lic3A (lex1_4). Interestingly, in strain 723 the acetylated hexose is also the site of PCho attachment (see Figure 2J). It is possible that differences in LOS produced by the strain C486 isolates were responsible for the lack of antibody production. PCho modification of LOS is phase variable in NTHi and has been shown to alter immune responses and inhibit bacterial clearance during disease.[33–36] Although the expression status of PCho biosynthesis genes was the same between all strain 723 variants tested herein, it is possible that the close proximity of acetylation may physically alter PCho relevant interactions. Future studies to determine the specific epitopes or sugar moieties recognized by anti-LOS antibodies are needed to fully determine the role of acetylation and/or PCho in this antibody response.

NTHi strain 285 isolates that express *oafA*, and therefore have acetylated LOS, are better protected against killing by NHS compared to *oafA* mutants.[15] However, prior to the current study, the significance of *oafA* expression, or lack thereof, had not yet been established using a live animal model. We were able to successfully measure this using a chinchilla model of otitis media, which led to the discovery that animals challenged with *oafA*-expressing NTHi strain 723 do not elicit a strong antibody response against LOS. Based on our data, we believe that acetylation of LOS affects both antibody specificity as well as antibody production in response to infection. Acetylation of LOS may block the predominant anti-LOS binding site, reducing the number of antibodies that can bind to LOS. Similarly, this acetylation may prevent the development of antibodies against unacetylated LOS due to steric constraints. Instead, antibodies may be produced against other parts of the LOS molecule that are not acetylated, such as Lipid A. Further testing would be required to determine which regions of LOS in strain 723 cf. the greatest antibody binding. Knowledge of the primary binding sites for antibodies against both the acetylated and unacetylated LOS could inform the development of therapeutic strategies that may improve treatment of NTHi-related diseases. A number of studies have already shown that immunization of animals with detoxified LOS conjugates induces production of LOS-specific antibodies and that preimmunization with these conjugates leads to increased NTHi clearance.[11–13] However, factors such as phase variation of biosynthesis genes and host modifications could significantly affect the efficacy of these LOS-based approaches. Future immunization studies that investigate the effect of phase variable genes, such as *oafA*, on antibody response and NTHi evasion are needed.

To determine if acetylation of LOS protects NTHi strain 723 from antibody-mediated opsonization and phagocytic killing, opsonophagocytosis by human HL-60 cells was assessed. NTHi with acetylated LOS were better protected from killing by HL-60s in the presence of anti-LOS antibodies compared to the other 2 variants that had unacetylated LOS. These results indicate that acetylation of LOS is beneficial to NTHi survival in the presence of antibodies that target LOS. Further studies will be necessary to determine the exact mechanism behind the observed phenotype. As discussed previously, expression of *oafA* in NTHi has been reported to be in the ON state during disease. It is therefore probable that protection against serum and opsonization mediated killing may drive selection for OafA expressing variants during disease. In contrast, acetylation by OafA may be detrimental to NTHi during asymptomatic colonization of the nasopharynx in healthy individuals. Acetylation may interfere with other evasive mechanisms such as host mimicry that allow NTHi to reside within the nasopharynx with minimal inflammatory response.

The work presented here has shown that acetylation of LOS by OafA affects both LOS antibody specificity and LOS antibody production during infection and leads to greater resistance to opsonophagocytic killing. As other strains of NTHi express different combinations of LOS biosynthesis genes and thus have different LOS structures, it will be important to determine how other LOS modifications, in addition to acetylation, impact antibody specificity and production. Such experiments could allow for development of therapies that target multiple epitopes on LOS thereby broadening treatments against multiple strains of NTHi.

