Non-typeable Haemophilus influenzae invasion and persistence in the human respiratory tract

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INTRODUCTION
Non-typeable Haemophilus influenzae (NTHI) is a small, acapsulate Gram-negative coccobacillus. NTHI is a common commensal of the human nasopharynx, but can cause opportunistic infection when the respiratory tract is compromised by infection or disease. Consequently, NTHI is the most common cause of exacerbations in chronic obstructive pulmonary disease (COPD), and is an important cause of exacerbations in cystic fibrosis (Sethi and Murphy, 2008). NTHI is also a leading cause of otitis media, sinusitis, and community-acquired pneumonia, often following viral respiratory infection (Murphy, 2003). A rising proportion of otitis media caused by NTHI has been attributed to widespread use of the pneumococcal conjugate vaccine, which was introduced in the year 2000 and has reduced the incidence of respiratory infections caused by Streptococcus pneumoniae (Block et al., 2004; Casey and Pichichero, 2004; Benninger, 2008).

CLINICAL, EXPERIMENTAL DATA SUPPORT AN INTRACELLULAR PHASE OR RESERVOIR OF NTHI
NTHI is classically considered to be an opportunistic extracellular pathogen and a cause of localized respiratory infections. However, NTHI infections frequently persist and recur despite antibiotic therapy, the development of bactericidal antibodies, and periods of asymptomatic, colonization, pathogenesis, invasion, trafficking, survival, epithelium, macrophage

RESPIRATORY EPITHELIUM
Host cells can offer temporary or long-term protection from immune clearance mechanisms. Intracellular NTHI was protected from antibiotics and bactericidal antibodies for at least 24 h in vitro (van Schilfgaarde et al., 1999). Respiratory epithelial cells...
play a dominant role in pathogen detection by pattern recognition receptors, and in pathogen clearance by recruiting leukocytes, inducing inflammation, and direct killing by up-regulating antimicrobial peptides (Evens et al., 2010). Evidence suggests that, while primarily the responsibility of resident macrophages and recruited leukocytes, especially neutrophils, a secondary role for respiratory epithelial cells might be to internalize and eliminate bacteria (Pier, 2000). Epithelial cells are shed during infection or injury, but normal turnover of pulmonary epithelial cells is infrequent, occurring only every 180 days, providing a long-lasting location for potential bacterial persistence, as in Escherichia coli bladder infections (Mysorekar and Hultgren, 2006; Rawlins and Hogan, 2008). Respiratory epithelial cells of lymphatic tissue, such as tonsils and adenoids, are thought to be uniquely active in antigen uptake and are loosely connected, allowing infiltration of macrophages, lymphocytes, and dendritic cells. In all, this suggests that respiratory epithelia provide a potential "haven" for NTHI.

**NTHI Colonization and Persistence Mechanisms**

As an opportunistic pathogen, NTHI colonization depends on a variety of host and bacterial factors. NTHI adheres to respiratory mucus and to an unidentified non-ciliated cell type(s) of the multiple cell types of the respiratory epithelium (Ketterer et al., 1999). Adherence is mediated by specific binding of NTHI adhesins to mucin, extracellular matrix proteins, and plasma membrane receptors (Swords et al., 2000; Ahren et al., 2001a,b; Fink et al., 2002; St Geme, 2002; Avadhanula et al., 2006; Jurcisek et al., 2007; Bookwalter et al., 2008; Pang et al., 2008a; Ronander et al., 2008).

