Haemophilus Sap transporter dependent mechanisms to resist host innate and nutritional immunity influences pathogenesis

Dissertation

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Abstract

Nontypeable *Haemophilus influenzae* (NTHI) is a commensal inhabitant of the human nasopharynx and a causative agent of otitis media, sinusitis and exacerbations in patients with chronic obstructive pulmonary disease and cystic fibrosis. Otitis media is classified as acute, characterized by rapid onset of pain and fever and is often accompanied by erythema and perforation of the tympanic membrane, or chronic, which is often asymptomatic, associated with persistent effusion in the middle ear, and often recalcitrant to antibiotic treatment. During colonization of the host NTHI must acquire essential nutrients and evade host immune clearance mechanisms. Commensal colonization by NTHI is asymptomatic, characterized by low levels of inflammation and sufficient nutrients in the nasopharynx. However, during colonization of the middle ear NTHI encounters a host environment initially depleted of nutrients yet prone to a rapid inflammatory response. We have previously described that the Sap (Sensitivity to Antimicrobial Peptides) transporter, an inner membrane protein complex, mediates NTHI resistance to antimicrobial peptides and heme-iron homeostasis. We therefore hypothesized that Sap transporter function is required for NTHI interactions with the host and establishment of colonization and persistence. We determined that the outer membrane of the sapA mutant is less permeable and more hydrophobic, yet lacks alterations to lipid A,
phospholipids and LOS compared to the parent strain. The outer membrane proteome of the sapA mutant is significantly different than the parent strain, suggesting surface protein changes likely influence outer membrane permeability and hydrophobicity. To investigate the contribution of Sap transporter function on the interactions with the host epithelium, we co-cultured the parent strain or the sapA mutant on the apical surface of polarized epithelial cells at an air-liquid interface. We determined that the sapA mutant was initially less adherent, yet significantly more invasive into the epithelial cells. Upon internalization into the epithelial cells, the parent strain was trafficked to membrane bound vacuoles while the sapA mutant appeared free in the cytoplasm, suggesting that the sapA mutant was able to escape endosomal trafficking. Additionally, we observed a decrease in pro-inflammatory cytokine production by epithelial cells challenged with the sapA mutant compared to those challenged with the parent strain. Consistent with the alterations in cytokine production in vitro, we observed an alteration in cytokine levels in chinchilla middle ear effusions. Furthermore, chinchilla middle ear challenged with the sapA mutant demonstrated a decrease in disease severity compared to middle ears challenged with the parent strain. The decrease in disease severity caused by the sapA mutant is similar to chronic otitis media suggesting that heme-restriction promotes an attenuation of disease severity. To investigate if there is an increase in intracellular populations of the sapA mutant in the middle ear, we used an ex vivo gentamicin protection assay to identify invasive populations. We determined, for the first time, intracellular populations in the middle ear during otitis media. Collectively, our data suggest
that nutrient availability and inflammatory status in the host drives NTHI behavior
toward more acute or chronic infection, such that, transient restriction of essential
nutrients promotes a more pathogenic lifestyle of NTHI.
Dedication

To my family
Acknowledgments

I would not have been able to make it this far without the invaluable support of my family and friends. I would like to thank my parents for listening when I shared the highs and lows and always offering fantastic advice. RJ Krom and Isaac Therneau for being some of the best guys out there, I could not ask for better friends. My brother for being such a great guy, one of the nicest people, and always keeping things interesting. The members of the Mason lab: Blake, Derek, Sam, Catie, Rachel. DeAnna Duvall for always being there and putting up with me no matter my mood, I love you. And Joanna Marshall, I could not imagine a better friend to be in the trenches with.

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1.1 Nontypeable *Haemophilus influenzae*

Nontypeable *Haemophilus influenzae* (NTHI) is a gram-negative coccobacillus that belongs to the family *Pasteurellaceae*. NTHI can grow as both an aerobe and a facultative anaerobe. Additionally, NTHI is a fastidious microorganism that requires environmental sources of nutrients such as beta-nicotinamide-adenine-dinucleotide and heme (81, 129). NTHI is a commensal microorganism of the human nasopharynx. Colonization with NTHI typically occurs early in life, often within the first few months of age. During the first year of life the nasopharyngeal colonization rate is approximately 20% and rises up to 80% in children between the ages of five and six (83, 101, 142, 171). Transient colonization with individual NTHI strains last from a few weeks to a few months. Subsequent exposure to other NTHI strains in the population however, leads to serial colonization (83, 285, 311). Children in day care represent a reservoir for NTHI, there is wide variation in rates of colonization. Individuals are colonized with genetically distinct strains, and over 20% of the strains are shared suggesting that high levels of transmission and sharing of strains is common in pediatric populations (85). Transmission occurs by contact with respiratory secretions or aerosolized
droplets containing NTHI. As a commensal, NTHI presents no known adverse effects during colonization in the nasopharynx, particularly in healthy patients. NTHI microcolony, biofilm formation and persistence in the nasopharynx is due in part to the ability of NTHI to adhere to mucin (200). Humans are the only known reservoir and natural host for NTHI; it is therefore important to understand the associations between the bacterium and the host in both the nasopharynx during commensal colonization and other anatomical locations such as the middle ear during pathogenic interactions.

1.2 Otitis media

Otitis media, or inflammation of the middle ear, is commonly caused by commensal inhabitants of the nasopharynx leading to opportunistic infections. These infections are primarily mediated by NTHI, Streptococcus pneumoniae, and Moraxella catarrhalis, preceded by compromise or impairment of the host mucosal clearance mechanisms, such as ciliastasis caused by viral infection (79). Colonization of the middle ear space can occur by infections with a single pathogen or multiple pathogens (24, 118, 240). Therefore, nasopharyngeal colonization, Eustachian tube dysfunction, and a viral infection of the upper respiratory tract are all factors that contribute to otitis media (29, 30, 57) (Fig. 1). The anatomy and function of Eustachian tube is one of the key factors in preventing colonization of the middle ear. The Eustachian tube is important for drainage, pressure equalization, and protection of the middle ear. However, the
FIG. 1. Commensal to pathogen transition
anatomical structure of the Eustachian tube in children is shorter and more horizontal compared to the adult Eustachian tube and is therefore not as efficient in bacterial clearance because there is less distance for the bacteria to travel and gravity cannot assist in clearance or ability to maintain homeostatic pressure of the middle ear (155, 308). Viral infections, such as those caused by the common cold influenza virus, respiratory syncytial virus, and other respiratory tract infections also predispose patients to otitis media infections. Viral respiratory tract infections can modify the host immune response, impair neutrophil and epithelial cell functions, and alter systemic immunity.

Otitis media has a drastic social and economic impact. Otitis media is one the most common diseases in children and is the leading cause for emergency room visits in pediatric patients and the number one reason for antibiotic prescription in children. It is estimated that annually otitis media costs associated with diagnosis and treatment are over $5 billion in the United States. Frequency of otitis media increases with age in pediatric patients, so that at the age of three, at least one incidence of otitis media is diagnosed in 50% to 85% of children, however six or more episodes are reported in 40% of children at this age (32, 270, 305). Otitis media is classified based on symptoms and duration. Acute otitis media is characterized by rapid onset of pain and fever and is often accompanied by tympanic membrane complications such as erythema or perforation (334). Chronic otitis media is often associated with persistent effusion in the middle ear and persists even after antibiotic treatment. Additionally, chronic otitis media is
characterized by recurrent middle ear infections despite culture negative middle ear effusion. The ability of bacteria to form biofilms in the middle ear space may contribute to the persistence of otitis media infections and failure of treatment and microscopic analysis of bacterial-host cell interactions during the chinchilla model of otitis media, indicate the presence of biofilms. Furthermore, biofilm formation also correlates with increased bacterial load and persistence (77, 135). Biofilms are able to contribute to the chronicity of middle ear infections by increasing the resistance to host immune cell mediated killing, preventing penetration and efficacy of antibiotics and host antimicrobial molecules (38, 94, 95, 312). Increase in mucin production is associated with effusions in the middle ear. Mucins protect the underlying epithelium, maintain mucociliary clearance, and interact with pathogens to prevent both adherence and invasion (73, 157). Production of mucins can affect the characteristics of the mucous layer, such as increasing or decreasing the viscosity of the mucous (156). Inflammatory cytokine production stimulates the production of mucins, indicating their importance in mediating disease (157, 158). Additionally, mucins are an important pathophysiologic mechanism of otitis media; high viscosity of mucous prevents mucociliary clearance (41, 170). It is therefore important to understand mucin regulation and its link towards pathophysiology of otitis media.

1.3 NTHI in chronic obstructive pulmonary disease, cystic fibrosis and rhinosinusitis
NTHI exacerbates patients with chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). COPD is defined as airflow limitation that is not fully reversible and is associated with persistent abnormality in the lung and lung function deterioration (202). In patients with COPD, there is a prominent influx of neutrophils and macrophages in the bronchialveolar lavage fluid, additionally there is an influx in T cells in the lung parenchyma, peripheral and central airways. The T cell populations contain more CD8+ T cells than CD4+ T cells, however the CD4+ T cell populations is hypothesized to be responsible for maintaining the abnormal inflammation. The CD4+ Th1 population is responsible for the chronic inflammation and the Th17 population is implicated in sustaining neutrophil activation in the lungs (272, 322). Additionally, B cells contribute to the chronic inflammation by specific antigen driven process (316). NTHI is the most common colonizing bacterium in patients with COPD, with up to 30% of patients colonized with NTHI in the lower respiratory tract, and colonization by NTHI can persist even after antimicrobial therapy and culture negative sputum (167, 213-215). Acquiring new strains of NTHI results in exacerbations of COPD symptoms, increases the frequency of exacerbations and a decline in lung function (167, 213, 336). Persistent colonization is likely due to the defective immune response; macrophages from patients with COPD are defective in phagocytosis (26). NTHI colonization can induce neutrophil influx, neutrophil necrosis, release of granules, and production of reactive oxygen species all of which damage the epithelial cell barrier (26, 161).
Cystic fibrosis is a recessive hereditary disorder caused by mutations in the CF transmembrane conductance regulator. These mutations cause a dysregulation of electrolytes across the epithelial cell membranes and lead to hyper-osmolar viscous mucous. The changes to the mucosal surface impair mucociliary clearance, and inhibit bactericidal molecule transport and immune cell migration (26). Therefore, CF patients are chronically colonized by pathogens early in life. These chronic infections and the host immune response cause irreversible lung damage (182). NTHI is isolated from the lung of CF patients early in life and multiple strains of NTHI can persist in the CF airways for years (203, 258).

Rhinosinusitis is a group of disorders characterized by inflammation of the nose and paranasal sinuses (25). Bacterial infections are usually secondary to a preceding self-limiting viral infection (25). Bacterial infections are more common in adults and acute bacterial sinus infections affect 0.5% to 2.0% of patients. Acute bacterial infections usually last less than 4 weeks while chronic sinus infections last longer than 12 weeks. A large percentage of rhinosinusitis patients have an associated biofilm in the sinus cavity suggesting an inability of the host to clear the biofilm in the sinus (22, 249, 274).

1.4 Colonization and Adherence

NTHI transmission occurs via inhalation of aerosolized droplets from an infected individual. Upon entry into the nasopharynx NTHI encounters the mucus layer
and the underlying epithelium. NTHI preferentially adheres to mucus, non-
ciliated epithelial cells, and areas of epithelial cell damage. NTHI adherence in
the nasopharynx is a mechanism to resist host clearance mechanisms, most
notably the host mucociliary clearance. To facilitate adherence to the mucosal
surface, NTHI employs a number of adhesin proteins. The NTHI type IV pilus is
a key contributor to biofilm formation both in vitro and in vivo. Additionally, the
PilA protein, the major type IV pilus subunit, is important for adherence to human
respiratory epithelial cells and biofilm formation in the chinchilla respiratory tract
(152). NTHI also expresses a number of type V secretion system, or
autotransporter adhesins. Autotransporter proteins are synthesized as
preproteins, containing at least three required functional domains: an N-terminal
signal sequence, a passenger, and C-terminal outer membrane translocator
domains (287). These domains may be located on separate proteins or all
contained in the same protein. As such, the N-terminal signal sequence directs
trafficking across the inner membrane and is subsequently cleaved; the C-
terminal outer membrane translocator domain assembles into a β-barrel structure
in the outer membrane and serves as the pore for the passenger domain (287).
The prototypic clinical isolate 86-028NP contains three such autotransporter
adhesins: Hap, HMW1, and HMW2. The Hap adhesin is encoded as a single
transcript, and therefore, the protein contains all three required domains. Once
the passenger domain facilitates transport of the Hap protein to the periplasm,
the protein is transported to the bacterial surface, the C-terminal outer membrane
domain inserts itself into the membrane and secretes the passenger domain,
after which there is an autoploteolytic cleavage event, resulting in the release of the passenger domain (Hap\textsubscript{s}) (127, 287). The Hap adhesive properties are tied to the cleaved domain and lead to a paradox in which the Hap\textsubscript{s} domain mediates adherence and invasion to epithelial cells, as well as aggregation and microcolony formation, yet is cleaved from the cell surface (127, 287). It is possible that the Hap\textsubscript{s} domain is secreted as a means to adsorb antibodies away from the bacterium, or the autoproteolysis of the Hap protein is dictated by environmental conditions, which would influence whether Hap\textsubscript{s} remains attached to the cell or is cleaved. 86-028NP also contains two closely related high-molecular-weight adhesins. The HMW adhesins are encoded in an operon composed of three genes, the first gene in the operon (HMW1A, HMW2A) encodes the passenger domain and N-terminal signal sequence and has the adhesive properties and is transported across the inner membrane via the Sec system (107, 287). The second gene in the operon (HMW1B, HMW2B) is analogous to the C-terminal outer membrane translocator domain in the Hap protein and therefore facilitates the transport of the adhesin across the outer membrane. The last gene in the operon (HMW1C, HMW2C) contains glycotransferase activity and mediates N-linked glycosylation of the adhesin (51). The two HMW systems likely originated from a gene duplication event and are very similar; overall the two systems are 71% identical (19, 20, 287). Despite the high levels of sequence identity, the two adhesins recognize different targets on the host cell; the HMW1 adhesin recognizes a glycoprotein receptor containing
an N-linked oligosaccharide chains with a sialic acid in the α2-3 configuration, while the target of the HMW2 adhesin remains unknown (286, 287).

1.5 Biofilms

Biofilms are described as multicellular microbial communities encased and interwoven with a polymeric matrix component (59, 60). Biofilms are composed of metabolically distinct bacterial populations due to heterogeneous penetration of essential metabolites through the extraceulluar matrix and other bacteria. Similarly, biofilms are inherently resistant to antimicrobial treatment, because of both the inability of the antibiotics to penetrate into the biofilm and the decreased metabolic activity (94, 117). Many aspects of the NTHI surface are important in biofilm formation. Sialylation of NTHI lipooligosaccharide (LOS) promotes biofilm formation in both static and continuous flow systems (108, 301). Additionally, phosphoryl choline moiety inclusion into the LOS increases in NTHI biofilms and promotes biofilm formation in a continuous flow system (136,137, 333). Importantly, phosphoryl choline has been shown to modulate the host immune response both in vitro and in vivo in the chinchilla model of otitis media (136, 333). Besides modifications to LOS, NTHI expression of type IV pili on its surface, is essential for both biofilm formation and transformation (13, 42, 151). The NTHI major subunit of the type IV pilus, PilA, has been identified as a promising anti-biofilm drug target; treatments of NTHI biofilms with antibodies directed against the pili are able to disrupt established bacteria (221).
Additionally, extracellular DNA (eDNA) is believed to be the polymeric matrix component of NTHI biofilms. eDNA produced by either NTHI or neutrophil extracellular traps have been identified in NTHI biofilms in the chinchilla middle ear (145, 151).

1.6 Invasion

Although NTHI has classically been characterized as an extracellular pathogen, there is increasing evidence that NTHI can reside intracellular or paracellular between epithelial cell layers. Chronic NTHI infections persist despite antibiotic treatment, the production of bactericidal antibodies, and remain asymptomatic through culture negative periods. It is unlikely that epithelial cell surface or mucous colonization would be undetectable, and it is therefore likely that NTHI resides in intracellular populations in addition to biofilms on the epithelial cell surface. NTHI populations have been identified within and between cell layers in vitro in a number of different cell types including human epithelial cells and macrophages (3, 206). Intracellular populations have also been observed in bronchial epithelial cells and macrophages from patients with chronic lung disease and in adenoid tissue from pediatric patients (17, 91, 130, 204). Multiple NTHI adhesins and surface molecules can promote invasion, however NTHI does not have a dedicated invasin protein. The Hap adhesin described above has been implicated in invasion and was identified as such using a genetic screen. A non-adherent and non-invasive NTHI strain was transformed with a
genomic library from a virulent clinical isolate and tested for its ability to invade using a gentamicin protection assay. The hap gene locus was identified as promoting moderate adherence and low levels on invasion (289). Protein D is a lipoprotein displaying glycerophosphodietster phosphodiesterase activity and has been found in all Haemophilus strains (147, 271). Additionally, Protein D has been implicated in decreasing cilia beating and promoting the loss of cilia in nasopharyngeal tissue and enhancing invasion into human monocytes (3, 147). Protein E, another ubiquitous adhesin, has also been implicated in invasion into respiratory epithelium (266). Furthermore, protein E induces a proinflammatory response in respiratory epithelium characterized by an increase in production and expression of IL-8 and intercellular adhesion molecule-1, leading to a more efficient attachment of neutrophils (266, 267). In addition to protein factors implicated in invasion into host cells, phosphoryl choline moiety incorporation into NTHI LOS also enhances invasion (299). LOS itself is required for adherence to epithelial cells and phosphoryl choline expression is required for high levels of adherence and to promote subsequent invasion. Loss of phosphoryl choline expression, due to phase variation or deletion of the enzymes required for synthesis, results in bacteria which are significantly less adherent than wild type NTHI or NTHI with phosphoryl choline phase variation locked on (299). Additionally, NTHI with LOS glycoforms containing phosphoryl choline colocalized with platelet-activating factor receptors on the epithelial cells surface and a platelet-activating factor receptor antagonists significantly inhibited invasion in a phosphoryl choline dependent manner (299). Furthermore,
phosphoryl choline dependent invasion into epithelial cells required platelet-activating receptor g-protein signaling cascade for internalization, and induced activation of phosphoinositide-3-kinase, which can initiate anti-inflammatory signaling cascades, ultimately leading to negative regulation of TLR-2, TLR-4 and TLR-9 expression suggesting NTHI must influence to host response and decrease inflammation (93, 299).

The multiple surface structures that NTHI has to invade host cells suggest that NTHI is able to initiate host cell signaling and invade through different mechanisms. NTHI lacks Type III and Type VI secretion system effector proteins suggesting that NTHI is unable to hijack the host cell signaling pathways. Many pathogens are able to directly alter host cellular processes to aid in adherence, invasion, replication and dissemination (27). Therefore, NTHI must exploit normal host cell processes to gain access to the intracellular compartment. NTHI has been demonstrated to invade epithelial cells by both actin and microtubule dependent mechanisms. NTHI induces membrane ruffling and the formation of lamellipodia in epithelial cells, which engulf the bacteria to form enclosed vesicles (3, 134, 160, 290, 300, 318). Actin polymerization is associated with this mechanism of invasion; polymerized actin has been observed to co-localize beneath adherent NTHI and surrounding internalized bacteria and the actin polymerization inhibitor cytochalasin D inhibits NTHI invasion (134, 160). Additionally, NTHI invasion can proceed through lipid raft-mediated endocytosis by tubulin polymerization and requiring phosphatidylyl-3-kinase signaling (206).
Internalized NTHI are trafficked to an acidic subcellular compartment that contains late endosome features yet lacks autophagy related markers, these NTHI were identified as non-replicative yet metabolically active (206). These data suggest that NTHI has mechanisms to influence endosomal trafficking. Consistent with these observations for both actin and tubulin mediated invasion, intracellular NTHI identified in adenoid and bronchial tissue samples are enclosed in a vacuole and suggest that invasion in vivo is a viable survival technique for NTHI to replicate and escape the extracellular environment (17, 91).

