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Biofilm Growth Increases Phosphorylcholine Content and Decreases Potency of Nontypeable *Haemophilus influenzae* Endotoxins

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Nontypeable *Haemophilus influenzae* (NTHI) is a common respiratory commensal and opportunistic pathogen. NTHI is normally contained within the airways by host innate defenses that include recognition of bacterial endotoxins by Toll-like receptor 4 (TLR4). NTHI produces lipooligosaccharide (LOS) endotoxins which lack polymeric O side chains and which may contain host glycolipids. We recently showed that NTHI biofilms contain variants with sialylated LOS glycoforms that are essential to biofilm formation. In this study, we show that NTHI forms biofilms on epithelial cell layers. Confocal analysis revealed that sialylated variants were distributed throughout the biofilm, while variants expressing phosphorylcholine (*P*Cho) were found within the biofilm. Consistent with this observation, *P*Cho content of LOS purified from NTHI biofilms was increased compared to LOS from planktonic cultures. Hypothesizing that the observed changes in endotoxin composition could affect bioactivity, we compared inflammatory responses to NTHI LOS purified from biofilm and planktonic cultures. Our results show that endotoxins from biofilms induced weaker host innate responses. While we observed a minimal effect of sialylation on LOS bioactivity, there was a significant decrease in bioactivity associated with *P*Cho substitutions. We thus conclude that biofilm growth increases the proportion of *P*Cho$^+$ variants in an NTHI population, resulting in a net decrease in LOS bioactivity. Thus, in addition to their well-documented resistance phenotypes, our data show that biofilm communities of NTHI bacteria contain variants that evoke less potent host responses.

Nontypeable *Haemophilus influenzae* (NTHI) is a human-specific respiratory commensal that is especially common in young children (12) and patients with impaired mucociliary clearance (36). In contrast with encapsulated *H. influenzae* strains that cause acute invasive infections, NTHI causes localized airway infections (36). These infections include otitis media (4, 47), bronchitis (45), and sinusitis (22), and are among the most common and costly public health problems worldwide.

Most of the NTHI "virulence factors" identified to date are determinants of asymptomatic carriage that allow the organism to persist in the airways. These include adherence factors (48), immunoglobulin A protease (55), and endotoxin (50). Like many bacteria adapted to mucosal surfaces, NTHI endotoxins are lipooligosaccharides (LOS) which lack polymeric O side chains (43, 51). Innate responses to the lipid A portion of LOS are mediated by Toll-like receptor 4 (TLR4) (39) and are important to the containment of *H. influenzae* infections in the airway (56) and in the generation of the inflammatory response that is the hallmark of chronic airway infections (10). Our previous work showed that production of the hexa-acylated lipid A recognized by TLR4 promotes persistent NTHI colonization (50), at least in part by increasing bacterial resistance to killing by defensins (46).

The carbohydrate portion of NTHI LOS is highly variable (31, 32, 41) and contains glycolipids that mimic host epitopes (29, 30). The decoration of bacterial surfaces with host components has long been thought to impede immune clearance by masking bacteria as "self" (33). *H. influenzae* scavenges choline (13) and sialic (*N*-acetylneuraminic) acid (NeuAc) (18) from mucosal surfaces, after which both structures are added to discrete oligosaccharides within the LOS by means of specific transferases (21, 27). In prior work, we showed that LOS containing phosphorylcholine (*P*Cho) promotes adherence in the upper airway (49), and that sialylated LOS promote biofilm formation and persistence in the middle ear and lung (52). Several groups confirm that NTHI in vivo express discrete lipooligosaccharide glycoforms, including LOS containing globoside trisaccharides (40), *P*Cho (54), and NeuAc (5).

Chronic otitis media, and possibly other NTHI infections, involve the formation of dense biofilm communities (9). Biofilms have been observed in the middle ears of NTHI-infected chinchillas and on tympanostomy drain tubes removed from children with chronic otitis (11, 42). Murphy and Kirkham showed that some NTHI isolates from chronic bronchitis also form biofilms, although there was no direct correlation between length of bacterial carriage and biofilm formation in vitro (38). We recently discovered that sialylation of the NTHI surface promotes biofilm formation in both static and continuous-flow systems in vitro (52). Recent work also shows that an α-2,6-linked sialic acid structure, likely assembled by the *siaA* sialyltransferase, promotes biofilm formation (14). Like NeuAc, *P*Cho is added to LOS oligosaccharides, usually as a terminal structure (27).

In this work, we show that biofilm growth results in an increase in *P*Cho$^+$ variants. Analysis of biofilms on host cell surfaces by confocal laser scanning microscopy revealed that these *P*Cho$^+$ variants are localized within the biofilm. LOS purified from NTHI biofilms or *P*Cho$^+$ strains was a significantly less potent inducer of inflammatory responses than LOS

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TABLE 1. Bacterial strains and phenotypes

Bacterial strain	Description	Reference(s)
NTHI 2019	Bronchial isolate	6
NTHI 2019 *siaB*	Asialylated mutant	21
NTHI 2019 *licD*	*P*Cho$^-$ mutant	49
NTHI 86-028NP	Otitis isolate	1, 34
NTHI 86-028NP *licD*	*P*Cho$^-$ mutant	This study

purified from planktonic cultures or *P*Cho$^-$ isogenic mutants. These data show that NTHI adaptations occurring during biofilm growth can weaken the activation of host innate defenses. These results may provide new insights into the long quiescent periods of low-level inflammation that are typical of chronic NTHI infections. Moreover, because *P*Cho surface moieties are a common theme among mucosal pathogens, these results could also have broad implications for host-pathogen interactions occurring in a wide spectrum of chronic infections.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The NTHI strains used in this study and their descriptions are provided in Table 1. Overnight cultures of nontypeable *H. influenzae* strains 2019 and 86-028NP were maintained at 37°C in brain heart infusion medium (Difco) supplemented with 10 μg/ml hemin chloride (ICN) and 10 μg/ml NAD (Sigma). Hereafter, this medium is referred to as supplemented BHI (sBHI).