Studies on the significance of NTHI–host cell interactions, and indeed all NTHI studies, are complicated by the aclonal nature of this bacterium. Enormous strain-to-strain heterogeneity exists in the possession, expression, and composition of many NTHI outer membrane molecules. Added complexity comes from variable expression and availability of certain host cell receptors and matrix proteins. These bacterial and host variations likely promote bacterial evasion of host clearance mechanisms. NTHI also encodes specific defense mechanisms. Protein D enhanced damage to a nasaephyrygal tissue culture model (Janson et al., 1999). The Sap transporter and lipo polysaccharide (LOS) phosphorylcholine (PCho) appear to protect NTHI against human antimicrobial peptides β-defensin and cathelicidin LL-37, respectively, which are important respiratory defense molecules (Lysenko et al., 2000; Mason et al., 2005). NTHI LOS glycosyltransferase Lic2B activity and the ability to bind host complement inhibitors C4 binding protein, factor H, and vitronectin promote NTHI evasion of complement-mediated killing (Hallstrom et al., 2007, 2008, 2009, 2010; Wong et al., 2011). NTHI also encodes IgA1 proteases to inactivate IgA1, likely facilitating colonization, although this is difficult to confirm in animal models since IgA1 is a human-exclusive antibody. However, human lactoferrin cleaves IgA1 proteases and the homologous NTHI adhesin, Hap, suggesting that the healthy host might neutralize at least some of these potential colonization factors (Plaut et al., 2000; Hendrixson et al., 2003).

Adherent aggregates of bacteria may mature into biofilm, an important and intensely studied form of NTHI persistence in vitro and in vivo (Armbruster et al., 2009; Hoa et al., 2009; Hong et al., 2009; Murphy et al., 2009; Juneau et al., 2011; Nistico et al., 2011). Much less is known about the potential for NTHI persistence by invasion of host tissue. NTHI invasion abilities are strain-variable (Ketterer et al., 1999; van Schilfgaarde et al., 1999; Swords et al., 2000; Hotomi et al., 2010). Invasion is promoted by adhesins Hap, PCho, Protein D, and Protein E (St Geme et al., 1994; Swords et al., 2000; Ahren et al., 2001a; Ronander et al., 2009). Binding to certain host plasma membrane receptors promotes NTHI invasion of human bronchial epithelial cells and macrophages in vitro, described below (Swords et al., 2000; Ahren et al., 2001b). However, no dedicated invasins that function solely to promote invasion have been identified.

Studies to identify genetic commonalities and potential virulence factors among pathogenic or persistent strains have yielded mixed results (Erwin and Smith, 2007). However, genomic and proteomic studies in our laboratory have identified genes of interest for further examination, including igaB, a novel type II IgA1 protease (Fernays et al., 2006a,b). Nearly all strains of NTHI contain the iga gene, encoding a type I IgA1 protease. The igaB gene is found more frequently in disease isolates than in nasopharyngeal isolates of NTHI, and is homologous to the IgA1 protease of pathogenic Neisseria, which cleaves lysosome associated membrane protein 1 (LAMP1) and promotes intracellular survival. Studies on the role of NTHI IgA1 proteases in adherence, invasion, trafficking, and persistence of NTHI in human respiratory epithelial cells are in progress in our laboratory. It would be interesting to conduct parallel studies of Hap, a ubiquitous NTHI adhesin shown to promote invasion, as it is highly homologous to IgA1 proteases (St Geme et al., 1994).

Certain pathogenic bacteria express type III or type IV secretion system effector proteins to initiate receptor-independent host cell signaling, actin-dependent membrane ruffling, and internalization of bacteria, and to manipulate trafficking to promote survival (Bhavsar et al., 2007). However, the NTHI genome does not encode such secretion systems, suggesting that this bacterium must rely on existing host pathways to invade or survive within host cells or tissues. Examples of well-studied host pathways exploited by pathogens include phagocytosis, macrophagocytosis, clathrin- or receptor-mediated endocytosis, lipid raft-mediated endocytosis, autophagy, secretion, transcytosis, and paracytosis. Since many of these pathways terminate in lysosomes, successful intracellular pathogens must find ways to avoid, escape, or neutralize this deadly fate. To more fully characterize the invasion, trafficking, and survival of NTHI in host cells, recent studies have employed invasion assays, or “gentamicin protection” assays, with and without inhibitors of well-characterized host pathways to determine the relative kinetics of NTHI invasion and survival, and confocal microscopy to visualize markers of these pathways in relation to NTHI.

