Host cell kinases, α5 and β1 integrins, and Rac1 signalling on the microtubule cytoskeleton are important for non-typable Haemophilus influenzae invasion of respiratory epithelial cells

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Non-typable Haemophilus influenzae (NTHi) is a common commensal of the human nasopharynx, but causes opportunistic infection when the respiratory tract is compromised by infection or disease. The ability of NTHi to invade epithelial cells has been described, but the underlying molecular mechanisms are poorly characterized. We previously determined that NTHi promotes phosphorylation of the serine-threonine kinase Akt in A549 human lung epithelial cells, and that Akt phosphorylation and NTHi cell invasion are prevented by inhibition of phosphoinositide 3-kinase (PI3K). Because PI3K-Akt signalling is associated with several host cell networks, the purpose of the current study was to identify eukaryotic molecules important for NTHi epithelial invasion. We found that inhibition of Akt activity reduced NTHi internalization; differently, bacterial entry was increased by phospholipase C1 inhibition but was not affected by protein kinase inhibition. We also found that α5 and β1 integrins, and the tyrosine kinases focal adhesion kinase and Src, are important for NTHi A549 cell invasion. NTHi internalization was shown to be favoured by activation of Rac1 guanosine triphosphatase (GTPase), together with the guanine nucleotide exchange factor Vav2 and the effector Pak1. Also, Pak1 might be associated with inactivation of the microtubule destabilizing agent Op18/stathmin, to facilitate microtubule polymerization and NTHi entry. Conversely, inhibition of RhoA GTPase and its effector ROCK increased the number of internalized bacteria. Src and Rac1 were found to be important for NTHi-triggered Akt phosphorylation. An increase in host cyclic AMP reduced bacterial entry, which was linked to protein kinase A. These findings suggest that NTHi finely manipulates host signalling molecules to invade respiratory epithelial cells.

INTRODUCTION

Non-typable (non-capsulated) Haemophilus influenzae (NTHi) is a Gram-negative human pathogen that asymptptomatically colonizes the nasopharynx of healthy individuals. Age, viral infections and a constant exposure to pollutants are risk factors for development of NTHi-triggered diseases, including otitis media, sinusitis, meningitis, septicaemia and respiratory infections (Foxwell et al., 1998; Rao et al., 1999). NTHi is the most frequently isolated bacterium in the lungs of patients suffering from chronic obstructive pulmonary

Abbreviations: ECM, extracellular matrix; FAK, focal adhesion kinase; GEF, guanine nucleotide exchange factor; GTPase, guanosine triphosphatase; HRP, horseradish peroxidase; NTHi, non-typable Haemophilus influenzae; PGE2, prostaglandin E2; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase; PLC, phospholipase C; PTK, protein-tyrosine kinase; siRNA, small interfering RNA.

Three supplementary figures are available with the online version of this paper.
disease (COPD) and it accounts for a significant number of bacterial COPD exacerbations (Sethi & Murphy, 2001, 2008; Sethi et al., 2002). The information available on the molecular mechanisms involved in the progression of NTHi infections is limited. There are reports indicating that identical NTHi strains are repeatedly isolated from the lungs of chronic respiratory patients in serial clinic visits, suggesting that NTHi is endowed with features promoting chronic infection (Murphy et al., 2004). In this context, cell invasion and intracellular location could contribute to the ability of NTHi to persist. Entering host cells is a strategy used by several pathogens to survive in the hostile host environment, and bacteria previously regarded as extracellular pathogens such as uropathogenic Escherichia coli and Staphylococcus aureus have demonstrated the ability to invade non-professional phagocytic cells and behave as opportunistic intracellular pathogens (Bower et al., 2005; Oviedo-Boyso et al., 2011). NTHi has been traditionally considered to be extracellular, but different reports have repeatedly shown evidence indicating that NTHi invades epithelial cells. Thus, NTHi clinical isolates have been found to invade cultured epithelial and endothelial cells, primary human bronchial epithelium and type II pneumocytes (Ahrén et al., 2001; Ketterer et al., 1999; St Gene & Falkow, 1990; Swords et al., 2000; Virji et al., 1991). Moreover, NTHi has been found intracellularly and viable in human adenoid tissue and in bronchial human biopsies (Bandi et al., 2001; Forsgren et al., 1994), and electron microscopy has shown that intracellular NTHi remains within membrane-bound vacuoles (St Gene, 2002).

In a previous study, we reported that NTHi invades and resides within human type II pneumocytes in an intracellular compartment keeping late endosome features (Morey et al., 2011). These observations have been reviewed, discussing how important it is to understand the molecular nature of the interaction between NTHi and the human respiratory epithelium, because of a possible correlation between intracellular NTHi and symptomatic infection, and because NTHi infections frequently persist and recur despite antibiotic therapy and development of bactericidal antibodies (Clementi & Murphy, 2011).

Little is known about the molecular mechanisms that underlie the interplay between NTHi and human airway epithelial cells, and one currently unresolved question is how NTHi enters epithelial cells. We previously found that inhibition of the polymerization of host microtubule cytoskeleton significantly decreases NTHi epithelial internalization, whereas inhibition of host actin cytoskeleton polymerization increases the number of internalized bacteria. We also found that the serine-threonine kinase Akt is phosphorylated on Ser473 in cells infected with NTHi, and that cell treatment with the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 reduces Akt phosphorylation and NTHI internalization (Morey et al., 2011).

PI3K is a lipid kinase which, when active, phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to produce phosphatidylinositol-3,4,5-triphosphate (PIP$_3$) (Pizarro-Cerdá & Cossart, 2004). PIP$_3$ is a lipid second messenger that binds several downstream target proteins, including Akt (Bunney & Katan, 2010; Engelman et al., 2006; Kim & Chung, 2002). PI3K-Akt takes part in several host cell signalling networks including integrin, focal contact and guanosine triphosphatases (GTPases) (Martin et al., 2002). Integrins are a family of $\alpha \beta$ heterodimeric cell surface receptors that mediate cell–cell and cell–matrix adhesion (Caswell et al., 2009; Huveneers & Danen, 2009). The multi-domain organization and diverse modes of integrin activation enable them to interact with and respond to a range of extracellular ligands and intracellular signals, playing roles in multiple biological processes. Indeed, members of the integrin family have been demonstrated to mediate the entry of several microbial pathogens into host cells (Hoffmann et al., 2011). Integrin activation stimulates signal transduction pathways that often rely on focal adhesion kinase (FAK) and Src tyrosine kinases, at dynamic groups of structural and regulatory signal transduction proteins known as focal contacts (Guarino, 2010; Mitra et al., 2005). FAK-Src signalling recruits and/or phosphorylates adaptors and signalling factors modulating several host cell functions (Guarino, 2010). Integrins and focal contact kinases signal, among others, to members of the Rho family of small GTPases (Caswell et al., 2009; Huveneers & Danen, 2009).

