Haemophilus influenzae Protein F Mediates Binding to Laminin and Human Pulmonary Epithelial Cells

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The mucosal pathogen nontypeable Haemophilus influenzae (NTHi) adheres to the respiratory epithelium or, in the case of epithelial damage, to the underlying basement membrane and extracellular matrix that, among other proteins, consists of laminin. We have recently identified protein F, an ABC transporter involved in NTHi immune evasion. Homology modeling of the protein F tertiary structure revealed a strong resemblance to the streptococcal laminin-binding proteins Lbp and Lmb. Here, we show that protein F promotes binding of NTHi to laminin and primary bronchial epithelial cells. Analyses with recombinant proteins and synthetic peptides revealed that the N-terminal part of protein F contains the host-interacting region. Moreover, protein F exists in all clinical isolates, and isogenic NTHi Δhpf mutants display significantly reduced binding to laminin and epithelial cells. We thus suggest protein F to be an important and ubiquitous NTHi adhesin.

Keywords. ABC transporter; adhesion; laminin; nontypeable Haemophilus influenzae; protein F; pulmonary epithelial cells; respiratory tract infection; virulence.

Nontypeable Haemophilus influenzae (NTHi) is a gram-negative opportunistic pathogen that colonizes the nasopharynx of humans. It is one of the leading causes of bacterial respiratory tract infections, such as acute otitis media in children and bronchitis, as well as exacerbations in patients with chronic obstructive pulmonary disease (COPD) [1–3]. To prevent clearance by the ciliated respiratory tract mucosal epithelium an important initial step of bacterial colonization is adherence to host tissue [4]. In patients with disruption of the epithelial integrity caused by viral infections, mechanical damage, or chronic inflammation, the underlying basement membrane is exposed to the lumen and forms a viable attachment site for pathogens [5].

The heterotrimeric glycoprotein laminin (Ln; approximately 800 kDa) is one of the major constituents of the extracellular matrix (ECM) and the basement membrane. It is involved in an array of physiological functions, such as cellular proliferation, migration, and structural scaffolding in tissues [6–8]. During bacterial pathogenesis, Ln is frequently targeted for adherence by respiratory tract pathogens [9–16]. NTHi has previously been reported to bind Ln via the Haemophilus adhesion and penetration protein (Hap) and protein E (PE) [17, 18]. In parallel, Hap and PE interact with host epithelial cells [19, 20]. These interactions are thus multifactorial and involve several adhesins that mutually promote bacterial host-adhesion.

We recently identified a virulence factor in NTHi that we designated as protein F (PF; Su et al, unpublished data). PF (approximately 30 kDa) is annotated as a bacterial metal-binding receptor, belonging to the adenosine triphosphate–binding cassette (ABC) transporter family. During structural analysis of PF, we noticed a strong structural resemblance to the
Ln-binding proteins of *Streptococcus pyogenes* (Lbp) and *Streptococcus agalactiae* (Lmb) [21, 22]. The structural similarity raised a hypothesis regarding the potential Ln-binding capacity of PF. In the present study, we show that PF is a novel surface-exposed Ln-binding protein that also promotes NTHi adherence to host epithelial cells. This is the first report that shows a direct interaction between a gram-negative ABC transporter protein and host components.

**METHODS**

**Bacteria, Eukaryotic Cells, Culture Conditions, and Reagents**

NTHi 3655 wild-type (wt), Δhap, and Δpe mutants, and clinical isolates KR217, KR314, KR315, KR336, KR385 obtained by nasopharyngeal swabbing from patients with upper respiratory tract infection, were cultured as described elsewhere [17]. Chloramphenicol (10 µg/mL) was used for selection of Δhpf mutants. *Escherichia coli* BL21 (DE3; Novagen) and DH5α (Invitrogen) were cultured in Luria-Bertani broth and solid-phase medium supplemented with kanamycin (50 µg/mL) or ampicillin (100 µg/mL). The type II alveolar A549 (American Type Culture Collection [ATCC] CCL-185) and bronchial epithelial NCI H292 (ATCC CRL-1848) cell lines were cultured in F-12 and Roswell Park Memorial Institute medium (Gibco), respectively, with 10% fetal calf serum. Primary bronchial epithelial cells were obtained from a healthy adult donor with no history of lung disease. This protocol was approved by the Swedish Research Ethical Committee in Lund (FEK 413/2008), and written consent was obtained from the closest relatives. Cells were maintained in BEGM medium (Clonetics). All bacteria and human cells were cultured at 37°C with 5% CO₂. Anti-PF polyclonal antibodies (pAb) were raised in rabbits and affinity purified using recombinant PF (rPF) or PF peptides [23].