**Invasion and Trafficking**

There is no single “gold standard” approach to studying microbial invasion and trafficking in host cells, given the dynamic and complex nature of these interactions. Thus, it is important to use multiple complementary methods and rigorous controls when visualizing pathway markers using antibodies or fluorescent fusion constructs, and when using inhibitors, including pharmacologic inhibitors, siRNA, and dominant negative mutant constructs. The
markers and inhibitors indicated for each host component, below, often represent only some of many available. Investigators should carefully determine the pros and cons of each, since their effects and utility can vary significantly depending on experimental conditions (Ivanov, 2008).

**CYTOSKELETAL REQUIREMENTS**

Live NTHI, but not killed NTHI, induce the extension of microvilli and lamellipodia from epithelial cells, which surround the bacteria to form an enclosed vesicle (St Geme and Falkow, 1990; Holmes and Bakaletz, 1997; Ketterer et al., 1999; van Schilfgaarde et al., 1999; Ahren et al., 2001b; Swords et al., 2001). Cytoskeletal rearrangements associated with NTHI internalization include actin strand formation beneath adherent bacteria and around intracellular bacteria (Holmes and Bakaletz, 1997; Ketterer et al., 1999). Cytochalasin D and colchicine each inhibits NTHI invasion of monocyctic and epithelial cells, suggesting roles for both the actin and tubulin cytoskeletons (St Geme and Falkow, 1990; Holmes and Bakaletz, 1997; Ahren et al., 2001b).

**PHAGOCYTOSIS AND MACROPINOCYTOSIS**

Studies of phagocytosis and macropinocytosis are complicated because these processes are largely characterized by membrane dynamics with limited distinguishing features (Jones, 2007; Kerr and Teasdale, 2009). Macropinocytosis is characterized by receptor-independent, actin-dependent membrane ruffling for bulk uptake of fluid. Depending on cell type, macropinocytosis may be constitutively active or initiated in a controlled manner. Phagosomes and macropinosomes are trafficked to the endolysosomal pathway, although inconsistently, as discrepancies have been observed. In studies using primary bronchial epithelial cells, some NTHI bacteria colocalized with fluorescently labeled dextran, a high molecular weight fluid-phase marker of macropinocytosis, suggesting that macropinocytosis represents at least one pathway of NTHI invasion (Ketterer et al., 1999). Future studies might benefit from visualization of SNX5, a marker of macropinosomes, or 5-(N-ethyl-N-isopropyl)amiloride (EIPA), an inhibitor of macropinocytosis.

**LIPID RAFT-MEDIATED ENDOCYTOSIS**

Lipid rafts are plasma membrane microdomains enriched in cholesterol, glycosphingolipids, and glycosylphosphatidylinositol (GPI)-anchored molecules, with roles in signal transduction and trafficking (Mayor and Pagano, 2007; Donaldson et al., 2009). Lipid raft-mediated endocytosis produces endosomes that are trafficked in different ways that may completely avoid the endolysosomal pathway. Thus, it has been proposed that lipid raft-mediated endocytosis is a less microbicidal type of internalization (Duncan et al., 2002).

Garmendia and colleagues examined NTHI invasion of mouse alveolar macrophages and human alveolar epithelial cells (Martí-Lliteras et al., 2009; Morey et al., 2011). NTHI invasion was significantly inhibited by certain lipid raft inhibitors: methyl-β-cyclodextrin (MβCD) and nystatin, but not filipin. Nystatin and filipin each bind to cholesterol, whereas MβCD extracts cholesterol from host cell membranes, which can impair membrane integrity and interfere with other pathways, such as clathrin-mediated endocytosis. Thus, it is worth building on this work by assessing host cell cytotoxicity for inhibitor studies with additional strains and cell types. Antibodies are available for immunofluorescence of several lipid raft markers including caveolin, flotillin, and the GM1 ganglioside. Cholera-toxin B-subunit (CTxB) fluorescent conjugates are also used to identify lipid rafts, as CTxB binds to GM1.

