Biofilm Biology and Vaccine Strategies for Otitis Media Due to Nontypeable *Haemophilus influenzae*

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Abstract

Otitis media (OM) is one of the most common diseases of childhood, and nontypeable *Haemophilus influenzae* (NTHI) is the predominant causative agent of chronic and recurrent OM, as well as OM for which treatment has failed. Moreover, NTHI is now as important a causative agent of acute OM as the pneumococcus. NTHI colonizes the human nasopharynx asymptomatically. However, upon perturbation of the innate and physical defenses of the airway by upper respiratory tract viral infection, NTHI can replicate, ascend the Eustachian tube, gain access to the normally sterile middle ear space, and cause disease. Bacterial biofilms within the middle ear, including those formed by NTHI, contribute to the chronic and recurrent nature of this disease. These multicomponent structures are highly resistant to clearance by host defenses and elimination by traditional antimicrobial therapies. Herein, we review several strategies utilized by NTHI to persist within the human host and interventions currently under investigation to prevent and/or resolve NTHI-induced diseases of the middle ear and uppermost airway.

Keywords
- phasevarion
- Type IV pilus
- PilA
- eDNA
- DNABII proteins
- integration host factor
- EPS

Otitis Media Burden

Otitis media (OM) is one of the most common diseases of children < 15 years of age, with peak incidence between 9 and 15 months.[1] As a result, OM is the primary cause for hearing loss in childhood, which can have a notable impact on behavior, language, and educational development.[2–5] In developed countries, therapeutic and prophylactic antibiotic treatment is typically relied upon for the management of acute OM, and clinical practice guidelines recommend a period of "watchful waiting" for children with less severe disease.[6–9] However, OM is the primary reason for a child to be prescribed an antimicrobial, a fact that is driving the emergence of antibiotic resistance among those bacteria frequently identified as disease-causing agents and not just those that are predominant pathogens of OM.[10–12] Worldwide, 709 million cases of acute OM, and 31 million cases of chronic suppurative OM occur yearly, and while morbidity is uncommon in developed countries, approximately 21,000 children die each year in developing countries as a consequence of this disease.[13]

Surgical management of OM involves insertion of tympanostomy tubes into the tympanic membrane and is the most common surgical procedure for children under the age of 15 in the United States.[14] While effective to relieve pressure and pain due to fluid accumulation in the middle ear space, tube insertion does not prevent OM. Moreover, between 10 and 70% of children develop post-tympanostomy tube otorrhea, a complication for which there is no consensus on effective treatment.[15,16] Therefore, there is an obvious need to develop more effective approaches to the management and prevention of OM. To do so requires an understanding of the strategies employed by potential otopathogens, including nontypeable *Haemophilus influenzae* (NTHI), that promote persistence within the human nasopharynx during colonization and survival in the middle ear upon induction of disease.

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Bacterial Biofilms

One strategy that promotes persistence of NTHI within its host is biofilm formation. The ability of NTHI to build a biofilm contributes to the chronic character of diseases caused by this bacterium, including bronchitis, exacerbations of chronic obstructive pulmonary disease, conjunctivitis, sinusitis, and OM. Moreover, biofilms are associated with prolonged drainage from the middle ear that results from the perforation of the tympanic membrane in chronic suppurative OM and following tympanostomy tube insertion.[15–18] Biofilms are characterized as a community of bacteria, single- or multispecies in nature, often adherent to a surface and encased in an extracellular polymeric substance (EPS).[19] Biofilm-resident bacteria exhibit a reduced metabolism and an altered proteome compared with their planktonic counterparts, features that contribute to their recalcitrance against typical antimicrobial therapies.[20] Clinically, biofilms are present within middle ear specimens and within the discharge collected from patients with otorrhea.[21–23]