**Structural Modeling and Bioinformatic Analyses**

Modeling of PF was performed using the Swiss-Model automated server against homologous templates available in the Protein Data Bank (PDB; available at: http://www.rcsb.org). Homologs were analyzed with BLAST (available at: http://www.ncbi.nlm.nih.gov/), and multiple alignment was performed using ClustalW (available at: http://www.ebi.ac.uk/). Three-dimensional models were prepared using PyMOL (available at: http://www.pymol.org/).

**Construction of NTHi Δhpf Mutants**

Upstream (621 bp) and downstream (997 bp) flanking regions of the *hpf* gene (CGSHi3655_02309) from NTHi 3655 genomic DNA (GenBank accession number AAZF00000000) and cat (chloramphenicol acetyltransferase; BAA78807) from pLysS (Novagen) were amplified (Supplementary Table 1). Thereafter, we produced a linear knockout vector, inserting cat between the flanking regions, using an overlap extension polymerase chain reaction (Supplementary Figure 1). The *hpf* gene in NTHi 3655, clinical isolates, and the isogenic NTHi 3655 Δpe was mutated as described elsewhere [24].

**Recombinant Proteins and Peptides**

We amplified full-length and truncated fragments of NTHi 3655 *hpf*, using specific primers (Supplementary Table 1). Restriction sites were introduced as indicated. pET26(b)+ (for purified recombinant proteins) or pET16b (Novagen; for surface expression of PF) were used and transformed into *E. coli* DH5α, followed by transformation into *E. coli* BL21 (DE3). Recombinant proteins were produced as described previously [25]. Peptides (approximately 25 amino acids long) spanning the entire mature PF and overlapping with neighboring peptides were from Innovagen. The radiolabeled peptide-binding assay was performed as described elsewhere [26].

**Flow Cytometry and Transmission Electron Microscopy**

For flow cytometry, stationary phase bacteria (10⁹ colony forming units/mL) were washed and resuspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) followed by addition of Ln (Engelbreth-Holm-Swarm murine sarcoma basement membrane, Sigma-Aldrich). The samples were incubated at 37°C for 1.5 hours. Bacteria were thereafter washed in PBS plus 1% BSA and were incubated with rabbit anti-Ln pAb (Sigma-Aldrich). After washing, fluorescein isothiocyanate–conjugated swine anti-rabbit pAb (Dako) were added. Finally, bacteria were analyzed by flow cytometry (EPICS XL-MCL, Beckman Coulter). Ten thousand events were measured for each sample. Transmission electron microscopy (TEM) was performed as described previously [17].

**Bacterial Adherence Assays**

Adherence of NTHi to immobilized Ln was studied by bacterial probing over Ln-coated glass slides [17]. NTHi adherence to mammalian cell lines and primary cells was analyzed using ['H]-thymidine-pulsed bacteria as described elsewhere [19]. Blocking was conducted with 20 µg of antibodies per well.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Laminin binding to rPF fragments was studied with an indirect ELISA as described previously [17]. Recombinant truncated PF fragments (50 nM) and synthetic PF peptides (1 µM) were immobilized on polysorp microtiter plates (Nunc). Blocking was done with PBS containing 5% milk for 1 hour at room temperature. For the binding-inhibition assay, Ln (10 nM) was preincubated with PF peptides (0–5 µM) at 37°C for 1.5 hours. The suspension was thereafter transferred to plates coated with rPF12-293, and the binding was measured as described above. In the direct cell ELISA, cells were grown to confluenf in 96-well plates (Nunc) and fixed with 2% formaldehyde for 30 minutes at room temperature. Thereafter, cells were incubated with PF and, subsequently, rabbit anti-PF
pAb. Horseradish peroxidase-conjugated anti-rabbit pAb (Dako) was used as the secondary layer for all ELISA.

**Statistical Analyses**

We used the Student t test and Mann–Whitney U test for statistical analyses of 2 parametrical sets of data and 2 nonparametric sets of data, respectively. Two-way analysis of variance was used for statistical analyses of several sets of data. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). A P value of ≤ .05 was considered statistically significant.