**CLATHRIN- OR RECEPTOR-MEDIATED ENDOCYTOSIS**

Clathrin-mediated endocytosis, or classical receptor-mediated endocytosis, produces endosomes that are typically trafficked by the endolysosomal pathway (Veiga et al., 2007). Specific inhibitors of the platelet activating factor receptor (PAFR) and the beta glucocan receptor (βGR) indicate that both receptors mediate NTHI internalization into respiratory epithelial cells, and that βGR, but not PAFR, mediates non-opsonic internalization of NTHI into macrophages (Swords et al., 2000, 2001; Ahren et al., 2001b). PAFR internalization is clathrin-dependent, so the internalization of at least some strains of NTHI is also expected to be clathrin-dependent, but this hypothesis has not been tested. Future studies might benefit from visualizing or inhibiting clathrin and associated proteins, including dynamin, AP180, and epsin. Pharmacologic inhibitors include chlorpromazine, monodansylcadaverine, and dynasore. Monodansylcadaverine significantly inhibited NTHI invasion of human monocytes (Ahren et al., 2001b).

While NTHI binds to βGR via an unknown bacterial ligand, NTHI binds to PAFR via PCho. PCho is a phospholipid acquired from the host cell surface and expressed on the bacterial cell surface of NTHI and several other diverse species of respiratory bacteria, indicating that this unusual bacterial structure is important for colonization of the respiratory tract. PCho has received increased attention for its contributions to NTHI colonization and persistence. Specifically, PCho reduces the potency of LOS as an endotoxin and promotes NTHI adherence, invasion, biofilm maturation, resistance to host antimicrobial peptide cathelicidin LL-37, and resistance to pulmonary clearance (Swords et al., 2000, 2001; West-Barnette et al., 2006; Hong et al., 2007a,b; Pang et al., 2008b). H. influenzae exhibits phase-variable expression of PCho (Weiser et al., 1997). The human respiratory tract appears to enrich for PCho⁺ H. influenzae variants, which likely represents bacterial molecular mimicry of PCho present on the host respiratory epithelium and mucus layer (Weiser et al., 1998). Interestingly, PCho enhances H. influenzae sensitivity to CRP-complement-mediated killing, which is predicted to enrich for PCho⁺ variants during clinically invasive infection or at sites of inflammation (Weiser et al., 1998). Immunomodulatory properties have also been observed for PCho found on the surface of filarial nematodes (Harnett and Harnett, 2010).

**HOST CELL SIGNALING**

Platelet activating factor mediates inflammatory and immune responses, and PAFR is a G protein–coupled receptor expressed on a variety of cell types, including the apical surface of polarized human bronchial epithelial cells in vitro (Swords et al., 2001). G protein-coupled receptor activation initiates signal transduction and receptor endocytosis. PAFR binding activates pertussis toxin-sensitive and -insensitive heterotrimeric G protein complexes, which activate phosphoinositide-3-kinase (PI3K) and
mitogen-activated protein kinase (MAPK) signaling, phospholipases A₂, C, and D, and the Jak/STAT signaling pathway. Phosphatidylinositol (PI) is a membrane-associated phospholipid, and its metabolized forms play key roles in the regulation of eukaryotic signaling, membrane dynamics, and trafficking (Weber et al., 2009). PI is metabolized by kinases, phosphatases, and phospholipases. Phospholipase C cleaves PI into diacylglycerol and inositol triphosphate (IP₃). IP₃ is the most common inducer of calcium release from intracellular stores into the cytoplasm, which further propagates signaling. PI3Ks play critical roles in bacterial engulfment via actin cytoskeleton rearrangement and membrane ruffling (class I PI3Ks) and in trafficking of proteins and vesicles (class III PI3Ks).

