Evidence for a non-replicative intracellular stage of nontypable *Haemophilus influenzae* in epithelial cells

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Nontypable *Haemophilus influenzae* (NTHi) is a Gram-negative, non-capsulated human bacterial pathogen, a major cause of a repertoire of respiratory infections, and intimately associated with persistent lung bacterial colonization in patients suffering from chronic obstructive pulmonary disease (COPD). Despite its medical relevance, relatively little is known about its mechanisms of pathogenicity. In this study, we found that NTHi invades the airway epithelium by a distinct mechanism, requiring microtubule assembly, lipid rafts integrity, and activation of phosphatidylinositol 3-kinase (PI3K) signalling. We found that the majority of intracellular bacteria are located inside an acidic subcellular compartment, in a metabolically active and non-proliferative state. This NTHi-containing vacuole (NTHi-CV) is endowed with late endosome features, co-localizing with LysoTracker, lamp-1, lamp-2, CD63 and Rab7. The NTHi-CV does not acquire Golgi- or autophagy-related markers. These observations were extended to immortalized and primary human airway epithelial cells. By using NTHi clinical isolates expressing different amounts of phosphocholine (PCho), a major modification of NTHi lipooligosaccharide, on their surfaces, and an isogenic *lic1BC* mutant strain lacking PCho, we showed that PCho is not responsible for NTHi intracellular location. In sum, this study indicates that NTHi can survive inside airway epithelial cells.

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INTRODUCTION

Haemophilus influenzae is a Gram-negative human pathogen that colonizes asymptotically the nasopharynx of healthy individuals. Disease occurs when the bacterium

reaches privileged anatomical sites under various predisposing conditions, including, among others, age, viral infections or a constant exposure to pollutants, leading to otitis media, sinusitis, meningitis, septicaemia or respiratory infections (Foxwell *et al.*, 1998; Rao *et al.*, 1999). Nontypable *H. influenzae* (NTHi) is the most frequently isolated bacterial pathogen in the lungs of patients suffering from chronic obstructive pulmonary disease (COPD), and furthermore, NTHi accounts for the majority of bacterial COPD exacerbation episodes (Sethi *et al.*, 2002; Sethi & Murphy, 2001, 2008).

One of the NTHi virulence factors that has been most extensively analysed is its lipooligosaccharide (LOS). It consists of lipid A, an inner core consisting of a

Abbreviations: COPD, chronic obstructive pulmonary disease; EEA1, early endosome antigen 1; FISH, fluorescent *in situ* hybridization; LOS, lipooligosaccharide; M β CD, methyl- β -cyclodextrin; NHBE cells, normal human bronchial epithelial cells; NTHi, nontypable *Haemophilus influenzae*; NTHi-CV, NTHi-containing vacuole; PCho, phosphocholine; PFA, paraformaldehyde; PI3K, phosphatidylinositol 3-kinase; SCV, *Salmonella*-containing vacuole; TEM, transmission electron microscopy.

Eight supplementary figures and a supplementary table are available with the online version of this paper.

phosphorylated 2-keto-3-deoxyoctulosonic acid (KDO) linked to three heptoses and an outer core consisting of a heteropolymer of glucose and galactose (Schweda *et al.*, 2007). Similar to other bacterial pathogens adapted to mucosal surfaces, such as *Neisseria* spp. and *Moraxella catarrhalis*, NTHi LOS lacks polymeric O-linked sugar chains (Schweda *et al.*, 2007; Virji, 2009). The carbohydrate portion of the NTHi LOS is highly variable, both among strains and within the same strain upon passage, as a result of differences in the composition and linkage of saccharides in the outer core (Schweda *et al.*, 2007). An additional source of LOS heterogeneity is the regulation by phase variation of genes encoding enzymes responsible for LOS modifications with di-galactose, sialic acid and phosphocholine (PCho) (Gilsdorf *et al.*, 2004; Power *et al.*, 2009). PCho has been shown to be involved in different aspects of NTHi pathogenicity, i.e. resistance to antimicrobial peptides and to serum-mediated killing (Lysenko *et al.*, 2000; Weiser *et al.*, 1998). PCho is also involved in the interplay of NTHi with cellular components of innate immunity, including adhesion to and entry into monocytic and epithelial cells (St Geme, 2002; St Geme & Falkow, 1990; Swords *et al.*, 2000). A number of reports associate PCho expression with NTHi biofilm growth, maturation and stability (Hong *et al.*, 2007a, b; West-Barnette *et al.*, 2006), and it has been shown to play a role in host colonization *in vivo*, both in a chinchilla model of otitis media and in a mouse model of pulmonary infection (Hong *et al.*, 2007b; Pang *et al.*, 2008).