1.7 Bacterial Trafficking

Phagocytosis and endocytosis are normal host cell processes in which a membrane domain invaginates and is cleaved, followed by transport of the vesicle within the cell (31, 54, 55). Phagocytosis is utilized by specialized immune cells, such as macrophages and monocytes, as a mechanism to eliminate pathogens and extracellular debris. Internalization is initiated by cell surface signaling receptors and transmitted into the cell through Rho family GTPases resulting in actin polymerization and depolymerization events leading to an actin driven protrusion from the cell surface. Following engulfment, the endosome is transported and fused with a lysosome to degrade the cargo by acidicification, acid hydrolases and proteases (72, 268). Endocytosis is mediated through four mechanisms: clathrin mediated uptake, caveolae mediated uptake,
clathrin-caveolae independent uptake and macropinocytosis. Macropinocytosis is mechanistically analogous to phagocytosis (31). Clathrin and caveolae mediated endocytosis is protein coat driven; clathrin and caveolae polymerize and coat the internal face of the plasma membrane to form a curved rigid structure mechanically inducing pit formation. Following pit formation dynamin cuts the neck of the vacuole and the cell internalizes the endosome (128, 315). Clathrin and caveolae independent endocytosis is lipid raft associated uptake. Lipid rafts are dynamic microdomains of the host cell membrane enriched in cholesterol, glycolipids and glycosylphosphatidylinositol-anchored proteins. Lipid rafts are important for host cell receptor clustering and signaling cascades (31). Intracellular pathogens exploit endocytosis machinery as a mechanism to escape the extracellular environment and exploit cellular functions to aid in survival and replication. Pathogens can invade host cells through two main mechanisms: protein injection and interaction with receptors. Type III secretion systems, such as those in *Salmonella* and *Shigella*, can directly inject effector proteins into the host cell cytoplasm to initiate Rho GTPase and Src signaling cascades leading to endocytosis. Other pathogens, such as NTHI, have a direct interaction between bacterial surface proteins and host cells receptors (300). In both mechanisms of invasion, the bacteria induce a reorganization of the host cell cytoskeleton (31). Following invasion into the host cell, the pathogen must escape the endocytic trafficking pathway and subsequent degradation. For example, *Listeria monocytogenes* escapes from the endosome to colonize the cytoplasm, *Mycobacterium tuberculosis* modifies the lysosome to prevent acidification, and
Salmonella arrests the endocytic pathway before lysosomal fusion (5, 39, 268, 296). Bacteria colonizing the cytoplasm must also be able to escape from autophagy. Autophagy is a homeostatic mechanism used by the host cell for bulk degradation and recycling of organelles and proteins (201, 342). Components targeted for autophagy are enclosed in a double membrane structure and directed to an autolysosome for degradation (4). Autophagy is an innate immune effector against intracellular pathogens and is further involved in the adaptive immune system because it is induced by interferon-\(\gamma\) and provides peptides for MHC class II presentation (4, 62, 71, 76, 115, 144, 218, 226). Therefore, NTHI must avoid autophagy to colonize in the cytoplasm.

1.8 Lipooligosaccharide

Lipooligosaccharide (LOS) is the major antigenic component of the NTHI outer membrane (307). LOS is analogous to LPS of enteric gram-negative bacteria, in that it contains lipid A linked by 3-deoxy-D-manno-octulosonic acid to a heterogeneous sugar polymer (102, 307). However, LOS does not contain a repeating O-antigen (187). NTHI mimics many host carbohydrates within LOS as a mechanism to evade immune clearance; these modifications include the addition of phosphoryl choline, N-acetyl-lactosamine, pK antigen, and sialylactosamine (300).

1.9 Phase variation
Phase variation is a random process in a clonal population in which there is a heterogeneous phenotype due to reversible genetic events. These random genetic events provide a mechanism to evade the host immune system or concurrently modify the outer membrane to colonize different anatomical locations, by interacting with a distinct complement of host receptors (307, 329). Therefore, at different locations, there is selection for a derivative that is most fit for that environment. Phase variation can occur through a number of mechanisms including slip-strand mispairing, site-specific recombination and epigenetic changes such as DNA methylation (245, 317, 327). In NTHI slip-strand mispairing occurs via an intra DNA event in which mispairing occurs at a site of short tetra-nucleotide tandem repeats caused by the local denaturation and displacement of DNA strands during DNA replication (176, 245, 327). These mispairing events lead to insertion or deletion of the tetra-nucleotide repeats resulting in either sense or missense RNA products. Additionally the number of tetra-nucleotide repeats can change the efficiency of transcription and the rate of phase variation, and therefore provides another level of regulation (67). A number of genes are associated with phase variation in NTHI and include genes important for iron and heme regulation and transport, membrane protein expression, LOS biosynthesis, epigenetic regulation, and IgA1 protease expression (56, 75, 209, 234, 235, 245, 331, 332). As a mechanism to evade host detection and bind to host receptors, a number of core LOS epitopes are expressed by highly phase variable genes (lic1-3, lgtC). Phase variation of lic1,
which is required for phosphoryl choline expression on LOS, is maintained in a
delicate balance between use for receptor mediated adherence and invasion via
phosphoryl choline- platelet activating factor receptor binding and a target for c-
reactive-protein and complement in serum and effusions (329, 330).
Additionally, lic2 and lgtC, which mediate the attachment of Galα1-4Gal to LOS,
are important for resistance to serum antibody bactericidal activity (330).

1.10 Immunoglobin A1 protease

IgA1 is the most abundant immunoglobin at the mucosal surface and protects the
host from invading pathogens by agglutinating bacteria to inhibit their interaction
with the epithelial cell surface, binding, neutralizing bacterial enzymes and toxins
(159, 164). The IgA1 protease was originally identified in Neisseria and
Streptococcus. The NTHI IgA1 protease is homologous to the Neisseria IgA1
protease. However, in NTHI, the type I IgA1 protease (iga1) is highly
antigenically variable, likely due to horizontal gene transfer and recombination
events between strains (179, 217). However, the high level of heterogeneity of
the IgA1 protease between NTHI strains likely facilitates immune system
evasion. Intriguingly, higher activity of the IgA1 protease correlates with disease
isolates, confirming the importance of evading immune detection (320). The
IgA1 protease is an autotransporter protein containing an N-terminal
trypsin/chymotrypsin-like serine protease domain and a β-helical spine, which
extends the N-terminal domain from the cell surface (149). Cleavage of IgA1
occurs at the hinge and separates the Fab domain from the Fc domain, and IgA proteases are designated as type I or type II based upon site of cleavage (163, 185, 199, 211, 212, 243, 244). The cleavage of IgA1 further serves to protect the bacteria; the Fab domain will still recognize and bind to epitopes on the bacterial surface masking them from detection by uncleaved immunoglobin. NTHI also produces a type II IgA1 protease (*igaB*) which is separate and distinct from the type I *iga1* (87). NTHI strains with the type II IgA1 protease are more likely to be isolated from adults with COPD and form a clonally related group adapted to colonize the COPD lung (87, 88).

### 1.11 Antimicrobial Peptides

Antimicrobial peptides are small cationic (+2 > +9) peptides produced by the immune system and have bactericidal activity at physiological concentrations. Antimicrobial peptides follow a standard structural principle, the amino acid organization in the folded peptide form clusters of hydrophobic and cationic amino acids, which are spatially separated, ensuring that the molecule is amphipathic (344). These small cationic peptides are derived from larger precursor propeptides, which are post translationally processed by splicing, glycosylation, carboxy-terminal amidation, amino acid isomerization, halogenation, or cyclation (281, 304, 344). Host cells and bacterial cells have different membrane properties and these differences are exploited by antimicrobial peptides; bacterial outer membranes have a net negative charge
due to exposed negatively charged phospholipids, while host cells only expose neutral phospholipids on their surface leading to selective targeting of the cationic peptide to the anionic bacterial surface (195, 344). Once bound to the bacterial membrane, the antimicrobial peptides form ion channels, transmembrane pores and ultimately membrane disruption leading to loss of membrane potential, pH gradient and eventual cell lysis. There are multiple suggested models for how antimicrobial peptides mediate cell lysis. The barrel stave model predicts that the peptides insert themselves vertically into the membrane so that the hydrophobic portion of the peptides aligns with the lipid bilayer and the hydrophilic region forms the pore (36, 225). In the carpet model, the hydrophobic regions of the peptides align along the surface of the membrane and disrupt the membrane similar to a detergent (36, 225). In the torodial pore model, the peptides cause the membrane to bend continuously through the pore such that the pore is lined with both the hydrophilic regions of the peptides and the lipid head groups (36, 143).

Humans produce two main classes of antimicrobial peptides, cathelicidins and defensins. Humans only produce one cathelicidin, LL-37. LL-37 is a linear peptide formed from the proteolysis of the CAP18 proprotein in both leukocytes and epithelial cells (1, 15). The bactericidal activity of LL-37 is dependent on the α-helical structure in hydrophobic environments and increased α-helical content correlates with increased activity (231). LL-37 also binds and neutralizes free LPS to protect from septicemia, serves as a chemoattractant for neutrophils,
monocytes, mast cells, T-cells, and induces degranulation of mast cells (16).
cCRAMP, the chinchilla ortholog of LL-37, is expressed in the chinchilla respiratory tissue, including the ciliated columnar epithelium of the Eustachian tube and middle ear, and expression is increased upon colonization by NTHI in vitro indicating that antimicrobial peptide production by the host is a clearance mechanism that NTHI must overcome to colonize the middle ear (198).

Defensins have three distinct families, but only two are produced in humans: α-defensins and β-defensins, which differ in length but share defining rigid anti-parallel β-sheets structurally maintained by three di-sulfide bonds between 6 cysteines (344). The third family member of defensins, θ-defensins, are not produced in humans, however there are six pseudogenes in the genome and some transcription of these pseudogenes has been observed (97). The θ-defensins are believed to be evolutionarily diverse from the other two defensins because there is splicing and cyclization of an α-defensin-like precursors which is absent in the other two members of the family (97). Both immune cells and epithelial cells abundantly produce defensins. α-Defensins have the highest concentration in granules of leukocytes, but are synthesized in precursor cells in the bone marrow as a tripartite prepropeptide composed of a signal sequence, anionic propiece, and c-terminal mature defensin (98, 99, 124, 313). Maturation of the peptide occurs in primary granuoles, which ultimately fuse with phagocytic vacuoles to mediate killing (86, 262). β-Defensins differ from α-defensins in the coordination of the cysteines, having no anionic propiece in the propeptide and
being produced in cells other than leukocytes, most notably they are secreted epithelial cells (97, 119). The local concentration of β-defensins secreted by epithelial cells can reach up to 100µg/ml (97, 280). Defensins also have chemoattractant properties and can recruit monocytes, T-cells and dendritic cells (50, 306, 339, 340).

SPLUNC1 is an antimicrobial peptide, in the bacterial/permeability increasing (BPI) fold-containing protein family found in the mucous of the respiratory tract, and has been demonstrated to kill bacteria, reduce biofilm formation and promote homeostatic mucosal liquid volume by protecting the sodium channel ENaC from cleavage, ultimately promoting mucociliary clearance (28, 100, 197, 345). It was further determined that chinchilla SPLUNC1 is required to maintain middle ear pressure and mucociliary clearance (197).

1.12 Antimicrobial peptide resistance in NTHI

Gram-negative bacteria possess two distinct membranes, each which have a dedicated function. Antimicrobial peptide bactericidal activity is associated with permeabilization of the inner membrane and subsequent loss of membrane integrity and cell lysis (175). It is therefore of upmost importance for bacteria to limit antimicrobial peptide interactions with the inner membrane. NTHI has multiple mechanisms to resist antimicrobial peptide killing including biofilm formation, electrostatic repulsion, and altering the biophysical properties of LOS.
NTHI biofilms do not contain unique polysaccharide such as those found in many other species, however the NTHI biofilm matrix is composed of outer membrane proteins, LOS, integration host factor, and extracellular DNA of both host and pathogen origin (106, 150, 152, 324). Biofilm formation itself serves as a mechanism of host innate immune system resistance, however components of the biofilm matrix have also been shown to promote resistance to antimicrobial peptides (61, 150). The extracellular DNA functions as a negatively charged sink for antimicrobial peptides, the cationic antimicrobial peptides interact with the anionic DNA and this interaction prevents antimicrobial peptide contact with the bacterial cells and reduces the killing potential of the peptides (150).

Antimicrobial peptide electrostatic repulsion by NTHI is mediated by the expression of the positively charged molecule phosphoryl choline on the LOS controlled by expression of the lic1 operon (183, 184). The LOS carbohydrate branches serve as the last line of defense before permeabilization of the outer membrane and there is a correlation between LOS truncation and sensitivity to antimicrobial peptide mediated killing, likely due to increased access to the outer membrane leading to an increase in inner membrane disruption (207).

Heptosyltransferase addition of L-glyco-D-manno-heptose to keto-deoxyoctulosonate by OpsX and glycotransferase addition of glucose and galactose to heptose I and heptose III by LgtF and LpsA respectively are required for resistance, indicating a contribution of the triheptose inner core, sugar extensions on heptose I and II, and digalactose in mediating resistance to antimicrobial peptides (68, 111, 188, 207). Additionally the stability and
hydrophobicity of the outer membrane influences resistance to antimicrobial peptides (341). In NTHI, hexaacylation of lipid A is required for resistance mediated by htrA and htrB (173, 291). Similar modifications to lipid A have been shown to be important in other pathogens; in *Salmonella* hyper-acylation of lipid A promotes resistance to killing and in *Pseudomonas* palmate, aminoarabinose, penta- and hexaacylation are all associated with increased resistance to antimicrobial peptides (78, 114).

1.13 Nutritional immunity

Host nutritional immunity is the delicate balance between limiting free transition metals to exploit the pathogen’s requirement of these ions and using the catalytic potential of the ions for bactericidal activity (138, 325, 326). Transition metals, most notably Fe, Mn, Zn, and Cu, are incorporated into metalloproteins, storage proteins and transcription factors and function as either a non-catalytic, redox catalyst, or non-redox catalyst (138). Fe is the most common redox active metal used in biological systems and plays an important role in DNA replication, DNA transcription and central metabolism, most common of which are oxidoreductases or electron transfer. Because of these important functions, Fe is required by all bacterial pathogens and must be acquired from the host. Therefore, the host has multiple protein systems to withhold Fe from bacteria. Fe is most commonly found associated with protoporphyrin IX (Fe^{2+}) in the macromolecule heme, the majority of heme is bound as a cofactor for oxygen
transport with hemoglobin and found in erythrocytes. Heme is also incorporated into cytochromes, is essential for respiration, metabolism, and biosynthetic processes (7). Free hemoglobin or heme is bound to haptoglobin and haemopexin respectively (138). Fe$^{3+}$ is stored and bound intracellularly by ferritin; Fe$^{3+}$ imported through phagocytosis and then pumped from the phagosome into the cytoplasm by natural resistance-associated macrophage protein 1 (90, 146). Fe$^{3+}$ is sequestered in serum by transferrin and in mucous by lactoferrin. Lactoferrin is also abundant in leukocyte granules, and sequester Fe$^{3+}$ during the mucosal immune response. Fe$^{2+}$ is also associated with bactericidal activity and drives the Fenton reaction, producing hydroxyl radicals (Fe$^{2+}$ + H$_2$O$_2$ > OH$^•$ + Fe$^{3+}$ + OH$^-$).

Mn$^{2+}$ and Zn$^{2+}$ are also actively sequestered from the extracellular environment. Zn is the most common non-redox transition metal in biological systems and coordinates with proteins for structural and catalytic functions (138). Zn is more commonly found as enzyme cofactors in bacteria and in transcription factors in eukaryotes (7). The S100 protein family is a small homodimer or heterodimeric protein complexes that bind two transition metal ions per protein. S100A7 and S100A12 secreted by keratinocytes at the epithelial cell surface, chelates Zn$^{2+}$ (104). The S100A8-S100A9 protein complex (calprotectin) chelates both Mn$^{2+}$ and Zn$^{2+}$ and is one of the most abundant proteins in neutrophils and is released during infections (58). The host transports Mn$^{2+}$ into the cytoplasm by the same
mechanism as Fe$^{3+}$. On the other hand, Zn$^{2+}$ is transported into the phagosome from the cytoplasm to kill engulfed bacteria (34, 35).

Cu$^{2+}$ has long been recognized as having bactericidal activity and accumulates at sites of infection (337). It is believed that part of the bactericidal activity of Cu$^{2+}$ is that catalyzes the Fenton reaction, similar to Fe$^{2+}$. Additionally, Cu$^{2+}$ can disrupt Fe-S clusters, leading to disruption of metabolic processes (138). Intracellularly, Cu$^{2+}$ accumulates in the phagosome after transport from the extracellular space through the Cu$^{+}$ transport protein 1, where it is shuttled to the ATP7A transporter by the ATOX1 protein and transported into the phagosome (165, 166, 335).

1.14 Sap Transporter

The Sap transporter genes were originally identified in *Salmonella* for its critical function of conferring resistance to the antimicrobial peptides protamine, melitin, and to neutrophil extracts (110, 232). Sequence analysis of the genes indicated homology to the permease and ATPase components of the Opp and SpoOK oligopeptide uptake ABC transporter systems (131, 132, 232, 237). The Sap transporter encodes the four-domain structure of an ABC transporter composed of the SapB and SapC permease components and the SapD and SapF ATPases, and a corresponding periplasmic binding protein SapA (Fig. 2). The Sap transporter system has been studied in a number of bacterial species in
FIG. 2. The Sap transporter
addition to *Salmonella* including *Erwinia, Vibrio, Proteus, Pasteurella, Actinobacter, Haemophilus ducreyi* and NTHI (48, 121, 180, 196, 233). In NTHI the Sap transporter is required for antimicrobial peptide resistance, in which the antimicrobial peptides are bound by the SapA periplasmic binding protein and translocated through the SapBC permease components (192, 279). Following translocation into the cytoplasm, the antimicrobial peptides are proteolytically degraded (279). In addition to energizing transport of antimicrobial peptides, the SapD ATPase mediates potassium influx and is utilized to maintain potassium homeostasis and counter the potassium efflux caused by antimicrobial peptide pore formation by providing ATP hydrolysis for the TrkA/TrkH system (192, 260, 294, 295). The SapA periplasmic binding protein shares homology with the DppA, NikA and HpbA periplasmic binding proteins, which bind heme but are absent from the NTHI genome, suggesting that it is involved in heme homeostasis. Concurrent with this hypothesis, the SapA periplasmic binding protein binds heme and transport is mediated by the SapBC permease components (194). The Sap transporter system is required for survival in the chinchilla model of otitis media, and data indicate that both heme transport and antimicrobial peptide resistance are essential functions *in vivo*. Antibody neutralization of the chinchilla β-defensin-1, the chinchilla ortholog of human β-defensin-3, restores the virulence phenotype of the sapA mutant indicating the essential role of Sap transporter dependent antimicrobial peptide resistance during disease progression (279). Additionally, Sap transporter function influences biofilm formation, development and architecture in a heme dependent
manner. Biofilms formed by the sapF mutant and heme starved NTHI biofilms contain more long chain length bacteria and increased filament production (302, 321). Additionally, heme starved NTHI inoculation into the middle ear results in a decrease in disease severity compared to non-starved NTHI, indication the essential role of heme availability in disease progression (302).