Swords et al. (2001) described the effects of NTHI infection on host cell signaling using human bronchial epithelial cells infected with an isogenic mutant lacking PCho and with chronic bronchitis clinical isolates that varied in their reactivity to PCho antiserum. PCho–PAFR binding initiated host cell signaling through a PTX-sensitive G protein complex that was required for invasion and for induction of phospholipase activity. NTHI-induced phospholipase activity did not correlate with or appear to be required for invasion. NTHI-induced calcium release appeared to be required for invasion by PCho⁺ strains. PCho⁻ strains induced phospholipase activity and calcium release in a PAFR-independent manner, and PI3Ks were more important for invasion by PCho⁻ strains than by PCho⁺ strains, suggesting that macropinocytosis might serve as the primary pathway for PCho⁻ NTHI invasion of bronchial epithelial cells. This also suggests that macropinocytosis is a distinct route of invasion that is either less efficient or more bactericidal than PCho–PAFR-mediated invasion, since PCho⁻ NTHI exhibit significantly lower invasion values.

Garmendia and colleagues similarly demonstrated that PI3K signaling is important for NTHI invasion of mouse alveolar macrophages and human alveolar epithelial cells (Marti-Lliteras et al., 2009; Morey et al., 2011). In contrast to Swords et al. (2000) PCho⁺ strains and a PCho⁻ isogenic mutant exhibited no significant differences in adherence or invasion. Such discrepancies could be due to differences in experimental conditions, including host cell types, bacterial strains, selected time points, and multiplicity of infection, highlighting a need for further study.

These studies by Garmendia and Swords have suggested separate and distinct pathways of NTHI-induced signal transduction and invasion. PCho–PAFR signaling might promote a more efficient invasion by NTHI. While it was largely unknown if PCho–PAFR signaling promotes inflammation or persistent colonization, recent studies indicate that it might not, as PAFR-deficient mice showed unaltered inflammation and clearance of NTHI from the respiratory tract (Branger et al., 2004). Several studies have described NTHI-induced pro- and anti-inflammatory signaling pathways, as discussed in recent reviews (Li, 2003; Erwin and Smith, 2007). These findings are worth building on and repeating with different strains and relevant host cell types given the dynamic nature of these interactions.

**ENDOLYSOSOMAL TRAFFICKING**

Using confocal microscopy, Garmendia and colleagues showed that NTHI in mouse alveolar macrophages, in human alveolar epithelial cells, and in primary normal human bronchial epithelial (NHBE) cells were trafficked by the endolysosomal pathway (Marti-Lliteras et al., 2009; Morey et al., 2011). NTHI was found within vesicles positive for early endosomal antigen 1 (EEA1), a marker of early endosomes, and in vesicles positive for LAMP1, LAMP2, or CD63, which are markers of late endosomes and lysosomes. Trafficking of a PCho⁻ isogenic mutant was comparable to wild type in human alveolar epithelial cells, suggesting that PCho might not significantly influence the trafficking of NTHI.

Intracellular NTHI must avoid, escape, or neutralize the lysosome to survive. To determine if NTHI traffics to and can survive in mature lysosomes, several approaches are available. Lysotracker is a marker of acidic compartments, but its sensitivity makes it a relatively non-specific marker of lysosomes and other less acidic vesicles. Alternatively, Cathepsin D is a protease that accumulates in lysosomes and can be used as a marker of endolysosomal fusion. A significantly lower percentage of intracellular NTHI colocalized with cathepsin D than a Salmonella typhimurium control strain known to traffic to the lysosome, suggesting that NTHI either does not traffic to or cannot survive in mature lysosomes (Morey et al., 2011). Lysosome acidification inhibitors, such as concanamycin A or bafilomycin A₁, could be used to indicate whether NTHI is killed in lysosomes, if intracellular NTHI survival improves as determined by gentamicin protection assays.