In this study, we considered whether host cell integrins, kinases and GTPases are important for NTHi internalization by A549 cells. We employed specific pharmacological inhibitors, blocking or activating antibodies, and transient transfection to a number of host molecules, and performed NTHi infection, comparing bacterial invasion into treated and untreated control cells. We report that Akt and integrins $\beta 1$ and $\alpha 5$ are important for A549 cell invasion by NTHi. NTHi internalization is also facilitated by FAK and Src, and by host signalling involving Rac1 and RhoA GTPases, which might display antagonistic roles in the invasion event. Finally, we show that the secondary messenger CAMP is involved in NTHi epithelial invasion.

**METHODS**

**Bacteria, media and growth conditions.** NTHi375 is an otitis media isolate (Bouchet et al., 2003; Morey et al., 2011). NTHi398, 1566, 1606 and 1619 are clinical isolates (Marti-Literas et al., 2009, 2011; Regueiro et al., 2009). NTHi was grown on chocolate agar plates (bioMerieux) or on brain heart infusion (BHI) agar plates supplemented with 10 $\mu$g haemin ml$^{-1}$ and 10 $\mu$g $\beta$-NAD ml$^{-1}$. Bacteria were grown at 37 °C and 5% CO$_2$. Escherichia coli was grown in Luria–Bertani (LB) broth or on LB-agar plates at 37 °C, using 100 $\mu$g ampicillin ml$^{-1}$ and 50 $\mu$g kanamycin ml$^{-1}$, when necessary.

**Antibodies.** Rabbit primary antibodies were anti-FAK (Cell Signalling), anti-Pak1 (Cell Signalling), anti-Op18/stathemin (Calbiochem), anti-Vav-2 (Cell Signalling), anti-Src pY418 (Invitrogen), anti-phospho Akt Ser473 (Cell Signalling) and anti-c-Src (sc-18, Santa Cruz). Mouse primary antibodies were anti-Rac1 clone 23A8 (Millipore) and anti-RhoA (sc-418, Santa Cruz). The goat primary antibody used was anti-$\beta 1$ integrin (N-20, Santa Cruz). Secondary antibodies were goat anti-rabbit...
conjugated to horseradish peroxidase (HRP) (Sigma), goat anti-mouse–HRP (Thermo Scientific) and rabbit anti-goat–HRP (sc-2768, Santa Cruz). Blocking/activating integrin antibodies were mouse monoclonal anti-β1 integrin activating TS2/16, anti-β1 integrin blocking Lial1/2, anti-β1 integrin Alex1/4, anti-α3 integrin VI1/6.1, anti-α4 integrin HP2/1 and anti-α5 integrin P1D6 (Arroyo et al., 1992; Burrows et al., 1999; Campanero et al., 1992; Maqueda et al., 2006; Yamada et al., 2008).

**Cell culture and bacterial infection.** Carcinomic human alveolar basal epithelial cells (A549, ATCC CCL-185) were maintained as described previously (Morey et al., 2011). Cells were seeded to 6 × 10^4 cells per well in 24-well tissue culture plates for 32 h, and serum-starved for 16 h before infection to synchronize cell growth. Confluence (90%) was reached at the time of infection. Bacteria were recovered with 1 ml PBS from a chocolate agar plate grown for 16 h. Bacterial suspension was adjusted to OD 600 1 (~10^9 c.f.u. ml^-1), and added to the cells 1 h before infection and removed before infection. Antibodies were kept during infection. RGD peptide was available with the online version of this paper).

When indicated, cells were pretreated with pharmacological inhibitors as follows: (i) 30 min with 75 μM genistein (Sigma), 10 μM Y-27632 (Calbiochem); (ii) 1 h with 40 μM PP2 (Gibco), 10 μM PP3 (Gibco), 10 μM PF-573228 (Sigma), 30 μM colchicine (Sigma), 10 μM Akt VIII inhibitor (Calbiochem), 10 μM H-89 (Santa Cruz), 10 μM forskolin (Sigma), 2 μM PGE2 (Sigma), 10 μM U-73122 (Sigma), 5 μM G60983 (Sigma), 1 μM Calphostin C (Sigma), 5 μM GF92032X (Sigma); (iii) 2 h with 100 ng toxin B from *Clostridium difficile* ml^-1* (Calbiochem), 10 μM CT04 (Calbiochem); (iv) 16 h with 100 μM NSC23766 (Calbiochem). Cell treatments, inhibitors and the concentrations of choice did not induce cytotoxicity on A549 cells, verified by measuring the release of lactate dehydrogenase and microscopy (data not shown). Drug exposure was maintained during bacterial contact. Drug exposure had no effect on bacterial viability under the conditions tested (Fig. S1, available with the online version of this paper).

Integrin activation was performed by cell pretreatment with 5 mM MnCl2 for 5 min, or cell incubation with anti-β1 integrin antibody TS2/16 (dilution 1:2) for 1 h before infection. Blocking β1 integrin was achieved by cell incubation with anti-β1 integrin antibody Lial1/2 (dilution 1:2) for 1 h before infection. Anti-β1 integrin antibody Alex1/4 was used as a control (dilution 1:2). α Integrin assessment was performed by cell incubation with anti-α3 VI1/6.1, anti-α4 HP2/1 or anti-α5 P1D6 function blocking antibodies, for 1 h before infection. Antibodies were kept during infection. RGPD was added to the cells 1 h before infection and removed before infection. All infections were carried out in triplicate and independently at least three times (n ≥ 9).