The EPS that surrounds and supports bacteria within a biofilm is complex in both molecular composition and structure. EPS shields bacteria from host immune responses and antimicrobials, mitigates the efficacy of surfactants, sequesters nutrients, concentrates cell-to-cell signaling molecules, and slows desiccation (see reviews,[24,25]). As such, development of therapeutic strategies to eradicate bacterial biofilms in the middle ear or the design of vaccines to prevent their formation requires a thorough understanding of the EPS structure and composition. Specific components of the EPS can vary among bacterial species; however, EPS is generally comprised of proteins, polysaccharides, and nucleic acids.[25–27] We and others have investigated the composition of the NTHI biofilm EPS and showed that NTHI proteins OMP P5 and Type IV pilus (Tfp), OMP P2 porin, OMP P6 lipoprotein, and lipooligosaccharide are distributed throughout biofilms formed in vitro and in vivo.[28–33] In addition, extracellular DNA (eDNA) is found in abundance within most bacterial biofilms and is thought to protect against host-derived antimicrobials and other cationic molecules.[34] Moreover, the abundance of eDNA and its unique lattice-like organization observed in vitro and within specimens collected from the middle ear during experimental NTHI infection (►**Fig. 1**) led to the discovery that eDNA also serves as a critical structural component of biofilms formed by NTHI and other medically- and environmentally important bacterial species.[29,35]

Key to the structural integrity of the eDNA lattice is a family of DNA-binding proteins, the DNABII family, which includes integration host factor (IHF) and histone-like protein (HU). Whereas IHF and HU are classically known to bind and stabilize prebent DNA and cruciform structures intracellularly,[36–41] they also play an important role in the stabilization of the eDNA structure within biofilms formed by NTHI and other bacterial species (►**Fig. 1**).[35,42] We have shown that antibodies directed against DNABII proteins induce catastrophic collapse of biofilms formed by many bacterial pathogens in vitro. This disruption is attributed to

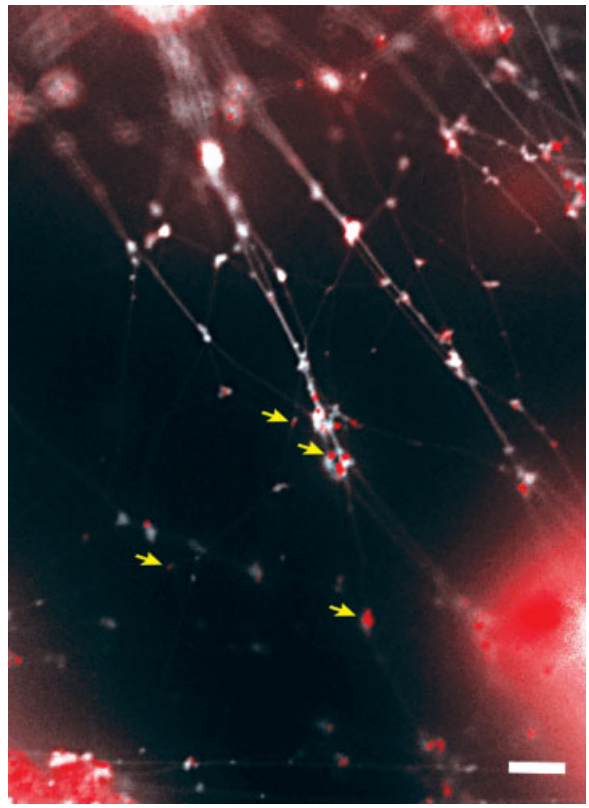

Fig. 1 Nontypeable *Haemophilus influenzae* (NTHI) biofilms formed within the chinchilla middle ear during experimental OM contain abundant extracellular DNA (eDNA) and DNABII proteins in association. Crossed strands of eDNA (white) form a lattice-like structure within the biofilm extracellular polymeric substance (EPS). The eDNA is stabilized by members of the DNABII family of DNA-binding proteins (red, indicated by yellow arrows) which bind at the vertices of DNA strands. Scale bar, 5 μm.

the sequestration of DNABII proteins from the extracellular milieu as the proteins rapidly cycle between "eDNA-bound" and "free" states.[31,33–41] The resulting equilibrium imbalance promotes dissociation of DNABII proteins from the eDNA matrix, destabilization of the eDNA lattice, and subsequent collapse of the biofilm structure (►**Fig. 2A**).[42] As biofilms are the preferred lifestyle for many bacterial species, including NTHI, efforts to understand the composition of the EPS and environmental factors that stimulate the formation of biofilms are an active area of investigation.