**RESULTS**

**PF Is Surface Associated and Has a Tertiary Structure Similar to That of Streptococcal Laminin-Binding Proteins**

We have recently identified PF, an NTHi protein involved in serum resistance (Su et al, unpublished data). The *hpf* gene encodes an uncharacterized 30-kDa iron-chelating protein and is one of 4 structural genes in an ABC transporter operon. The operon is present in all 20 available NTHi genomes in GenBank and is highly conserved (>98% identity; data not shown). To experimentally examine the subcellular localization of PF and determine whether it is exposed on the bacterial surface, we constructed an isogenic NTHi 3655 Δ*hpf* mutant (Supplementary Figure 1A). The physiological fitness of the mutant did not differ from the parental strain with regard to the whole cell protein profile, but a slightly slower growth was observed in soluble medium (data not shown). Transcription analysis showed that the other genes in the operon remained actively expressed in the isogenic mutant (data not shown). By use of TEM, gold-labeled anti-PF pAb recognized PF at the surface of NTHi 3655, but it did not recognize the PF-deficient mutant (Figure 1A). Since the affinity-purified anti-PF pAb were highly specific for PF (Supplementary Figure 1B), this experiment suggested that PF is a surface-exposed protein.

To elucidate the characteristics of the protein, we analyzed the tertiary structure of PF by using homology modeling. The manganese-binding protein MntC of cyanobacteria *Synechocystis* species (PDB code 1XVL) was found to be the closest structure-solved homolog with a 343-bit alignment score (52.1% identity/67.0% similarity) and was therefore used as the modeling template. The superimposition of PF with MntC is shown in Figure 1B. The structural model revealed that PF is constituted by distinct N- and C-terminal globular domains that are interlinked by a long helix backbone. A metal-binding active site is present between both lobes. Since the Ln-binding proteins Lbp, of *S. pyogenes* (PDB code 3GI1), and Lmb, of *S. agalactiae* (PDB code 3HJ7), belong to the same bacterial ABC transporter family, we superimposed PF with their crystal structures. Interestingly, despite the weak primary sequence similarity (Lbp, 28.3% identity/46.9% similarity; Lmb, 28.0% identity/46.3% similarity), we observed a strong resemblance in the secondary structure and folding of PF and Lbp/Lmb (Figure 1C) [21].

**PF Is a Ubiquitous Laminin-Binding Protein of *H. influenzae***

The structural resemblance between PF and Lbp/Lmb prompted us to investigate the potential Ln-binding capacity of PF. To study the PF-Ln interaction at the bacterial surface, we incubated NTHi 3655 wild-type and the PF-deficient NTHi 3655 Δ*hpf* with increasing concentrations of soluble Ln. The unbound Ln fraction was thereafter washed away, and bacterial Ln binding was analyzed by flow cytometry. Our data showed that the isogenic Δ*hpf* mutant bound significantly less Ln as compared to the NTHi 3655 wild-type (*P* ≤ .05; Figure 2A and 2C).

To prove the Ln-binding property of PF at the bacterial surface, we introduced the *hpf* open reading frame into a heterologous *E. coli* host. After induction of *hpf* expression, we observed a Ln-binding phenotype in the PF-producing *E. coli*, compared with the control *E. coli* containing the empty plasmid (Figure 2B and 2C). Further analysis of the Ln interaction at the bacterial surface was conducted using TEM. The isogenic NTHi 3655 Δ*hpf* showed a marked reduction in Ln binding as compared to the parental strain (Figure 2D). Moreover, co-localization of gold-labeled Ln (10 nm) and PF (detected by gold-labeled anti-PF pAb; 5 nm) was observed at the surface of both PF-producing NTHi 3655 and *E. coli* (Figure 2E).

To study the functional bacterial binding to immobilized Ln, which is more representative of the in vivo basement membrane, Ln was coated on glass slides and incubated with various samples of NTHi and *E. coli* adjusted to the same cell density (optical density at 600 nm = 1.0). NTHi 3655 Δ*hpf* displayed a markedly weaker adherence to immobilized Ln as compared to the NTHi 3655 wild-type (Figure 2F). In parallel, PF-producing *E. coli* adhered to the Ln-coated glass slides, whereas the control *E. coli* did not. Taken together, the co-localization of PF and Ln at the surface of NTHi 3655, in addition to the PF-dependent Ln binding of heterologous host *E. coli*, suggest that PF is directly involved in Ln binding at the bacterial surface.