Relatively little is known about the interplay between NTHi and the airway epithelium. Even though NTHi has traditionally been considered as an extracellular pathogen (Moxon *et al.*, 2008), a number of reports suggest that this bacterium might also reside within non-phagocytic cells. Wild-type NTHi clinical isolates have been found, *in vitro*, to adhere to and invade Chang epithelial cells, cultured human endothelium, primary human bronchial epithelium and type II pneumocytes (Ahrén *et al.*, 2001; Ketterer *et al.*, 1999; St Geme, 2002; St Geme & Falkow, 1990; Swords *et al.*, 2000; Virji *et al.*, 1991). Early experiments using Chang epithelial cells and primary human bronchial epithelial cells revealed the accumulation of actin strands beneath adherent bacteria and lamellipodia formation at the site of bacterial entry (Holmes & Bakaletz, 1997; Ketterer *et al.*, 1999). *Ex vivo*, NTHi has been found to appear in an intracellular and viable form in human adenoid tissue and in bronchial human biopsies (Bandi *et al.*, 2001; Forsgren *et al.*, 1994). Transmission electron microscopy (TEM)-based experiments have shown that intracellular NTHi remains within membrane-bound vacuoles (St Geme, 2002). In view of these earlier reports, we based our work on the hypothesis that NTHi might be able to survive within host cells.

We report here that NTHi resides within airway epithelial cells without proliferating. The pathogen survives in a unique intracellular compartment which keeps late endosome features. The modification of LOS with PCho is not a

requirement for NTHi localization inside subcellular compartments.

METHODS

Bacterial strains, media and growth conditions. NTHi strain 375 is an otitis media isolate (Bouchet *et al.*, 2003). NTHi 2019 is a COPD isolate (Swords *et al.*, 2000). NTHi 398 (formerly known as 157925) is a COPD isolate (Hospital Son Dureta, Spain) (Regueiro *et al.*, 2009). NTHi 375 Δ lic1BC is a mutant strain lacking PCho and has been described previously (Martí-Llitas *et al.*, 2009). NTHi strains were grown on chocolate agar plates (bioMérieux) or on brain heart infusion (BHI) agar plates supplemented with 10 μ g haemin ml⁻¹ and 10 μ g NAD ml⁻¹. Bacteria were grown at 37 °C and 5% CO₂, using 10 μ g erythromycin ml⁻¹ when necessary. *Salmonella typhimurium* SL1344 Δ invA has been described elsewhere (Galán *et al.*, 1992; García-del Portillo & Finlay, 1994), and was transformed with the invasin-encoding plasmid pRI203 (invasin) (Isberg *et al.*, 1987). *Escherichia coli* and *S. typhimurium* Δ invA (invasin) were grown in Luria-Bertani (LB) broth or on LB agar plates at 37 °C, using antibiotics at the following concentrations, when necessary: ampicillin, 100 μ g ml⁻¹; erythromycin, 150 μ g ml⁻¹; chloramphenicol, 25 μ g ml⁻¹.