Environmental Factors Influence Biofilm Formation

As we and others examine strategies to break down or prevent bacterial biofilms, it is important to understand the factors that influence the formation of these structures, particularly those relevant to the human host. The majority of work on biofilm biology is performed under standard laboratory conditions (i.e., 37°C, 5% CO_2, humidified atmosphere, rich medium) to promote bacterial growth in vitro. However, in the human host, bacteria resident within the nasopharynx experience an average temperature of 34°C, neutral pH, and mechanical and shear stresses due to air and liquid movement

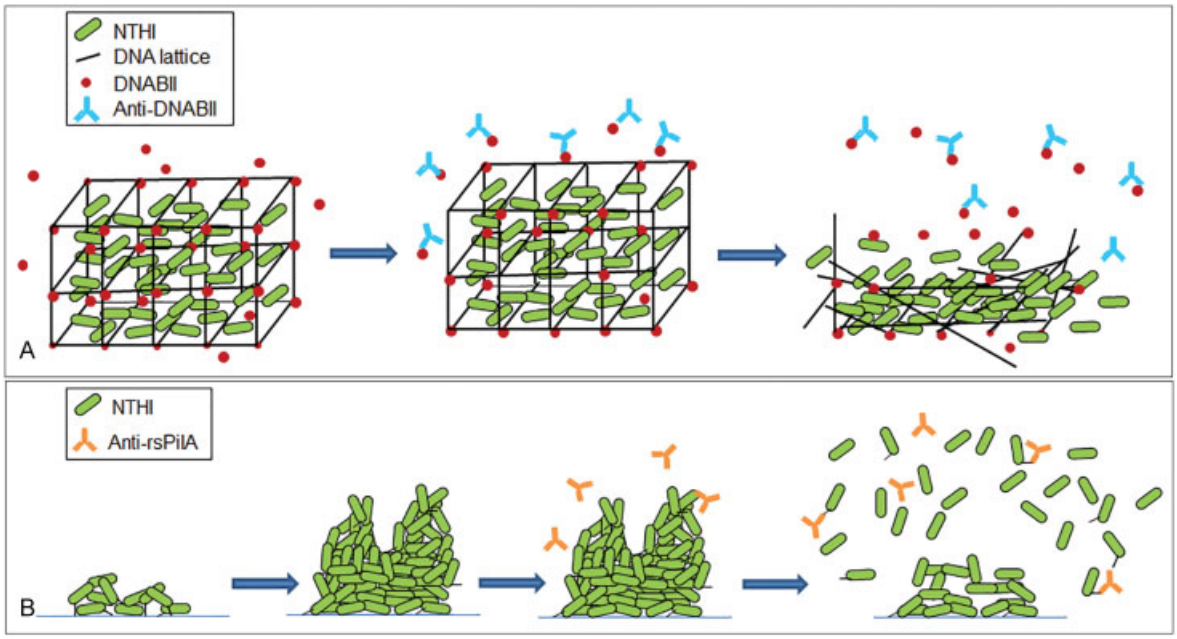

Fig. 2 Two distinct mechanisms to disrupt nontypeable *Haemophilus influenzae* (NTHI) biofilms. (**A**) Biofilms formed by many bacterial human pathogens, including NTHI incorporate extracellular DNA (eDNA) (black lines) and DNABII proteins (red circles) within the extracellular polymeric substance (EPS). Antibodies against DNABII proteins (blue) bind and sequester DNABII molecules from the extracellular milieu and induce an equilibrium imbalance. Release of DNABII proteins from the eDNA scaffold results in catastrophic collapse of the biofilm structure and exposure of resident bacteria. (**B**) NTHI utilize Type IV pilus (Tfp) to adhere, organize, and form a biofilm. Antibodies directed against the majority subunit of NTHI Tfp, PilA (orange) induce a "top-down" dispersal event that is dependent on quorum signaling.

in addition to nutrient limitation.[43–45] In contrast, at the site of disease in the middle ear, the temperature is typically 37°C or greater if fever is present, and middle ear effusions from patients with chronic OM are uniformly alkaline in pH.[46,47] Marks et al observed temperature-dependent variations in transformation efficiency and biofilm dispersal by the nasopharyngeal commensal bacterium and OM pathogen, *Streptococcus pneumoniae*.[48,49] As NTHI also colonizes the human nasopharynx, we examined whether the three-degree temperature difference between 34°C and 37°C affected the expression kinetics of the NTHI Tfp.