We further wanted to evaluate the Ln-interacting role of PF in a series of clinical NTHi isolates from patients with upper respiratory tract infection. PF-deficient mutants were produced in 5 clinical isolates, and Ln binding was assessed by flow cytometry (Figure 3A). A consistent decrease in Ln binding was observed in all strains in the absence of PF. The results indicate that PF is important for the NTHi-Ln interaction in several clinical isolates.

**PF Contributes to the Multifactorial NTHi-Dependent Laminin Binding**

Currently, there have been 2 reports regarding Ln-binding proteins in NTHi [17, 18]. To evaluate the relevance of PF in
comparison to PE and Hap for the NTHi-Ln interaction, NTHi 3655 wild-type and knockout mutants were incubated with soluble Ln, and binding was measured with flow cytometry (Figure 3B). Our data demonstrated that all mutants had decreased Ln binding as compared to their wild-type counterparts, showing that PE, Hap, and PF contribute to the Ln interaction.

The N-terminal Region Lys23-Glu48 of PF Interacts With the C-terminus of the Laminin α-Chain

To study the PF-Ln interaction at the molecular level, a series of recombinant PF (rPF) fragments were produced (Figure 4A and Supplementary Table 2). The putative Ln-binding region of PF was determined by incubating immobilized truncated rPF fragments with increasing concentrations of Ln. Recombinant PF$_{12-293}$ and rPF$_{12-98}$ bound significantly better when compared to the nonbinding fragment rPF$_{61-144}$ ($P \leq .001$; Figure 4B). The N-terminal rPF$_{12-98}$ also exhibited saturable and dose-dependent Ln binding, whereas no binding to the negative control fragment rPF$_{129-177}$ was detected (Figure 4C).

The Ln-binding region of PF was further analyzed in detail by using 13 synthetic peptides that span the entire PF molecule (Figure 5A). PF$_{23-48}$ was identified as the major Ln-binding region ($P \leq .001$) in an indirect ELISA. Moreover, PF$_{23-48}$ exhibited saturable and dose-dependent binding to Ln, whereas the non–Ln-binding peptide PF$_{124-148}$ did not (Figure 5B). The specificity of the interaction with Ln was evaluated with a binding inhibition assay in which Ln was preincubated with various PF peptides prior to addition to
microtiter plates coated with rPF12-293 (Figure 5C). The peptide PF23-48 inhibited Ln binding to rPF12-293, proving that the Pf23-48-Ln interaction was specific. Although Pf184-209 bound Ln (Figure 5A and 5B), the peptide did not inhibit Ln binding to immobilized rPF12-293 (Figure 5C). The N-terminal peptide PF23-48 was thus identified as the main Ln-binding region.

To determine the PF-binding domain of the Ln molecule, TEM with Ln and gold-labeled rPF12-293 was conducted. As seen in Figure 5D, PF consistently bound to the C-terminal part of the cruciform Ln molecule. When the length of the molecule was measured, PF appeared to bind to the C-terminal globular domains (LG1–5) of the Ln α-chain (Figure 5D and 5E). We conclude that the N-terminal region of PF interacts with the C-terminus of the Ln α-chain, as revealed by the peptide mapping approach and TEM.