1.15 Therapeutic intervention and Vaccine

Biofilm formation in the middle ear presents a clear problem for treatment. Biofilms are recalcitrant to antibiotic treatment and are believed to be the basis for chronic and recurrent otitis media. Effusions from middle ear infections can be sterile, yet bacterial DNA and RNA can be readily detected indicating that there are metabolically active bacterial communities (254). Prophylactic antibiotic treatment can reduce otitis media occurrence, frequency of recurrence, and increase time between recurrent infections (49). However, over prescription of antibiotics can cause many problems by disrupting normal flora and can drive antibiotic resistance. Drug delivery for middle ear infections can be administered by a number of means including topical application, systemic injection (intravenous), transtympanic, and via the Eustachian tube. Localized treatment through the Eustachian tube allows for targeted delivery to the middle ear space and is less prone to side effects, however there is not enough current evidence to support nasal passage delivery through the Eustachian tube (140, 252). Immunization to prevent middle ear infections is currently under intense
investigation and a few promising vaccines have been developed. Because biofilms are concurrent with middle ear infections, adhesins expressed during biofilm growth have been the target of the vaccines. Vaccines generated from recombinant proteins or peptides of outer membrane protein P5 or the type IV pilus major pilin subunit PilA are protective in the chinchilla model of otitis media (13, 96, 148, 151, 152, 216, 221, 282). Furthermore, α-PilA disrupts biofilm formation in vitro further indicating the potential of this vaccine. Additionally, vaccination against protein D, conjugated to pneumococcal polysaccharides, reduced episodes of acute otitis media in infants and this vaccine had a 35.3% efficacy against NTHI (248). Biofilms present a challenge for antibody-mediated clearance by cloaking the bacteria in an extracellular polymeric matrix, therefore alternatives to antibody mediated clearance must be developed. Other vaccines are currently being developed and immunize against the P4, OMP26, and P6 of the NTHI outer membrane. P4 is a phosphomonoesterase and immunization is protective in mice (141). OMP26 is a member of the Skp protein family and is protective in chinchillas and mice (162). P6 is a peptidoglycan associated lipoprotein and is protective in multiple models and provides a protective response in humans (162). It was recently determined that micro RNA silencing regulates otitis media production and inflammatory process in vitro and may play a role in otitis media inflammation and could indicate potential targets to reduce inflammation during otitis media (284).

1.16 Remaining questions
Many questions remain pertaining to NTHI pathogenesis, specifically how does the host environment influence biofilm formation and persistence in the middle ear. Learning more about NTHI survival during pathogenesis will identify new targets for therapeutic intervention, potentially leading to novel antibiotic agents and vaccine targets. Importantly, virulence determinants, which promote chronic and persistent infections, remain to be characterized. It is becoming evident that NTHI invasion into epithelial cells and between epithelial cells layers may be an important niche for NTHI survival and may provide a mechanism to potentiate chronic infections. However, it is unknown if these intracellular populations persist in the respiratory tract and if there is a specific relationship between epithelial cell invasion and pathogenesis. Additionally, it will be important to determine if NTHI invades respiratory tissue, how long NTHI survives in epithelial cells, what is the mechanism of NTHI invasion and how does NTHI avoid immune clearance mechanisms after invasion.

1.17 Project goals and Hypothesis

Recent research has highlighted the link between metabolism and virulence. NTHI is unable to synthesize heme and must acquire it from the host. The Sap transporter mediates heme transport across the inner membrane, therefore inactivation of the Sap transporter to prevent heme transport provides a unique mechanism to investigate the role of essential nutrient uptake during bacterial
pathogenesis. We hypothesize that heme transport provides NTHI with a microenvironmental cue, and that fluctuations in heme availability would serve as a signal for the temporal and spatial localization of NTHI in the respiratory tract. Additionally, the Sap transporter mediates antimicrobial peptide resistance by transporting the antimicrobial peptide from the periplasm into the cytoplasm preventing inner membrane lysis. During commensal colonization in the nasopharynx there must be sufficient heme for NTHI colonization and low levels of inflammation, yet heme must be at a low enough local concentration to prevent bacterial outgrowth and inflammation must be at a low enough level to prevent clearance of commensal bacteria. We hypothesize that low levels of nutrients and inflammation would maintain NTHI in a commensal interaction with the host leading to chronic and subclinical infections in the middle ear, however insufficient nutrients and high levels of inflammation would cause a shift towards acute infections in the middle ear. Additionally, chronic middle ear infections are associated with recurrent episodes of otitis media despite antibiotic treatment and culture negative effusions. Invasive populations of NTHI could be protected from antibiotic treatment and host clearance mechanisms and we hypothesize that the NTHI sapA mutant may invade epithelial cells as a mechanism to evade antimicrobial peptides and gain access to intracellular heme. Therefore, the goals of the studies presented herein were to: (i) study the interactions between the sapA mutant and epithelial cells in vitro, (ii) identify changes to inflammation caused by the sapA mutant, (iii) investigate disease severity caused by the sapA mutant during otitis media, (iv) investigate the role of the Sap transporter in
mediating invasion by NTHI.
Chapter 2. The *Haemophilus* Sap Transporter Mediates Bacterial-epithelial Cell Homeostasis

Abstract

Nontypeable *Haemophilus influenzae* (NTHI) is a commensal inhabitant of the human nasopharynx, and causative agent of otitis media and other diseases of the upper and lower human airway. During colonization within the host, NTHI must acquire essential nutrients and evade immune attack. We previously demonstrated that the NTHI Sap transporter, an inner membrane protein complex, mediates resistance to antimicrobial peptides and is required for heme homeostasis. We hypothesized that Sap transporter functions are critical for NTHI interaction with the host epithelium and establishment of colonization. Thus, we co-cultured the parent or the sapA mutant on polarized epithelial cells grown at an air-liquid interface, as a physiological model of NTHI colonization, to determine the contribution of the Sap transporter on bacterial-host cell interactions. Although SapA-deficient NTHI were less adherent to epithelial cells, we observed a significant increase in invasive bacteria compared to the parent strain. Upon internalization, the sapA mutant appeared free in the cytoplasm whereas the parent strain was primarily found in endosomes, indicating differential subcellular trafficking. Additionally, we observed reduced
inflammatory cytokine production by the epithelium to the sapA mutant strain compared to the parental strain. Furthermore, chinchilla middle ears challenged with the sapA mutant demonstrated a decrease in disease severity compared to ears challenged with the parental strain. Collectively, our data suggest that NTHI sense host environmental cues via Sap transporter function to mediate interaction with host epithelial cells. Epithelial cell invasion and modulation of host inflammatory cytokine responses may promote NTHI colonization and access to essential nutrients.

2.1 Introduction

Nontypeable *Haemophilus influenzae* (NTHI) is a commensal inhabitant of the human nasopharynx, yet can cause opportunistic infections in compromised upper and lower respiratory tracts. As such, NTHI is a leading cause of otitis media, sinusitis, and community-acquired pneumonia and is also commonly associated with exacerbations of chronic obstructive pulmonary disease and cystic fibrosis (109, 168, 214, 215, 265, 277, 288). Commensal and opportunistic colonization of the host requires NTHI to overcome a myriad of host defense mechanisms such as production of bactericidal proteins, physical mucociliary clearance and sequestration of essential nutrients (14, 58, 74, 104, 138, 276, 325, 326). NTHI have developed several successful strategies to evade host immunity and equip for survival *in vivo* including production of IgA1 protease, expression of aggregative adhesins, formation of biofilms and modification of surface lipooligosaccharide (LOS) (2, 9, 120, 135-137, 152, 200, 212, 220, 228,
Gram negative outer membrane components such as LOS can be potent stimulators of the host inflammatory response and serve as binding targets for immunoprotective antibodies, complement proteins and antimicrobial peptides (137, 264, 338). Modification of LOS provides a first line of defense for NTHI, and as such, a mechanism to evade the host innate immune system. For instance, addition of sialic acid to LOS prevents complement deposition and phosphorylcholine (ChoP) modification inhibits the binding of immunoprotective antibodies, further serving to mask bacterial surface charge and minimize antimicrobial peptide binding (9, 301, 333, 338). In addition, LOS modifications induce changes in host epithelial responses. ChoP decorated LOS facilitates attachment to and invasion of the epithelium and signaling through the platelet activating factor receptor (PAFr). ChoP activation of the PAFr signaling cascade down regulates host expression of TLR-2, TLR4 and TLR-9 and promotes NTHI invasion of host epithelial cells (136, 137, 299, 300). Traditionally, NTHI has been categorized as an extracellular pathogen; however, invasion of host epithelial cells can offer a temporary or long-term respite from the host immune response and counter active nutrient depletion by the host. The process of active nutrient depletion, termed “nutritional immunity", is a host mechanism to inhibit microbial growth by sequestering essential nutrients (i.e. iron, zinc, manganese) (58, 104, 138, 325, 326). Recalcitrance to antibiotic therapy, persistence in the presence of protective antibodies and culture negative clinical status suggest that biofilm formation and development of bacterial reservoirs within host cells may contribute to the chronic nature of NTHI infections by...
providing a protected reservoir which can seed subsequent infections (43, 53, 109, 116, 203, 210, 215, 246).

Bacterial mechanisms to acquire essential nutrients and evade innate immune responses are essential for NTHI survival as a commensal in the nasopharynx and pathogen at privileged sites in the host. We have previously demonstrated an essential role for the Sap transporter, a multifunctional inner-membrane ABC transport complex, in resistance to antimicrobial peptide killing and the transport of the essential heme-iron (191-194). Antimicrobial peptides are transported into the bacterial cytoplasm in a Sap-dependent manner and are subsequently degraded by cytoplasmic peptidase activity (279). Similarly, the Sap transporter functions in the uptake of heme-iron. First bound by the SapA periplasmic binding protein, heme-iron is delivered for transport across the cytoplasmic membrane through the SapBC permease complex (194). In addition to the multifunctional roles of Sap transporter function in innate immune resistance and nutrient acquisition, Sap transporter function influences NTHI biofilm development and architecture. NTHI deficient in the SapF ATPase protein developed a more robust biofilm compared to that of the parental strain, coincident with morphological plasticity of NTHI, including increased chain length and filament production within biofilm architecture (321). We have further demonstrated that neutralization of host antimicrobial peptides restores virulence to the sapA mutant in vivo, suggesting an essential role for Sap-dependent antimicrobial peptide resistance during acute phase of disease in the host (279).
Sap transporter function thus serves to maintain NTHI heme-iron homeostasis and persistence in the host, providing mechanisms to resist antimicrobial peptides, aid in nutrient acquisition and influence NTHI biofilm formation.

Due to these essential roles, we investigated the consequence of loss of Sap transporter functions on colonization of epithelial cells. We determined that NTHI deficient in SapA were less adherent to epithelial cells yet were associated with membrane ruffling and epithelial cell destruction. In addition, we observed SapA-deficient NTHI were more invasive and had a decreased immune-stimulatory effect on host epithelium compared to host responses induced by the parental strain. Collectively, these data support an important role for Sap transporter function in NTHI interaction with host epithelial cells. We propose that NTHI utilizes the Sap transporter to sense microenvironmental cues such as heme-iron limitation and antimicrobial peptide production to modulate the host environment, gain access to essential nutrients and evade the innate immune system. Additionally, these data suggest that NTHI residence in the epithelial cytoplasm may function as a bacterial reservoir during chronic infections.

2.2 Materials and Methods

Bacterial Strains and Growth Conditions

The parental NTHI strain 86-028NP::rpsLA128G is a streptomycin resistant strain constructed as previously described (309). Construction of an unmarked, nonpolar deletion mutant of the sapA gene was performed by the recombineering
strategy as previously described (309, 321). Briefly, primers 5'-
AAGTGCAGATGATTATTTGACGAA -3' and 5'-
ACGAGTAATATGATCCGCTTTGT -3' were used to amplify sapA and 1kb of
the flanking DNA both 5' and 3' to sapA. The subsequent amplicon was ligated
into the pGEM-T Easy vector (pFR001) and transformed into E. coli strain
DY380. In parallel, primers, 5'
TAATATGCCTTACAATTTGACACATAATTATCACAATGCATTGTATG 3'
and 5'
CAGAATATGGCGAAGAACCGACCAGAACATTAGTGTTCCTCTGAATAAA 3',
each containing 50 base pairs of DNA homologous to the 5' and 3' ends of the
sapA gene were used to amplify the spec-rpsL cassette from pRSM2832 (309).
This amplicon was then electroporated into strain E. coli DY380/pFR001 to form
strain DY380/pFR002, in which the sapA gene in pFR001 has been replaced by
the cassette. The plasmid pFR002 was then used to transform NTHI 86-
028NP::rpsLA128G and transformants were selected by growth on spectinomycin-
containing chocolate II agar plates. To generate a nonpolar deletion mutant, the
sapA mutant was transformed with plasmid pRSM2947, grown at 32°C, and FLP
expression was induced using anhydrotetracycline. The cells were cured of the
plasmid by growth at 37°C. GFP expressing parent and sapA mutant strains
were created by electroporation of pGM1.1 as published previously (193).

Bacterial strains were grown overnight on Chocolate II agar (Becton Dickinson,
Sparks, MD) and then subcultured into pre-warmed brain heart infusion broth
supplemented with 2 µg heme/mL (Becton Dickinson, Sparks, MD) and 1 µg NAD/mL (Becton Dickinson, Sparks, MD) (sBHI). Cultures were normalized to \(OD_{490}=0.65\), diluted 1:6 in sBHI and grown for three hrs to logarithmic phase at an \(OD_{490}=0.65\). Logarithmic phase bacteria were inoculated onto epithelial cells at a MOI=50.

**Epithelial Cell Adherence Assay**

Adherence of the parent strain and the sapA mutant were determined on epithelial cell monolayers in a 96 well plate. Two µl logarithmic phase bacteria (MOI 50) were inoculated onto confluent monolayers of Chinchilla Middle Ear epithelial cells, A549 human adenocarcinoma epithelial cells (American Type Tissue Collection, Manassas, VA) or Normal Human Bronchial epithelial cells (American Type Tissue Collection, Manassas, VA). After 30, 60, and 90 minutes the cell culture media was removed and the epithelial cell layers were washed three times with 200ul Dulbecco’s phosphate buffered saline (DPBS) (Mediatech, Manassas, VA) followed by a 3 minute incubation with 0.25% Trypsin 2.21mM EDTA in HBSS (Mediatech, Manassas, VA). Cell suspensions were serially diluted and adherent bacteria colony forming units were determined by plating on Chocolate II agar. Adherent bacteria were calculated as a percent of the inoculum. The adherence assay was repeated for a total of three biological replicates on each cell type and significance for CMEE cells was determined by a two-tailed Student’s T-test and two biological replicates for NHBE cells.
Transwell Model of Respiratory Epithelial cell Growth

Normal Human bronchial epithelial cells, Chinchilla Middle Ear epithelial cells and Primary Human Airway epithelial cells were seeded onto transwell membranes and grown to confluency. Confluent monolayers were determined by measuring a resistance of greater than 1000 ohms/4.5 cm$^2$ across the transwell membrane using an Epithelial Voltohmeter (World Precision Instruments, Sarasota, FL). After confluency, cell culture media on the apical surface was removed and epithelial cell growth and differentiation was monitored for two weeks prior to inoculation. Twenty eight point three µl (MOI=50) of logarithmic phase bacteria were inoculated onto the apical surface of the transwell grown epithelial cells in 300 µl DPBS for one hour after which non-adherent bacteria were removed and the epithelial cell surface was washed once with 500 µl DPBS. After 24 hrs, spent media was collected from the basolateral surface and the apical surface was washed once with DPBS and collected. The transwells were fixed for electron microscopy in 2.5% gluteraldehyde (Electron Microscopy Sciences, Hatfield, PA) in DPBS for Scanning Electron Microscopy or 2.5% gluteraldehyde 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) DPBS or treated with TRIzol (Invitrogen, Carlsbad, cA) for RNA isolation.

Scanning Electron Microscopy

After fixation, cells were washed two times in 0.2M sodium cacodylate buffer, followed by subsequent incubation with 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) for 2 hrs, 1% thiocarbohydrazide (Electron Microscopy Sciences, Hatfield, PA) for 2 hrs, and 1% uranyl acetate for 30 mins. After fixation, cells were washed two times in 0.2M sodium cacodylate buffer,
Microscopy Sciences, Hatfield, PA) for 30 minutes, and 1% osmium tetroxide to
enhance electron contrast of osmiophilic structures on the cell’s surface. The
samples were then dehydrated in a graded series of ethanol, followed by critical
point dehydration in hexamethyldisilazane (Electron Microscopy Sciences,
Hatfield, PA) for 15 and then 10 minutes. Between each step the samples were
washed 5 times with double distilled water. Samples were air dried overnight
and adhered to SEM specimen mount stubs with colloidal silver (Electron
Microscopy Sciences, Hatfield, PA). Images were obtained on a Hitachi S4800
Scanning electron microscope at 3kV.

**Transmission Electron Microscopy**

For ultrastructural analysis, transwells were fixed in 2% paraformaldehyde/2.5%
glutaraldehyde in PBS for 1 hr at room temperature. Samples were washed in
phosphate buffer and postfixed in 1% osmium tetroxide (Polysciences Inc.,
Warrington, PA) for 1 hr. Samples were then rinsed extensively in dH2O prior to
en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA)
for 1 hr. Following several rinses in dH2O, samples were dehydrated in a graded
series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Ultrathin
sections of 90 nm were obtained with a Leica Ultracut UCT ultramicrotome (Leica
Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate,
and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA
Inc., Peabody, MA).
NTHI inoculation onto epithelial cell monolayers for microscopy

Normal Human Bronchial Epithelial cells and Chinchilla Middle Ear epithelial cells were grown to confluence on a glass cover slip. Epithelial cells were inoculated with 3.3 µl of mid-logarithmic phase bacteria (MOI 50) in 100µl DPBS. One hour after inoculation, the coverslips were flooded with 1ml DPBS and replaced with cell culture medium. After 24, 72, or 96 hrs, the cells were fixed in 2.5% gluteraldehyde in DPBS for Scanning Electron Microscopy or 2% paraformaldehyde in DPBS for fluorescence microscopy. For fluorescence microscopy epithelial cell membranes were labeled with WGA-Alexafluor 594 (Life Technologies), DNA was counter stained with Hoescht 34580 (Life Technologies. For immunofluorescence microscopy, NTHI was labeled with rabbit α-OMP and detected with α-rabbit GFP.