Limitations of the study

Due to the outbred nature of the chinchillas, two animals challenged with the same NTHi variant could elicit distinct adaptive immune responses. However, such responses may better represent those observed within the general human population. Due to the lack of antibody production in animals infected with C486, the role of OafA in antibody response was only able to be defined in one strain, 723. Furthermore, this study was limited to studying the effect of one LOS biosynthesis gene on adaptive immune response.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107785.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.L.B.; Methodology, B.M.W., P.G., Q.D., and K.L.B.; Formal Analysis, B.M.W., P.G., and Q.D.; Investigation, B.M.W., P.G., Q.D., and K.L.B.; Writing – Original Draft, B.M.W. and K.L.B.; Writing – Review & Editing, P.G., Q.D., and J.G.M.; Visualization, B.M.W., P.G., and Q.D.; Supervision, J.G.M. and K.L.B.; Funding Acquisition, J.G.M. and K.L.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal to Lipid A	abcam	Cat#ab8467, RRID:AB_306573
Goat Anti-Mouse IgG H&L (HRP)	abcam	Cat#ab205719, RRID: AB_2755049
Goat Anti-Mouse IgG H&L (HRP)	abcam	Cat#ab205719, RRID: AB_2755049
Bacterial and virus strains		
Non-typeable Haemophilus influenzae Strain 723	Finnish Otitis Media study group	SAMN02595605
Non-typeable Haemophilus influenzae Strain 86-028NP	Columbus Children's Research Institute	SAMN02603157
Non-typeable Haemophilus influenzae Strain C486	Dr. Arnold Smith	SAMN02595604
Non-typeable Haemophilus influenzae Strain R2866	Drexel University College of Medicine	SAMN02604259
Non-typeable Haemophilus influenzae Strain 477	Finnish Otitis Media study group	SAMN02595602
Non-typeable Haemophilus influenzae Strain 1209	Finnish Otitis Media study group	SAMN02595603
Non-typeable Haemophilus influenzae Strain 86-028NP	Columbus Children's Research Institute	SAMN02603157
Non-typeable Haemophilus influenzae Strain C486	Dr. Arnold Smith	SAMN02595604
Non-typeable Haemophilus influenzae Strain R2866	Drexel University College of Medicine	SAMN02604259
Non-typeable Haemophilus influenzae Strain 477	Finnish Otitis Media study group	SAMN02595602
Non-typeable Haemophilus influenzae Strain 1209	Finnish Otitis Media study group	SAMN02595603
Chemicals, peptides, and recombinant proteins		
Hemin	Sigma Aldrich	Cat#H9039
β-Nicotinamide adenine dinucleotide hydrate	Sigma Aldrich	Cat#N7004-5G
N-Lauroylsarcosine Sodium Salt	Sigma Aldrich	Cat#L5125-50G
Thermolabile Proteinase K	New England Biolabs	Cat#P8111S
Proteinase K from Tritirachium album	Sigma Aldrich	Cat#P2308-10MG
RPMI-1640 Medium	ATCC	Cat#30-2001
Gelatin Veronal Buffer	Sigma Aldrich	Cat#G6514-50ML
β-Nicotinamide adenine dinucleotide hydrate	Sigma Aldrich	Cat#N7004-5G
N-Lauroylsarcosine Sodium Salt	Sigma Aldrich	Cat#L5125-50G
Thermolabile Proteinase K	New England Biolabs	Cat#P8111S
Proteinase K from Tritirachium album	Sigma Aldrich	Cat#P2308-10MG
RPMI-1640 Medium	ATCC	Cat#30-2001
Gelatin Veronal Buffer	Sigma Aldrich	Cat#G6514-50ML
Critical commercial assays		
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Fisher Scientific	Cat#34580
1-Step Turbo TMB-ELISA Substrate Solution	Thermo Fisher Scientific	Cat#34022
CloneJET™ PCR Cloning Kit with DH10B Competent Cells	Thermo Fisher Scientific	Cat#K123120
1-Step Turbo TMB-ELISA Substrate Solution	Thermo Fisher Scientific	Cat#34022
CloneJET™ PCR Cloning Kit with DH10B Competent Cells	Thermo Fisher Scientific	Cat#K123120
Experimental models: Cell lines		
HL-60	ATCC	Cat#CCL-240

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Chinchilla lanigera	Rauscher's Chinchilla Ranch	N/A
Oligonucleotides		
Primers for PCR	See Table S1	N/A
Software and algorithms		
Prism 9	GraphPad 9.5.0	N/A
DNAStar	SeqBuilder Pro 17	N/A
FreeStyle	Thermo Fisher Scientific	N/A
Pyteomics	See mass spectrometry in STAR Methods	N/A
spectrum_utils	See mass spectrometry in STAR Methods	N/A
matplotlib4	See mass spectrometry in STAR Methods	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kenneth L. Brockman (kbrockman@mcw.edu).