**Transient transfections.** A549 cells were seeded into 24-well tissue culture plates to 4 × 10^3 cells per well, to reach 40% confluence in 24 h. Cells were washed twice with PBS; transfections were carried out in 500 μl Opti-MEM reduced-serum medium (Invitrogen) with 1 μg DNA using PolyFect transfection reagent following the manufacturer’s recommendations (Qiagen). Cells were infected 48 h post-transfection. An amplicillin-resistant vector encoding a kinase-inactive c-Src variant (SrcK297M) (Agarwal et al., 2010) and a kanamycin-resistant vector encoding Src–GFP were used. FAK, Pak1 and Op18/stathmin silencing gene expression was achieved using synthetic small interfering RNA (siRNA) purchased from Qiagen: for FAK, S100287791 and S106222130; for Pak1, S100301056 and S100301063; for Op18/stathmin, S100301875 and S105391379. Rac1, RhoA and Vav-2 silencing gene expression was achieved using synthetic SMART pools (Thermo Scientific), each comprising four proprietary siRNA sequences: for Rac1, ON-TARGET plus SMART pool L-003560-00-0005; for RhoA, ON-TARGET plus SMART pool L-003860-00-0005; for Vav-2, ON-TARGET plus SMART pool siRNA L-005199-00-0005. β1 Integrin silencing gene expression was achieved with β1 integrin siRNA (h) sc-35674 (Santa Cruz). AllStars Negative Control siRNA (Qiagen) was used as non-silencing control interference RNA. siRNA (20 nM per well) was used for transfection using Polyfect Transfection Reagent as described above. Cells were infected 48 h post-transfection. RNA interference efficiency was assessed at the protein level by Western blotting of cell lysates prepared at 48 h post-transfection. Knocked down and subsequent infection assays were carried out in triplicate at least three times (n ≥ 9).

**Western blotting.** A549 cells were seeded on six-well tissue culture plates to 4.8 × 10^5 cells per well for 32 h and serum-starved 16 h before infection. Cells were infected, washed three times with cold PBS, scraped and lysed with 100 μl lysis buffer [1 % SDS sample buffer, 62.5 mM Tris/HCl pH 6.8, 2 % (w/v) SDS, 10 % glycerol, 1/500 mM DTT, 0.01 % w/v bromophenol blue] on ice. Samples were sonicated, boiled (100 °C, 10 min) and ice cooled. Extracts were separated by 10 % SDS-PAGE, transferred to nitrocellulose by using a semi-dry transfer and membrane blocked overnight with 4 % skimmed milk in Tris-buffered saline (TBS). Immunodetection was performed with primary rabbit anti-FAK (1:20 000), anti-Pak1 (1:50 000), anti-Op18/stathmin (1:10 000), anti-Vav2 (1:2500), anti-c-Src (1:20 000) and secondary goat anti-rabbit–HRP (1:1000) antibodies. Alternatively, immunodetection was performed with primary mouse anti-Rac1 (1:1000), anti-RhoA (1:500) and secondary goat anti-mouse–HRP (1:1000) antibodies, or with primary goat anti-β1 integrin (1:1000) and secondary rabbit anti-goat–HRP (1:5000) antibodies. For Src tyrosine phosphorylation immunodetection, cells were seeded on 60 × 15 mm tissue culture dishes to 9.6 × 10^5 cells per dish for 32 h, serum-starved, infected and processed as above. Proteins were transferred by using a semi-dry transfer to a PVDF membrane and blocked with 4 % skimmed milk in TBS. Immunodetection was performed with primary rabbit anti-Src [pY416] (1:20 000) and secondary goat anti-rabbit–HRP (1:10 000) antibodies. For detection of Akt phosphorylation, cells were seeded on six-well tissue culture plates to 1.6 × 10^5 cells per well for 32 h and serum-starved 16 h before infection. Cells were infected, processed, transferred to a nitrocellulose membrane and blocked. Akt phosphorylation was detected with primary rabbit anti-phosphoSer473 Akt (1:1000) and secondary goat anti-rabbit–HRP (1:1000) antibodies. Tubulin was detected with primary mouse anti-tubulin (1:3000) and secondary goat anti-mouse–HRP (1:1000) antibodies. An ECL advance Western blotting detection (GE HealthCare) or Supersignal West dura extended duration substrate (Thermo Scientific) kit was used. Western blots were extended out at least three times by using independently generated cell extracts. Representative immunoblots are shown.

**Rac1 activation assay.** Rac1 activation was determined with the Rac1 activation assay kit (Millipore) according to the manufacturer’s instructions. A549 cells were grown to ~10^6 cells in 25 cm^2 tissue culture flasks and infected with NTHi375 for 1 or 2 h. Cells were washed twice with ice-cold TBS and resuspended in the assay buffer provided with the kit. Protein concentration was determined by using the BCA protein assay kit (Thermo Scientific). Lysates (200 μg) were mixed with Pak1 PBD agarose slurry (10 μg) and incubated for 1 h at 4 °C with gentle rocking. Beads were collected by centrifugation (14 000 g, 5 s), washed three times with the assay buffer provided by the kit, and resuspended in lysis buffer (see above). Activated Rac1 was immunodetected using primary mouse anti-Rac1 (clone 23A8, provided by the kit) and goat anti-mouse–HRP antibodies (1:1000).
Immunofluorescence microscopy. Cells were seeded on 12 mm circular coverslips in 24-well tissue culture plates. Transfections and infections were performed as described above. When indicated, cells were washed three times with PBS and fixed with 3.7% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature. NTHi was stained with rabbit anti-NTHi serum (1:800). β1 Integrin was stained with goat anti-β1 integrin (1:10). DNA was stained with Hoechst 33342 (Invitrogen) (1:2500). Secondary antibodies used were donkey anti-rabbit and donkey anti-goat conjugated to Rhodamine or Cy2 (1:200) (Jackson Immunological). Staining was carried out as described previously (Morey et al., 2011).

Statistical methods. Error bars in the graphs indicate SEM. The number of bacteria recovered from cells after each treatment was compared with its untreated control, carried out in parallel in each experiment. Statistical analyses were performed using Student’s t-test, followed by the Mann–Whitney U-test. A P-value of <0.05 was considered statistically significant. Analyses were performed using Prism4 for PC (GraphPad Software).

RESULTS

Akt activity is important for NTHi internalization by A549 epithelial cells

We have shown previously that cell treatment with the PI3K inhibitors LY294002 and wortmannin reduces NTHi invasion, and that NTHi infection promotes phosphorylation (thus activation) of the PI3K downstream effector Akt via PI3K (Morey et al., 2011). To explore whether Akt is involved in NTHi internalization, we treated human-lung-derived A549 cells with Akt inhibitor VIII (AktVIII), a selective inhibitor of the main isoforms Akt-1 and Akt-2, and evaluated the number of intracellular bacteria by gentamicin protection assays with NTHi strain 375 (hereafter NTHi375) (Morey et al., 2011). Our results indicate that AktVIII caused a significant reduction of NTHi375 invasion compared with untreated control cells (Fig. 1a). NTHi375 adherence to cells was unaffected by AktVIII (Fig. 1b), as was the case previously when LY294002 or wortmannin were used (Morey et al., 2011).