Tfp are essential for NTHI adherence, twitching motility, and biofilm formation in vitro and within the middle ears of chinchillas during experimental NTHI-induced OM.[50–53] An additional function attributed to expression of Tfp is competence, and the presence of each gene in the *pil* and *com* operons is required for the uptake of exogenous DNA.[54] Antibodies against an N-terminally truncated, recombinant variant of NTHI PilA, (called rsPilA, for recombinant and soluble PilA), prevent adherence of NTHI to human respiratory tract epithelial cells and inhibit biofilm formation in vitro.[55] Moreover, incubation of preformed NTHI biofilms with anti-rsPilA antibody induces a "top-down" dispersal of bacteria that is dependent on quorum signaling, a process of bacterial communication facilitated by secretion and detection of self-produced signaling molecules (►**Fig. 2B**).[56,57] Of note, the mechanism for anti-rsPilA-induced biofilm "top-down" dispersal is distinct from catastrophic biofilm collapse via anti-IHF antibodies (compare ►**Fig. 2A** and **B**).

Tfp expression (as estimated by *pilA* promoter activity) is significantly greater in biofilms formed at 34°C compared with those formed at 37°C (►**Fig. 3**).[55] Moreover, twitching motility mediated by Tfp is also significantly increased at 34°C compared with that observed at 37°C. Thus, temperature likely contributes to the regulation of Tfp expression and twitching motility and facilitates NTHI adherence and organization into a biofilm under conditions that mimic the dynamic and stressful environment of the human nasopharynx. This conclusion is further supported by evidence that a clinical isolate of NTHI, strain 86–028NP, colonizes the nasopharynx of chinchillas during experimental OM significantly longer than its isogenic *pilA* mutant.[50]

NTHI Tfp expression and twitching motility are also induced under alkaline conditions. This result is particularly relevant in OM, as the pH of chronic middle ear effusions is typically greater than 8.0.[46,47] Interestingly, alkaline pH also provides an optimal environment for mixed NTHI-*S. pneumoniae* biofilms. Tikhomirova et al showed that whereas both bacterial species thrived within a mixed biofilm when grown in medium at a pH of 8.0, NTHI did not survive coculture with *S. pneumoniae* at a pH 7.4.[58] The interaction between NTHI and *S. pneumoniae* is complex, however, and displays either synergy or antagonism, depending on the model system and growth conditions. In addition to pH, nutrient availability and the growth phase of the bacterial inoculum also affect NTHI survival.

NTHI has an absolute requirement for iron to survive; however, the human host normally sequesters this molecule such that it is not freely available. This iron-restricted status changes upon host inflammatory response due to infection, as damage or death of host immune or epithelial cells results in release of iron into the microenvironment. Szelestey et al