Materials availability

This study did not generate new unique reagents. Any bacterial strains generated as part of this work are available from the lead contact upon request and consistent with required terms of institutional Material Transfer Agreement (MTA).

Data and code availability

This paper does not report sequencing data or original code. Original SDS-PAGE and western-blot images reported in this paper are included in the Supplemental Materials; any additional information required to reanalyze the data reported in this paper are available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Adult chinchillas (*Chinchilla lanigera*) were supplied by Rauscher's Chinchilla Ranch, LaRue, OH. Chinchillas were not sex-differentiated and were classed as adult if weighing between 500–700 grams. Chinchillas were allowed to acclimate in the vivarium for 7 days before beginning the study. All challenge cohorts were mixed sex and established based on weight, sex, and pre-immune status (as applicable) to provide as equivalent of groups as possible. There was no clear correlation between sex or weight and any of the outcomes measured. All protocols were approved by the Medical College of Wisconsin's Animal Care and Use Committee, in accordance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals.

METHOD DETAILS

Bacterial strains and culture

NTHi were routinely cultured in brain heart infusion broth supplemented with hemin (2 μg/mL) and NAD (2 μg/mL) or on chocolate agar and grown at 37°C with 5% CO_2.

Generation of mutants

To generate a chromosomal knock-out of *oafA*, a kanamycin cassette flanked by sequences homologous to the upstream and downstream regions of the *oafA* gene were synthesized (IDT). The fragment was ligated into pJET1.2 using the CloneJet PCR Kit (Thermo). *E. coli* DH10B cells were transformed with the ligated DNA and desired clones were screened via agarose gel electrophoresis. Plasmids isolated from positive transformants were used as the template to amplify the linear DNA for transformation of NTHi. Plasmids were amplified using pJET 1.2 fwd and rev primers (Table S1). The resulting DNA was confirmed by sequencing (MCLAB) and was then transformed into NTHi via the MIV method (Poje and Redfield, 2003). Mutations were confirmed via PCR using the oafA-F and oafA-R primers, followed by sequencing (Table S1).

Chinchilla model of NTHi-induced otitis media

Adult chinchillas (*Chinchilla lanigera*) were supplied by Rauscher's Chinchilla Ranch, LaRue, OH. Chinchillas were not sex-differentiated and were classed as adult if weighing between 500–700 grams. Chinchillas were allowed to acclimate in the vivarium for 7 days before beginning the study. Prior to challenge, blood serum was collected from each animal. Cohorts of either 2 or 3 chinchillas were established, then challenged transbullarly with either: (1) *oafA* OFF, (2) *oafA* ON, or (3) *ΔoafA* at a challenge dose of ~750 CFU. Delivered doses were confirmed by dilution plate counts of inocula on chocolate agar. The animals were monitored daily via video otoscopy and tympanometry for 14 days. On day 14 (unless removed from the study early owing to morbidity), blood serum was collected from anesthetized chinchillas, anesthetized chinchillas were euthanized, and the bullae were dissected from the skull. Bullae from each animal were aseptically opened and any middle ear fluids (MEFs) present were collected. All protocols were approved by the Medical College of Wisconsin's Animal Care and Use Committee, in accordance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals.

Sequencing LOS biosynthesis genes

NCBI's BLAST tool was used to search published gene sequences for known LOS biosynthesis genes in the NTHi complete genome. Depending on the strain, a total of 4-6 LOS biosynthesis genes encoding tetrameric repeats were identified using a Tandem Repeats Finder (https://tandem.bu.edu/trf/trf.html). Genomic DNA isolated from our laboratory variants of NTHi was amplified via custom primers specific to the repeat regions of the identified LOS biosynthesis genes (Table 1). The resulting PCR products were sequenced (MCLAB) and sequences were used to determine the number of tetrameric repeats in each gene for all of the variants used in the study. Based on the number of repeats, the gene was determined to be either in-frame (ON status) or out of frame (OFF status).