Phospholipase C (PLC)-γ 1 is another PI3K downstream effector (Uliczka et al., 2009). We examined its role in NTHi invasion by treating A549 cells with the PLC inhibitor U73122. The number of internalized NTHi was higher in cells treated with this inhibitor than in control untreated cells (Fig. 1c). Differently, NTHi375 adherence to cells treated with U73122 (Fig. 1d), PLC-γ 1 generates inositol 1,4,5-triphosphate and diacylglycerol (DAG) after PIP 2 hydrolysis. DAG recruits protein kinase (PKC) isoenzymes (Uliczka et al., 2009). We sought to determine whether PKCs are involved in NTHi invasion, by treating A549 cells with the PKC inhibitors G6983, Calphostin C and GF109203X. These treatments affected neither adhesion nor invasion of cells by NTHi375 (Fig. 1c, d). In summary, our data show that NTHi internalization is facilitated by a balance between the PI3K effectors Akt and PLC, which might display antagonistic roles.

Integrins β1 and α5 are involved in NTHi invasion of A549 epithelial cells

Integrins are sensors of the extracellular matrix environment (ECM) which play essential roles in cell migration, growth and survival (Martín et al., 2002). Integrin activation induces its clustering, leading to recruitment of signalling molecules to the cytosolic tail, and activation of transduction pathways including PI3K-Akt (Caswell et al., 2009; Huveneers & Danen, 2009). Having established the importance of PI3K-Akt for NTHi invasion, we explored whether integrins play a role in this process. We first asked whether integrin activation would facilitate NTHi invasion. Integrins can be activated from outside the cells by divalent cations, mostly Mn 2+ (Maqueda et al., 2006; Muenzner et al., 2010). When A549 cells were treated with Mn 2+, an increase in the number of internalized bacteria was observed, whereas bacterial adhesion was not affected (Figs 2a and S2a). A549 cells have been shown to preferentially express β1 integrin (Guo et al., 2009). To explore if β1 integrin plays a role in NTHi invasion, we pre-incubated cells with the β1 integrin-activating antibody TS2/16 or with the β1 integrin-blocking antibody Lia1/2, and quantified intracellular bacteria. Untreated cells and cells pre-incubated with the control antibody Alex1/4 were
Fig. 2. Involvement of integrins in NTHi invasion. (a) A549 cells were treated with Mn^{2+} and NTHi375 invasion was determined. (b) Cells were left untreated or were preincubated with control anti-β1 integrin Alex1/4, activating anti-β1 integrin TS2/16 or blocking anti-β1 integrin Lia1/2 antibodies. NTHi375 invasion was determined in each case. (c) Cells were treated with a pool of three siRNAs to β1 integrin, and infected with NTHi375 to determine invasion. Inset, immunoblot analysis of integrin β1, shown in control (AS-CON) and siRNA-treated cells; tubulin was a loading control. (d) Integrin β1 is recruited to the site of bacterial adhesion. A549 cells were infected with NTHi375. NTHi (DNA) was stained with Hoechst 33342 (blue). Integrin β1 was stained with goat anti-β1 integrin and Cy2-conjugated donkey anti-goat antibodies (green). Image was taken at 120 min post-infection. α5 Integrin is involved in NTHi375 epithelial infection. (e, f) Cells were preincubated with increasing concentrations of blocking anti-α5 P1D6 antibody, and bacterial invasion (e) and adhesion (f) were determined. (g, h) Cells were preincubated with increasing concentrations of RGD peptide. NTHi invasion (g) and adhesion (h) were determined. (i) α5 Integrin is involved in epithelial infection by a set of NTHi clinical isolates. Cells were left untreated (U) or were preincubated with 20 μg anti-α5 P1D6 antibody ml^{-1} and 5 μM RGD peptide. Bacterial adhesion was determined for NTHi398, 1566, 1606 and 1619. *P<0.05 compared with cells infected in the absence of treatment. Error bars, SEM, CON, Untreated cells.
used as controls. TS2/16 increased, whereas Lia1/2 decreased the number of internalized bacteria. These effects were not observed in control untreated or Alex1/4-treated cells (Fig. 2b). Bacterial adhesion was not affected compared with untreated cells (Fig. 2b). Next, cells were transfected with a pool of three siRNAs to integrin β1, and infected after 48 h. NTHi375 invasion into cells transfected with integrin β1 siRNAs was lower than that into cells transfected with AllStars control siRNA, although bacterial adhesion was not altered by integrin β1 interference (Figs 2c and S2e). Integrin β1 interference was confirmed by Western blot (Fig. 2c). To support these observations, we used immunofluorescence labelling to find out whether integrin β1 is recruited around adhering NTHi in infected A549 cells. As shown in Fig. 2(d), integrin β1 could be observed around adhering bacteria.

Next, we sought to determine which α integrin subunit is implicated in NTHi infection by using blocking antibodies against integrins α3, α4 or α5. We found that only the α5 integrin-blocking antibody P1D6 decreased both bacterial invasion and adhesion in a dose-dependent manner (Figs 2e, f and S1d, e). To confirm the role of α5 integrin in NTHi infection, competition experiments were carried out with a synthetic peptide containing a RGD sequence, which mimics the physiological α5 integrin ligand fibronectin (Zimmermann et al., 2010). RGD peptide decreased bacterial invasion and adhesion in a dose-dependent manner (Figs 2g, h). To expand this observation, we carried out blocking experiments with P1D6 antibody and RGD peptide and a set of NTHi clinical isolates. As shown in Fig. 2(i), NTHi398, 1566, 1606 and 1619 displayed a reduced adhesion when A549 cells were pre-incubated with P1D6 antibody or RGD peptide.

Together, these results indicate that integrins containing β1 and α5 are important for NTHi entry into A549 cells. Moreover, α5 integrin might be a cell surface structure to which bacteria attach.

**Epithelial cell invasion by NTHi relies on protein-tyrosine kinase (PTK) activities**

Integrins contribute to the dynamic turnover and remodelling of adhesion complexes by activating FAK and Src tyrosine kinase signalling (Martin et al., 2002). Having shown that β1 and α5 integrins are important for NTHi internalization, we assessed whether host PTKs play a role in this event. We incubated A549 cells with genistein, a broad PTK inhibitor, and evaluated the number of intracellular bacteria. NTHi internalization was significantly reduced when cells were incubated with genistein, compared with the untreated control (Fig. 3a). Next, we explored the effect of a specific FAK inhibitor. PF-573228 cell exposure reduced internalized bacteria (Fig. 3a). To further sustain FAK involvement in NTHi invasion, cells were transfected with two siRNAs to FAK, a and b, and infected 48 h after transfection. NTHi375 invasion into cells transfected with FAK siRNAs was lower than that into cells transfected with AllStars control siRNA (Fig. 3c). FAK interference was confirmed by Western blot (Fig. 3e, upper).