Figure 2. Protein F (PF) mediates laminin (Ln) binding at the surface of bacteria. A and B, Flow cytometry analysis shows significant differences in the Ln binding of nontypeable Haemophilus influenzae (NTHi) 3655 wild-type and NTHi 3655 Δhpf (A) and PF-producing Escherichia coli (B) acquire previously nonexistent Ln binding, as measured by mean fluorescence intensity/bacterium. E. coli containing an empty plasmid was used as a control. The mean of 3 separate experiments is plotted, and error bars indicate the standard error of mean (A and B). Statistical analysis was performed using 2-way analysis of variance, and P ≤ .05 was considered statistically significant. **P ≤ .01; ***P ≤ .001. C, Histogram of a data set from A and B. D, Transmission electron microscopy shows a marked decrease of bound Ln (gold-labeled) at the surface of NTHi 3655 Δhpf as compared to the parental wild-type strain. E, Arrows point to bound gold-labeled Ln (10 nm) that is colocalized with PF detected by gold-labeled anti-PF polyclonal antibodies (5 nm) at the bacterial surface. The bar indicates 100 nm. F, PF promotes NTHi 3655 and E. coli-PF adherence to immobilized Ln in a basement membrane-mimicking setting, whereas non-PF-producing bacteria are seen to exhibit markedly less binding to the Ln-coated surface.
Figure 3. Protein F (PF) mediates laminin (Ln) binding in clinical isolates and contributes to the nontypeable Haemophilus influenzae (NTHi)–dependent Ln binding by an interaction analogous to that of PE and Hap. A, Flow cytometry analysis show that 5 clinical isolates display markedly decreased Ln-binding capacity when PF is knocked out, compared with the PF-producing parental wild-type (wt) strains. B, Comparison of PF-, PE-, and Hap-deficient mutants shows that the absence of all 3 proteins causes a decline in Ln binding (Ln concentration, 2.5 nM). The mean of 3 separate experiments is plotted in both graphs, error bars indicate the standard error of mean, and statistical differences are between wild-type and mutants. Statistical analysis was performed using analysis of variance, and $P \leq .05$ was considered statistically significant. *$P \leq .05$; **$P \leq .01$.

Figure 4. Recombinant protein F (rPF) binds laminin (Ln) via a region in PF$^{12-98}$. A, Results of Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis, showing the affinity purified recombinant truncated PF-fragments. B and C, rPF$^{12-98}$ contains the Ln-binding region and binds to Ln in a dose-dependent and saturable manner. Statistical analysis was performed using the Mann–Whitney U test. The mean of 3 separate experiments is plotted in B. In C, 1 set of data from 3 similar experiments is shown. Error bars indicate the standard error of mean in all experiments.
Protein F (PF) interacts with the C-terminus of the cruciform laminin (Ln) macromolecule α-chain. A, To more precisely define the Ln-binding region of PF, a series of synthetic peptides spanning the entire PF molecule were produced. Two potential Ln-binding regions, PF23-48 (consistent with the data from the recombinant fragments) and PF184-209, were identified using an indirect enzyme-linked immunosorbent assay. B, Ln binds to PF23-48 and, to a lesser degree, PF184-209 in a dose-dependent and saturable manner. C, Only PF23-48 binds specifically to Ln and inhibits the interaction with recombinant PF (rPF12-293). D, Electron microscopy showing gold-labeled rPF12-293 binding to the Ln α-chain C-terminus (as indicated by white arrows). E, Schematic representation of the Ln heterotrimer, showing the α-, β-, and γ-chains that form the cruciform molecule. The PF-binding α-chain C-terminal globular domains are indicated by an arrow. Statistical analysis was performed using the Mann–Whitney U test, and error bars indicate the standard error of the mean for all experiments. The mean of 3 separate experiments are shown in A and C, whereas 3 separate experiments were performed with the same outcome for B, of which 1 set of data is shown. $P \leq .05$ was considered statistically significant. ***$P \leq .001$. 

**Figure 5.** Protein F (PF) interacts with the C-terminus of the cruciform laminin (Ln) macromolecule α-chain. A, To more precisely define the Ln-binding region of PF, a series of synthetic peptides spanning the entire PF molecule were produced. Two potential Ln-binding regions, PF23-48 (consistent with the data from the recombinant fragments) and PF184-209, were identified using an indirect enzyme-linked immunosorbent assay. B, Ln binds to PF23-48 and, to a lesser degree, PF184-209 in a dose-dependent and saturable manner. C, Only PF23-48 binds specifically to Ln and inhibits the interaction with recombinant PF (rPF12-293). D, Electron microscopy showing gold-labeled rPF12-293 binding to the Ln α-chain C-terminus (as indicated by white arrows). E, Schematic representation of the Ln heterotrimer, showing the α-, β-, and γ-chains that form the cruciform molecule. The PF-binding α-chain C-terminal globular domains are indicated by an arrow. Statistical analysis was performed using the Mann–Whitney U test, and error bars indicate the standard error of the mean for all experiments. The mean of 3 separate experiments are shown in A and C, whereas 3 separate experiments were performed with the same outcome for B, of which 1 set of data is shown. $P \leq .05$ was considered statistically significant. ***$P \leq .001$. 