Cytokine Array

Spent media was collected from the basolateral surface of the transwell model 24 hrs after inoculation with NTHI. Cytokine secretion into the spent media was measured using the Proteome Profiler Human Cytokine Array Kit (R&D Systems, Minneapolis MN). Briefly, media samples were incubated with biotinylated detection antibodies. The cytokine and detection antibody complex was then bound to a cognate antibody immobilized on a nitrocellulose membrane. Relative amounts of the cytokine were detected by measuring streptavidin-HRP chemiluminescence and the fold change in cytokine production was determined by measuring pixel density at each cognate antibody spot.
**Gentamycin Protection Assay**

Invasion of the parent strain and the sapA mutant were determined on epithelial cell monolayers grown to confluence in a 96 well plate. Two µl logarithmic phase bacteria (MOI 50) were inoculated onto confluent monolayers of Normal Human Bronchial epithelial cells. After adherence for one hour the cells were washed once with DPBS to removed non-adherent bacteria. 24 hrs after inoculation the wells were washed once with DPBS and then treated with 50µg/mL gentamycin (Sigma Aldrich) in tissue culture media for one hour. Following gentamycin treatment, the epithelial cells were lysed in 0.1% Triton X-100 (Fischer Scientific, Fair lawn, NJ) in DPBS and number of invaded bacteria were determined by protection from gentamycin killing and enumerated by serial dilution and plating. The gentamycin protection assay was repeated for a total of three biological replicates and significance was determined by a two-tailed Student’s T-test.

**Animal Studies**

Healthy adult chinchialls (*Chinchilla lanigera*) purchased from Rauscher’s Chinchilla ranch (LaRue, OH) were used to assess disease progression after inoculation with either the parent strain or the sapA mutant. Chinchillas were anesthetized with xylazine (2mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and ketamine (10mg/kg, Phoenix Scientific Inc., St. Joseph, MO) and middle ears were challenged with 2500 CFU/ml of either the parent strain or the sapA mutant by transbullar inoculation. Three days post inoculation the animals were
sacrificed and the middle ears were fixed for histological examination. Middle ear samples were fixed in 4% paraformaldehyde in DPBS, decalcified, paraffin embedded, section and stained with hematoxylin and eosin.

2.3 Results

Loss of SapA decreases adherence to epithelial cells

Sap transporter function maintains NTHI heme-iron homeostasis and persistence in the host, providing mechanisms to resist antimicrobial peptides, acquire heme and influence NTHI biofilm formation (191-194, 279). The host microenvironment at the mucosal surface challenges NTHI survival through nutrient limitation (i.e. heme-iron) and bactericidal innate immune effectors (257), thus we hypothesized that Sap transporter functions are critical for NTHI interaction with the host epithelial cells and establishment of colonization. To investigate the influence of Sap transporter function on this critical initial interaction we monitored NTHI adherence to anatomically and species variant primary epithelial cells: normal human bronchial epithelial (NHBE) cells and chinchilla middle-ear epithelium (CMEE). The chinchilla has been used extensively as a model for NTHI-mediated otitis media and cells established from chinchilla middle ear explants have been used to study NTHI-host epithelial interactions (33, 222). NHBE cells are derived from donor epithelial tissue and used as a physiological model to study upper respiratory tract infections (257). The parent and sapA mutant strains were evaluated for adherence to epithelial cell monolayers. Independent of epithelial cell source, we observed a reduction in the ability of a sapA mutant
to adhere to epithelial cells compared to adherence of the parent strain (Fig. 3). NTHI has been shown to preferentially adhere to non-ciliated cells of the respiratory tract, and utilize host structures such as ICAM-1, CEACAM1, sialic acid-containing lactosylceramide, glycoproteins related to heparan sulfate and mucin and plasma membrane receptors (2, 11, 33, 89, 92, 152, 169, 220, 228, 267, 286, 287, 299, 314). Therefore, the disparity in NTHI adherence to these different cell types was not unexpected. In fact, the higher binding efficiency to the CMEE cells may have allowed for better detection of changes in adherence in the absence of SapA to these cells. These results demonstrate that the Sap transporter function can influence adherence of NTHI to epithelial cells.

**Loss of SapA perturbs NTHI-epithelial cell interaction**

Since we observed a reduction in the adherence properties of a mutant deficient in SapA, we sought to determine the effects of this mutation on colonization and biofilm formation on the surface of host epithelial cells. The importance of biofilm formation in the resistance to antimicrobial peptides and antibiotic therapy has been well established and contributes to persistence in the host (40, 247, 301). In addition, biofilm formation is influenced by host microenvironments limited in available iron, particularly important as the host sequesters free iron as a means to limit bacterial growth (6, 18, 205, 223, 224, 230, 256, 261, 343). We have demonstrated that transient restriction of heme-iron enhances biofilm structural complexity and peak height in wild-type NTHI (302). Since microenvironmental cues can influence biofilm formation, we hypothesized that Sap transporter
FIG. 3. Adherence and epithelial cell surface remodeling in the absence of SapA. NHBE (A) and CMEE (B) cells were inoculated with either the parent strain (white bars) or the sapA mutant strain (black bars). Numbers of bacteria adherent to the epithelial cell monolayers were determined at each time point and depicted as the mean adherent bacteria ± standard deviation of triplicate wells from three (B) or two (A) independent experiments. The asterisk depicts a significant change in adherence between the sapA mutant and the parent strain ($P<0.05$). The parent strain (C,E) or the sapA mutant (D,F) GFP reporter strains (green) were inoculated onto CMEE cell monolayers (red), incubated for 72 hrs and monitored for colonization and epithelial cell surface changes by confocal microscopy. Panels E and F are three-dimensional rendered optical sections of the bacterial-epithelial cell interface.
functions contribute to NTHI colonization and biofilm formation on host epithelial cells. NTHI inhabits a number of different types of epithelium in the host ranging from the oropharynx, middle ear and lung epithelium. The best representative cultured cell condition to model the privileged middle ear is CMEE cells. Since we observed a more pronounced adherence defect by the sapA mutant on CMEE monolayers (Fig. 3), we examined colonization and biofilm formation by the parental or sapA mutant strain on these cells. To that end, parent or sapA mutant GFP reporter strains were co-cultured with CMEE cell monolayers for 72 hrs, washed to remove non-adherent bacteria, fixed, and monitored for community development and epithelial cell membrane perturbations. Epithelial cell membranes were labeled with Alexafluor 594-conjugated wheat germ agglutinin (WGA) and visualized for bacterial interaction by confocal microscopy. We observed that the parental strain was primarily cell-surface associated as a biofilm (Fig. 3C). In addition, three-dimensional rendered optical sections of the bacterial-epithelial cell interface revealed little perturbation of the epithelial cell membrane as indicated by green (bacteria) and red (epithelial cell membrane) pseudocolor in different focal planes (see orthogonal view, Fig. 3E) supporting surface colonization by the bacteria. In contrast, the sapA mutant remodels the epithelial cell surface resulting in membrane perturbations that enveloped the bacteria (Fig. 3D, F). In fact, the sapA mutant was observed in the same focal plane as the epithelial cell membrane, in many cases extending 20 microns above the planer surface, suggestive of epithelial cell membrane ruffling and compromise of the epithelial cell membrane integrity (see orthogonal view, Fig.
We observed similar results when the parent strain or sapA mutant was co-cultured with NHBE cell monolayers (Fig. 4). These observations were thus independent of epithelial cell type and initial levels of adherence (compare Fig. 3A, B, and Fig. 4).

The membrane changes associated with colonization by the sapA mutant suggested the sapA mutant had penetrated the epithelial cell membrane and colonized the cytoplasm. To more closely examine the cellular localization of NTHI on and within epithelial cells, NTHI were co-cultured with NHBE monolayers for 24 hrs, washed to remove non-adherent bacteria and surface exposed NTHI were immuno-labeled. Invasive bacteria, which were inaccessible to immuno-labeling, were subsequently identified by DNA counter labeling. Thus, surface-associated bacteria were detected by punctate fluorescence (pseudocolored green), whereas internalized bacteria were visualized as white. Epithelial cell membranes were labeled with WGA and visualized for bacterial localization by immunofluorescence microscopy. We observed the parental strain colonizing the surface of the epithelial cells, predominantly visualized by punctate green fluorescence (Fig. 5B). In contrast, we observed that the sapA mutant localized to both the surface and cytoplasm of the epithelial cells (Fig. 5C). Cytoplasmic localization of the sapA mutant was indicated by white DNA counter labeling throughout the cytoplasm of the epithelial cells. The fluorescence of the bacteria in the cytoplasm in conjunction with the absence of Moreover, the intensity and proximity of the signal is indicative of bacterial
FIG. 4. Sap dependent epithelial cell surface remodeling. The parent strain (A, C) or the sapA mutant (B, D) GFP reporter strains (green) were inoculated onto NHBE cell monolayers (red), incubated for 96 hrs and monitored for colonization and epithelial cell surface changes by confocal microscopy.
**FIG. 5. Epithelial cell invasion in the absence of SapA.** NHBE cell monolayers (A, D) were inoculated with the parent strain (B, E) or the sapA mutant strain (C, F), incubated for 24 hours and monitored for colonization by scanning electron microscopy (A-C) or fluorescence microscopy (D-F). Scale bars represent 10 µm (panels A-C) or 20 µm (panels D-F). NTHI cells were labeled with a α-OMP antibody and detected by α-rabbit FITC antibody (green). Epithelial cell membranes were stained with Wheat Germ Agglutinin conjugated to Alexfluor-594 (red) and DNA was counter stained with Hoescht 34580 (white). White arrows indicate cytoplasmic NTHI.
antibody labeling is highly suggestive for internalization of the sapA mutant. Microcolonies within the cytoplasm. Collectively, these data suggest that Sap transporter function mediates NTHI adherence, influences colonization of epithelial cells and maintains epithelial cell membrane homeostasis.

Next, we sought to better define bacterial-host interactions in conditions that more closely mimic bacterial infections of the respiratory tract (257). Polarized, differentiated NHBE cells were grown on semipermeable transwell membrane supports at an air-liquid interface; similar to epithelial cells of the respiratory tract, the NHBE formed polarized semi-stratified cell layers that produce mucus and express microvilli and cilia. Therefore, bacteria co-cultured with the apical surface of the epithelial cells must colonize at the air-exposed surface and acquire nutrients from the epithelial cell, unlike colonization of epithelial cell monolayers which are submerged in nutrient rich media (257). Thus, to determine the influence of the Sap transporter on colonizing the nutrient limited, apical surface of epithelial cells we exposed polarized NHBE cells to either the parental strain or the sapA mutant strain for 24 hrs and bacterial-host cell interactions were visualized by SEM. The parental strain colonized the apical surface by formation of microcolonies or larger biofilm communities (Fig. 6C, E). We typically observed small clusters of the parent strain in patches on the epithelial cell surface; these patches appear to form in minor lesions on the epithelial cell surface. The lesions are absent in the control cells (Fig. 6A, B) suggesting that they are triggered by colonization of the bacteria. Despite the
presence of small lesions there was very little disruption of the epithelial cell layer integrity (i.e. the surface appears relatively smooth). In contrast, although able to colonize the apical surface, the biofilms formed by the sapA mutant were more dense and coincided with evidence of epithelial cell destruction associated with bacterial colonization (Fig. 6D, F). In contrast to the parental strain (Fig. 6G), populations of the sapA mutant were often associated with “honey comb”-like structures which seemed to have been left behind by bacteria that were disassociated during the processing of the samples (Fig. 6H). The “honey comb”-like structures appeared to be epithelial cell in origin. Consistent with our observations with polarized NHBE cells, we also observed enhanced biofilm formation and paracellular localization by the sapA mutant on CMEE cells (Fig. 7). Thus, our observations of colonization on polarized NHBE cells were similar to our observations on epithelial cell monolayers, both strains colonized the surface of the epithelial cells and the sapA mutant modified the epithelial cell surface and was associated with epithelial cell membrane perturbations. However, we observed small lesions induced by both the parental strain and the sapA mutant in this nutrient restricted air exposed microenvironment. Furthermore, the biofilms formed by the sapA mutant were associated with epithelial cell destruction and invasion by the sapA mutant. Thus, the polarized tissue culture model enabled us to study colonization of NTHI using a more relevant, nutrient-restricted microenvironment. Further, the presence of NTHI within adenoids and bronchial epithelium suggests that an invasive phenotype (17, 91, 130, 204, 206). Invasion of epithelial cells could provide NTHI with
FIG. 6. SapA mediates epithelial cell surface colonization by NTHI. Polarized epithelial cells grown at an air-liquid interface (A, B) were inoculated with the parent strain (C, E, G) or the sapA mutant strain (D, F, H) and incubated for 24 hours. NTHI-epithelial cell interactions were monitored by scanning electron microscopy.
FIG. 7. SapA mediates epithelial cell surface colonization by NTH on polarized CMEE cells. Polarized CMEE cells grown at an air-liquid interface (A, B) were inoculated with the parent strain (C,D) or the sapA mutant strain (E,F) and incubated for 24 hours. NTHI-epithelial cell interactions were monitored by scanning electron microscopy.
may coincide with the chronic nature associated with NTHI-mediated diseases an
environment rich in nutrients and a refuge from immune pressures. Our previous
data indicates an increased propensity for the sapA mutant to disrupt the
epithelial permeability barrier and invade bronchial epithelial cells (Fig. 7C).
Therefore, we sought to further investigate this hyper-invasive phenotype and
determine the ultrastructural localization of the parental strain or the sapA mutant
following co-culture on the apical surface of polarized NHBE cells by examining
cross sections of the epithelium by transmission electron microscopy. We
observed that the parental strain primarily colonized the apical surface of the
epithelial cells with little evidence of membrane perturbations (Fig. 8A,C),
consistent with our previous results (Fig. 3C, 5C, 5E, 5G). This rather benign
surface association did not preclude the ability of NTHI to invade as the parent
strain was also observed to localize to the cell cytoplasm, typically in membrane
enclosed vacuoles (Fig. 8C). Although the fate of these intracellular bacteria
appeared to be detrimental, as observed by loss of bacterial density and
membrane integrity within the presumed phagosomal vacuoles, these data are
consistent with previous observations of NTHI intracellular invasion (257). In
contrast, although the sapA mutant also colonized the apical surface, we
observed enhanced epithelial cell membrane perturbations, disruption of the
epithelial membrane barrier and populations of the sapA mutant in the cytoplasm
of epithelial cells lacking intact cell membranes or between the epithelial cell
layers of bacteria in the cytoplasm of the epithelial cells (Fig. 8B,D).
Interestingly, these invasive bacteria did not appear contained within a membranous compartment but appeared to colonize the host cytoplasm, in most cases retaining bacterial density and membrane integrity (Fig. 8B,D). Similarly, we observed invasive phenotypes of the parent and sapA mutant in primary differentiated chinchilla nasopharyngeal epithelial cells (data not shown), suggesting differential mechanisms of intracytoplasmic trafficking and survival of the sapA mutant compared to the parental strain. The hyper-invasive phenotype of the sapA mutant was confirmed by gentamycin protection demonstrating a significant increase in invasion by the sapA mutant compared to the parental strain (Fig. 8E). In addition, we observed outer membrane vesicle (OMV) production by NTHI at the epithelial cell surface (Fig. 8E) by the parent strain and both within and between NHBE cells by the sapA mutant strain (Fig. 8F). These data are consistent with recent observations of NTHI OMV production following long-term (5 day) colonization of EpiAirway cells, a commercially available cell model system (257). However, our observations indicate that OMVs are not only produced rapidly, one day following infection of polarized NHBE cells, but are shed differentially at subcellular locations of NHBE cells. Previously, we demonstrated that NTHI OMVs are internalized by host epithelial cells and trigger host cell signaling and cytokine production (278). These data, in addition to observed hyper-vesiculation of the SapA-deficient strain when compared to that of the parental strain in vitro (SWS and KMM, unpublished), implicate an important role for OMV production in NTHI-host cell interactions, currently under investigation in our laboratory.
Collectively, these data indicate that loss of Sap transporter function promotes a hyper-invasive phenotype of NTHI, and further suggests a role for the function of the Sap transporter in mediating a homeostatic interaction with host epithelial cells. Invasion into epithelial cells may benefit survival of NTHI in nutrient restricted microenvironments as a mechanism to gain access to available nutrients in the cytoplasm of the host epithelium. Our observations suggest that invasion of the sapA mutant may compensate for the loss of Sap transporter function to counter nutrient starvation.

**Sap transporter function mediates epithelial cell stimulation and cytokine response to colonization**

The epithelium provides the host with a first line defense to the external environment (153). In addition to providing a selective barrier, the epithelium is capable of mounting an inflammatory response by the secretion of antimicrobial molecules and the production of cytokines after stimulation by conserved microbial structures. Signaling pathways are often manipulated by bacteria to gain access to the cytoplasm in non-phagocytic cells (84). Our data suggest that the sapA mutant compensates for the loss of Sap transporter function via altered host cell membrane interactions and invasion of epithelial cells. The epithelial cell membrane perturbations and hyper-invasion of the epithelial cells by the sapA mutant suggest and alteration in epithelial cell homeostasis. To investigate this change in epithelial cell homeostasis we monitored cytokine secretion by the
FIG. 8. Loss of SapA promotes a hyperinvasive phenotype. Polarize epithelial cells grown at an air-liquid interface were inoculated with the parent strain (A,C) or the sapA mutant strain (B,D) and incubated for 24 hours. The subcellular ultrastructural localization of the parent strain or the sapA mutant was determined by transmission electron microscopy. (A-D) Black arrows indicate bacteria that colonized the epithelial cell surface and white arrows indicate bacteria present in the cytoplasm of the epithelial cells. (E) The parent strain produced outer membrane vesicle at the epithelial cell surface and (F) the sapA mutant strain produced outer membrane vesicles both within and between NHBE cells by, black triangles indicate vesicles. (G) Gentamycin protection assay depicts a ~2 fold increase in invasion of the sapA mutant compared to the parent strain when comparing the mean number of bacteria protected from gentamycin lethality ± standard deviation of triplicate wells performed in three independent experiments. Asterisks indicates a significant increase in survival of the sapA mutant compared to that of the parent strain (P<0.05).
epithelial cell in response to colonization by the parent strain or the sapA mutant. Polarized epithelial cells were co-cultured with either the parent strain or the sapA mutant strain at the air exposed apical surface of NHBE cells and assessed for fold change in basolateral cytokine production. We observed a decrease in pro-inflammatory cytokine and chemokine production by polarized normal human bronchial epithelial cells when exposed to the sapA mutant strain for 24 hrs compared to those exposed to the parental strain (Table 1). We extended our analysis to other cell types including: CMEE cells, primary human alveolar epithelial (HAE) cells from human lung explants. We again observed a decrease in pro-inflammatory cytokine and chemokine production on these other primary cell types (Table 1). It is intriguing to note that IL-25 was elevated due to exposure of the parental strain in all three cell types tested. IL-25 stimulation of epithelial cells triggers the production of the type 2 cytokine response, characterized by the secretion of IL-4, IL-5 and IL-13, which were also increased in production in response to the parent strain (239). These data indicate that loss of Sap transporter function decreases NTHI stimulation of epithelial cell cytokine production. Further NTHI sensing of host microenvironmental cues (heme-iron or antimicrobial peptides) via Sap transporter function may ultimately result in a decrease in epithelial cell cytokine production and thus contribute to the chronicity of NTHI-mediated diseases.
### Table 1. Cytokine production by respiratory epithelial cells stimulated by NTHI.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>NHBE</th>
<th>CMEE</th>
<th>HAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>2.21</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>I-309</td>
<td>3.37</td>
<td>2.56</td>
<td>ND</td>
</tr>
<tr>
<td>IL-23</td>
<td>2.99</td>
<td>3.87</td>
<td>ND</td>
</tr>
<tr>
<td>IL-16</td>
<td>1.95</td>
<td>ND</td>
<td>3.29</td>
</tr>
<tr>
<td>CD40 L</td>
<td>10.39</td>
<td>ND</td>
<td>2.08</td>
</tr>
<tr>
<td>IL-25</td>
<td>2.59</td>
<td>3.31</td>
<td>2.88</td>
</tr>
<tr>
<td>IL-1a</td>
<td>ND</td>
<td>2.54</td>
<td>4.81</td>
</tr>
<tr>
<td>IL-1b</td>
<td>ND</td>
<td>3.51</td>
<td>7.15</td>
</tr>
<tr>
<td>IL-2</td>
<td>ND</td>
<td>2.52</td>
<td>1.97</td>
</tr>
<tr>
<td>IFN-g</td>
<td>ND</td>
<td>2.52</td>
<td>2.00</td>
</tr>
<tr>
<td>IL-17</td>
<td>ND</td>
<td>2.62</td>
<td>10.63</td>
</tr>
<tr>
<td>IL-4</td>
<td>ND</td>
<td>3.71</td>
<td>3.40</td>
</tr>
<tr>
<td>IL-13</td>
<td>ND</td>
<td>ND</td>
<td>8.09</td>
</tr>
<tr>
<td>MCP-1</td>
<td>ND</td>
<td>ND</td>
<td>3.68</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>2.38</td>
<td>ND</td>
</tr>
<tr>
<td>IL-12 p70</td>
<td>ND</td>
<td>2.86</td>
<td>ND</td>
</tr>
<tr>
<td>IL-5</td>
<td>ND</td>
<td>4.85</td>
<td>ND</td>
</tr>
<tr>
<td>IL-6</td>
<td>ND</td>
<td>2.44</td>
<td>ND</td>
</tr>
<tr>
<td>IP-10</td>
<td>ND</td>
<td>4.05</td>
<td>ND</td>
</tr>
<tr>
<td>MIP-1a</td>
<td>ND</td>
<td>4.27</td>
<td>ND</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>ND</td>
<td>5.74</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data represents the fold increase in production of the listed cytokines stimulated by the parent strain compared to the mutant strain; cytokines not listed had a fold change less than 1.5. ND indicates no difference in cytokine production stimulated by the parent strain and the sapA mutant strain.
**Sap Transporter function modulates severity of experimental otitis media**