Preparation of outer membrane components (OMPs)

NTHi variants were grown overnight in sBHI broth (500 ml) at 37°C, 5% CO_2 without shaking. Cells were pelleted at 12,000 x g for 15 minutes at room temperature, resuspended in 10 mM HEPES-NaOH pH 7.40, and OMPs were prepared as described previously (Murphy et al., 1983). Briefly, cells were lysed by sonication and debris was pelleted as described above. Supernatants were collected and the centrifugation was repeated once more. The pellets were resuspended in 4 mL 1% sarcosyl-10 mM HEPES, pH 7.40 and incubated at 24°C, 60 rpm for 1 hour. Samples were then centrifuged at 10,400 x g for 30 minutes, washed with 200 μL DEPC H_2O, and resuspended in 400 μL DEPC H_2O. OMP concentrations were determined by Qubit™ Protein Assay according to the manufacturer's instructions (Invitrogen™). As indicated, OMPs were treated with of 0.12 units of Thermolabile Proteinase K (NEB P8111S) at 37°C for 24 hours, heat inactivated at 55°C for 10 minutes, then stored at -80°C for future use.

Isolation of LOS

NTHi were grown overnight in sBHI broth at 37°C, 5% CO_2 without shaking. Cells were centrifuged at 4000 x g for 10 minutes at 4°C and LOS was extracted following standard protocol (Apicella, 2008). Briefly, cells were resuspended in buffer containing 10 mM Tris-Cl (pH 8.0), 2% SDS, 4% β-mercaptoethanol, and 2 mM $MgCl_2$ and heated until completely resuspended. Samples were treated with proteinase K (100 μg/μL, Sigma) and digestion was carried out at 65°C for 1 hour followed by overnight incubation at 37°C. Samples were ethanol precipitated 3 times then resuspended in 9 mL of 10 mM Tris-HCl and treated with 0.5 mL DNase I (100 μg/mL, Sigma) and 0.5 mL RNase (25 μg/mL, Sigma). Samples were digested for 4 hours, cooled in an ice bath, and then phenol extraction was performed twice. Aliquots of each extraction were collected, run on a 4–20% Mini-PROTEAN® TGX Stain-Free Protein Gel (100V, 70 min, Bio-Rad), and silver stained (Pierce) to confirm purity and estimate concentration. Samples were concentrated with 3K MWCO concentrators (Pierce), dialyzed in 3.5K MWCO cassettes using UHPLC-MS water (Thermo Scientific), and then stored at 4°C.

Mass spectrometry

Purified LOS was subjected to weak hydrolysis of the LipA group by dissolution in 1% acetic acid in water and heated to 100°C for two hours, resulting in liberated oligosaccharide (OS). After hydrolysis, the samples containing OS were dried in a speed vac for two hours at a temperature of 30°C and a pressure of 800 millibar. After all water and acid was removed, the LOS was dissolved in 50% acetonitrile, 50% water with 0.05% ammonium formate, at a concentration of 1 μg/μl. Samples were directly infused using an UltiMate 3000 HPLC system while running constant solvent of 50% acetonitrile, 50% water with 0.2% formic acid at a rate of 0.5 μl/min. Infused samples were nano-electrosprayed into an Orbitrap Exploris 240 mass spectrometer with a Nanospray Flex ESI ion source. ESI-MS was run in both positive and negative mode at a resolution of 240,000 to obtain survey scans of potential LOS species. ESI-MS/MS was performed in positive mode targeting the relevant masses of interest for Q1 selection before higher energy collisional dissociation (HCD) with an normalized collision energy of 20%. Fragment ions were measured with the orbitrap set at a resolution of 240,000. Data was analyzed using ThermoScientific FreeStyle, Pyteomics (Goloborodko et al., 2013; Levitsky et al., 2019), spectrum_utils (Bittremieux, 2020), and matplotlib4 (Hunter, 2007). Each spectrum is the average of 338 spectra, representing 1.5 minutes of direct infusion.

Western blots

OMPs (2.5 μg) or LOS samples were separated on 4–20% Mini-PROTEAN® TGX Protein Gels (Bio-Rad), transferred to nitrocellulose membranes on an iBlot II system (Thermo), and blocked with 3% skim milk. Membranes were probed with a 1:200 dilution of chinchilla serum

followed by 3 washes. Membranes were then probed with a 1:5000 dilution of Protein A-HRP (Invitrogen 101023) and washed 3 times. Blots were developed with a SuperSignal™ West Pico PLUS Chemiluminescent Substrate kit according to the manufacturer's instructions (Thermo Scientific). Developed membranes were imaged on an Azure Biosystems c600 Gel Imaging System at an exposure time of 20 seconds.