**AUTOPHAGY**

Autophagy is a eukaryotic pathway induced during stress, such as starvation or infection, in which a double membrane bound vesicle, or autophagosome, is formed around cytosolic contents and trafficked to lysosomes for degradation. Autophagosomes provide a potential haven for pathogens, and certain pathogenic bacteria can modify this pathway to prevent trafficking to the lysosome (Kirkegaard et al., 2004; Deretic and Levine, 2009). Few studies have examined the role of autophagy in NTHI infection. NTHI did not colocalize with autophagy marker LC3 in alveolar epithelial cells, or in cells treated with rapamycin, an autophagy inducer (Morey et al., 2011). Additional inducers, inhibitors, such as 3-methyladenine, and markers, such as monodansylcadaverine or LC3-LAMP1 colocalization, and the distinct double membrane appearance of autophagosomes in electron microscopy are available for future studies.

**EUKARYOTIC SECRETION (ENDOPLASMIC RETICULUM, GOLGI)**

Eukaryotic secretion is the pathway by which proteins and lipids are modified and packaged through the endoplasmic reticulum (ER) and Golgi for intracellular or extracellular destinations. Certain bacterial pathogens engage this pathway to reach their destination or to form a replicative intracellular niche (Salcedo and Holden, 2005). Little is known about the role of eukaryotic secretion in NTHI infection. NTHI did not colocalize with Golgi resident proteins GM130 and TGN46 in alveolar epithelial cells (Morey et al., 2011). For future studies, several ER and Golgi markers are available, and inhibitors include brefeldin A.

**TRANSCYTOSIS AND PARACYTOSIS**

Morphological comparisons of intracellular and intercellular bacteria in transmission electron microscopy images suggest...
that intracellular bacteria appear degraded, whereas intercellular bacteria appear intact (van Schilfgaarde et al., 1995, 1999). This suggests that NTHI in host tissue might survive by transcytosis or paracytosis, meriting further investigation of these poorly defined pathways that could provide a means of NTHI escape, survival, or reemergence.

Transcytosis of NTHI through host cells might be an important mechanism by which NTHI gains access to subepithelial spaces. Interestingly, PCho binding to PAFR mediates transcytosis of *S. pneumoniae*, and binding to CEACAM1 mediates transcytosis of pathogenic *Neisseria*, which is also promoted by a neisserial IgA1 protease (Ring et al., 1998; Wang et al., 1998; Hopper et al., 2000). This neisserial IgA1 protease is highly homologous to an NTHI IgA1 protease (Fernaays et al., 2006a,b). Furthermore, NTHI binds to PAFR and CEACAM1 via PCho and P5, respectively, however, it is not yet known if any of these factors similarly mediate transcytosis of NTHI into subepithelial spaces (Swords et al., 2000, 2001; Hill et al., 2001).

Paracytosis, or penetration between host cells, was evident in microscopy studies that revealed clusters of NTHI between and beneath infected polarized human bronchial epithelial cells *in vitro* with visibly intact intercellular contacts, and between and beneath cells in respiratory tissue from patients with end stage pulmonary disease (van Schilfgaarde et al., 1995, 1999; Moller et al., 1998). Studies of NTHI paracytosis are few, but have identified hypothetical proteins that might serve as paracytins (van Schilfgaarde et al., 2000).

Summaries of the invasion and trafficking studies, above, are presented in Figure 1; Table 1.

**ASSESSING INTRACELLULAR SURVIVAL**

**CULTURE-BASED SURVIVAL ASSAYS**

van Schilfgaarde et al. (1999) demonstrated that a significant number of NTHI are capable of invading H292 human bronchial epithelial cells, a number that fell only slightly at 7 days post-infection. Similarly, Ahren et al. (2001b) investigated the ability of non-opsonized NTHI and encapsulated *H. influenzae* type b (Hib) to enter monocytic macrophages. An increasing number of intracellular NTHI, but not Hib, were cultured, and an even greater number were cultured from primary blood monocytes.