The decrease in epithelial cytokine response to the sapA mutant suggested that there would be an alteration in disease progression in the middle ear. To characterize middle ear mucosal changes, chinchilla middle ear inferior bullae were examined histologically four days following infection with either the parental strain or the sapA mutant. We observed capillary dilation, erythema, hemorrhagic foci, host immune cell infiltrate in middle ears inoculated with the parent strain (Fig. 9B) compared to naïve middle ears (Fig. 9A). Upon closer examination of the middle ear mucosa, we observed mucosal epithelial cell thickening and edema (Fig. 9D). Middle ears inoculated with the sapA mutant also presented similar markers of middle ear disease; however, there is a clear decrease in the disease severity compare to the middle ears inoculated with the parent strain (Fig. 9C, E) despite no significant difference in bacterial burden compared to the parental strain (data not shown). These observations, concurrent with diminished cytokine production, suggest that the sapA mutant is less immunostimulatory than the parent strain, likely compensating for the increased susceptibility to antimicrobial peptides and thus, decreasing epithelial cell stimulation.

**2.4 Discussion**

Elucidation of NTHI pathogenic survival strategies will provide the necessary platform for the development of novel treatment modalities that will ultimately
FIG. 9. Loss of SapA attenuates NTHI-induced disease severity. Naïve chinchilla middle ears (A) were inoculated transbularly with the parent strain (B) or the sapA mutant strain (C). Three days after inoculation the middle ear inferior bullae were excised, embedded and sectioned. Sections were H&E stained to monitor middle ear mucosae inflammation and NTHI biofilm formation in the middle ear cavity. L indicates the middle ear lumen, I indicated infiltrating leukocytes, M indicates mucosa, B indicates the bone and Bf indicates biofilm.
reduce the burden of NTHI-mediated diseases. NTHI is exposed to complex host microenvironments that are typically nutrient-limited due to host sequestration strategies, such as limitation of available iron, and are replete with bactericidal immune molecules. Our previous work describes an essential role for Sap transporter function in the acquisition of heme-iron and resistance to host antimicrobial peptides (191-194, 279, 321). Therefore, we exploited the use of NTHI deficient in Sap transporter function as a means to elucidate the consequences of microenvironmental cues on NTHI-host interactions that modulate disease severity. Here, we demonstrated increased ruffling of the epithelial membrane when cells were co-cultured with NTHI deficient in SapA indicating an altered interaction with the host cell surface. The increase in membrane ruffling coincides with an increased propensity of the sapA mutant to invade the apical layers of polarized, differentiated epithelial cells, in contrast to that of the parental isolate which primarily associated with the cell surface and did not induce membrane ruffling. This hyper-invasive phenotype was coincident with a dysregulation in epithelial cell cytokine production, dampened in inflammatory cytokine production in vitro and further, less inflammatory in vivo, compared to the responses observed to the parental strain. Bacteria invasion has been shown to increase or dysregulate epithelial cytokine production (153, 275, 297). Although we observed a decrease in cytokine production following invasion our data supports a dysregulation in cytokine production by the epithelial cells following invasion. Collectively, our data suggest a dynamic interplay between NTHI and the host that is mediated, at least in part, by host microenvironmental
cues. Thus, microbial sensing of nutrient availability influences interactions with host cells. It has previously been demonstrated that the Ami oligopeptide ABC transporter in *Streptococcus* influences interaction with epithelial cells, hypothesized to be regulated by oligopeptide uptake and contribution to global metabolic gene regulation, thus dependent upon nutrient availability (52, 65). Our data are consistent with this hypothesis suggesting that NTHI utilizes the Sap transporter to sense host-derived molecules that can differ in availability in distinct microenvironments of the respiratory tract. Our data further indicate that NTHI invades the epithelium, likely to gain access to essential nutrients, evade the innate immune response and provide a bacterial population that upon reemergence may seed recurrent infections that contribute to chronic otitis media.

NTHI colonization is dependent on bacterial adherence to the mucosal surface. Additionally, epithelial cell invasion has been shown to be mediated by Hap, Protein D, and Protein E adhesins (2, 286, 299) and ChoP moieties on NTHI LOS (73, 74). Our observations indicated that loss of SapA reduced initial adherence of NTHI, yet did not affect colonization of the cell surface. In fact, loss of SapA promoted invasion of epithelial cells. We do not observe alterations in ChoP expression in the LOS or changes in the expression of known adhesins in the outer membrane of the sapA mutant (251). Thus, these data suggest a previously uncharacterized mechanism for NTHI invasion. Further, the hyper-invasive phenotype of the sapA mutant suggests that this process may be
regulated by the function of the Sap transporter and therefore influenced by host microenvironmental cues such as heme limitation and antimicrobial peptide production during pathogenesis. It is interesting to note, that although predominately surface associated, the parent strain also invaded the epithelial cells. However, the parent strain was observed in membrane bound vacuoles, in contrast to localization of the sapA mutant in the cytoplasm suggesting differential trafficking once internalized by the epithelial cells. Additionally, the fate of the internalized bacteria was different between the parent strain and the sapA mutant. The parent strain lost electron density and membrane integrity within the phagosomal vacuoles, consistent with previous observations of NTHI intracellular invasion. In contrast, the sapA mutant invasive bacteria did not appear contained within phagosome but appeared to colonize the host cytoplasm, in most cases retaining bacterial density and membrane integrity. Invasion into epithelial cells provides an additional mechanism for resisting host nutritional and innate immune responses. It has been previously demonstrated that auxotrophic E. coli mutants are unable to survive in the extracellular environment but were able to survive in the cytoplasm of host epithelial cells (46, 105). The survival of auxotrophic E. coli in the cytoplasm suggests increased access to nutrients in the cytoplasm compared to the extracellular environment of the host. The clustering of the cytoplasmic NTHI is suggestive of intracellular growth. These data suggest that during colonization of the host in a permissive microenvironment NTHI primarily colonizes the epithelial cell surface, and the few bacteria that are able to invade the epithelial cells are readily cleared likely
by lysosomes. However, when NTHI encounters a non-permissive microenvironment high in antimicrobial peptide concentration and heme-iron sequested, similar to the environment experienced by the sapA mutant, it is able to invade the epithelial cells, escape the endosome and replicate in the cytoplasm. Interestingly, NTHI does not have a dedicated invasion or secretion system to initiate epithelial cell receptor independent invasion which suggests that NTHI must exploit host cell signaling to gain access to the cytoplasm, mechanisms currently under investigation in our laboratory. It is also interesting to note the presence of NTHI membrane vesicles in our polarized NHBE model (Fig. 8). We have previously determined that NTHI vesicles are able to stimulate epithelial cells (278) and we are currently investigating their relevance in NTHI-epithelial cell homeostasis.

We examined the contribution of Sap transporter function on NTHI colonization of polarized, differentiated epithelial cells grown at an air-liquid interface, a physiological model of epithelial cells of the upper respiratory tract. Here, we observed that the sapA mutant formed a dense biofilm on the apical surface and invaded into the apical most cell layers. Biofilms provide a mechanism for survival in a nutrient limited environment and resistance to bactericidal molecules (40, 247, 301). Biofilm survival strategies are mediated by the expression of quorum sensing regulated genes, increase in persister cell formation, metabolic heterogeneity and secretion of extracellular polymeric structures. This suggests that the sapA mutant may compensate for the inability to acquire heme-iron and
resist antimicrobial peptides by preferentially forming biofilms on the epithelial cell surface to survive in the host epithelial cell microenvironment. Host microenvironmental cues may influence a similar phenotype during NTHI colonization. In fact, we have demonstrated that transient restriction of heme-iron mediates NTHI morphological changes that influence biofilm architecture, attenuates the host response and thus promotes NTHI persistence in the middle ear (302), suggesting that at least the heme-iron acquisition function of SapA contributes to the phenotypes described here. Both the increased propensity for biofilm formation and invasion of the epithelial cells could represent survival strategies utilized by NTHI in vivo.

Epithelial cells respond to bacterial colonization and invasion by the secretion of cytokines and chemokines that serve to initially attract innate immune leukocytes and subsequently B-cells and T-cells to remove the invading pathogens. We determined that epithelial cell colonization by the sapA mutant stimulated decreased production of inflammatory cytokines compared to those produced in response to colonization by the parent strain. In order to determine if this differential host response to colonization would alter disease progression we monitored the middle ear mucosal of chinchillas inoculated with either the parent strain or the sapA mutant. Histological analysis of middle ears infected with parental strain demonstrated severe edema, capillary dilation, erythema, hemorrhagic foci, host immune cell infiltrate and mucosal epithelial cell destruction 3 days after inoculation. In contrast, middle ears inoculated with the
sapA mutant demonstrated a striking decrease in epithelial inflammation that maintained an intact mucosal surface, despite no significant difference in bacterial burden compared to the parental strain as evidenced by biofilm formation and leukocyte influx in the middle ear. These data suggest that NTHI utilizes the Sap transporter to sense the host microenvironment and mediate interactions with the host mucosal surface. In fact, we have determined that transiently heme-iron restricted NTHI elicited a similar reduction in inflammation and epithelial cell damage that enhanced NTHI persistence in the middle ear (302). Therefore, NTHI will decrease the stimulation of epithelial cells resulting in a decreased expression and production of cytokines and ultimately alter disease progression.

Our results highlight the delicate balance maintained between the host epithelium and commensal bacteria at the epithelial cell interface. Here we demonstrated that the Sap transporter function influenced NTHI interaction with host epithelium, revealing a mechanism by which NTHI can sense the host epithelial cell microenvironmental cues that impact NTHI behavior. Our data suggest that NTHI colonization of the nasopharynx is dictated by host microenvironmental cues that limit host cell interaction to thus establish a commensal relationship in this environment. However, upon transition to other host sites, NTHI senses changes in nutrient availability and innate immune pressures, which triggers a more pathogenic lifestyle which coincides with enhanced biofilm community development, modulation of epithelial cell responses. The lifestyle changes by
NTHI in these environments will promote survival and resistance to clearance mechanisms. The information gathered here provides new avenues of investigation to determine the NTHI and epithelial cell factors that NTHI will decrease stimulation of epithelial cells to diminish the immune response and alter disease.

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Chapter 3: *Haemophilus* Sap transporter function modulates outer membrane biophysical properties and alters cytokine production *in vivo*

Abstract

Nontypeable *Haemophilus influenzae* (NTHI) infections are preceded by commensal colonization in the nasopharynx. During commensal colonization, NTHI must be in a microenvironment with sufficient essential nutrients and low inflammation to persist in the host. Upon transition to other anatomical sites of the respiratory tract, NTHI encounters environments with varying levels microenvironmental cues; such as heme availability and antimicrobial peptide production. We have previously determined that NTHI utilized Sap transporter function to sense heme and antimicrobial peptides and mediate invasion into the epithelium. We, therefore, hypothesized that the Sap transporter dependent alteration with the host are mediated by phenotypic changes to the NTHI outer membrane. We determined that the sapA mutant formed taller biofilms and is more aggregative in suspension. Additionally, the outer membrane of the sapA mutant is less permeable and more hydrophobic, however we observed no alterations to lipid A, phospholipids or lipooligosaccharide, yet significant changes to the outer membrane proteome. We have previously shown that the sapA mutant is hyper-invasive, is less immnuo-stimulatory and induces a
decrease in disease severity *in vivo*. Consistent with these observations, we demonstrate that there are significant invasive populations in the middle ear during otitis media and there are Sap transporter dependent changes to cytokine production in middle ear effusion. Our data suggest that NTHI sense host environmental cues via Sap transporter function to mediate interaction with host epithelial cells by manipulation of the outer membrane and that these changes may promote chronic or acute infections.

### 3.1 Introduction

Nontypeable *Haemophilus influenzae* (NTHI) is a commensal inhabitant of the human nasopharynx. However, it is able to cause opportunistic infections of the upper and lower respiratory tract and, as such, is one of the leading causes of pediatric otitis media (OM) as well as conjunctivitis, sinusitis, pneumonia and exacerbations in patients with chronic obstructive pulmonary disease and cystic fibrosis (109, 168, 214, 215, 265, 277, 288). Colonization of these privileged sites coincides with changes in the host microenvironment, including the production of cytokines, chemokines, infiltration of leukocytes and most notably, host sequestration of essential nutrients, termed nutritional immunity, to restrict bacterial growth (44). Nutrient restriction, specifically the effects of iron, have previously demonstrated a large range of physiological changes including increased resistance to antibiotics, alterations in virulence factor expression as well as induction biofilm formation (18, 205, 255, 323). Consistent with other bacterial infections, NTHI mediated OM is associated with biofilm formation in the
middle ear space. In addition to requiring iron for growth, NTHI must acquire the iron containing molecule heme, yet lacks the biosynthetic pathways to generate protoporphyrin IX and therefore must acquire heme from the host (227). We have previously demonstrated that environmental heme-iron restriction or genetic mutations that confer a heme-iron starved phenotype alter NTHI biofilm structure and architecture. Loss of Sap transporter function, an inner membrane ABC transporter required for efficient heme iron transport into the cytoplasm, influences changes in biofilm architecture to an openwork, filamentous phenotype (321). Moreover, transient heme iron restriction results in physiological changes that modify NTHI biofilm structure and architecture, which is dependent on changes in bacterial morphology (302). We demonstrated that, loss of Sap transporter function attenuates survival in NTHI chinchilla middle ear and nasopharynx, highlighting the essential function of the Sap transporter to counter host nutritional immunity (122, 191, 192, 208). We also observed that, transient heme iron restriction of NTHI altered disease severity in chinchilla middle ears, characterized by reduction in inflammation and tempered mucosal epithelial cell damage (251, 302). Despite the key role of biofilm formation during OM and the heme dependent alterations in biofilm structure and architecture, the presence and characterization of heme dependent alterations in bacterial surface structures and effects on membrane composition have not been determined.

NTHI has been traditionally described as an extracellular pathogen and many studies investigating the interaction between the bacteria in the host have
focused on the interplay between bacteria and the surface of the host epithelium. However, there is increasing evidence that populations of NTHI may reside within of the host epithelium (3, 160, 287, 290, 299, 319). Previous work from our lab has shown that the loss of Sap transporter function increases the propensity for NTHI to invade into both human and chinchilla epithelial cells (251). Additionally, NTHI restricted for heme-iron are also hyperinvasive (302). Small invasive populations of NTHI have been found within both adenoid and bronchial human tissue samples, suggesting their link to chronic and recurrent infections (17, 91). However, the ability of NTHI to form viable intracellular populations during OM has not yet been determined.

To determine heme-iron acquisition dependent changes in NTHI outer membrane characteristics upon deficiency in heme-iron uptake we used a SapA deficient NTHI strain, which is unable to acquire heme (192). We determined that loss of the SapA dependent transport resulted in biofilms which were structurally distinct from the parent strain, potentially due to increased autoaggregation. Furthermore, we observed that loss of SapA decreased outer membrane permeability and increased hydrophobicity, and this phenotype was recapitulated in wild-type bacteria that were heme-iron starved. We did not observe alterations in lipid A, phospholipids, or lipoligosaccharides between the parent and mutant strains. However, there were significant changes in the outer membrane proteins in the SapA deficient strain. We also observed significant changes in cytokine levels in effusions from our experimental model of pediatric otitis media,
consistent with our previous observations that loss of Sap transporter function or transient heme iron restriction results in reduced disease severity. Lastly, previous work has demonstrated enhanced intracellular populations of NTHI due to both loss of Sap transporter function or transient heme iron restriction, and here we demonstrate for the first time populations of intracellular bacteria during our experimental model otitis media. Therefore, intracellular populations of NTHI must be considered when determining treatment modalities for chronic otitis media because they are more resistant to antibiotic treatment and can serve as a reservoir to seed recurrent infections.

3.2 Materials and Methods

Bacterial strains and growth conditions: NTHI strain 86-028NP is a minimally passaged clinical isolate, which has been characterized both in vitro and in vivo as a model pathogen to study otitis media. The parent strain (86-028NP::rpsLA128G) and the sapA mutant strain (86-028NP::rpsLA128G sapA) are streptomycin-resistant strains generated by the recombineering strategy as previously described (251, 309, 321). The sapA::kan insertion mutant in 86-028NP was constructed as previously described (191). Complementation of the sapA non-polar deletion mutant in the parent strain was constructed as previously described (191). Green fluorescent protein (GFP)-expressing parent and sapA mutant strains were generated as previously described (193). The licD ON mutant was a kind gift from WE Swords (137).
The NTHI strains were grown on chocolate II agar (Becton, Dickinson, Sparks, MD) overnight at 37°C and 5% CO₂. After overnight grown, NTHI was subcultured into prewarmed brain heart infusion (sBHI) supplemented with 2 µg/ml heme (Becton, Dickinson, Sparks, MD) and 1 µg/ml NAD (Becton, Dickinson, Sparks, MD). Cultures were normalized to an optical density of OD₄₉₀=0.65, diluted 1:6 in prewarmed sBHI and grown for three hours to logarithmic phase to an optical density of OD₄₉₀=0.65. Heme restricted NTHI were subcultured from overnight growth on chocolate II agar into chelated defined iron-source medium with (Heme-iron replete) or without (Heme-iron restricted) 2 µg/ml heme and normalized to an optical density of OD₄₉₀=0.65, and grown statically for 24 hours to deplete internal stores of heme (194, 302, 321). Following overnight growth cultures were normalized to an OD₄₉₀=0.37.