To prepare NTHI biofilm cultures, we used a silicon tubing continuous-flow system, as we have described previously (53). NTHI from overnight broth cultures were diluted to an optical density at 600 nm of 0.150 (10^8 CFU/ml) in sBHI broth, and 200 μl was inoculated into sterile silicon tubing and incubated for 2 h without medium flow for initial adherence. Thereafter, a continuous flow of sBHI (1 drop per 4 to 5 seconds) was initiated through the tubing and maintained for 96 h. Afterward, a 9-in. (ca. 25-cm) section of tubing was excised approximately 3 in. below the inoculation site, opened, and scraped to remove adherent bacteria, which were then suspended in sterile phosphate-buffered saline. Aliquots of the biofilms were plated to confirm lack of contamination. For the CFU counts, the bacteria recovered from the biofilms were serially diluted and plated onto sBHI agar.

NTHI biofilm formation under static conditions was done using a previously described static biofilm formation assay (53). NTHI (5×10^6 CFU) were inoculated into a 96-well dish and incubated at 37°C. At the indicated time points, the wells were washed with double-distilled H_2O and stained with 0.1% crystal violet solution. After additional washing, the remaining crystal violet was solubilized in ethanol and the absorbance at 540 nm was read using a microplate reader (Labsystems).

Lipooligosaccharide purification and analysis. LOS was isolated from *H. influenzae* using a modified proteinase K procedure (17, 21). Briefly, NTHI cells were harvested from plate, broth, or biofilm cultures as indicated in the text, diluted in sterile PBS, pelleted, and then lysed in 2.0% sodium dodecyl sulfate, 10 mM EDTA, 0.06 M Tris (pH 6.8). After overnight treatment with proteinase K (Sigma), the lysates were boiled for 5 min and digested overnight with 10 units staphylococcal nuclease (Sigma). LOS was precipitated with sodium acetate-ethanol, dialyzed overnight, and lyophilized. LOS was analyzed by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19, 26) and immunoblotting (6) as indicated in the text. Quantities of LOS were standardized by silver stain and *Limulus* assay (BioWhittaker).

Cell culture. The immortalized mouse monocyte/macrophage lines HeNC2 (TLR4$^+$) (20) and GG2EE (TLR4$^-$) (3) were cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum. LOS treatments were performed as indicated in the text, after which the level of nitrite (as an indicator of nitric oxide) was measured using Greiss reagent (Sigma), and the levels of tumor necrosis factor alpha (TNF-α) or interleukin-1β (IL-1β) were measured by capture enzyme-linked immunosorbent assay using commercial antibody sets (Pharmingen/Becton Dickinson).

Immortalized human middle ear epithelial cells (8) were provided by David Lim and Jian-Dong Li (House Ear Institute, CA), and were cultured in bronchial epithelial growth medium (Clonetics) using all of the vendors' recommended supplements except the gentamicin-amphotericin. Immortalized 16HBE14o$^-$ human airway epithelial cells (15) were cultured in Eagle's modified essential medium with 10% added fetal bovine serum. For infection studies, both cell lines were grown in air-liquid interface cultures (23) on sterile membrane inserts (BioCoat, 1-μM pore size).

The electrical resistance of representative layers was measured using an EVOM "chopstick" voltohmmeter (World Precision Instruments, Sarasota, FL); only layers with resistance measurements of >250 Ω were used in the infection studies. The cell layers were infected with overnight plate cultures of NTHI 2019 (1:1 multiplicity of infection). At 48 h postinfection, the cell layers were washed and fixed using 1% paraformaldehyde in phosphate-buffered saline and stained for immunofluorescent analysis using polyclonal rabbit antiserum against NTHI (21), anti-*P*Cho monoclonal antibody TEPC-15 (49), or *Limax flavus* lectin-rhodamine conjugate (EY Laboratories), as indicated in the text. *Limax flavus* lectin recognizes sialic acid (24). Specimens were visualized by fluorescent antibody conjugates specific for mouse immunoglobulin A (TEPC-15) or rabbit immunoglobulin G (polyclonal rabbit anti-NTHI). The stained samples were viewed using a Zeiss 510 confocal laser scanning microscope, and three-dimensional images were constructed using stacked Z-series measurements.

For scanning electron microscopy, the infected cell layers were fixed overnight in 2.5% glutaraldehyde and treated with 2% tannic acid solution, followed by a graded ethanol dehydration series and osmium tetroxide fixation. The membranes were then excised, adhered to scanning electron microscopy stubs, and sputter coated with palladium prior to viewing with a Philips 515 scanning electron microscope.