To explore the involvement of PTKs belonging to the Src family in NTHi invasion, we used PP2, a specific Src family PTK inhibitor, and evaluated the number of intracellular bacteria. NTHi internalization was significantly reduced when cells were incubated with PP2, compared with the untreated control (Fig. 3a). Control experiments showed that PP3, a structurally related molecule that does not inhibit Src PTKs, did not affect NTHi invasion (Fig. 3a). The activity of Src family PTKs is upregulated by tyrosine phosphorylation in their catalytic region. Full Src catalytic activity requires its phosphorylation (Guarino, 2010). Immunoblot analysis showed that NTHi invasion induces phosphorylation of Src on Tyr418 in a time-dependent manner (Fig. 3b). To investigate whether Src activity is involved in NTHi invasion, a kinase-inactive (dominant negative) form of c-Src (c-SrcK297M) was transiently overexpressed in A549 cells (Fig. 3i, lower). Under these conditions, a reduction of NTHi internalization was observed (Fig. 3f) compared with control non-transfected cells. To support these observations, we used immunofluorescence labelling to assess whether Src is recruited around bacteria in infected A549 cells. As shown in Fig. 3(h), Src–GFP recruitment around adhering bacteria could be observed.

Bacterial adhesion was not affected by cell incubation with genistein, PF-573228, PP2 or PP3 (Fig. S2f). FAK and Src knocked down did not modify NTHi375 adhesion (Fig. 3d–g). Together, these experiments show that FAK, Src family PTKs, and Src in particular, are important for NTHi A549 cell invasion.

**Involvement of Rho small GTPases in NTHi invasion of airway epithelial cells**

Integrins signal to members of the Rho family of GTPases Cdc42, Rac and Rho (Caswell et al., 2009; Huyrveers & Danen, 2009). Several bacterial pathogens manipulate the cytoskeleton to get inside host cells by targeting Rho GTPases (Carabeo, 2011; Yoshida & Sasakawa, 2003). We tested the importance of these molecules in NTHi invasion. We firstly analysed the effect of C. difficile toxin B, an inhibitor of Cdc42, Rac and Rho (Mejia et al., 2008), on NTHi A549 cell invasion. This broad inhibitor did not affect either the adhesion or the invasion of NTHi (Figs 4a and S3a). Next, we determined the importance of Rho GTPases in NTHi invasion by individual blocking. We incubated A549 cells with NSC23766, a specific Rac1 inhibitor, and evaluated the number of intracellular bacteria. NTHi375 internalization was significantly decreased when cells were incubated with NSC23766, compared with the untreated control. In contrast, treatment with CT04, a specific RhoA inhibitor, led to an increase in the number of intracellular bacteria (Fig. 4a). Bacterial adhesion was not affected by NSC23766 or CT04 cell incubation (Fig. S3a).
Rac1 signalling is important for NTHi invasion of airway epithelial cells

Rac1 role on NTHi invasion was further studied by knocking down its expression using siRNA, following the approach previously reported by Aiastui et al. (2010). NTHi375 invasion of Rac1 knocked-down cells was significantly lower than that of cells transfected with AllStars control siRNA (Fig. 4b). Interference was assessed by Western blot (Fig. 4c, upper). We then asked if NTHi infection promotes activation of Rac1 in A549 cells. Pull-down assays revealed that Rac1–GTP levels were higher in NTHi375 infected cells than in control non-infected cells (Fig. 4d). These data show that NTHi375 activates Rac1 upon infection, and that this GTPase is important for NTHi cell invasion.

Vav2 is a Rac1 guanine nucleotide exchange factor (GEF) which is activated by Src-dependent phosphorylation...
Having established that Src and Rac1 are involved in NTHi invasion, we asked whether Vav2 is linked to Rac1 during NTHi infection. As shown in Fig. 4(e), NTHi375 invasion was significantly reduced in cells where Vav2 was knocked down by siRNA, using a previously reported approach (Boettcher et al., 2010). The level of Vav2 interference was assessed by Western blot (Fig. 4f).

Paks are a family of serine/threonine-specific protein kinase downstream effectors of Rac1 (Van den Broeke et al., 2010). Given the result obtained by Rac1 interference, we hypothesized that Paks could be important for NTHi invasion. We assessed the role of Pak1 on NTHi invasion by transfection with two siRNAs, a and b. NTHi375 invasion was reduced in A549 cells transfected with Pak1 siRNAs (Fig. 4b). siRNAs reduced Pak1 abundance, as shown in Fig. 4(c) (lower panel).

(Marignani & Carpenter, 2001). Having established that Src and Rac1 are involved in NTHi invasion, we asked whether Vav2 is linked to Rac1 during NTHi infection. As shown in Fig. 4(e), NTHi375 invasion was significantly reduced in cells where Vav2 was knocked down by siRNA, using a previously reported approach (Boettcher et al., 2010). The level of Vav2 interference was assessed by Western blot (Fig. 4f).

Paks are a family of serine/threonine-specific protein kinase downstream effectors of Rac1 (Van den Broeke et al., 2010). Given the result obtained by Rac1 interference, we hypothesized that Paks could be important for NTHi invasion. We assessed the role of Pak1 on NTHi invasion by transfection with two siRNAs, a and b. NTHi375 invasion was reduced in A549 cells transfected with Pak1 siRNAs (Fig. 4b). siRNAs reduced Pak1 abundance, as shown in Fig. 4(c) (lower panel).