**Chamber slide biofilm growth:** Logarithmic phase parent or sapA mutant strains expressing GFP were diluted 1:2500 in prewarmed sBHI, and 200 µl were added to well of an 8-well chamber slide (Nunc, Lab-Tek) and grown for 24 hours at 37°C and 5% CO₂. After biofilm growth, the wells were washed twice with PBS and fixed in 4% paraformaldehyde overnight at 4°C. Following fixation the slide was prepared and visualized as previously described (321). Biofilms were imaged and analyzed with an Axiovert 200M inverted epifluorescent microscope with an Apotome attachment and Axiocam MRM CCD camera (Carl Zeiss Inc., NY). To quantify biofilm height, the distance from the base of the biofilm to the peak was measured in 10 random fields of view in three independent biofilms for
each strain. A two-tailed Student $t$ test was used to determine a significant
difference in peak height.

**Bacterial aggregation:** Aggregation of either the parent strain or the sapA mutant was determined as previously described (69, 178). Briefly, logarithmic bacteria were agglutinated on a slide with either 0.5M or 2.0M ammonium sulphate. Bacterial aggregations were visualized on an inverted light microscope at 63x magnification.

**NPN uptake assay:** Membrane permeability of the NTHI strains was determined as previously described (126, 310). Briefly, logarithmic parent sapA mutant, heme-iron replete, and heme-iron restricted cultures were pelleted by centrifugation (3220xg for 10 minutes) and resuspended in 5mM HEPES buffer. 100µl of the bacterial suspensions or buffer alone were added to wells of a 96 well microtitre plate (Fischer, NJ). The wells were supplemented with 50 µl of 40 µM NPN (0.5mM NPN in acetone diluted in 5mM HEPES to 40 µM). NPN was excited at 355nm and emission was measured at 405nm and relative fluorescent units (RFU) was calculated by subtracting the fluorescence in the buffer alone from the bacterial suspension samples. RFU was determined in 4 independent experiments with 6 wells per sample in each replicate and a two-tailed Student $t$ test was used to determine a significant difference in permeability.

**Hexadecane Hydrophobicity Assay:** The hydrophobicity of the parent, sapA mutant, starved and non-starved was determined by their ability to adhere to
hexadecane droplets as previously described (298). Briefly, logarithmic parent sapA mutant, heme-iron replete, and heme-iron restricted cultures were pelleted by centrifugation (3220xg for 10 minutes) and concentrated 5x in DPBS. 1ml of the sample was added to 13mm glass tubes (3 tubes/strain). Optical density (OD$_{620}$) was measured, followed by the addition of 200µl of hexadecane. The samples were vortexed for 30 seconds and incubated at room temperature for 15 minutes. Following incubation the OD$_{620}$ of the aqueous layer was measured. Percent adherence to hexadecane droplets was determined by calculating the difference between the initial OD$_{620}$ subtracted by the OD$_{620}$ after mixing with the hexadecane divided by the initial OD$_{620}$ [(R$_{initial}$-R$_{hexadecane}$)/(R$_{initial}$) *100]. Hexadecane adherence was determined in 3 independent experiments with 3 tubes per strain in each replicate and a two-tailed Student t test was used to determine a significant difference in hydrophobicity.

**Lipid A and Phospholipids:** Parent, sapA mutant, the sapA complement and *E. coli* controls strains (W3110, WD101) were grown to logarithmic phase in the presence of 5.0 µCi/ml $^{32}$Pi (PerkinsElmer). The $^{32}$P-labelled lipid A and phospholipids were isolated as previously described (Tran, 2004). Samples were spotted onto a Silica Gel 60 TLC plate (EMD) at 2500 cpm/lane. Lipids were separated using chloroform, pyridine, 88% formic acid and water (50:50:16:5). Lipid species were visualized by overnight exposure to a phosphoimager screen and visualization with a Bio-Rad Molecular Imager phosphoimager.
LOS analysis: Logarithmic cultures of the parent, sapA mutant, the sapA complement and controls (LT2 S. typhimurium and W3110 E. coli) were pelleted by centrifugation at 16,000 x g and washed once with PBS. Pellets were resuspended in 100µl LDS sample buffer (Invitrogen) 4% BME. Cell suspensions were boiled from 10 minutes. After the samples cooled, 125 ng/µl proteinase K (New England Biolabs) was added and incubated for 16 hours at 55°C. Following incubation the proteinase K was heat inactivated, and the samples were resolved by gel electrophoresis using a 16% Tricine-Glycine Gel. LPS and LOS were visualized by silver stain.

Phosphoryl choline expression: Phosphoryl choline moiety incorporation into the LOS of the parent and sapA mutant strain was analyzed by colony dot blot. Briefly, 2 µl of logarithmic cultures of either the parent strain, sapA mutant, or a licD ON strain were spotted onto a nitrocellulose membrane, dried, blocked with 5% BSA in TBST and probed with the TEPC 15 antibody (Sigma Aldrich, MO). Following anti-mouse-HRP incubation the samples were incubated with ECL, and exposed to X-ray film. The pixel densities of the spots were measured by densitometry.

Outer membrane proteomics: The outer membranes of the parent or sapA mutant were isolated by French press lysis followed by sucrose density centrifugation. Logarithmic cultures were pelleted by centrifugation at 5000 x g for 20 minutes, washed with 10mM HEPES and pelleted again. The pellets were
concentrated 5x in 20% sucrose 10mM HEPES and passed once through the French press (SLM Aminico) at 14,000 psi. 4.0 ml of the samples were added onto 6.25 ml of 25% sucrose 10mM HEPES and 2.0 ml of 60% sucrose 10mM HEPES. Membrane fractions were isolated at the interface of the 25% sucrose 10mM HEPES and 60% sucrose 10mM HEPES with a syringe after centrifugation at 40,000 rpm in a SW 41 Ti rotor for three hours. The samples were diluted with 10mM HEPES to a refractive index of 1.365 using a refractometer. 2 ml of the samples were added onto a step gradient (0.5ml of 67%, 1.0ml of 55%, 2.4ml of 50%, 2.4ml of 45%, 2.4ml of 40%, 1.4ml of 35% and 1.0ml of 30% sucrose in 10mM HEPES) and centrifuged at 40,000 rpm for 16 hours at 4°C in a SW 41 Ti rotor. Fractions were collected with syringe through the bottom of the tube. The outer membrane enriched fraction was isolated at 47.5% sucrose 10mM HEPES.

**Animal Studies:** Health adult chinchillas (*Chinchilla lanigera*) were purchased from Rauscher’s chinchilla ranch (LaRue, OH) and were used to assess cytokine production and intracellular NTHI populations during experimental otitis media. Chinchillas were anesthetized with 2 mg/mg xylazine (Fort Dodge Animal Health, IA) and 10 mg/kg ketamine (Phoenix Scientific Inc., MO) and middle ears were transbullarly inoculated with 2500 CFU of the parent or *sapA* mutant as previously described (Raffel, 2013). At 2 days post inoculation, the animals were sacrificed and the middle ear mucosa and effusions were collected. Four ears were inoculated with each strain tested.
**Cytokine analysis:** The cytokine profile in the middle ear effusions was measured using the Proteome Profiler human cytokine array kit (R&D Systems, MN). Briefly, 50 µl of effusion were incubated with biotinylated detection antibodies, and the complex was bound to a cognate antibody immobilized on a nitrocellulose membrane. Relative amounts of the cytokine were detected by chemiluminescence generated by streptavidin-horseradish peroxidase activity. Fold change in cytokine production was determined by densitometry at each cognate antibody spot. Effusions from four ears were measured for each strain and a two-tailed Student t test was used to determine a significant difference in cytokine production.

**NTHI inoculation onto epithelial cell monolayers for microscopy**

Normal Human Bronchial Epithelial cells monolayers in a chamber slide were inoculated at an MOI 50 with GFP-expressing parent strain, sapA mutant, non-heme starved and heme starved NTHI in DPBS. One hour after inoculation, the coverslips were washed once with 300ul DPBS, treated with 100 µg/ml gentamicin in DPBS for one hour, washed once with 300ul DPBS and fresh media was added. After 4 or 24 hours, the cells were fixed in 2% paraformaldehyde in DPBS. Early endosomes and lysosomes were labeled with an α-EEA1 antibody (Santa Cruz Biotechnology, sc-6415) and α-Lamp1 antibody (Developmental Studies Hybridoma Bank, University of Iowa), respectively. DNA was counter stained with Hoescht 34580 (Life Technologies).
**Ex vivo gentamicin protection assay:** Gentamicin protected populations were measured in the middle ear mucosa of chinchillas inoculated with the parent, sapA mutant or sapA complemented strains. The middle ear mucosa was washed with 1 ml 0.9% saline to remove not adherent NTHI, peeled from the bone, homogenized in 1000 µl 0.9% saline and then diluted 1:1 in either 0.9% saline 1% glucose, 0.9% saline 1% glucose 100 µg/ml gentamicin, or 0.9% saline 1% glucose 100 µg/ml gentamicin 0.2% Triton X-100. After a 1 hour incubation the samples were serially diluted and plated on chocolate II agar. Colonies were quantified after overnight incubation at 37°C 5% CO₂. % Protected population was determined by calculating the difference between the saline CFU subtracted by the CFU after gentamicin treatment divided by the saline CFU [(CFU_{saline} - CFU_{gentamicin})/(R_{saline}) *100], the % Triton X-100 was calculated in the same fashion. Four middle ear samples were used for each strain tested and a two-tailed Student t test was used to determine a significant difference in gentamicin protection.

### 3.3 Results

**Biofilm and Aggregation**

We have previously shown that Sap transporter function is required to maintain NTHI heme iron homeostasis and persistence in the host by facilitating the transport of heme iron across inner membrane and providing a mechanism to resist antimicrobial peptides (191-194, 279). Additionally, we have shown Sap transporter dependent alterations in biofilm formation utilizing the sapF ATPase
mutant (321). We have previously shown that the Sap transporter contributes to NTHI colonization of epithelial cells in vitro, and the sapA mutant remolds the epithelial cell surface (251). We, therefore, hypothesized that the sapA mutant, which would prevent heme and antimicrobial peptide delivery to the permease, would similarly form an altered biofilm. Biofilms grown for 24 hours were monitored for peak height and architecture. We observed that the sapA mutant formed a significantly taller and less dense compared to the parent strain by fluorescent microscopy (Fig. 10A, B). To better define the differences in the biofilms between the parent strain and the sapA mutant we quantitatively evaluated the height of the biofilms. We measured peak height of the biofilms from the glass surface to the highest biofilm associated signal in the observed field of view. We determined that the biofilms formed by the sapA mutant were significantly taller than the parent strain, confirming that Sap transporter function influences biofilm structure and architecture (Fig. 10C).

Bacterial cell-cell interactions are an important step for biofilm initiation. Because of the differences in biofilm height and architecture between the parent strain and sapA mutant we hypothesized that this may be due to differences in aggregation between the two strains. Using a bacterial precipitation technique we determined that the sapA mutant formed larger bacterial aggregates compared to the parent strain suggesting that initial cell-cell interactions and may influence biofilm structure and architecture in NTHI (Fig. 10D, E, F, G) (69, 178). Multiple cell surface properties can influence cell-cell interactions in bacteria such as surface
FIG. 10. Biofilm formation and aggregation in the absence of SapA. The parent (A) or the sapA mutant (B), each expressing GFP, were grown in chamber slide wells for 24 hours and biofilm structure and development were monitored by fluorescent microscopy. (C) Biofilm height of the parent and sapA mutant was quantified by measuring peak height from the slide surface and depicts a ~2 fold increase in peak height by the sapA mutant compared to the parent strain when comparing the mean peak height (*, p-value<0.05). Aggregation of either the parent (D, E) or the sapA mutant (F, G) was determined by agglutination on a slide with either 0.5M (D, F) or 2.0M (E, G) ammonium sulphate.
charge, hydrophobicity and protein interactions. To address potential global changes in the outer membrane components between the parent strain and the sapA mutant we measured both the permeability and hydrophobicity of the outer membrane. To measure outer membrane permeability we utilized a 1-N-phenyl-naphthylamine (NPN) uptake assay. In this assay, the molecule NPN is utilized as a fluorescent probe for its ability to selectively fluoresce in phospholipid environments, therefore when the outer membrane is more permeable there will be a measureable increase in fluorescence (126). We determined that the sapA mutant is significantly less permeable to NPN compared to the parent strain (Fig. 11A). Outer membrane hydrophobicity is implicated in promoting interactions between bacteria and attachment to host cells. Because we observed larger bacterial aggregates formed by the sapA mutant compared to the parent strain, we hypothesized that the sapA mutant outer membrane would be more hydrophobic. To determine changes in hydrophobicity between the parent strain and sapA mutant strain we measured the ability of the bacteria to adhere to hexadecane droplets and observed that the sapA mutants was significantly more adherent to these hexadecane droplets (Fig. 11B). These data suggest that Sap transporter function influences global changes in outer membrane components.

The Sap transporter is required for efficient heme transporter across the inner membrane of NTHI and is therefore essential for maintaining heme homeostasis. Loss of Sap transporter functions results in bacteria that are genetically heme-
iron starved. We have previously shown that we can environmentally heme-iron starve NTHI and these heme-iron restricted NTHI form with increased peak height, altered architecture, and filamentous bacteria (302). We therefore hypothesized that environmental heme-iron restriction would alter the biophysical properties of NTHI. Utilizing the same methodology as outlined above, we determined that heme starved NTHI, similar to the sapA mutant, were significantly less permeable and significantly more hydrophobic (Fig. 11C, D). These data suggest that the ability of the Sap transporter to transport heme into the cytoplasm alters the biophysical properties of the outer membrane. We determined that the sapA mutant and heme-iron restricted NTHI are more hydrophobic and less permeable than the parent strain or heme-iron replete NTHI.

**Outer membrane modifications**

NTHI employs many methods to alter its outer membrane as a mechanism to protect itself from host clearance mechanisms. Among these modifications are the addition of phosphoryl choline (ChoP) and sialic acid residues to the lipooligosaccharide (LOS). ChoP decorated LOS promotes the attachment of NTHI to the host epithelium, stimulates signaling through the platelet activating factor receptor and induces uptake of the bacteria into the epithelial cells (299, 300). ChoP decoration also inhibits the binding of immunoprotective antibodies and promotes biofilm formation and maturation (136, 137). Sialylation of NTHI LOS has also been shown to prevent complement deposition and promote
FIG. 11. Heme-iron transporter dependent changes to outer membrane hydrophobicity and permeability. The outer membrane permeability differences of the parent strain and sapA mutant (A) or heme-iron replete and heme-iron restricted NTHI (C) was determined by measuring the difference NPN fluorescence. The hydrophobicity of the parent strain and sapA mutant (B) or heme-iron replete and heme-iron restricted NTHI (D) was measured by the ability to adhere to hexadecane droplets, data represents percent of initial suspension adherent to the hexadecane droplets. (*, p-value<0.05)
biofilm formation and adherence (301, 314). Additionally, $htrB$ expression and hexaacylation of lipid A are required for antimicrobial peptide resistance (291). To investigate potential SapA dependant changes to the outer membrane; lipid A, phospholipids and LOS samples were analyzed for differential modifications between the parent and sapA mutant. We determined that there was no modification of the lipid A, phospholipids and LOS between the parent strain and sapA mutant (Fig. 12A, 10B, 10C). However, small modifications may not be resolved liquid chromatography or gel electrophoreses and ChoP expression is phase variable. To determine if there was a change in ChoP decoration we spotted mid-log bacterial strains on nitrocellulose and probed for ChoP using an $\alpha$-ChoP antibody. We observed no difference in ChoP decoration between the parent and Sap transporter deficient strain (Fig. 12D). Therefore, despite biophysical differences in the outer membrane of the sapA mutant, we did not observe a difference in lipid A, phospholipids or LOS and further observed that both the parent strain and the sapA mutant are in the OFF conformation for ChoP moiety expression.

Since we were unable to identify biochemical modifications to the outer membrane lipid bilayer components, we next investigated changes in the outer membrane proteome. Many previously described factors, such as the Hap, protein D, protein E, PilA, and HMW1/2 have been shown to be critically important in biofilm formation, adherence to biotic or abiotic surfaces and in some cases mediate invasion. However, it is unknown if these factors could play a role
FIG. 12. There are no sapA dependant changes to lipid A, phospholipids or lipooligosaccharide. The lipids from the Parent, sapA mutant, the sapA complement and E. coli controls strains (W3110, WD101) were separated using thin layer chromatography and visualized after overnight exposure to a phosphoimager screen (A,B). LOS species from the parent, sapA mutant, the sapA complement and controls (LT2 S. typhimurium and W3110 E. coli) resolved by gel electrophoresis and visualized by silver stain (C). (D) Phosphoryl choline moiety incorporation into the LOS of the parent and sapA mutant strain was analyzed by colony dot blot.
in changing the hydrophobicity and permeability of the outer membrane of NTHI. Previous work from our lab demonstrated that loss of Sap transporter function confers a heme and iron starved phenotype by microarray. We therefore hypothesized that loss of the SapA periplasmic binding protein would cause differential protein expression in the outer membrane. In order to identify changes to the outer membrane proteome in the Sap deficient strain, we isolated outer membrane enriched fractions by density centrifugation and then subjected the samples to MS-MS mass spectrometry. We compiled a list of all of the proteins in which there was a significant difference in expression (Table 2). Although we did not observe a specific protein previously identified to play a role in biofilm formation or adherence, we discovered a number of interesting changes. We observed a differential change in heme-iron uptake mechanisms, suggesting that HemY and the Hemoglobin-haptoglobin binding protein B, which were increased in expression in the Sap deficient strain, are more efficient for heme-iron uptake than the Heme-hemopexin utilization protein C under heme-restricted conditions. Additionally, the lipoprotein NlpI has been shown to be a key player in the release of extracellular DNA (eDNA), which is incorporated into NTHI biofilms and is likely the main component of the biofilm matrix (137, 145, 273). NlpI is a negative regulator of eDNA release in E. coli, such that over expression of nlpI decrease eDNA release, while a nlpI mutant increases eDNA release (273). Alternatively, in Salmonella enterica serovar Typhimurium, nlpI was shown to decrease biofilm formation in a CsgD dependant manner (269). Since we observed a decrease in NlpI localization to the outer membrane in the
Table 2. Outer membrane protein production differences between the parent strain and sapA mutant

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fold decrease in ΔsapA</th>
<th>Fold change (ΔsapA/ΔsapA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophanase</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c552</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>F0F1 ATP synthase subunit delta.</td>
<td>∞</td>
<td></td>
</tr>
<tr>
<td>Thiamine biosynthesis lipoprotein ApbE</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>F0F1 ATP synthase subunit beta</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Hypothetical protein NTHI1140</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>IgA-specific serine endopeptidase</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Heme-hemopexin utilization protein C</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>5,10-methylenetetrahydrofolate reductase</td>
<td>∞</td>
<td></td>
</tr>
<tr>
<td>Lipoprotein NlpI.</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>Outer membrane protein P2.</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>F0F1 ATP synthase subunit alpha.</td>
<td>3.3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fold increase in ΔsapA</th>
<th>Fold change (ΔsapA/rpsL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA translocase FtsK</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>HemY</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Multidrug resistance protein A</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>ATP-dependent protease La</td>
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</tr>
<tr>
<td>HflK</td>
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<td></td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin-haptoglobin binding protein B</td>
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<td></td>
</tr>
<tr>
<td>Hypothetical protein NTHI1930</td>
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</tr>
<tr>
<td>Penicillin-binding protein 5</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Fumarate reductase iron-sulfur subunit</td>
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<td></td>
</tr>
<tr>
<td>Transketolase.</td>
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<td></td>
</tr>
</tbody>
</table>

Data represents the fold change in production of proteins in outer membrane enriched samples from the parent strain or sapA mutant. Proteins not listed were not significantly different.
sapA mutant, it suggests that there may be more eDNA in the SapA deficient biofilm, which could account for the structural differences between the parent strain and the sapA mutant biofilms. Furthermore, NlpI has been implicated in maintaining membrane integrity under hydrostatic pressure, and is hypothesized to assist in repairing damage to the cell envelope (47, 186). The sapA mutant is more susceptible to antimicrobial peptide killing and it is therefore likely that it is utilizing other systems besides nplI to counteract antimicrobial peptide activity.