RESULTS

NTHI forms biofilms on epithelial cell surfaces. Culture of epithelial cells in air-liquid interface systems elicits more differentiated, stratified, and polarized cell layers that more accurately model host epithelial surfaces in vivo (23). Therefore, physiologically relevant immortalized human epithelial cell lines (HMEEC-1 middle ear epithelial cells and 16HBE14o$^-$ airway epithelial cells) were cultured at an air-liquid interface on permeable membrane inserts (BioCoat, Becton Dickinson; 1-μM pore size) as we have described previously (50). The cells were infected with NTHI 2019 and incubated for 48 h, with daily washing to remove nonadherent bacteria. By 48 h postinfection, scanning electron microscopy analysis revealed significant biofilm formation on the apical surfaces of air-liquid interface cell cultures (Fig. 1).

LOS phosphorylcholine content increases during biofilm growth. We have established that bacteria within NTHI biofilms contain sialylated LOS forms that are essential for biofilm formation (53). *P*Cho is also added to specific LOS oligosaccharides. The genetic determinants of *P*Cho uptake and assimilation into LOS are phase variable (57), and a considerable body of work shows that *P*Cho$^+$ variants predominate during infection (2, 54, 58, 59). We therefore asked whether LOS from biofilms contain increased proportions of *P*Cho$^+$ glycoforms. *P*Cho content of LOS from biofilm and planktonic cultures of NTHI 2019 and 86-028NP was compared by immunoblot analysis (Fig. 2). The data show a significant increase in *P*Cho$^+$ glycoforms associated with biofilm growth. Assays for biofilm formation under static and continuous-flow conditions showed that *P*Cho additions are not required for biofilm formation (Fig. 3 and Table 2).

***P*Cho$^+$ variants are localized within biofilms.** We have previously established that biofilms contain different populations of NTHI variants than planktonic cultures, including sialylated variants (53). We next asked how NTHI variants were distributed within the biofilms using immunofluorescent staining and confocal laser scanning microscopy. As shown in Fig. 4, *P*Cho$^+$ NTHI variants are found within the interior portion of biofilm communities on immortalized HMEEC-1 cell surfaces. NTHI

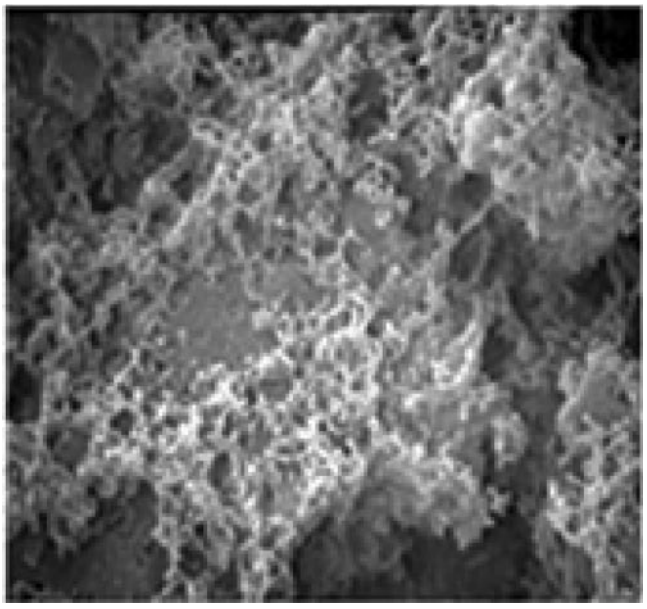

FIG. 1. NTHI 2019 forms biofilms on epithelial cell surfaces. Immortalized human middle ear epithelial cells (HMEEC-1) were cultured at an air-liquid interface on semipermeable plastic membrane inserts and infected with NTHI 2019 for 48 h. The infected cells were fixed, processed, and analyzed by scanning electron microscopy.

2019 formed dense biofilms that extended >30 μm above the HMEEC-1 cell layers (Fig. 4A). Staining for *P*Cho revealed that these variants are contained within the biofilm community (Fig. 4B and merged image in 4C). Images of a vertical Z-series (1 μm/slice) were used to compile a vertical Z-slice image that confirms that *P*Cho$^+$ variants are contained within mature biofilms (Fig. 4D). Comparable results were obtained using air-liquid interface cultures of 16HBE14o$^-$ immortalized human airway epithelial cells (data not shown).

In contrast, sialylated NTHI were distributed throughout the biofilm, as depicted in Fig. 5. Reactivity with the sialic acid-specific lectin from *Limax flavus* (red) was observed in all strata of biofilms cultured on HMEEC-1 or 16HBE14o$^-$ host cell surfaces.

LOS from biofilms elicit diminished inflammatory responses from immortalized mouse macrophages. Since *H. influenzae* bio-

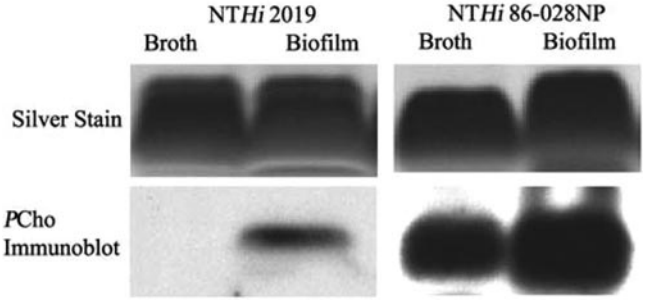

FIG. 2. LOS phosphorylcholine content increases during biofilm growth. Lipooligosaccharides were purified from biofilm or broth cultures and analyzed by Tricine-SDS-PAGE, followed by silver staining and immunoblot using an anti-*P*Cho monoclonal antibody, as described in Materials and Methods.