**FIGURE 1 | Model of NTHI colonization and invasion of epithelial cells.**

NTHI adheres to mucus and non-ciliated epithelial cells. NTHI aggregates mature into a biofilm composed of bacterial and host components. NTHI has been observed within, between, and beneath epithelial cells *in vitro and ex vivo*. It is clear that NTHI are internalized by macropinocytosis and are trafficked to vesicles that are positive for endolysosomal markers. It is unclear what role(s) are played by other host internalization and trafficking pathways (noted by question marks), or how these pathways affect NTHI viability. Further examination of these pathways in relation to NTHI is needed to fully characterize the journey and fate of intraepithelial NTHI.
Studies with a greater number of time points and bacterial strains are needed to provide a broader understanding of the kinetics of NTHI invasion and survival in relevant cell types. To more specifically track the survival of NTHI using an approach that is more akin to a clinical situation, future gentamicin protection assays should use a constant application of gentamicin beginning at, for example, 24 h post-infection to eliminate the possibility of continued infection or reinfection and to focus on the fate of intracellular bacteria. Garmentia and colleagues described a similar method that resulted in almost total clearance of NTHI from mouse alveolar macrophages and alveolar epithelial cells, suggesting that NTHI does not survive in these cell types (Martí-Lliteras et al., 2009; Morey et al., 2011). However, more rigorous survival assays should be performed to determine if intracellular NTHI actually dies, or if it enters an altered growth state. For example, NTHI is normally cultured on chocolate agar at 35–37°C, with 5% CO₂, but NTHI is a facultative anaerobe, and if intracellular NTHI switches to anaerobic growth, perhaps a greater number of colonies could be cultured using anaerobic culture methods, as shown in a Campylobacter jejuni survival study (Watson and Galan, 2008).

**NON-CULTURE-BASED SURVIVAL ASSAYS**
Routine culture methods, such as those used for gentamicin survival assay colony counts, are important initial measures of bacterial viability, but what is the viability and survival of NTHI relative to its location in the host? Methods to determine bacterial location include Gram staining, immunohistochemistry, electron microscopy, immunofluorescence microscopy, bacterial expression of green fluorescent protein (GFP), and fluorescent in situ hybridization (FISH). For FISH, a labeled nucleotide probe is used to target and illuminate a specific nucleic acid sequence, usually rRNA, in single bacterial cells either isolated from a sample or present within a sample (in situ), such as biofilm or host tissue.

### Table 1 | Current knowledge of NTHI invasion and trafficking*

| Common host cell components exploited by pathogens | Evidence for NTHI interaction | Remaining questions |
|--------------------------------------------------|-------------------------------|---------------------|
| Cytoskeleton                                      | Cytochalasin D and colchicine inhibit NTHI invasion of monocytes and epithelial cells, suggesting roles for actin and tubulin. | What is the role of lipid rafts? |
| Phagocytosis                                      | NTHI has been observed within monocytes and macrophages. | What is the role of clathrin? |
| Macropinocytosis                                  | Electron microscopy – epithelial plasma membrane projections appear to engulf NTHI. Fluorescent microscopy – NTHI colocalization with HMW dextran. Signaling evidence (below) – suggests that macropinocytosis is a distinct and less efficient/more bactericidal route of invasion. | Can PCho−PAFR signaling significance be extended to additional strains, host cell types? How do phospholipase inhibitors affect invasion by PCho− and PCho+ wild type and mutant strains? How do intracellular calcium chelators affect invasion by PCho− and PCho+ wild type and mutant strains? Do other signaling pathways play significant roles in invasion? |
| Lipid raft-mediated endocytosis                   | Methyl-β-cyclodextrin and nystatin, but not filipin, inhibited NTHI invasion of alveolar epithelial cells. | |
| Clathrin/receptor-mediated endocytosis            | PAFR-mediated in bronchial epithelial cells. βGR-mediated in alveolar epithelial cells and in monocytes. Monodansylcadaverine inhibited NTHI invasion of monocytes. | |
| Host cell signaling and NTHI invasion             | PCho−PAFR signaling appears to be required for optimal and efficient invasion; discrepancies have been observed. PCho+ strains require PCho−PAFR signaling to optimally induce phospholipase activity. PCho− strains also induce phospholipase activity. Phospholipase activity does not seem to be required for invasion. PCho+ and PCho− strains induce increases in cytosolic calcium. It is unclear if PCho−PAFR induced calcium is required for invasion. PI3K signaling is important for invasion by all strains, but appears to be more important for invasion by PCho− strains, suggesting that PCho− strains rely more heavily on macropinocytosis. | |
| Endolysosomal trafficking early endosome late endosome/lysosome mature lysosomes | NTHI colocalizes with EEA1. NTHI colocalizes with LAMP1, LAMP2, CD63. NTHI exhibits less colocalization with Cathepsin D than a positive control strain of Salmonella. | Does NTHI traffic to mature lysosomes? Is NTHI killed in mature lysosomes? |
| Autophagy                                         | NTHI did not colocalize with LC3 in untreated cells or in cells treated with rapamycin. | What is the role of autophagy? |
| Eukaryotic secretion pathway                      | NTHI did not colocalize with cis-Golgi marker GM130 or trans-Golgi marker TGN46. | Does NTHI interact with the eukaryotic secretion pathway? |
| Transcytosis, paracytosis                         | NTHI visible between and beneath cells of respiratory tissue in vitro and ex vivo. | Are transcytosis and paracytosis significant for NTHI invasion and survival? If so, how? |