**Protein F Mediates NTHi Adhesion to the Host** • **JID 2013:207 (1 March) • 809**
PF Mediates Adhesion of NTHi to Pulmonary Epithelial Cells via a Conserved N-Terminal Domain

Since several bacterial Ln-binding proteins, including the aforementioned PE, Hap, Lmb, and Lbp, have been reported to interact with epithelial cells [15, 19, 20, 27, 28], we analyzed the cell-adhesive capacity of PF. Epithelial cells were immobilized on a solid surface and incubated with increasing concentrations of rPF12-293 (Figure 6A). Recombinant PF bound to both NCI H292 and A549 cell lines in a dose-dependent and saturable manner. To evaluate the influence of PF on bacterial adhesion, NTHi 3655 wild-type and the PF-deficient Δ hpf mutant were pulsed with [3H]-thymidine and incubated with pulmonary epithelial cells, and adhesion was measured after washing (Figure 6B). The adherence of NTHi 3655 Δ hpf to NCI H292 and A549 was reduced by 32.9% and 64%, respectively, compared with the PF-producing wild-type. Importantly, the isogenic mutant also showed a significant reduction (36.6%) in binding to primary bronchial epithelial cells obtained from a healthy adult donor (P ≤ .05; Figure 6B).

To investigate the putative cell-adhesive region of PF, cell lines were probed with iodine-labeled synthetic PF peptides, followed by washes and measurement of binding (Figure 6C). Interestingly, the Ln-binding region PF23-48 (Figure 5A) also displayed the strongest binding to epithelial cells. Moreover, the bacterial-host cell interaction was significantly reduced (P ≤ .05) when bacteria were preincubated with anti-PF23-48 pAb but not with the control anti-PF44-68 (Figure 6D). These results thus suggest that NTHi uses the same N-terminal PF region for both binding to Ln and attachment to epithelial cells.

Bioinformatic analyses and a detailed database search using BLAST showed that PF orthologs exist in a number of other pathogens. The partial alignment of PF23-48 with these is shown in Figure 7A. The orthologs present in several members of the family Pasteurellaceae and in other pathogens, such as Eikenella corrodens and Yersinia pestis, have highly similar N-termini (Figure 7A). In addition, S. pyogenes and S. agalactiae Lbp/Lmb N-terminal sequences display partial similarity to PF23-48 (Figure 7B). The 3D model of the cell- and Ln-binding region was analyzed in detail and revealed to comprise 2 β-sheets and 1 α-helix, connected by 3 loops (Figure 7C). Amino acid residues Lys23 and Lys25 in loop-1, Gln34, Asp35, Gln38, Asn39, and Asn43 in the α-helix, and Thr46 and Glu48 in β-sheet 2 are exposed on the protein surface, as predicted by the model (Figure 7D and 7E). Thus,

Figure 6. Protein F (PF) promotes nontypeable Haemophilus influenzae (NTHi) adherence to pulmonary epithelial cells via the N-terminus. A, A direct enzyme-linked immunosorbent assay shows that PF binds to fixed and immobilized NCI H292 and A549 in a saturable and dose-dependent manner. B, Deletion of hpf results in a marked decrease in the adhesion of NTHi to pulmonary epithelial cell lines, as well as to primary bronchial epithelial cells from a healthy adult donor. C, PF23-48 shows the strongest binding to both cell lines, as revealed by a radiolabeled peptide adherence assay. D, The NTHi interaction with A549 cells can be inhibited by epitope-specific anti-PF23-48 polyclonal antibodies but not with anti-PF44-68. The mean of 3 separate experiments is used for all panels, and error bars indicate the standard error of the mean in all experiments. The Student t test was used for statistical analyses in panel B and for analysis of variance in panel D. P ≤ .05 was considered statistically significant. *P ≤ .05; ***P ≤ .001. Abbreviation: NS, not significant.
the structural analysis shows that the experimentally defined host component-binding region Lys23-Glu48 contains several conserved and charged residues that are accessible for interaction with Ln and host epithelial cells.

**DISCUSSION**

NTHi has recently been reported to bind to ECM proteins such as Ln and vitronectin [17, 18, 29]. Here, we report that the NTHi ABC transporter protein PF to be a novel Ln- and cell-binding adhesin. ABC transporters constitute a large family of proteins that play important roles for bacterial pathogenesis [30]. Although previous reports have suggested similar proteins to be implicated in interactions between gram-negative bacteria and the host [31–35], this is the first study that shows a gram-negative ABC transporter protein directly mediating bacterial adherence to host components.