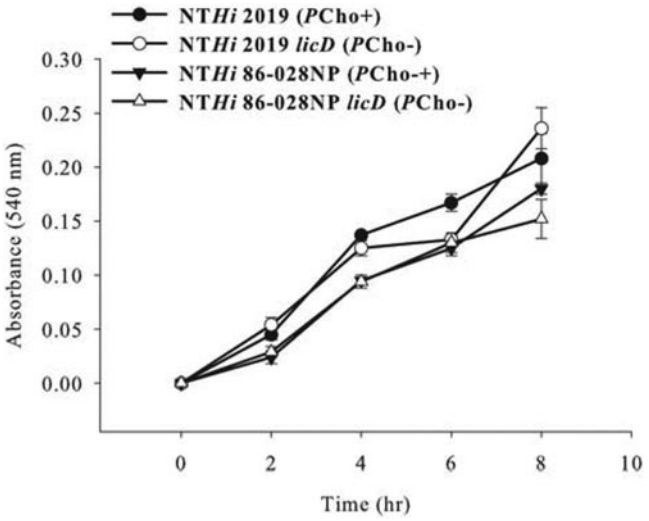

FIG. 3. *P*Cho does not effect biofilm formation in a static assay system. Biofilm formation was compared using the crystal-violet microtiter dish assay as described in Materials and Methods and in previous work (53).

films contain LOS of different composition than planktonic cultures (14, 53), we asked whether innate responses to LOS from biofilm and planktonic cultures differ. Immortalized HeNC2 mouse macrophages were treated with various doses of LOS purified from planktonic and biofilm cultures of NTHI 2019; nitric oxide and cytokine levels in culture supernatants were then measured (Fig. 6). The results show that LOS from biofilm cultures elicited significantly less release of nitric oxide (A), IL-1β (B), and TNF-α (C) from the macrophages than LOS from planktonic cultures. Comparable results were obtained with LOS purified from biofilm and planktonic cultures of NTHI 86-028NP (data not shown).

As responses of the TLR4$^-$ cells were at baseline levels (data not shown), we concluded that differences in bioactivity were due to differences in LOS, and were not influenced by contaminants that may activate other TLRs.

Effect of sialylation on inflammatory responses to NTHI LOS. Because our recent work showed that biofilm cultures contain increased proportions of sialylated LOS (53), we next asked whether LOS sialylation changes its potency. No detectable differences were observed in the amount of nitric oxide (A), IL-1β (B), and TNF-α (C) release from mouse macro-

TABLE 2. Quantitation of bacteria recovered from a continuous-flow biofilm growth assay

Bacterial strain	Log_{10} CFU recovered[a]	*P*
NTHI 2019	8.17	
NTHI 2019 *licD*	7.87	0.29
NTHI 86-028NP	7.94	
NTHI 86-028NP *licD*	7.84	0.24

[a] Bacterial counts were obtained from biofilms recovered from silicon tubing using the continuous-flow biofilm system described in Materials and Methods. Data are means of two independent experiments. *P* values were derived from nonparametric *T* test comparison of CFU counts of biofilms formed by parental and *licD* strains.