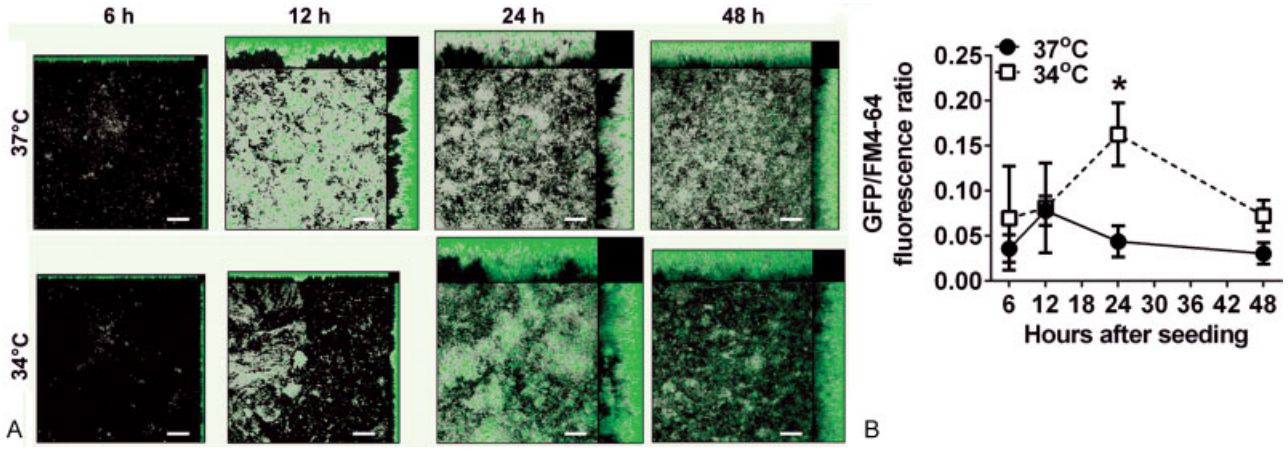

Fig. 3 Expression of Type IV pilus (Tfp), as estimated by *pilA* promoter activity, reaches a significantly higher maximum value in biofilms formed at 34°C compared with biofilms formed at 37°C in vitro. (**A**) Biofilms formed with nontypeable *Haemophilus influenzae* (NTHI) wherein the *pilA* promoter drives expression of green fluorescent protein. Total biomass (as indicated by FM-464 fluorescent membrane stain) is shown in gray, and green areas indicate *pilA* promoter activity. Promoter activity is greatest near the base of the biofilms early on, but as the biofilms mature, regions of intense fluorescence become more prevalent toward the apex of towers. (**B**) Peak *pilA* promoter activity is 3.7 times higher in biofilms formed at 34°C versus 37°C as measured by fluorescent intensity, and this effect is independent of the amount of biomass. $^{*}p \leq 0.05$. Scale bars, 20 µm. Reproduced with permission from American Society for Microbiology, [Journal of Bacteriology, 198, 2016, 2619–2630. doi: 10.1128/JB.01022-15].

examined the outcome of shifts in iron availability specific to NTHI biofilm formation in vitro and in vivo.[59] NTHI initially cultured in medium depleted of heme-iron, then transitioned into medium supplemented with heme-iron (i.e., transiently iron-restricted) formed biofilms with a substantially greater peak height and increased architectural complexity compared with NTHI grown continuously in supplemented medium in vitro. Inoculation of chinchilla middle ears with transiently iron-restricted NTHI mixed 1:10 with bacteria maintained in supplemented medium revealed persistence of 99% of the transiently iron-restricted population after 4 days with less severe middle ear pathology, due to an observed increase in number of intracellular NTHI, compared with middle ears inoculated with NTHI maintained in supplemented medium. These results indicated that changes in heme-iron availability can alter the phenotype of NTHI biofilms and promote NTHI survival and persistence in vivo.

It is clear that traditional culture methods that utilize standard laboratory conditions of temperature, pH, and nutrient availability designed to maximize bacterial growth do not faithfully replicate the environments in which bacterial pathogens exist in vivo, and that changes in these environmental factors can significantly influence biofilm biology and virulence factor expression. To fully understand the survival strategies and expression of virulence determinants by organisms such as NTHI, temperature, pH, and nutrient availability must be considered, particularly in the analysis of potential vaccine targets.

Regulation of Biofilm and Virulence Determinants

NTHI utilizes a variety of mechanisms to regulate the expression of virulence factors required for colonization, immune evasion, and biofilm formation. One important mechanism involves transcriptional regulators that alter bacterial gene expression in response to key environmental changes such as oxidative stress. Reactive oxygen species (ROS), such as hydrogen peroxide and superoxide, are among the primary antimicrobial agents produced by macrophages and neutrophils to kill respiratory tract pathogens. In addition, bacteria themselves produce hydrogen peroxide as a respiratory byproduct, which can reach toxic levels at high bacterial density, such as in a biofilm. In response to oxidative stress, the transcriptional regulator OxyR upregulates the expression of proteins that mitigate damage due to ROS. Proteins regulated by OxyR include catalase, an enzyme that breaks down hydrogen peroxide and Dps which protects DNA from damage by oxygen radicals.[60–62] OxyR is well-characterized as an oxidative stress-responsive transcriptional regulator in many bacteria and is important for NTHI survival and pathogenesis in animal models of disease.[61–63]