Fig. 4. Rho GTPases are coordinately required for NTHi invasion. (a) Invasion was monitored in the absence or presence of the Rho GTPase inhibitors C. botulinum toxin B, NSC23766 and CT04. (b) Rac1 and Pak1 are implicated in NTHi invasion. A549 cells were treated with siRNAs to Rac1 and Pak1, and infected with NTHi375 to determine invasion. (c) Interference of Rac1 (upper) and Pak1 (lower) was evaluated by Western blotting of cell lysates from control (AS-CON) and siRNA-treated cells. Tubulin was a loading control. (d) Rac1 activation, detected by the amount bound to the GST-Pak Rac1 interaction binding site, was determined in A549 cells uninfected (–) or infected for 60 or 120 min with NTHi375. Tubulin was a loading control before the pull-down. (e) Vav2 GEF interference reduces NTHi invasion. A549 cells were knocked down with siRNA to Vav2 and infected with NTHi375 to determine invasion. (f) Vav2 interference was evaluated in cell lysates from control (AS-CON) and siRNA-treated cells. Tubulin was a loading control. (g) Op18/stathmin interference increases NTHi invasion. A549 cells were treated with two siRNAs to Op18/stathmin and infected with NTHi375 to determine invasion. (h) siRNA interference of Op18/stathmin was evaluated in cell lysates from control (AS-CON) and siRNA-treated cells; tubulin was a loading control. *P<0.05 compared with cells infected in the absence of treatment. Error bars, SEM. CON, Untreated cells.
Promotion of microtubule cytoskeleton polymerization is a dynamic process dependent on Rac1; Rac1 promotes microtubule growth at the leading edge of migrating cells through Pak1, which in turn inhibits the microtubule-destabilizing protein Op18/stathmin (Raftopoulou & Hall, 2004). Op18/stathmin is a ubiquitous phosphoprotein with tubulin-binding and microtubule-destabilizing activities (Wittmann et al., 2004). The importance of microtubule polymerization on NTHi invasion was suggested by our previous finding showing that nocodazol and colchicine, two inhibitors of microtubule polymerization, reduce NTHi375 invasion of A549 cells (Morey et al., 2011). To explore the importance of Op18/stathmin on NTHi invasion, Op18/stathmin levels were reduced by transfecting cells with two siRNAs to Op18/stathmin, a and b. siRNAs reduced the abundance of Op18/stathmin, as shown by Western blot (Fig. 4h). NTHi375 invasion was increased in Op18/stathmin knocked-down cells, compared with cells transfected with AllStars siRNA (Fig. 4g). NTHi375 adhesion to A549 cells was not altered by interference of Rac1, Vav2, Pak1 or Op18/stathmin, compared with control cells (Fig. S3b–d).

Together, these results show that Vav2, Rac1, Pak1 and Op18/stathmin are involved in NTHi375 internalization by A549 cells, suggesting that NTHi may modulate host microtubule polymerization dynamics to facilitate its entry into the host cell.

cAMP is important for NTHi internalization by A549 cells

Secondary messengers, chiefly cAMP, have effects on multiple cellular functions. cAMP has been shown to regulate Rac1 activity negatively in a process involving the protein kinase A (PKA) (Howe, 2004; Nagasawa et al., 2005; O’Connor & Mercurio, 2001; Waschke et al., 2004). This evidence, and the requirement of Rac1 for NTHi invasion shown above, prompted us to analyse cAMP and PKA involvement in NTHi invasion. We compared internalized bacteria into the control and into A549 cells treated with forskolin, a broad spectrum activator of adenylate cyclases, mediators of ATP conversion into cAMP (Song et al., 2007). Forskolin treatment reduced bacterial invasion, compared with control untreated cells, suggesting that an increase in intracellular cAMP dampens NTHi375 entry (Fig. 5a). Pursuing this finding, we tested the effect of prostaglandin E2 (PGE2) on NTHi invasion. PGE2 activity is mediated by E prostanoid receptors (EP1–EP4), shown to be present in A549 cells (Fang et al., 2004; Vancheri et al., 2004). EP2 and EP4 activation increases intracellular cAMP (Vancheri et al., 2004). As shown in Fig. 5(a), cell incubation with PGE2 reduced the number of intracellular NTHi375. Forskolin treatment and PGE2 addition did not affect bacterial adhesion (Fig. 5b). Having observed that increasing cAMP causes a decrease in the number of internalized bacteria, we explored the importance of its downstream effector PKA in this process. Based on the notion that PKA blocks Rac1, we hypothesized that PKA inhibition may favour NTHi invasion. Indeed, cell treatment with H-89 dihydrochloride hydrate, a specific PKA inhibitor, increased the number of intracellular NTHi375, compared with control untreated cells (Fig. 5a). NTHi375 adhesion to A549 cells was not altered by treatment with H-89 compared with control cells (Fig. 5b).

In summary, these data suggest that the increase in the cAMP level and the activation of PKA contribute to the prevention of NTHi epithelial invasion.

Akt phosphorylation on Ser473 in A549 cells infected with NTHi depends on Src and Rac1

Src can play a direct role in PI3K activation (Guarino, 2010). Having determined the implication of Src in NTHi invasion, we explored whether NTHi-induced Akt phosphorylation is dependent on Src kinases. To do so, we analysed the level of Akt phosphorylation on Ser473 in cells infected with NTHi by using a phospho-Akt antibody which recognizes Akt phosphorylated at Ser473. As shown in Fig. 6, treatment with H-89 dihydrochloride hydrate, a specific PKA inhibitor, increased the number of intracellular NTHi375, compared with control untreated cells (Fig. 6a). NTHi375 adhesion to A549 cells was not altered by treatment with H-89 compared with control cells (Fig. 6b).

In summary, these data suggest that the increase in the cAMP level and the activation of PKA contribute to the prevention of NTHi epithelial invasion.
infected with NTHi375, in the absence or presence of PP2 inhibitor. PP2 treatment reduced the level of NTHi-triggered Akt phosphorylation (Fig. 6a). In contrast, the FAK inhibitor PF-573228 did not affect infection-induced Akt phosphorylation (data not shown).

Rac1 has also been reported to regulate phosphoinositide dynamics during bacterial uptake processes (Wong & Isberg, 2003). Having established the importance of Rac1 in NTHi invasion, we determined whether NTHi-induced Akt phosphorylation is dependent on Rac1. To do so, we analysed the level of Akt phosphorylation on Ser473 in cells infected with NTHi375, in the absence or presence of NSC23766 inhibitor. Levels of phosphorylated Akt were lower in infected cells treated with NSC23766 than in infected control cells (Fig. 6b). These data show that NTHi-promoted phosphorylation of Akt on Ser473 depends on Src PTKs and on Rac1 GTPase.

**RhoA involvement in NTHi invasion of airway epithelial cells**

To sustain a positive effect of RhoA inhibition on NTHi invasion (Fig. 4a), RhoA levels were diminished by using siRNA; the siRNA pool reduced RhoA abundance, as shown by Western blot (Fig. 7c). In agreement with the results obtained using a CT04 inhibitor, the c.f.u. count of intracellular NTHi was higher in cells transfected with RhoA siRNA than in cells transfected with AllStars control siRNA (Fig. 7a). NTHi375 adhesion to A549 cells was not altered by RhoA interference compared with control cells (Fig. 7b). We next studied the importance of the RhoA effector ROCK in NTHi invasion. We incubated A549 cells with Y-27632, a specific ROCK inhibitor, and evaluated the number of intracellular bacteria. Y-27632 increased the number of internalized bacteria compared with untreated control cells (Fig. 7d). NTHi375 adhesion to A549 cells was not altered by Y-27632 treatment compared with control cells (Fig. 7e). The data indicate a connection between RhoA-ROCK signalling and microtubule cytoskeleton dynamics, supporting the notion that ROCK can reduce microtubule stability (Takesono et al., 2010). We speculated that ROCK inhibition may rescue the negative effect of microtubule destabilization on NTHi invasion. Indeed, the inhibition of NTHi375 invasion by colchicine was abolished by co-treatment with Y-27632. As previously shown, bacterial adhesion remained unaltered (Fig. 7d, e).