*For discussion and references, see text.*
Non-culture methods to determine bacterial viability include reverse transcription PCR (RT-PCR), which is considered to be the “gold standard” but provides no information about bacterial location. Efforts have been made to adapt FISH for detection of mRNA in situ, but the challenges of this adaptation have limited its use.

To determine bacterial location and viability, several methods are available. Membrane integrity can be assessed using fluorescent, membrane impermeant dyes, such as propidium iodide, ethidium monoazide, or propidium monoazide, which selectively permeate bacteria with compromised membranes and intercalate into their DNA. These dyes are often used in combination with fluorescent, membrane permeant dyes, such as SYTO-9 (Invitrogen LIVE/DEAD®), to stain intact bacteria. However, these dye combinations cannot be used to determine the viability of bacteria inside eukaryotic cells since the cell-permeant dyes will enter the eukaryotic cell and intracellular bacteria, and the cell-impermeant dyes will not have access to intracellular bacteria.

Determining bacterial location and viability inside eukaryotic cells and tissues or other environmental matrices is a challenging task that requires methods and reagents capable of accessing the bacteria without falsely altering their viability. Sophisticated techniques have been developed to determine bacterial identity, viability, and, in some cases, in situ location (Amann and Fuchs, 2008; Nielsen et al., 2010). These techniques were originally developed to study bacteria in complex environmental samples, but might prove valuable for infection models and host tissue samples. Many of these methods assess the incorporation of stable or radioisotopes or labeled substrates and are often based on or combined with FISH, but some are based on different principles. For example, bacterial incorporation of bromodeoxyuridine (BrdU) is an indicator of DNA replication that can be visualized using anti-BrdU antibody conjugates. This method was recently used to identify and locate viable intracellular Staphylococcus epidermidis in biopsies of infected tissues surrounding biomaterial implants in mice and humans (Broekhuizen et al., 2010). Such techniques could have applications for NTHI infection models or patient samples.

CONCLUSION

It is clear that NTHI invades and survives in human respiratory tissue, but a better understanding is needed regarding the role of intracellular NTHI in the pathogenesis of infection. Significant progress has been made, but multiple approaches with rigorous controls will be required to draw solid conclusions about these dynamic, complex interactions. Future studies (Table 1; Box 1) should make broad and careful selections from among the many and varied bacterial strains, host cell and tissue types, and immunologic, pharmacologic, and molecular tools. Understanding NTHI invasion and survival in human respiratory tissue is critical to elucidating the pathogenesis of infection caused by this organism. Learning the mechanisms of invasion and intracellular survival by NTHI will identify key opportunities for the development of interventions that might include novel antimicrobial agents, immunomodulators, and vaccines.

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