In addition to active gene regulation and environmental sensing, human-adapted pathogens including NTHI have genes that undergo phase variation, a random and reversible change in gene expression (see review,[64]). Phase variation of virulence factors allows NTHI to rapidly adapt to microenvironmental changes and evade host immune defenses. Similar to antigenic variation, phase variation results in a subset of bacteria with a unique phenotype not present within the greater population. Dependent on the mutation and microenvironmental stresses, one phenotype will be more advantageous than the other. Phase variable virulence factors include the Hia autotransporter and high molecular weight (HMW) 1 and 2 adhesive proteins.[65–68] Whereas increased expression of Hia facilitates NTHI adherence and nasopharyngeal colonization in experimental models, reduced Hia expression protects the bacterium against opsonophagocytic killing.[67] Similarly, the phase variable promoter region of the NTHI HMW genes controls the expression of these adhesive proteins, which in turn influences bacterial adherence.[68,69] While variable expression of virulence factors is beneficial to the bacterium,

phase variation of potential vaccine targets can greatly decrease vaccine efficacy.

NTHI has additionally evolved a novel epigenetic system of rapid adaptation which regulates a switch in the expression of multiple virulence factors simultaneously. This unique mechanism is employed by multiple human pathogens and is termed the phasevarion, for *phase vari*able regul*on*.[70] Whereas phase variation results in a change in the expression of a single gene, the phasevarion simultaneously regulates the expression of many genes across the genome. This occurs by phase variation in a single DNA methyltransferase (ModA) independent of environmental cues. When expressed, this methyltransferase binds to and methylates sequence-specific sites on the bacterial chromosome, which, in turn, alters expression of genes in the regulon. The result is two phenotypically distinct subpopulations, *modA* ON and *modA* OFF. Within a collection of over 200 NTHI clinical isolates retrieved from the nasopharynx and/or middle ears of healthy and OM-prone children, we identified 21 distinct *modA* alleles.[71] Five phase variable *modA* alleles accounted for over two-thirds of clinical isolates. As *modA2* was the most prevalent allele, we focused our studies thereon.

The phasevarions of multiple NTHI strains control the expression of several outer membrane adhesive proteins, including HMW proteins.[71] Transcriptional analysis of the NTHI strain 723 ModA2 phasevarion also revealed regulation of multiple genes required for iron uptake.[71] NTHI has a strict requirement for heme-iron and maintenance of iron homeostasis is required for survival and pathogenesis in vivo.[62,63] As discussed, availability of heme-iron also influences biofilm formation.[59] In a chinchilla model of experimental OM, Atack et al showed a clear selection for the *modA2* ON subpopulation within the middle ear.[71] Furthermore, a shift from *modA2* OFF status to *modA2* ON status within the chinchilla middle ear results in significantly greater disease severity compared with populations that do not shift status.[72] Middle ears in which NTHI shifted *modA2* status had increased mucosal hyperplasia and edema, and significantly greater NTHI biofilm biomass. Work to identify phasevarion-specific regulation of virulence determinants that contribute to biofilm formation and pathogenesis is necessary and ongoing.

As we continue to develop new methods to treat and prevent bacterial infections, including those due to NTHI, it is critical to understand the regulation of potential vaccine targets under physiological- and disease-relevant conditions. Mechanisms such as phase variable regulation of individual virulence factors and genome-wide regulation by the phasevarion must be considered. Much is still not known about the phasevarions of NTHI and other human pathogens, and phase variation of a vaccine target can severely limit its effectiveness. Continued studies in this exciting new area will be crucial for future vaccine development.

Vaccine Strategies for NTHI-Induced OM

At present, an NTHI-specific vaccine for OM is not yet available; however, prevention of OM has the potential to not only limit disease but also avert the development of OM-associated sequellae.[73–75] Many NTHI surface-exposed proteins, or portions thereof, and lipooligosaccharides are under investigation as potential vaccine candidates. These include several NTHI adhesin proteins (OMP P5, Tfp, Protein E, HMW 1 and 2, and Hia), major NTHI porin protein (OMP P2), outer membrane lipoproteins (OMP P6 and Protein D), and a Skp-like chaperone protein (OMP 26) (see review,[76]). The Pneumococcal Otitis Efficacy Trial (POET) pediatric clinical trial, wherein NTHI Protein D served as a carrier molecule for a pneumococcal conjugate vaccine (PCV), showed 35.3% vaccine efficacy against OM due to NTHI,[77] and whereas these data were the first to demonstrate that immunization against NTHI-induced disease was possible, they also indicated that additional antigens, or combinations thereof, may be necessary to achieve greater protection.