**Fig. 6.** Akt phosphorylation during NTHi infection depends on Src PTKs and on Rac1 GTPase. (a) Src PTKs modulate Akt activation upon NTHi infection. Detection of Akt-Ser473 phosphorylation by immunoblot. Extracts were prepared from cells non-infected (0) or infected for 30, 60 and 120 min with NTHi375, in the absence (–) or presence (+) of the Src PTK inhibitor PP2; tubulin was a loading control. (b) Rac1 modulates Akt activation upon NTHi infection. Detection of Akt-Ser473 phosphorylation. Extracts were prepared from cells non-infected (0) or infected for 30, 60 and 120 min with NTHi375, in the absence (–) or presence (+) of the Rac1 inhibitor NSC23766; tubulin was a loading control.

**Fig. 7.** RhoA and ROCK diminish the recovery of intracellular NTHi. (a) A549 cells were treated with siRNA to RhoA and infected to determine invasion. (b) Cells were treated with siRNA to RhoA and infected to determine adhesion. (c) RhoA interference was evaluated by Western blotting of cell lysates from control and siRNA-treated cells; tubulin was a loading control. (d) ROCK inhibition by cell treatment with Y-27632 increases bacterial invasion; invasion was restored to control levels in cells pretreated with Y-27632 before colchicine addition. (e) NTHi adhesion was not altered, independently of the absence or presence of colchicine and Y-27632. *P<0.05 compared to cells infected without treatment. Error bars, SEM. AS-CON or CON, Untreated cells.
DISCUSSION

The ability of NTHi to enter non-phagocytic cells has been reported, but is poorly understood mechanistically (Clementi & Murphy, 2011; Morey et al., 2011). Considering that NTHi intracelluar location may provide a niche favouring its persistence, a comprehensive understanding of the mechanisms used by this pathogen to access such a subcellular location may be crucial in the development of strategies to counteract NTHi infection. In this study, we identified a number of host molecules and signalling events important for NTHi invasion of A549 airway epithelial cells (Fig. 8). Following previous observations regarding the importance of PI3K on NTHi invasion and on bacterially promoted Akt phosphorylation (Morey et al., 2011), we show that Akt is also important for NTHi invasion. Conversely, PLC-γ1 may dampen NTHi invasion, most likely by limiting PIP3, a PI3K substrate required for subsequent Akt activation. Our data also indicate that integrins α5 and β1 are important for NTHi invasion. These host cell receptors could be engaged by NTHi, triggering activation of downstream tyrosine kinases. We show that FAK and Src kinases are involved in NTHi epithelial entry, and that signalling events involving the GEF Vav2, the GTPase Rac1 and the effector Pak1 favour bacterial invasion. This cascade may lead to the inactivation of the microtubule destabilizing agent Op18/stathmin, hence facilitating the polymerization of the microtubule cytoskeleton and epithelial invasion by NTHi. We speculate that NTHi could manipulate microtubule dynamics through the inactivation of Op18/stathmin, upon activation of Rac1-Pak1. This signalling event may be counteracted by RhoA-ROCK signalling. We also found that the increase in host cytosolic cAMP dampens bacterial invasion, and that NTHi–induced Akt phosphorylation depends on Src and Rac1.

To our knowledge, and taking into account limitations of the approaches used, this study presents a number of previously unknown host molecules and signalling events involved in NTHi epithelial invasion. It should be considered that our data do not exclude the participation of molecules and signalling events additional or alternative to those shown here.

This study was carried out with A549 cells, a model system extensively used in host–pathogen interaction studies (Byrd et al., 2010; Chakrabarti et al., 2010; Hammerschmidt et al., 2005; Mata et al., 2011), and mostly with strain NTHi375. Phosphocholine (PCho)-platelet associated factor receptor (PAF-R) and OmpP5-carcinoembryonic antigen–related cell adhesion molecule-1 (CEACAM1) are bacterial adhesion–host receptor binding partners identified for NTHi (Hill et al., 2001; Swords et al., 2000). Adhesion to and invasion of A549 cells by NTHi375 mutants lacking PCho (NTHi375Δlic1BC) and OmpP5 (NTHi375ΔompP5) were decreased by incubation of cells with anti-α5 integrin antibody or RGD peptide compared with control untreated cells, as observed for the wild-type strain, excluding those bacterial molecules as α5 integrin ligands (A. López-Gómez, unpublished data). NTHi genetic content is highly variable among isolates (Gilsdorf et al., 2004; Martí-Lliteras et al., 2011); putative bacterial ligands might be identified by phenotypic comparison from a panel of strains with genome sequences.

Integrins are critical for a variety of events: the active, high affinity conformation of integrins can be stabilized by extracellular ligands, a feature exploited by several bacteria (Hoffmann et al., 2011). Two strategies are known: (i) expression of microbial proteins directly binding to integrins (invasion of Yersinia enterocolitica or Yersinia pseudotuberculosis, Ipa proteins of Shigella flexneri, CagL of Helicobacter pylori); (ii) expression of microbial proteins binding ECM proteins, which then associate with integrins (Staphylococcus aureus, Streptococcus pyogenes, Neisseria gonorrhoeae, Neisseria meningitidis, Bartonella henselae, Mycobacterium leprae). These strategies are not mutually exclusive. Then, Y. enterocolitica uses invasion for direct integrin β1 binding and YadA for binding via ECM proteins (Hoffmann et al., 2011). We show here that β1 and α5 integrins are important for NTHi

Fig. 8. Model showing signalling events triggered by NTHi375 upon infection of A549 cells. Molecules in black may contribute positively to NTHi375 entry into A549 cells, given that their inhibition decreases bacterial invasion; molecules in red may prevent NTHi375 invasion, because their inhibition enhances bacterial entry; molecules in blue may prevent NTHi375 invasion, because their activation reduces bacterial entry. Black arrows are activation events, red arrows are inhibition events.
epithelial invasion, and that NTHi may be endowed with an α5 integrin ligand(s). Additional indirect bacterial binding cannot be excluded, given that NTHi has been shown to bind ECM proteins fibronectin, laminin, collagen IV and vitronectin (Fink et al., 2002; Hallström et al., 2011). Future studies will aim to identify NTHi integrin ligands.