Collectively, we observed an alteration in expression of key heme-iron uptake systems, as well as, changes to known virulence factors used to combat the innate immune system and a decrease in expression of a lipoprotein that has previously been described to be involved in regulating biofilm formation and maintaining membrane integrity, suggesting that the sapA mutant is compensating for the loss of SapA dependent transport of heme-iron and antimicrobial peptides.

**Sap Transporter and Heme dependent alterations to cellular trafficking**

We have previously shown that the sapA mutant is hyper invasive into epithelial cells and that transient heme-iron restriction similarly promotes hyper-invasion and large colony formation in epithelial cells (Chapter 2) (302). The hyper-invasion into the epithelial cells, in conjunction with the observation that the sapA mutant was not observed enveloped by a membrane inside the epithelial cells, suggested that the sapA mutant and transiently heme starved NTHI were able to escape the endosomal trafficking pathway and survive in the cytoplasm (Fig.
Therefore, we monitored co-localization of invaded NTHI with markers for early endosomes (EEA-1) and lysosomes (Lamp1). After four hours we observed that the parent strain and continuously heme exposed NTHI co-localized with EEA-1 labeling suggesting that the parent strain and continuously heme-exposed NTHI were in endosomes, by twenty hours we observed similar co-localization with Lamp1 suggesting that NTHI containing endosomes fused with lysosomes. In contrast to the parent strain and continuously heme-exposed NTHI, the sapA mutant and transient heme-restricted NTHI were minimally co-localized with EEA-1 labeling and Lamp1 labeling (Fig. 13). These data further suggest that NTHI can disrupt epithelial cell endosomal trafficking and that the Sap transporter function and heme-iron availability can influence intracellular survival and localization.

**in vivo Interactions**

The epithelium serves as the first line of defense against invading pathogens and sensor to the external environment (153). We previously determined that there was a decrease in proinflammatory cytokine and chemokine production by epithelial cells stimulated by the sapA mutant compared to those exposed to the parent strain (251). Coincident with the decreased cytokine production we also observed that the sapA mutant induced a decreased level of disease severity in the chinchilla model of otitis media. Middle ears inoculated with the parent strain were characterized by many markers of inflammation including capillary dialation, While many of these markers were also observed in ears inoculated with the
FIG. 13. Sap and heme-iron dependent alterations to endosomal trafficking.
(A) The parent strain or the sapA mutant were inoculated onto NHBE epithelial cell monolayers and co-localization with EEA-1 and Lamp-1 was monitored 4 and 20 hours after inoculations. (B) Continuously heme exposed and transiently heme-restricted NTHI were inoculated onto NHBE epithelial cell monolayers and co-localization with EEA-1 and Lamp-1 was monitored 4 and 20 hours after inoculations.
sapA mutant there was a clear decrease in their severity despite no difference in bacterial numbers (251). The reduced levels of disease severity in vivo suggested that there would be reduced levels of inflammatory cytokines and chemokines produced in the middle ear in response to colonization by the sapA mutant. To address this hypothesis we inoculated chinchilla middle ears with either the parent or sapA mutant strains and two days after inoculation, excised the bullae and collected the effusion. Cytokine and chemokine levels were measured in the effusions and we determined that there were significant changes to the cytokine profile induced by the sapA mutant compared to the parent strain (Fig. 14A). Similar to our previous data we observed a significant increase in CD40L in response to colonization by the parent strain compared to the mutant strain. CD40L, a co-stimulatory molecule that induces activation of antigen presenting cells in association with TCR stimulation and mediates both humoral and cellular immune responses, induces the production of IL-8 (250). Consistent with this function we did observe an increase in IL-8 production, although not significant, by the parent strain compared to the mutant strain. Additionally, we observed a significant increase in production of IL-1ra, which competitively blocks the both IL-1r I/II to balance IL-1 mediated inflammation and prevent chronic infection, further suggesting that the sapA mutant alters signaling pathways to influence the balance of inflammation. In contrast to our previous data, we did observe a significant increase in production of IFN-γ after colonization with the sapA mutant compared to the parent strain. However, IFN-γ is a key signaling molecule for coordinating protective immunity against
intracellular pathogens and increasing lysosomal activity. This is intriguing because we have previously shown that the sapA mutant is hyper invasive in vitro, and suggests that the mutant may be forming similar intracellular communities during otitis media. NTHI has been characterized as an extracellular pathogen, however recent observations have increased the evidence that NTHI may have an intracellular lifestyle (53). Intracellular populations have been observed within adenoids and bronchial patient samples suggesting that invasion may contribute to the chronic nature of NTHI infections (17, 91). We have shown that the sapA mutant is hyperinvasive on both chinchilla middle ear epithelial cells and normal human bronchial epithelial cells grown as monolayers or on a transwell membrane, however invasion populations have not been identified during otitis media (251). As mentioned previously, we measured an increase in IFN-γ production after sapA mutant inoculation into chinchilla middle ears, we therefore hypothesized that the sapA mutant may be forming more intracellular communities in vivo compared to the parent strain. To measure intracellular populations in vivo we, designed an ex vivo gentamicin protection assay; chinchilla middle ears were inoculated with either the parent strain the sapA mutant, or sapA mutant complement, two days post infection the middle ears were excised, the middle ear mucosa was peeled from the bone, homogenized, and then treated with either saline, saline and gentamicin or saline, gentamicin and triton X-100. We determined that there were significant intracellular populations in all three strains tested, these gentamicin protected populations comprised 20%-40% of the adherent, non-planktonic population and were
FIG. 14. NTHI cytokine stimulation and invasive populations in the chinchilla middle ear. (A) Data represents the fold change in the listed cytokines in the effusions from chinchilla middle ears 2 days after challenged with either the parent or sapA mutant using a cytokine array from four independent ears. Cytokines from the array that are not listed had a fold change less than 1.5 (*, p-value<0.05). (B) Gentamicin protected populations were identified in chinchilla middle ears two days after inoculation. Data represents the average percentage of the saline control protected from gentamicin (average % protected) or after treatment with both gentamicin and Triton X-100 (average % Triton X-100) from four independent ears. There is a significant gentamicin protected population that is lost after Triton X-100 treatment (*, p-value<0.06) (**, p-value<0.04).
significantly greater than those samples treated with gentamicin and triton X-100 (Fig. 14B). These data suggest that intracellular populations may play a critical role during NTHI mediated otitis media.

3.4 Discussion

NTHI is the causative agent of many acute and persistent upper airway infections. NTHI mediated acute airway infections are often symptomatic, characterized by middle ear inflammation, fever and pain, and are easily treated with an antibiotic regime. However, due to the nature of chronic and recurrent NTHI infections, they are either not symptomatic or are able to resist antibiotic therapy. These infections are primarily associated with middle ear effusion in the absence of inflammation or observable tympanic membrane pathology (103, 229). NTHI colonizes most individuals asymptomatically in the nasopharynx, suggesting that it has mechanisms to limit the host immune response in that niche. Therefore, there must be certain changes in the host that alter this benign bacterial-host interaction in the middle ear. We have shown that transient heme starvation, or Sap transporter inactivation, skew the bacterial-host interactions towards the less symptomatic, while continuous heme exposure or a functional Sap transporter results in a more clinical manifestation of acute otitis media (251, 302). These data suggest that environmental signals, such as heme availability, can serve as a mechanism for NTHI to change its interactions with the host. During persistent infections, computational modeling predicts that bacteria colonize both intra- and extracellular compartments, suggesting that both
populations are able to cause chronic infections (80). We have previously shown that transient heme starvation and loss of Sap transporter function promote invasion into epithelial cells, further supporting the chronic subclinical modeling seen during experimental otitis media (251, 302). Therefore, we exploited the use of NTHI deficient in Sap transporter function as a means to determine the influence of microenvironmental cues on NTHI outer membrane composition and bacteria-host interactions during otitis media. Here, we demonstrated a Sap transporter and heme dependent decrease in outer membrane permeability and increase in hydrophobicity. These outer membrane changes were not due to modifications to LOS, lipid A or phospholipid content. However, the change to the outer membrane biochemical properties coincided with significant changes to the outer membrane proteome. We previously observed that Sap transporter deficient and heme starved NTHI induced a less severe disease state during otitis media, our data presented here suggested that this may be due to alterations in protein expression on the outer membrane. To further investigate the reduction in disease severity we monitored cytokines secreted into the middle ear effusion and determined a significant change in the cytokine profile. This reduction in disease severity was not coincident with an increase in invasion populations, however we demonstrate for the first time invasion populations during experimental otitis media. Collectively, our data suggests that heme availability plays a profound effect on NTHI-host cell interactions, mediated by the outer membrane protein components of NTHI. Our data and work done by others have shown that ABC transporter systems can modulate physiological
properties of bacteria, which are involved in biofilm formation and adherence, bacterial stress survival, and host cell signaling and cell death. These interactions with the host seem to be regulated by the nutrient transport and metabolism of the bacteria and the subsequent stress response during nutrient limitation (45, 52, 63, 241, 303). Our data is consistent with this hypothesis, and suggest that NTHI utilizes the Sap transporter as an environmental sensor, to monitor the availability of essential nutrients and the host immune status. Environmental sensing through the Sap transporter conveys information about the temporal and special localization for NTHI in the respiratory tract. During commensal colonization in the nasopharynx, NTHI resides in a low inflammation, nutrient sufficient environment, such that there are low concentrations of antimicrobial peptides at the mucosal surface and essential nutrients is at tolerable levels. However, during the transition from the nasopharynx to the middle ear NTHI encounters altering microenvironmental concentrations of antimicrobial peptides and heme. Accent of the Eustachian tube is coincident with decrease concentrations of heme and upon arriving at the middle ear space NTHI encounters a nutrient depleted environment, maintained by the host’s nutritional immunity. Following colonization, NTHI induces severe inflammation during acute infections leading to a large influx of immune cells and subsequent release of high concentrations of antimicrobial peptides. Additionally, colonization and inflammation of the host mucosa releases nutrients, including heme, into the middle ear lumen. Therefore, during acute infections, NTHI encounters temporal and special changes in antimicrobial peptide and heme.
concentrations. As presented earlier, this hypothesis further explains chronic and subclinical NTHI infections, if NTHI does not encounter these inflammatory and nutrient fluctuations it does not alter its commensal behavior when colonizing the middle ear.

NTHI colonization of the epithelium is dependent on cell-cell interactions between the bacterium and the epithelial cell followed by biofilm formation as a mechanism to resist clearance by bactericidal molecules and survive in a nutrient limited environment (40, 301, 314). Biofilm formation in NTHI is regulated through quorum sensing systems, leading to enhanced survival in vivo by increasing persister cell formation, inducing metabolic heterogeneity, and secretion of extracellular polymeric structures (9). Here, we show that the sapA mutant forms a structurally different biofilm than the parent strain, the sapA mutant biofilm is less dense, but taller than the parent strain. These changes in the sapA mutant biofilm are likely heme dependent, and in fact, we have demonstrated that transient heme starvation mediates NTHI morphological changes, leading to tower formation in the biofilms (302). Biofilms are initiated by aggregations of individual bacteria forming colonies on a surface; we investigated the aggregative properties of the sapA mutant as a potential initial mechanism to explain the alterations in biofilm morphology. We determined that the sapA mutant was more aggregative using a “salting out” method, which precipitate bacterial aggregations based upon their hydrophobicity. This methodology suggested that the sapA mutant is more hydrophobic than the parent strain, and
here, we demonstrated that the sapA mutant is more adhesive to hexadecane droplets confirming that it is more hydrophobic. Hydrophobicity is believed to aid bacterial colonization of host cells by facilitating contact with the host cell surface (219). Consistent with this hypothesis, we have observed that the sapA mutant induces membrane ruffling when inoculated onto epithelial cells and is hyper-invasive. Furthermore, we have previously determined that there is no change in adhesin expression in the sapA mutant, which suggests that the increase in hydrophobicity plays a key role in this phenotype (251)(Chapter 2).

Bactericidal agents must be able to penetrate the gram negative cell wall to exert their activity on either the inner membrane or cytoplasmic targets. Antimicrobial peptides disrupt the structure of the outer membrane, however their bactericidal activity is dependent on disruption of the inner membrane or interactions in the cytoplasm. As such, outer membrane physiochemical properties of the lipid and proteins are key players in the resistance to antimicrobial peptide mediated killing (238). We determined that loss of Sap transporter function decreased membrane permeability in NTHI. It is intriguing to hypothesize that the decrease in membrane permeability is a counter active measure to compensate for the loss of the Sap transporter. Because the Sap transporter is located in the inner membrane, we believe that it serves as a last line of defense; if the antimicrobial peptide concentration reaches a critical threshold, the resistance mechanisms in the outer membrane are overcome and the Sap transporter is utilized to decrease the antimicrobial peptide concentration in the periplasm. Loss of the
SapA periplasmic binding protein abrogates this last line of defense, so macromolecule changes in the outer membrane may be utilized to compensate. Previous work has shown that, reduction in the surface charge, increase in hyrophobicity and decrease in permeability all contribute to antimicrobial peptide resistance by repulsing the peptides or diminishing electrostatic and hydrophobic interactions at the cell membrane (238). For example, in *S. enerica* aminoarabinose and palmitate incorporated into the lipid A lead to a reduction in net negative charge of the outer membrane and increase in hydrophobic interactions between neighboring LPS molecules respectively (70, 113, 114, 238). Due to these previously described changes to lipopolysaccharide in other species we investigated for the presence of similar modifications in the sapA mutant. Here, we show that there are no modifications to lipid A, LOS or phospholipid content in the outer membrane of the sapA mutant compared to the parent strain. Apart from LOS and phospholipids, proteins make up a significant percentage of the outer membrane. We determined that there is a significant difference in the outer membrane protein profile between the sapA mutant and the parent strain. Unsurprisingly, we observed a change in expression of heme uptake systems, likely as a mechanism to combat Sap transporter deficiency. Additionally, we observed an alteration in virulence factor expression. Multidrug resistance efflux pumps have been previously implicated in antimicrobial peptide resistance (23, 175, 242, 263, 293). It is therefore intriguing that we observed an increase in production of a multidrug resistance protein in the sapA mutant,
although its role in antimicrobial peptide resistance in NTHI has not been determined.

Recent work has shown intracellular populations of NTHI, residing in both endosomes and cytoplasm of host cells, which are surviving in this niche and are metabolically active (206, 251) (Chapter 2). Invasion into host cells could protect NTHI from host humoral and cellular immune response and provide a barrier to antibiotic treatment. During in vitro infection studies, NTHI has been detected in both the endosomal compartment and the cytoplasm (206, 251) (Chapter 2). Importantly, there may be an increase in available nutrients in the host cells, especially in the cytoplasm, which would be actively sequestered from extracellular populations. Therefore, it is plausible that intracellular communities could contribute to chronic NTHI infections of the respiratory tract and is supported by evidence of NTHI within adenoids and bronchial tissue samples (17, 91, 130, 204). These previous observations suggested that populations of NTHI might reside in the intracellular niche during middle ear infections. Here, we demonstrated intracellular populations of NTHI two days post inoculation. We did not observe a Sap transporter dependant change in invasion despite previous in vitro data indicating that the sapA mutant in hyper invasive. We have previously demonstrated that transient heme starvation leads to a hyper invasive phenotype similar to the sapA mutant (302). Intriguingly, the heme dependant hyper invasion appears to lead to larger intracellular populations suggesting a
stronger phenotype (302). Future studies will determine if transient heme starvation leads to hyper invasion in the middle ear.

Treatment of these chronic and recurrent NTHI infections has put a large burden on the administration of antibiotics. Middle ear infections are among the top reasons for antibiotic prescriptions in pediatric patients and are therefore one of the leading causes for the development of antibiotic resistance bacterial strains. For example, overuse of antibiotics, improper dosage, and horizontal gene transfer have all reduced the efficacy of antimicrobial agents. Because conventional agents target essential cell processes, drug resistance is acquired quickly. The strong selective pressure for resistance to inhibition of essential processes enriches for activation of alternate pathways which may be utilized to compensate for the loss of function, upregulation of efflux pumps may be used to export the drug out of the cell, decrease uptake of the drug or compartmentalize the drug all of which render the agent ineffective. However, inhibition of virulence rather than viability provides a way to attenuate the disease without exerting a strong selective pressure for resistance because essential processes are not inhibited. Because of this, the development of novel antibiotics to treat these chronic infections without applying environmental pressures leading to resistance is essential. Previous work from our lab has demonstrated the essential role of the Sap transporter in mediating heme uptake and antimicrobial peptide resistance in NTHI and ultimately biofilm formation, invasion and disease severity (191-194, 251, 279, 302, 321). Because of the essential role of the Sap
transporter for sensing and mediating interactions with the host microenvironments, we believe that small molecule inhibition of Sap transporter function would be an effective way to clear both acute and chronic NTHI mediated middle ear infections.

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4.1 Nasopharyngeal colonization

Bacteria rapidly colonize the normal upper airway after birth. The colonizing bacteria are in a constant state of flux due to ongoing turnover, in the case of NTHI, strains can persist for weeks to months but are eventually replaced with another strain (189). Early colonization with potentially pathogenic bacteria, such as NTHI, Streptococcus pneumonia, and Moraxella catarrhalis, represent a risk factor for upper respiratory tract infections (82, 172). The reservoir of bacteria in the nasopharynx has been identified as the population seeding middle ear infections, the same Streptococcus capsular type isolated from the nasopharynx was simultaneously isolated from the middle ear at the onset of otitis media (8, 10, 154, 174). Nasopharyngeal colonization with these bacteria precedes disease, however there is little damage to the host during nasopharyngeal colonization (8) (Fig. 1). Although nasopharyngeal colonization is asymptomatic, inoculation of Streptococcus into the nasopharynx of mice is associated with an inflammatory response characterized by the opening of the epithelial cell barriers, cytokine production and neutrophil infiltration, suggesting that commensal colonization of the nasopharynx does induce inflammation (328). Furthermore, inflammation induced by commensal colonization is enhanced by competition in
the nasopharynx. Co-inoculation or sequential challenge of potentially pathogenic strains either leads to inflammation and clearance or co-existence. Competition between NTHI and *Streptococcus* leads to an increase in neutrophil influx that is strain specific (189, 253). The neutrophil influx and concurrent increase in inflammation can damage the mucosal epithelium and impair the innate mucosal defense mechanisms. The impairment of the innate mucosal defense mechanisms is hypothesized to be a predisposing factor for disease (259). Although the process of early colonization by commensal bacteria is not symptomatic, changes in the host nasopharyngeal epithelium can predispose patients for later pathogenic transitions.