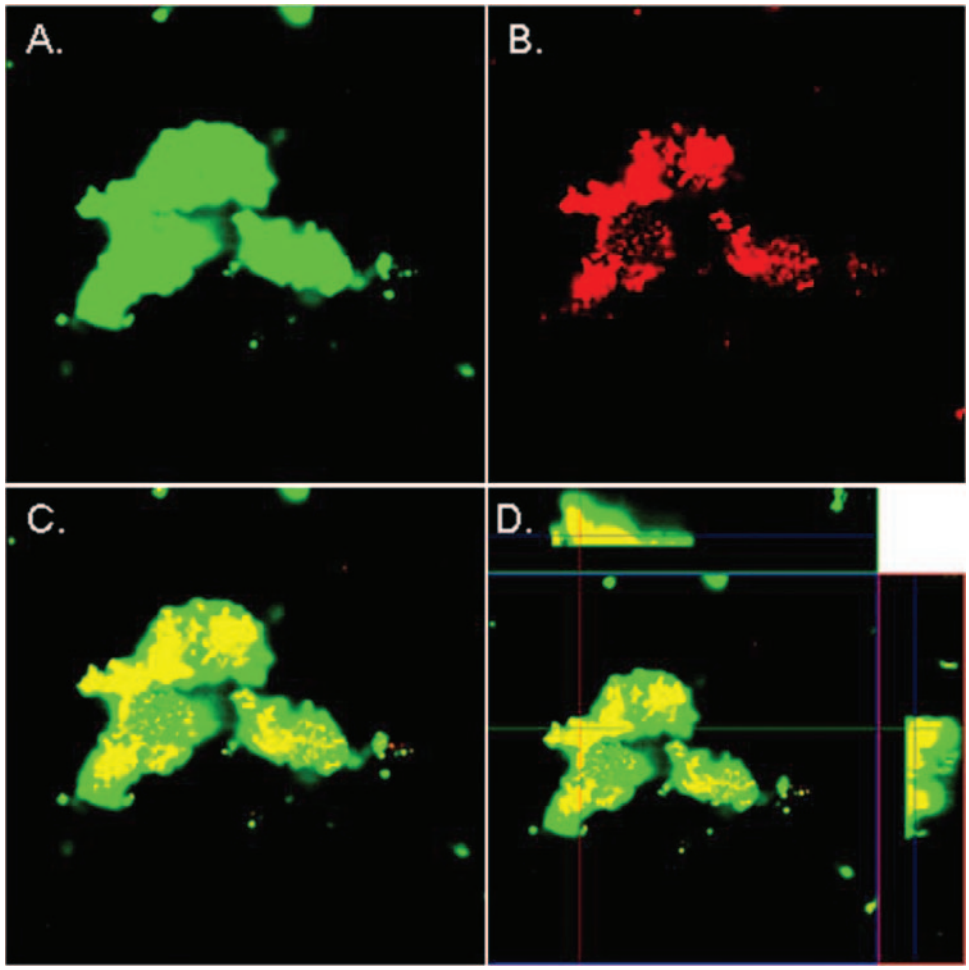

FIG. 4. Distribution of *P*Cho$^+$ variants within NTHI 2019 biofilms on immortalized epithelial cells. Immortalized HMEEC-1 human middle ear epithelial cells were cultured at an air-liquid interface on semipermeable membrane inserts (see Materials and Methods), infected with NTHI 2019 as in the preceding figure, fixed, and analyzed by immunofluorescent staining and confocal laser scanning microscopy. NTHI cells were labeled with a rabbit polyclonal antibody (A; green), and *P*Cho$^+$ variants were stained with TEPC-15 (B; red). As shown in the merged image (C), *P*Cho$^+$ variants are localized within the biofilm. Panel D shows a horizontal Z slice (center) and a reconstructed vertical cross section (margins) of an NTHI biofilm grown on HMEEC-1 cells.

phages after treatment with LOS from NTHI 2019 and an isogenic NTHI 2019 *siaB* mutant lacking all sialylated glycoforms (Fig. 7). Although statistical analyses revealed no difference in release of nitric oxide in accordance with changes in sialylation, statistically significant differences in IL-1β release were observed at 100 ng/ml ($P = 0.003$) and 1,000 ng/ml ($P = 0.041$) LOS doses, and a difference in TNF-α release was observed at 100 ng/ml LOS ($P = 0.038$). The magnitude of the observed differences was also markedly less than the differences in bioactivity we had observed for LOS purified from broth and biofilm cultures. We thus concluded that the observed biofilm-related changes in bioactivity are caused by LOS modifications distinct from sialylation.

FIG. 5. Distribution of sialylated variants within NTHI biofilms on immortalized epithelial cells. HMEEC-1 cells were cultured and infected with NTHI as in the preceding figure, and stained with polyclonal rabbit antiserum against all NTHI (green) and *Limax flavus* lectin-rhodamine conjugate (EY Labs) recognizing sialic acid (red).

Effect of *P*Cho on inflammatory responses to NTHI LOS. Our data show a significant reduction in innate responses to LOS from NTHI biofilms, and an increase in *P*Cho$^+$ LOS forms during biofilm growth. We thus hypothesized that *P*Cho additions to LOS may moderate its bioactivity. Therefore, we treated HeNC2 macrophages with LOS purified from plate cultures of NTHI 2019 (*P*Cho$^+$) and NTHI 2019 *licD* (*P*Cho$^-$), and compared the release of NO and TNF-α. The results (Fig. 8) show that *P*Cho decreases LOS bioactivity by approximately 100-fold. Furthermore, complementation of the *licD* mutant restored *P*Cho additions, and resulted in LOS