Integrin clustering triggers the assembly of protein complexes on the cytosolic side of the plasma membrane (Caswell et al., 2009). Here we show that FAK and Src are important for NTHi invasion. These kinases are frequent orchestrators of the linkage between integrins and signal transduction (Guarino, 2010; Mitra et al., 2005), and have been shown to be involved in epithelial invasion by several bacterial pathogens (Agerer et al., 2003, 2005; Cho et al., 2010; Ozeri et al., 2001; Xue et al., 2010). Lipid raft stabilization through integrin signalling also facilitates Rac1 coupling to downstream targets such as Paks (del Pozo et al., 2004). We showed previously that, cholesterol, a component of host membrane lipid rafts, is important for NTHi epithelial invasion (Morey et al., 2011). We show here that NTHi infection stimulates Rac1 activation, and that Rac1 and Pak1 play a positive role in bacterial invasion. A number of GEFs (Vav2, DOCK180, Tiam) activate Rac1. Considering that activated Src promotes Vav protein activation (Guarino, 2010), and that Src is important for NTHi epithelial internalization, we assessed Vav2 involvement in NTHi invasion. Vav2 interference confirmed that this molecule is likely to participate in NTHi invasion, supporting the notion that it may be a GEF for Rac1 during NTHi invasion. Rac1 signalling has been reported to be a key player at the leading edge of migrating cells by modulating actin dynamics at lamellipodia (Heasman & Ridley, 2008). Integrin α5β1-Rac1 signalling has been reported to play a role in Y. pseudotuberculosis cell invasion, which involves an integrin-Rac1-Arp2/3 pathway leading to actin cytoskeleton remodelling (Cossart & Sansonetti, 2004). Importantly, Rac1 also modulates cell microtubule dynamics by promoting their elongation, through Pak-dependent inactivation of Op18/stathmin (Ridley et al., 2003). Our findings suggest that NTHi may manipulate Rac1 activity to ultimately enhance its own entry by favouring microtubule assembly by Pak1-mediated Op18/stathmin inactivation. A pathway linking Rac1 to Op18/stathmin contributing to microtubule network regulation has been shown to regulate neuronal polarity during axon development (Watabe-Uchida et al., 2006). To our knowledge, this is the first study showing a role for Op18/stathmin in host–pathogen interplay. Together, the existing information suggests that different pathogens may differently subvert integrin α5β1-Rac1 signalling to ultimately enter host cells.

Rac1 also associates and induces PIP5K, which is involved in PIP2 formation. PIP2 is converted into PIP3 by PI3K (Weernink et al., 2004), and PIP3 activates Akt. Given that NTHi-promoted Akt phosphorylation is diminished in the presence of the Rac1 inhibitor NSC23766, Rac1-PIP5K-PI3K-PIP3 signalling may be involved in Akt phosphorylation upon NTHi infection. Another observation is that RhoA and ROCK inhibition increased NTHi invasion. These data follow our previous observation regarding an increase of NTHi invasion upon cell treatment with actin depolymerizing agents (Morey et al., 2011). RhoA has been highlighted as a player preventing N. gonorrhoeae entry in epithelial cells via a pathway where phosphorylated caveolin-1 blocks bacterial uptake by inducing Vav2-RhoA-mediated actin rearrangement (Boettcher et al., 2010). Mechanistically, integrin signalling can suppress RhoA activity via an Src-dependent mechanism (Arthur et al., 2000); Rac1 itself can keep RhoA activity low (Huveneers & Danen, 2009), and RhoA GEF-H1 availability is regulated by its interaction with assembled microtubules (Krendel et al., 2002). Although the molecular mechanisms modulating RhoA activity upon NTHi infection are currently unknown, NTHi invasion appears as reminiscent of the crosstalk between integrins, kinases and Rho GTPases at the heart of cell adhesion, spreading and migration processes (Huveneers & Danen, 2009). In summary, Rho GTPases seem to be coordinate involved in NTHi invasion; this could explain the absence of a detectable effect on bacterial entry upon cell treatment with toxin B. Moreover, our data suggest a crosstalk RhoA-Rac1, given that ROCK inhibition rescues the blocking effect of colchicine. This ROCK–microtubule link has been observed in a process regulating the migratory polarity of leukocytes (Takehono et al., 2010).

Finally, our data suggest that the increase in host cytosolic cAMP reduces NTHi invasion. Our data support the notion that cAMP activates PKA, which may inhibit Rac1, thus reducing NTHi entry, a signalling pathway shown to modulate uropathogenic E. coli (UPEC) epithelial invasion (Song et al., 2007). With the evidence that many pathogens seek refuge inside cells at some stage in the infectious process, pharmacotherapies increasing intracellular cAMP may lead to clinical benefits in persistent infections. This seems to be the case for UPEC, given that mice treatment with forskolin has been shown to be effective in the clearance of urinary tract infection by this pathogen (Song et al., 2007). Interestingly, a phosphodiesterase 4 inhibitor which increases intracellular cAMP is the basis of an anti-inflammatory therapy administered to chronic obstructive pulmonary disease (COPD) patients, frequently colonized by NTHi (Barnes, 2006). The fact that cAMP upregulation limits NTHi invasion suggests that phosphodiesterase inhibitors could reduce internalized NTHi, which may influence NTHi infection associated with COPD. Future clinical trials should consider this outcome.

ACKNOWLEDGEMENTS

We thank Dr Hammerschmidt (Uni. Griefswald) for pSrcK297M and Drs García-Pardo (Centro Investigaciones Biológicas, CIB-CSIC) and Sánchez-Madrid (Hospital Universitario la Princesa, Madrid) for anti-integrin antibodies. We thank Drs Toledo-Arana (Instituto Agriobiotecnología-CSIC) and Veiga (Centro Nacional Biotecnología, CNB-CSIC) for help with microscopy. We thank members of the Garmendia lab for helpful discussion. A.L.G. is a recipient of a
Predoctoral Fellowship from Govern Illes Balears, Spain. This work has been funded by grants to J.G. from Instituto de Salud Carlos III (PS09/00130) and from Fundación Mutua Madrileña. CIBERES is an initiative from Instituto de Salud Carlos III, Spain.

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Edited by: D. W. Hood