NTHi primarily colonizes the upper respiratory tract of humans and causes a range of airway infections. A significant rise in the occurrence of invasive NTHi infections has recently been observed, possibly due to a niche opening after the introduction of a capsule polysaccharide-conjugate vaccine against...
To disseminate from the primary site of infection, bacteria need to colonize and breach the basement membrane barrier. Pathogen interactions with ECM proteins are particularly important after epithelial disruption (such as that due to chronic inflammation and viral infections), which leaves the basement membrane exposed [5]. This is supported by the fact that NTHi infections are commonly identified in patients with COPD or during coinfections with viral pathogens [3, 38, 39]. Other respiratory tract pathogens, such as S. pneumoniae, S. pyogenes, Moraxella catarrhalis, and Mycobacterium species, have also been reported to interact with Ln and thereby augment their virulence [9–16]. Binding to ECM components such as Ln is thus most likely an important mechanism for bacterial pathogenesis.

In the present study, NTHi Δhpf mutants were shown to lose a significant part of their Ln-binding capacity as compared to the parental PF-producing wild-type strains. Moreover, expression of hpf in the heterologous host E. coli verified the role of PF as a Ln-binding protein, and colocalization of PF and Ln could be observed at the surface of PF-producing bacteria. In addition, rPF was shown to bind to both alveolar and bronchial epithelial cell lines, and NTHi 3655 Δhpf had a significant reduction in adherence to the cells as compared to the parental wild-type strain. Since NTHi 3655 Δhpf also displayed a significant loss of binding to primary cells, our collective data indicate that PF may be clinically relevant for the course of infection by promoting bacterial adherence to the host epithelium and basement membrane during colonization.

The NTHi adhesins PE (a 16-kDa lipoprotein) and Hap (a 155-kDa autotransporter) have previously been shown to interact with Ln [17, 18]. These proteins share no structural or primary sequence similarity with PF or each other, demonstrating the multifariousness of the NTHi-Ln interaction. We show that PF contributes to NTHi Ln binding to approximately the same degree as PE and Hap (Figure 3B). Furthermore, our data show that PF interacts with the C-terminus of the Ln α-chain (Figure 5D and 5E), the same region that PE binds to [17]. Because PE and PF are not similar, these data could possibly hint at a general pattern-specific binding region in the Ln molecule. The presence of additional Ln-binding factors in NTHi remains to be elucidated.

PF (also annotated as a Y. pestis yfA homologue) has been identified among other H. influenzae proteins to be upregulated during physiological conditions [40, 41]. Culturing NTHi in sputum obtained from COPD patients resulted in a 3.9-fold increase in PF concentration. Moreover, during heme- and iron-restricted growth, H. influenzae Rd KW20 was reported to enhance the hpf-operon transcription up to 11.8-fold. As the iron-concentration is low in the epithelial mucosa [42], an iron-dependent upregulation of PF may enhance the adhesive capacity of NTHi during the initial colonization of the host. These data suggest that PF is important for NTHi in vivo, possibly mediating adherence and metal ion transport.

The 30-kDa PF shares a strong structural resemblance with streptococcal Ln-binding proteins Lbp and Lmb (Figure 1B), although the primary sequence similarities are relatively weak. Preliminary unpublished data from our laboratory indicate that PF chelates Zn²⁺ via an interaction analogous to that of Lbp/Lmb [21, 22]. Assuming that the proteins share the same Ln-binding mechanism, the interactions could either be mediated by several separate but conserved sites in the tertiary structure, or they could be confined to a small local region that is highly similar in both proteins. Concurrent Zn²⁺-binding can be an additional mechanism, as has been suggested for S. pyogenes Lbp [21], since Ln is also known to bind Zn²⁺ [43]. However, we found that the PF-Ln interaction was unaffected by the divalent cation binding of PF (data not shown), suggesting that the mechanism behind the PF-Ln binding is a protein-protein interaction.

In conclusion, PF is characterized as a novel NTHi adhesin that mediates binding to the basement membrane glycoprotein Ln, as well as to human pulmonary epithelial cells. Our data shed light on the host colonization strategies of the multifaceted and important respiratory tract pathogen NTHi and will be the impetus for further studies.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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