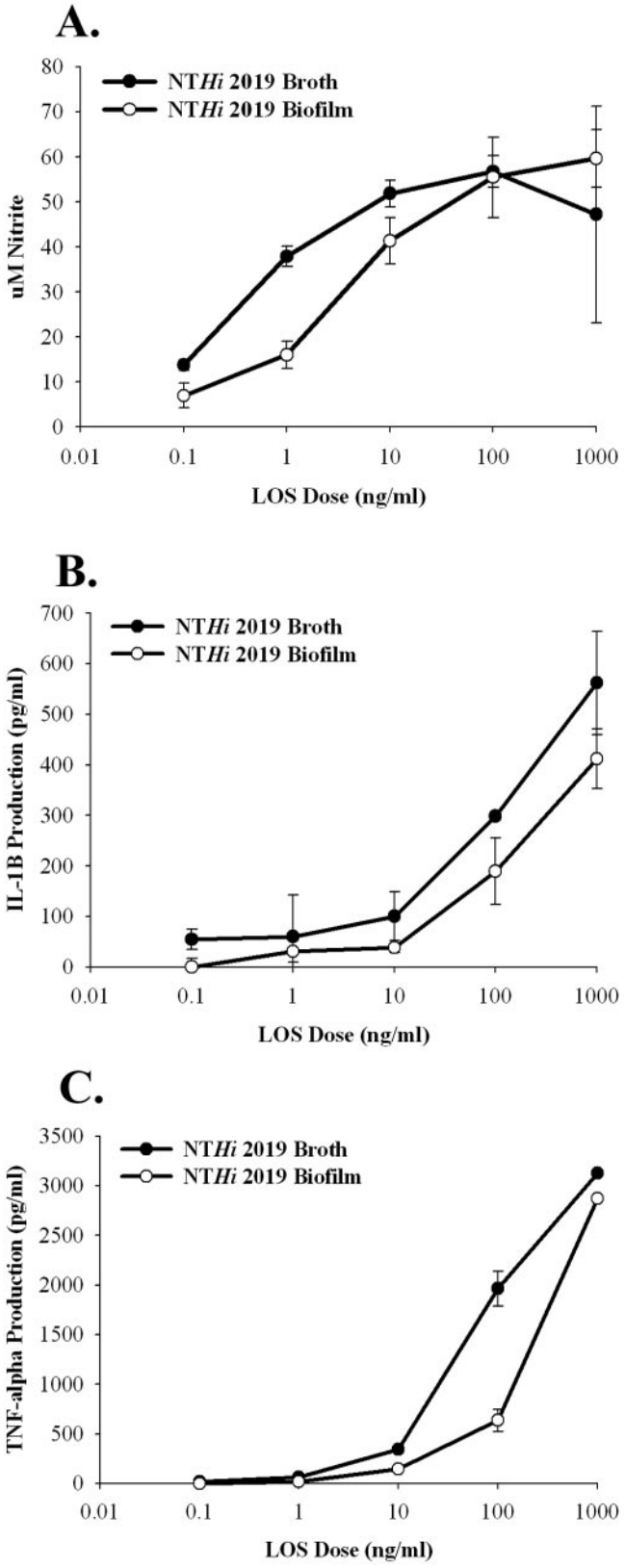

FIG. 6. Lipooligosaccharides purified from NTHI biofilms elicit lower release of inflammatory mediators from immortalized mouse macrophages. Nitric oxide (A), IL-1β (B), and TNF-α (C) were measured in supernatants of HeNC2 (TLR4$^+$) immortalized mouse macrophages ~24 h after treatment with LOS purified from broth (solid circles) or biofilm (open circles) cultures of NTHI 2019, using commercial assays according to the manufacturer's instructions (see Materials and Methods). All experiments were performed in the presence of 10% heat-inactivated fetal bovine serum (HyClone).

bioactivity that was comparable with the parental strain (data not shown).

We also compared host responses to LOS purified from biofilms formed by NTHI 2019 *licD* with LOS from planktonic cultures of this strain. Because these strains lack *P*Cho, this offered a test of the hypothesis that *P*Cho is a determinant of the biofilm-related decrease in LOS bioactivity. The results showed that LOS produced by a mutant lacking *P*Cho has equal potency during biofilm and planktonic growth (Fig. 9). While there were statistically significant differences in nitrite concentrations at some individual endotoxin doses (0.1 and 10 ng/ml), there was no evidence of a dose-dependent difference in potency. We thus conclude that *P*Cho, or a *P*Cho-related LOS modification, contributes significantly to the observed reduction in LOS bioactivity that occurs during biofilm growth.

DISCUSSION

NTHI primarily resides as a commensal in the human nasopharynx and is one of the most common constituents of the normal flora of the human upper airways. Such organisms are by necessity highly adapted for persistence in vivo, and in certain settings can cause chronic airway infections that are difficult to eradicate. Opportunistic infections caused by NTHI almost invariably originate from impairment of host clearance and/or innate defenses and are a major public health problem. In these settings, bacterial factors that promote persistence in vivo may contribute to virulence by impeding host clearance and increasing bacterial colonization.

The data presented in this study show that *P*Cho modifications are increased during biofilm growth. Confocal analysis revealed that *P*Cho$^+$ bacteria were found within the central portion of a mature biofilm. Our prior work showed that the majority of NTHI bacteria adhering to human airway cells are *P*Cho$^+$ variants (49, 50). Thus, it will be important in future studies to define the distribution of *P*Cho$^+$ variants through the course of microcolony formation and subsequent biofilm maturation. It is also possible that environmental conditions within the biofilm promote the establishment and/or maintenance of a *P*Cho$^+$ phenotype. In this respect, it is noteworthy that recent work from another group shows that both the synthesis of acceptor LOS oligosaccharides and addition of *P*Cho are regulated via an oxygen-sensitive regulatory circuit (61). Because available evidence indicates that the interior of biofilm communities is microaerophilic or even anaerobic (60), these data may be consistent with our findings.

While we found no evidence that *P*Cho promotes biofilm formation, these data do not exclude a role for *P*Cho in later stages of biofilm maturation. By analogy, while alginate production is increased in mature *P. aeruginosa* biofilms, and contributes to biofilm density and resistance phenotypes (16), biofilm formation is not dependent on alginate (62). Ongoing