ELISAs

LOS samples were analyzed following a standardized ELISA protocol. Briefly, Nunc Maxisorp plates (Thermo) were coated with 50 μL of purified LOS and incubated at 37°C for 1 hour. Plates were washed twice with 300 μL of wash buffer using a BioTek ELx50 plate washer. Wells were then blocked with 200 μL 0.5% bovine serum albumin in 1X DPBS at 37°C for 1 hour. Plates were washed again and the LOS was probed with 50 μL of a 1:200 dilution of either anti-Lipid A antibodies (Abcam Cat# ab8467, RRID:AB_306573) or chinchilla serum. Samples were incubated for 1 hour and then washed 3 times. 50 μL of a 1:3000 dilution of anti-Ms IgG-HRP (Abcam Cat# ab205719, RRID: AB_2755049) or HRP-Protein A (Invitrogen 101023) were used to detect Lipid A and chinchilla antibodies, respectively. Samples were incubated for 1 hour and then washed 3 times as described previously. 1-Step™ Turbo TMB (100 μL, Thermo 34022) was added to each well to visualize the HRP conjugated antibodies. The reaction in wells containing Lipid A + Goat pAb to Ms IgG was stopped with 100 μL 2M H_2SO_4 after 2 minutes. Wells containing chinchilla post-serum + Protein A-HRP were stopped with 100 μL 2M H_2SO_4 based on the *oafA* OFF samples, up to 5 minutes. Absorbance at 450 nm was measured with an H1MG plate reader (BioTek).

Opsonophagocytosis assay with HL-60 cells

The HL-60 human promyelocytic cell line (ATCC CCL-240) was cultured and cells were differentiated to assess opsonophagocytosis of NTHi as described previously (Winter and Barenkamp, 2006; 2009). Briefly, HL-60 cells were differentiated for 5-7 days at a density of $1x10^6$ cells/mL in RPMI 1640 medium (ATCC) with 15% fetal bovine serum (ATCC) and 100 mM N,N-dimethylformamide (Sigma). The opsonophagocytic assay was carried out at an effector (HL-60 cells)/target (NTHi) ratio of 100:1. HL-60 cells were resuspended in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (Gibco) prior to the assay. The cells were pelleted and resuspended in opsonophagocytosis buffer (Gelatin Veronal buffer, Sigma) just before use in the assay. Human pooled complement serum (Innovative Research) was used as the complement source and was passed through a protein-G affinity column (Cytiva) to remove serum IgG. An aliquot of the antibody depleted complement serum was heat inactivated for 30 min at 56°C to serve as a complement-free control. Chinchilla pre- and post-serum were used as the source of antibodies. The chinchilla serum was heat inactivated before use in the assay to inactivate intrinsic complement activity and then diluted tenfold in opsonophagocytosis buffer. The diluted chinchilla serum was further serially diluted two-fold in opsonophagocytosis buffer in a 96-well microtiter plate to test various concentrations of the serum. The bacterial suspension was prepared by diluting log-phase cultures of NTHi in opsonophagocytosis buffer. Diluted cultures were added to the serum in a microtiter plate at a density of $5x10^3$ CFU per well and incubated at 37°C and 5% CO_2 for 15 minutes. After the incubation period, 1.5 μL of complement source (or heat-inactivated control) was added to each well followed by immediate addition of differentiated HL-60 cells at a density of $5x10^5$ cells per well. The assay plate was then incubated at 37°C and 5% CO_2 for 90 min with horizontal shaking (180 RPM) to promote phagocytosis. At the end of the incubation period, aliquots from each well were serially diluted and plated on chocolate agar. Percent killing was calculated by dividing the CFU values for each dilution of the serum by that of the respective heat-inactivated control.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data are reported as the mean ± standard deviation. Data were analyzed and plotted with GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA) and figures were constructed using Adobe Photoshop (Adobe Inc., San Jose, CA, USA). Group differences were analyzed using a one-way ANOVA and differences with P-values <0.05 were considered significant.