A long-standing approach for vaccine development by our laboratory is to target both adhesive proteins expressed by this bacterium and those proteins essential to the formation and structural stability of its biofilms.[78–82] We have developed three immunogens that demonstrate efficacy against NTHI both in vitro and preclinically in animal models of disease. These include (1) an NTHI Tfp-derived recombinant protein called "rsPilA", which is designed to inhibit NTHI adherence, twitching motility, and biofilm formation;[29,50–52,55,57,83] (2) a chimeric immunogen that targets both NTHI OMP P5 and Tfp, called "chimV4", which is designed to block adherence and pathogenesis of NTHI as mediated by two important adhesive proteins/virulence determinants[83–86] and (3) IHF, a DNA-binding protein that serves as a critical structural element to the eDNA scaffold within the EPS incorporated into biofilms formed by many bacterial species.[17,35,42,87–93]

To date, we have shown that parenteral immunization with rsPilA or chimV4 prevents experimental OM caused by NTHI, likely due to inhibition of adherence and twitching motility, which is mediated by pilus- and OMP P5-specific antibodies present at the respiratory mucosal surface.[83] As an alternative, but potentially equally efficacious strategy, we have also explored the utility of transcutaneous immunization (TCI), the placement of vaccine formulations on to intact skin. TCI offers multiple advantages as an immunization strategy. TCI induces both systemic and mucosal immune responses, an important feature as the mucosae represent critical defensive barriers that also respond immunologically to insult.[94–98] It is also noninvasive, which may aid in acceptance and compliance. With all of these advantages, TCI could promote vaccine distribution beyond developed countries.[99,100] Thus, TCI exhibits potential as an efficacious and simple method to induce protective immune responses and thereby limit disease.

We considered practical application of TCI to humans, particularly to very young children, and envisioned the use of a small adhesive bandage to administer vaccine formulations on to intact skin. In animal models, the postauricular region (skin just behind the ear) was specifically targeted as the anatomical location for the placement of a circular bandaid. The *stratum corneum* at this location is uniquely organized in a vertically linear stacked arrangement, in contrast to the more typical "brick-and-mortar" stratification found in skin elsewhere on the body (►**Fig. 4**).[101,102] TCI via bandaid to