The majority of research to date has focused on disease progression and treatment with an emphasis on understanding the risk factors for colonization by pathogens and predisposing conditions such as age, gender, breast-feeding, daycare and exposure to smoke (236). Therapeutic interventions have also been a major focus of previous work with several groups investigating vaccine development and the impact of disease treatment such as antibiotic administration (236). However, there has been a move towards understanding nasopharyngeal colonization in the context of a polymicrobial community and commensal colonization. There is a dynamic interaction in the nasopharynx in regards to persistent colonization and clearance of bacteria and the role this may play in disease progression or asymptomatic colonization (240). However, inflammation in the nasopharynx predisposes for disease, so control of
inflammation may be a new target to prevent disease progression. The microbial community of the upper respiratory tract has a role in the regulation of virulence (236). Commensal bacteria in the nasopharynx can regulate the microenvironmental cues to reduce inflammation, such as maintaining pH and available nutrients. Unfortunately, antibiotic treatment can change key members of the normal respiratory flora, which can impact the normal host defense. These changes can impact disease development and even promote subsequent infections (236).

4.2 NTHI colonization in the nasopharynx and middle ear

NTHI is exposed to complex host and microbial microenvironments in the nasopharynx. Colonization in the nasopharynx depends on out-competing other bacteria at the mucosal surface, obtaining sufficient nutrients and resisting bactericidal molecules produced by the host. Therefore, we believe that the nasopharynx is an environment in which the host immune response is maintained at low levels such that there is a consistent turnover of bacterial colonizers. In this environment, it is possible that free essential nutrients are sufficient for minimal growth but not abundant enough to permit rapid expansion of bacterial populations. The host permits colonization of the nasopharynx, however colonization of other anatomical locations, such as the middle ear, by NTHI is actively inhibited. The Eustachian tube prevents bacterial ascension into the middle ear and maintains the middle ear pressure. Mucociliary clearance is a mechanical mechanism of the Eustachian tube epithelium to prevent bacterial
translocation; cilia on the epithelial cells beat and push mucous away from the middle ear. Therefore, this mechanism must be impaired for NTHI to colonize the middle ear. Viral infections often precede middle ear infections and this is attributed to viral inhibition of cilia function, loss of cilia on the epithelial cells and immune system impairment. In addition to mechanical clearance, antimicrobial peptides and nutritional immunity inhibit bacterial growth and kill ascending bacteria. Therefore, when bacteria colonize the middle ear space they encounter an environment that is markedly different from the nasopharynx. In the middle ear there are no competing bacteria, nutrients are actively sequestered, and the host utilizes many inflammatory processes to eliminate the bacterial invaders.

Colonization of the middle ear space can lead to two different disease states; symptomatic and asymptomatic (Fig. 15). Acute infections of the middle ear are associated with middle ear inflammation, fever and pain; thus, the intense host response to colonization leads to symptomatic infections. In contrast chronic and subclinical infections of the middle ear space are often associated with antibiotic resistant and asymptomatic infections, with middle ear effusions in the absence of inflammation or observable tympanic membrane pathology (103, 229). We know that both challenge by bactericidal molecules and nutrient availability can alter bacterial response and colonization. During commensal colonization in the nasopharynx, NTHI is asymptomatic, suggesting that in this environment NTHI NTHI to maintain the commensal interactions with the host in the nasopharynx are unknown. Additionally, competition in the nasopharynx can promote or inhibit
FIG. 15 Acute and Chronic Disease model
the pathogenic nature of NTHI. Because competition in the nasopharynx leads to inflammation, competition itself could be a mechanism for NTHI to ascend the Eustachian tube. Inflammation due to competition could affect essential nutrient availability by increasing the presence of essential transition metal ion chelators. The Sap transporter is an ABC transporter required for efficient heme transport across the inner membrane and important for preventing antimicrobial peptide-mediated cell lysis by transporting the peptides into the cytoplasm, thereby mitigating their destructive effects. Because NTHI requires an environmental source of heme, and the Sap transporter is required for heme transporter across the inner membrane, we were afforded a unique opportunity to investigate how nutrient availability can influence NTHI-mediated otitis media. *In vitro* and *in vivo* results demonstrate that NTHI experiencing transient heme restriction or NTHI lacking Sap transporter-mediated heme acquisition (*sapA* mutant) induce less inflammation in the middle ear. Conversely, heme replete and wild-type NTHI induce severe inflammation in the middle ear with disease markers associated with acute otitis media (302) (Chapter 2). It is therefore plausible that the nutritional status in the nasopharynx can play a key role in defining the inflammatory status during colonization of the middle ear. In this model, NTHI ascension after colonization of an environment that is nutrient limiting would lead to an asymptomatic or chronic infection, however colonization of the middle ear during continuous essential nutrient availability would lead to high levels of inflammation and acute infections. This enforces the important link between metabolism and virulence, likely mediated by the stress response (45, 52, 64,
Additionally, sensing of the host microenvironment may mediate bacterial-host interactions. Environmental sensing through the Sap transporter conveys information to NTHI on both the availability of essential nutrients as well as the inflammatory status of the host. By sensing these markers NTHI would be able to differentiate between colonization in the nasopharynx and colonization of bacteria restricted environments of the respiratory tract. For example, during commensal colonization of the nasopharynx, NTHI must be in an environment containing enough essential nutrients for survival and low enough local concentrations of bactericidal molecules to resist clearance. This would suggest that in the nasopharynx, NTHI is able to sense and transporter sufficient heme through the Sap transporter and does not have to use the Sap transporter for antimicrobial peptide resistance. As NTHI transitions to the middle ear, the local concentrations of heme decrease and the concentration of antimicrobial peptides increase in the Eustachian tube. Upon arrival into the middle ear space, the environment is nutrient depleted and high amounts of antimicrobial peptides are secreted after the epithelium senses colonization. Following colonization, an acute infection would stimulate high levels of inflammation leading to a rapid and large influx of immune cells and subsequent release of high concentration of antimicrobial peptides, epithelial cell damage and nutrient release. Therefore, during acute infections NTHI encounters a rapid change in the local concentration of heme and antimicrobial peptides, and senses the change by transport of these molecules through the Sap transporter. In contrast, during chronic and subclinical colonization of the middle ear, inflammation is not as
severe. Therefore, there is not a rapid change in the local concentrations of heme and antimicrobial peptides, as we hypothesize that the environment in the middle ear resembles the nasopharynx, such that a stress response and increase in virulence are not required by the bacteria to maintain colonization. Ultimately, NTHI can use the Sap transporter to sense the temporal and spatial localization in the respiratory tract by transport of heme or antimicrobial peptides. Because of the stark difference in these environments between the nasopharynx and the middle ear it is intriguing to imagine that other potential pathogens, such as *Streptococcus* and *Moraxella* may potentially use similar sensing techniques to ultimately mediate interactions with the host.

4.3 NTHI lifestyles during subclinical and chronic infections

Because of the drastic differences between chronic-subclinical middle ear infections and acute middle ear infections, NTHI has alternative lifestyle choices during each infection. In contrast to acute infections it is predicted that during chronic or persistent infections NTHI would reside in the intracellular as well as extracellular spaces. Utilization of game theory provided a simple heuristic model to predict host-pathogen interactions during persistent infections and it was estimated that bacteria would colonize and survive in both the intracellular and extracellular spaces during persistent infections (80). Additionally, subclinical and chronic infections are associated with less inflammation and symptoms; therefore it is likely that the bacteria would use different mechanisms for survival. Consistent with the predicted computational modeling, we have
determined using a model of in vitro infection on respiratory epithelial cells that transient heme restriction or an NTHI sapA mutant were hyperinvasive and colonized both the extracellular space and intracellular niche during infection (302) (Chapter 2). The hyperinvasion of the sapA mutant was preceded by epithelial cell membrane ruffling (Chapter 2). The differential localization between the parent and mutant strain suggests that NTHI does have a mechanism to escape the endosomal pathway and colonize the cytoplasm. The parent strain was able to invade into epithelial cells, however all incidences of invasion were associated with an endocytic vacuole, loss of electron density and loss of bacterial membrane integrity, suggesting that invasive parent strain bacteria were effectively cleared following epithelial cell invasion and prevented from colonizing the cytoplasm. It is currently unknown where in the endocytic pathway these NTHI become cleared and efforts are ongoing on identifying the compartment NTHI localize to. Work by Morey et. al. demonstrate that tubulin dependent invasion into epithelial cells traffic NTHI to an acidic subcellular compartment that contains late endosome features yet lacks autophagy related markers (206). In contrast to the parent strain the sapA mutant was effectively able to colonize and survive in the cytoplasm of the epithelial cells. Large populations of the sapA mutant were observed in the cytoplasm and were not associated with the endocytic pathway, suggesting that NTHI has a mechanism to escape the endosome and colonize the cytoplasm. However, it is not known how NTHI can escape the vacuole; surface proteins likely mediate this process through mechanisms such as membrane disruption, modification of membrane
domains, or location and movement as NTHI lacks traditional secreted effectors (112). Further, this suggests that NTHI has mechanisms to prevent degradation by autophagy and prevent identification by cytoplasmic Nod-like receptors (190)). The populations of invaded sapA mutant were not single cells in the cytoplasm, but large populations indicating that replication was occurring in the cytoplasm. Because of these large populations of the sapA mutant in the cytoplasm, quantification of the invading bacteria was challenging. Traditional methods to identify colony-forming units of invasive bacteria would vastly underestimate the number of bacteria present per cell. We were able to quantify a two-fold increase in the number of invasive sapA mutant compared to the parent strain, however, by microscopy we identified a much larger population of the sapA mutant in the cytoplasm compared to the parent strain. Similarly, the invasive populations of NTHI identified in vivo suggest that there is no difference in invasion between the parent and the sapA mutant. However, large populations of the sapA mutant in single cells would be measured as a single colony-forming unit because the epithelial cells were not lysed before enumeration. Therefore, we are likely underestimating the invasive populations in the middle ear. Identification of epithelial cell invasion events during chronic or subclinical infections in pediatric patients is problematic because patients with subclinical infections would be unlikely to visit the doctor and even if tissue samples were taken from chronic infecting patients, the tissue sample would likely be too small to readily identify intracellular populations. However, significant invasive populations should be
considered during treatment as they would be more antibiotic resistant and could seed subsequent infections.

NTHI has no known mechanism for escape from the endocytic trafficking pathway, however the IgA1 protease may be at least one such mechanism for modifying the lysosome. Previous work has identified that the IgA1 protease can cleave LAMP1, which leads to destabilization of the lysosome and subsequently increases invasion of host cells (12, 125, 139, 177). Additionally, the IgA1 protease can inhibit TNF-α mediated apoptosis by cleavage of the TNF receptor II, delaying apoptosis of the host cell to increase the time for colonizing the cytoplasm (21). It is therefore plausible that the IgA1 protease in NTHI could serve a similar function in host cells. We have identified through mass spectrometry that the IgA1 protease is increased in the outer membrane of the parent strain when grown under laboratory conditions. However, the IgA1 protease is a secreted protein, therefore, it is possible that secretion is more efficient in the sapA mutant so less is detected in the outer membrane. If the IgA1 protease does indeed assist survival in the intracellular populations, it would suggest that there is an increase in production of this molecule by the sapA mutant because it is hyper-invasive and able to form large populations in the cytoplasm of epithelial cells. We do not know if the expression of the IgA1 protease is increase in expression in the sapA mutant or how the expression of the IgA1 protease is regulated. However, low pH is a plausible signal for expression, which would increase IgA1 protease secretion in acidic
compartments in the host cell. On the other hand, IgA1 protease secretion can induce the expression of inflammatory cytokines by the host, such as TNF-α, IL-1β, IL-6 and IL-8, leading to unwanted recruitment of leukocytes. The stimulation of expression of these cytokines is independent of the proteolytic activity of the protease (181). Therefore, it is likely that there is a decrease in expression of the IgA1 protease during commensal colonization of the host. Together, this suggests that during commensal and chronic infections of the host, IgA1 is expressed at low levels, however upon onset of inflammation and subsequent increase in IgA1 production or after invasion of host cells, there is an increase in production of the IgA1 protease as a mechanism to resist clearance by the host. There, the ability to spatially and temporally regulate production of IgA1 may facilitate bacterial manipulation of host endocytic vesicular trafficking without inducing inhibitory levels of pro-inflammatory cytokines.

4.4 NTHI biofilm contributions to disease

Biofilms represent a highly conserved survival mechanism facilitating bacterial survival in nutrient-limited environments and providing increased resistance to bactericidal molecules. Biofilm survival strategies are mediated by the expression of quorum sensing regulated genes, increase in persister cell formation, metabolic heterogeneity, and secretion of extracellular polymeric structures. We observed that prior environmental heme-iron restriction induced a heritable change in biofilm formation associated with increased peak height and bacterial filamentation dependent on DNA-methylase epigenetic changes (302).
Genetic heme-restriction (sapA mutant) similarly altered biofilm formation (Chapter 3). These data suggest that heme-restriction modifies biofilm structure and that host microenvironmental cues may influence a similar phenotype in vivo. In fact, we have demonstrated that both prior genetic or environmental heme-restriction attenuates the host response during experimental otitis media (302) (Chapter 2). Middle ears inoculated with either genetic or environmental heme-restricted NTHI demonstrated a decrease in epithelial cell inflammation and an intact mucosal surface despite no difference in bacterial numbers. The epigenetic changes to biofilm structure and architecture observed in the transient heme-restricted NTHI may contribute to chronic NTHI infections. Chronic NTHI infections persist despite antibiotic treatment and host clearance mechanisms, implicating biofilm and intracellular populations as mechanisms of resistance. This suggests that during chronic infections NTHI is able to maintain a specific survival program in the face of fluctuating nutrients and bactericidal molecules. Therefore, epigenetic changes would maintain expression of specific proteins to dictate biofilm structure and hyper-invasion and prevent NTHI from shifting between the commensal-like colonization of the middle ear during chronic infections to pathogenic acute middle ear infections. However, pathogenic bacteria use metabolic fluctuations in the host for spatial and temporal regulation of virulence factor expression (283). Changes in nutrient levels, such as amino acids and fatty acid limitation, can also activate virulence factor expression through the stringent response (66). Therefore, it is necessary to investigate the expression and protein production profiles of NTHI during acute and chronic
infections to determine the relative contributions of inherited gene expression and metabolic regulation of gene expression.

Biofilms are heterogeneous populations of bacteria; gradients of nutrients and oxygen decrease away from the biofilm surface and are associated with decrease in metabolic activity and increased doubling time. The decrease in metabolic activity and increase in doubling time are attributed to some of the inherent antibiotic resistance of bacteria growing in a biofilm (133). NTHI is commonly the initial organism isolated from patients with CF and evidence of NTHI biofilms in CF can be detected from bronchiolalveolar lavage fluid (292). Importantly, NTHI colonization and biofilm formation induces an inflammatory response; therefore, it is plausible that the early interactions between NTHI and the CF lung may enhance the cyclical nature of inflammation and tissue damage.

Similarly, NTHI is a chronic pathogen in patients with COPD. The abnormal immune system activation and chronic tissue damage in both patients with COPD and CF likely contribute to NTHI colonization of the lung. Chronic inflammation in these patients causes an interesting paradox; neutrophils and other leukocytes actively chelate and sequester essential transition metal ions as a mechanism of the host innate immune defense, however the chronic inflammation during COPD or CF causes epithelial cell damage leading to a release of essential nutrients. Because pathogens are able to colonize the lungs of patients with COPD and CF there must be a positive net release of nutrients from the epithelial cells to provide nutrients for colonization. Additionally
antimicrobial peptides are salt and pH sensitive; therefore the bactericidal activity of these molecules would likely be decreased during COPD or CF (37). Therefore, biofilm formation and colonization of patients with NTHI is facilitated by a chronic, abnormal immune response, a surplus of free nutrients and decreased activity of antimicrobial peptides. Biofilm formation is likely selected for to counter the constant inflammation and reduce clearance. Although NTHI colonization of these patients is unlikely to be life threatening, treatment of early NTHI infections during COPD and CF could reduce the initial levels of tissue damage, thus potentially slowing the progression of disease.

Therapeutics targeted at essential prokaryotic cellular processes are attractive because of their high efficacy and specificity. However, because essential processes are targeted, resistance is quickly acquired. Resistance to therapeutics can arise through a variety of mechanisms; bacteria may employ alternative pathways to accomplish the same essential functions, upregulate expression of drug efflux pumps, decrease drug uptake or compartmentalization the compound in a manner which renders the therapeutic infective. Inhibition of virulence rather than inhibition of viability provides a way to attenuate disease without exerting a strong selective pressure. Small molecule inhibition of the Sap transporter would render NTHI more susceptible to antimicrobial peptide mediated killing, prevent essential nutrient acquisition and drive infections towards asymptomatic colonization, thereby reducing host tissue damage. Importantly, inhibition of the Sap transporter would be effective at clearing both
acute and chronic infections. During acute infections NTHI is largely
extracellular, therefore inhibition of the Sap transporter would render the bacteria
more susceptible to antibiotic mediated clearance and reduce inflammation.
During chronic infections, inhibition of the Sap transporter would prevent
essential nutrient acquisition ultimately starving the bacteria and promoting
clearance. Our data suggest that inhibition of the Sap transporter would promote
intracellular populations of NTHI; it is therefore necessary to further investigate
the pathogenic lifestyle of heme-iron restricted NTHI. This strategy of employing
small molecule inhibitors to target essential nutrient acquisition or host
bactericidal molecule resistance could be effective at promoting clearance of
non-life-threatening bacterial diseases in an immunocompetent patient.

The ability of many bacterial pathogens to survive as either biofilm or sessile
populations poses a challenge for successful vaccination. Significant phenotypic
differences exist between planktonic and biofilm associated bacteria and
vaccines are usually effective against one of these life styles (123). Biofilm
growths are recognized as the major mode of infection and up to 80% of all
bacterial infections are mediated by biofilms (123). Development of vaccinations
aimed at preventing NTHI-mediated disease has focused on outer membrane
proteins involved in biofilm formation and adherence to promote clearance
subsequent to establishment of colonization. However, there are a number of
problems with this approach; NTHI commensal colonization in the nasopharynx
is likely biofilm mediated and NTHI biofilms in the nasopharynx may prevent
colonization of more pathogenic bacteria, in which case eradication of this bacterium from the airway is unlikely to be of benefit. NTHI colonization in the middle ear may be in concert with other pathogens such as *Streptococcus pneumonia* or *Moraxella catarrhalis* leading to biofilms where the structure of facilitated by intermicrobial and host interactions. Additionally, biofilm formation in the middle ear is likely preceded by planktonic colonization of the Eustachian tube and middle ear. Therefore, single planktonic bacteria likely accomplish NTHI translocation into the middle ear through the Eustachian tube and only a few bacteria ultimately make it to the middle ear and establish communities. Therefore, it would be valuable to develop vaccines which target these planktonic bacteria in the Eustachian tube because it would prevent damage to the middle ear, circumvent biofilm mediated resistance and leave the commensal population in the nasopharynx unharmed.

4.5 Concluding remarks

It is clear that there are many facets of NTHI pathogenesis that remain to be determined. Despite the paucity of missing information, we have a better understanding about how the host nutritional and innate immunity can influence pathogenesis. We have determined that the Sap transporter and transient heme-iron restriction decrease inflammation and promote invasion into the epithelium. It is becoming evident that intracellular NTHI may play a key role in NTHI survival during chronic and subclinical infections. However, it remains to be determined if the intracellular niche is utilized during commensal colonization in the
nasopharynx. The work presented here provides the ground work to continue studying NTHI invasive populations and chronic infections of the middle ear and it will be important to determine the contribution of the intracellular populations for the treatment of NTHI mediated diseases.
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