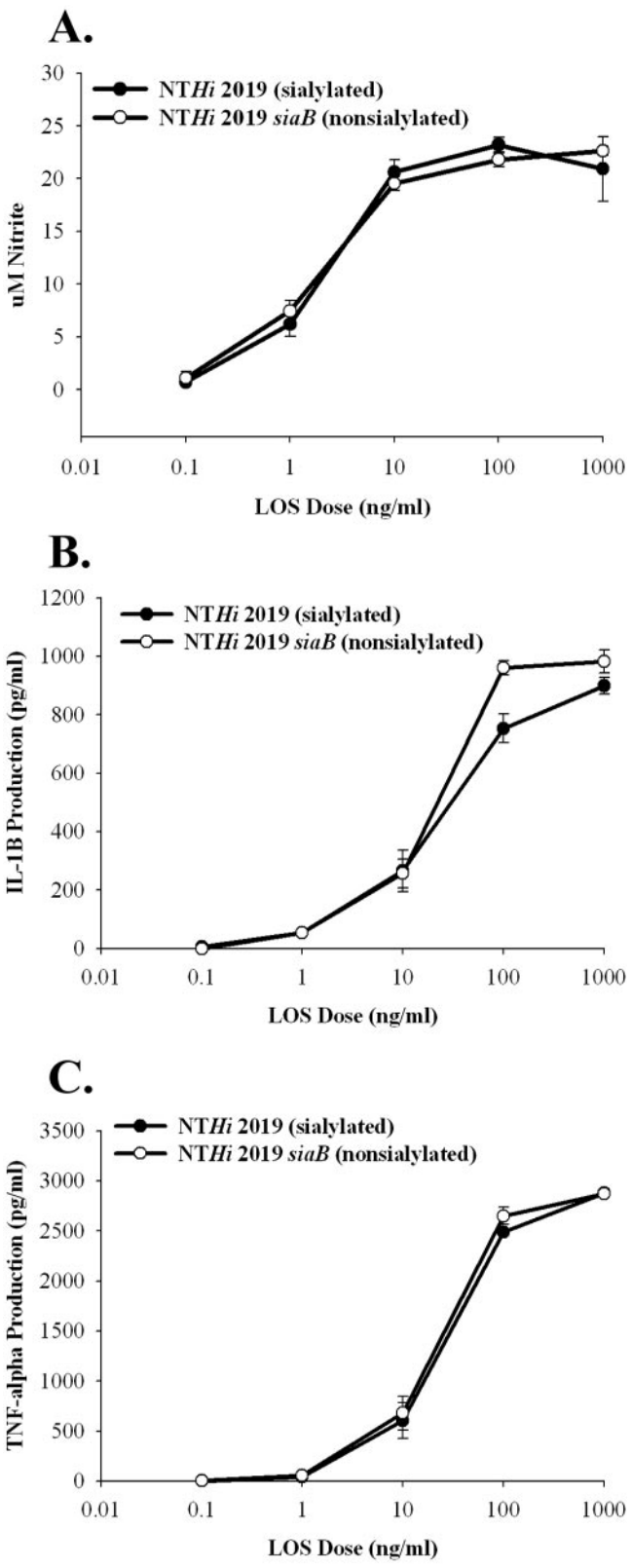

FIG. 7. Effect of sialylation on inflammatory responses to NTHI LOS. Endotoxins were purified from plate cultures of NTHI 2019 (solid circles) and an isogenic *siaB* mutant, which produces an asialyated LOS population (open circles). Nitric oxide (A), IL-1β (B), and TNF-α (C) levels were measured as in the preceding figure.

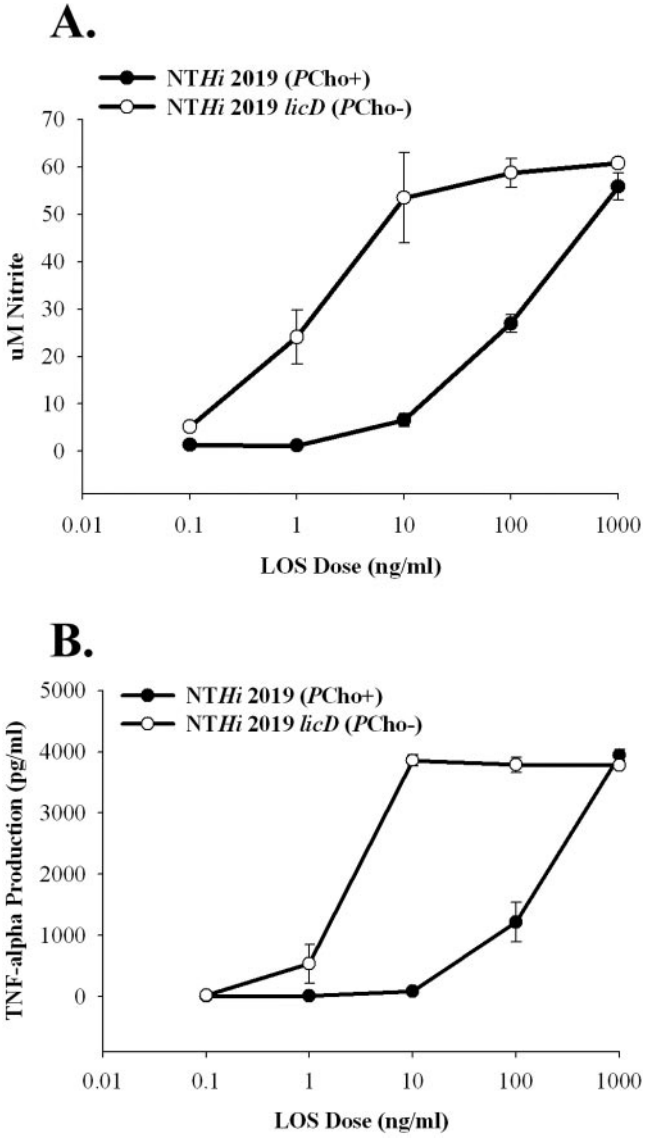

FIG. 8. Effect of *P*Cho on inflammatory responses to NTHI LOS. Endotoxins were purified from plate cultures of NTHI 2019 (solid circles) or an NTHI 2019 *licD* mutant lacking all *P*Cho$^+$ glycoforms (open circles). Nitric oxide (A) and TNF-α (B) levels were measured as in preceding figures.

work in our laboratory will address the role(s) for *P*Cho$^+$ LOS forms in *H. influenzae* biofilms.