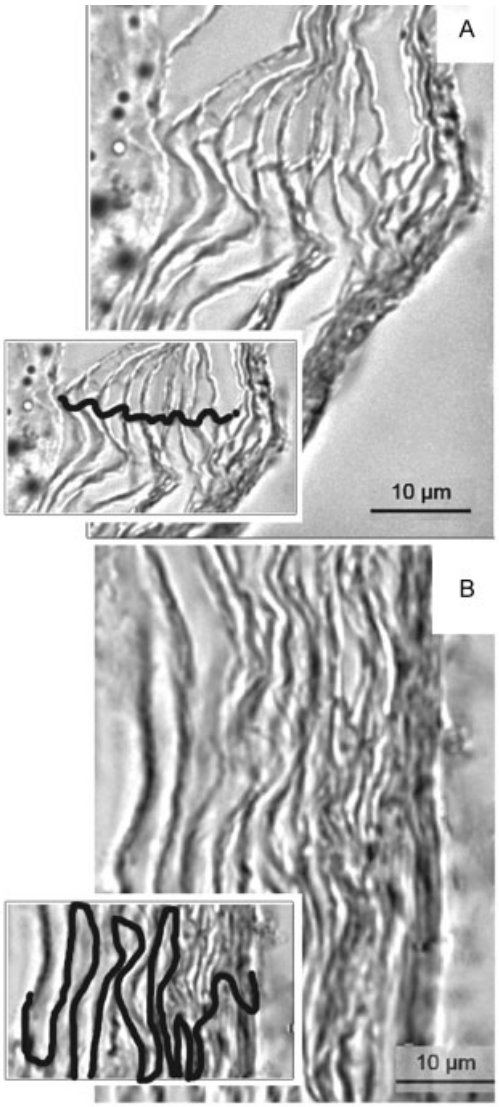

Fig. 4 The stratum corneum at the postauricular region is uniquely organized. To understand any differences in efficacy achieved by transcutaneous immunization via bandaid as related to anatomical placement, skin from the post-auricular region and the nape of the neck on chinchillas were collected and the organization of the cells within the *stratum corneum* was examined by microscopy.[106] (**A**) The corneocytes at the postauricular region were linearly aligned, whereas (**B**) at the nape, a classic "brick-and-mortar" arrangement was observed. Insets, visualization of cell stratification with cellular junctions traced. Scale bars, 10 μm. Reproduced with permission from American Society for Microbiology, [Clinical and Vaccine Immunology, 22, 2015, 867–874. doi:10.1128/CVI.00090-15].

deliver chimV4, rsPilA, or IHF admixed with a potent adjuvant, a derivative of *Escherichia coli* heat-labile enterotoxin LT(R192G/L211A) or "dmLT" to potentiate the immune response induced by these antigens[103] induces an immune response that resolves NTHI-induced OM.[35,57,104–106] Equally important is that TCI via bandaid with chimV4, rsPilA, or IHF also prevented the onset of OM in a polymicrobial chinchilla model that mimics the natural progression of disease in children wherein an upper respiratory tract viral infection predisposes to the development of bacterial OM.[107–110]

The means for TCI-induced efficacy are multifold. Placement of the immunizing bandaid at the postauricular region takes advantage of an atypical cellular arrangement at this anatomical location that permits underlying antigen-presenting cells greater access to topically applied antigens.[111,112] Directed migration of activated antigen-presenting cells, specifically dermal dendritic cells, to the rodent equivalent of the human Waldeyer's ring of lymphoid tissues in the nasopharynx (the nasal-associated lymphoid tissue or NALT) facilitates the induction of an immune response in close proximity to the site of disease within the middle ear.[113] Secretion of interferon-gamma and interleukin-17A by activated $CD4^+$ T-cells within the NALT promotes antibody production by plasma cells and chemotaxis of neutrophils to sites of inflammation (i.e., the infected middle ear) subsequent to bacterial clearance.[7,114,115] Our strategy of using a traditional small circular bandaid placed directly onto the intact skin just behind the ear as a delivery device may provide the opportunity to expand the reach of vaccines against NTHI-induced diseases.

Conclusion

Whereas the licensure and broad use of several pneumococcal conjugate vaccines (PCVs) have indeed had an impact on preventing acute OM due to those serotypes of *S. pneumoniae* included in the vaccine formulations, the impact of these vaccines on OM due to NTHI is much more limited. In some studies, when PCVs are delivered early in life, prevention of first episodes of AOM has been shown to limit subsequent more complex OM due to NTHI.[73] However, if these vaccines are given after the first episode of OM, there is no measurable effect on NTHI-induced OM, and in fact, PCVs are not designed to prevent NTHI-induced OM. Thereby, a broadly protective vaccine to prevent NTHI-induced OM is still of critical need, as is the development of novel therapeutic approaches to treat NTHI-associated diseases of the upper respiratory tract. NTHI is highly adept at biofilm formation, a phenotype that contributes significantly to the chronic, recurrent, and recalcitrant nature of the OM induced by this highly heterogeneous gram-negative bacterium. As studies being conducted by laboratories all over the world contribute to our improved understanding of the unique pathobiology of NTHI-induced OM, including how it adheres, builds biofilms, and responds to both unique microenvironmental cues encountered as it ascends from the nasopharynx to the middle ear as well as to host immune effectors, the knowledge gained will foster the ability to develop highly targeted approaches for disease prevention and treatment. Multiple technological advances, including genomics, proteomics, metabolomics, transcriptomics, high-resolution imaging, more sophisticated animal modeling, and appreciation for the polymicrobial nature of OM, in addition to many others, make this an exciting time for OM-focused research.

Competing Interest

L.A.N., K.L.B., E.M.M., and J.A.J. have no competing interests. L.O.B. is an inventor of technology related to PilA-

derived immunogens, which is licensed to GlaxoSmith-Kline Biologicals. L.O.B. is an inventor of technology related to the DNABII proteins.

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