Another key finding in this study is that addition of *P*Cho to LOS weakens pattern recognition and subsequent innate responses. It is not presently clear how this decrease in LOS bioactivity occurs, although the finding that TLR4-dependent responses occurring by separate intracellular pathways are proportionally affected suggests that *P*Cho impedes an early stage in endotoxin recognition or signaling. One possible explanation is that the net reduction in oligosaccharide length associated with *P*Cho substitutions (28) changes the properties of LOS in aqueous solution. This is not an unprecedented concept, as there have been reports of reduction in endotoxin bioactivity associated with truncations of the O side chains of

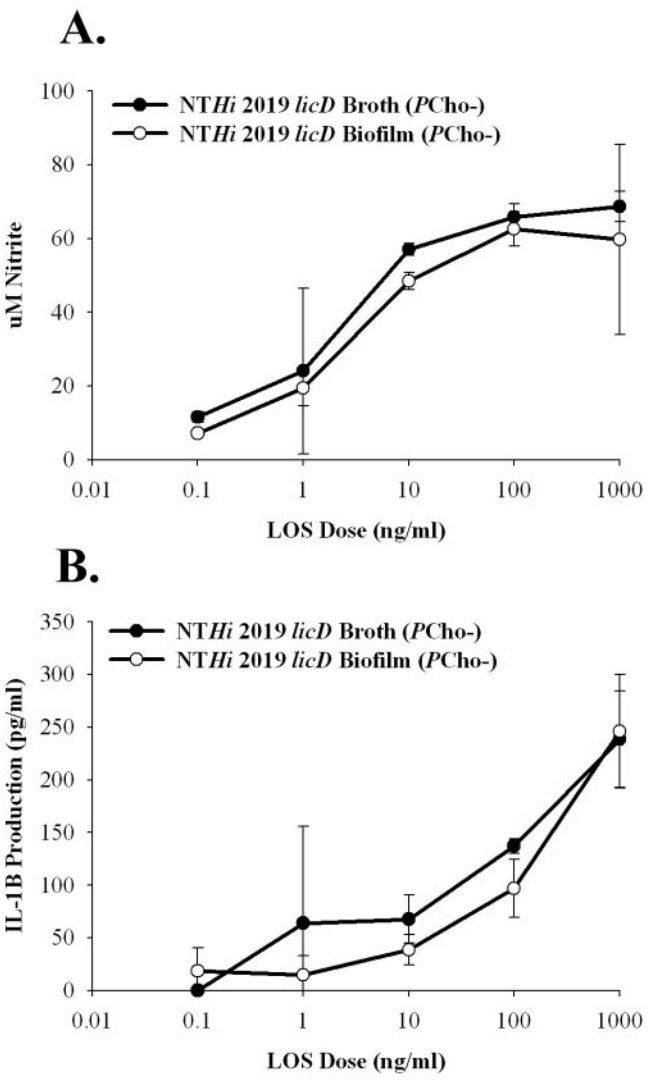

FIG. 9. *P*Cho is required for the shift in responses mediated by biofilm growth. Endotoxins were purified from NTHI 2019 *licD* (*P*Cho$^-$) broth (solid circles) and biofilm (open circles) cultures. The LOS were used to treat HeNC2 macrophages, and nitric oxide (A) and IL-1β (B) levels were measured as described in the text.

lipopolysaccharide (35). Our findings that sialylation does not impact LOS bioactivity would seem to contradict this interpretation, as does recent work with meningococcal endotoxin variants showing that truncation of LOS oligosaccharides does not affect bioactivity (63). Alternatively, it is possible that *P*Cho$^+$ LOS could activate a separate signaling pathway that reduces TLR signaling. Our previous work clearly shows that *P*Cho$^+$ glycoforms elicit signaling in airway epithelial cells via a platelet-activating factor receptor dependent route (52).

A large body of work, including our own, shows that *H. influenzae* populations in vivo are predominantly *P*Cho$^+$ (54, 58, 59). These findings have generally been interpreted to suggest some fitness advantage for *P*Cho$^+$ NTHI variants in the airways. Our data show that biofilm-associated changes in LOS composition blunt host pattern recognition of LOS. Because it is well established that the TLR4 pathway is crucial to clearance of *H. influenzae* from the lung (56), these data imply that *P*Cho$^+$ bacteria might outcompete *P*Cho$^-$ bacteria in vivo. Recent structural characterization of commensal NTHI strains showed that commensal strains tended to produce LOS with high *P*Cho content (25). Clinical epidemiology studies indicate that persistent strains of NTHI and other bacteria are less frequently associated with inflammatory exacerbations of chronic bronchitis than newly acquired strains (37, 44). More recently, Chin et al. observed weaker inflammatory responses to NTHI isolates from prolonged carriage in primary human airway cells and mouse lung (7). In concert with these findings, our data raise the intriguing possibility that modification of NTHI endotoxins with *P*Cho is a bacterial adaptation that impedes host innate responses and clearance.

Bacteria in biofilm communities are generally thought to persist in vivo because of their extreme resistance to immune or pharmaceutical clearance. An important conclusion from this work is that in addition to this resistant phenotype, bacteria within NTHI biofilms are less potent inducers of host innate responses. These responses, which for gram-negatives are largely directed against endotoxin, mediate clearance and containment of the bacterial infection and also initiate the inflammatory responses that underlie most of the pathology associated with chronic airway infections. Our ongoing efforts will address how NTHI populations are shaped by innate responses and what bacterial adaptations promote persistence in the airways. These insights may eventually be used to guide rational design of strategies to treat or even prevent chronic NTHI infections.

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