Colony immunoblotting. The assessment of PCho levels on the surface of NTHi strains was carried out by colony immunoblotting (Swords *et al.*, 2000). Briefly, NTHi strains were plated on supplemented BHI agar plates. Plates with approximately 100–300 colonies were used for transfer onto a nitrocellulose membrane. The membrane was washed twice for 15 min in TSBB buffer (0.5 M NaCl, 0.5% Tween 20, 10 mM Tris/HCl, pH 8.0) and incubated in TSBB for 16 h at 4 °C with monoclonal anti-PCho TEPC-15 (Sigma) diluted 1:10,000, an IgA, κ with a specificity for PCho obtained from the TEPC-15 tumour line in BALB/c mice. Membranes were washed five times for 5 min with fresh TSBB and incubated for 16 h with TSBB containing goat anti-mouse IgA conjugated to anti-alkaline phosphatase antibody (Sigma) diluted 1:10,000. Finally, membranes were washed five times with TSBB, incubated for 10 min in AP buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris/HCl, pH 9.5), and developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma) and nitro blue tetrazolium (Sigma) in AP buffer.

Cell culture and bacterial infection. Carcinomic human alveolar basal epithelial cells (A549, ATTC CCL-185) were maintained in RPMI 1640 tissue culture medium supplemented with 1% HEPES, 10% heat-inactivated fetal calf serum (FCS) and antibiotics (penicillin and streptomycin) in 25 cm² tissue culture flasks at 37 °C in a humidified 5% CO₂ atmosphere. Primary normal human bronchial epithelial cells (NHBE cells; Lonza) were grown in the bronchial epithelial cell growth medium BEBM (Bronchial Epithelial cell Basal Medium, Lonza) supplemented with 0.5 ng human epidermal growth factor ml⁻¹, 0.5 μ g hydrocortisone ml⁻¹, 5 μ g insulin ml⁻¹, 10 μ g transferrin ml⁻¹, 0.5 μ g epinephrine ml⁻¹, 6.5 ng triiodothyronine ml⁻¹, 50 μ g gentamicin ml⁻¹, 50 ng amphotericin B ml⁻¹, 52 μ g bovine pituitary extract ml⁻¹ and 0.1 ng retinoic acid ml⁻¹, at 37 °C with 5% CO₂. The 25 cm² tissue culture flasks were coated with collagen from calf skin (Sigma).

For A549, cells were seeded to a density of 10⁵ cells per well in 24-well tissue culture plates for 24 h. Cells were then serum-starved for 16 h before infection by replacement of the medium with supplemented RPMI lacking FBS. A confluency of 90% was reached at the time of the bacterial infection. For NHBE cells, cells were seeded at a density of 10⁵ cells per well and grown until 80% confluency was reached in collagen-coated 24-well tissue culture plates using 1 ml BEBM per well. For NTHi infection, bacteria were recovered with 1 ml PBS from

a chocolate agar plate grown for 16 h. The bacterial suspension was adjusted to OD_{600} 1 ($\sim 10^9$ c.f.u. ml^{-1}). A549 cells were infected in 1 ml Earle's balanced salt solution (EBSS) with 50 μl bacterial suspension to get an m.o.i. of 100:1. NHBE cells were infected on 1 ml BEBM with 30 μl of this suspension to get an m.o.i. of 100:1. *S. typhimurium* $\Delta invA$ (invasin) was grown overnight to stationary phase in LB at 37 °C. The culture was then diluted 1:33 and grown for 3.5 h to exponential phase. A 30 μl volume of this suspension was added to each well to get an m.o.i. of 100:1. Infection was carried out for 15 min, and wells were then washed three times with PBS and incubated for up to 1 h with RPMI 1640 containing 10% FCS, HEPES and 100 μg gentamicin ml^{-1} .

For adhesion experiments, cells were infected for 1 or 2 h, washed five times with PBS and lysed with 300 μl of PBS/0.025% saponin for 10 min at room temperature, and serial dilutions were plated on supplemented BHI agar plates. Adhesion data are presented as c.f.u. per well. For invasion assays, cells were infected for 2 h and washed three times with PBS. Cells were then incubated for an additional 1 h with RPMI 1640 containing 10% FCS, HEPES and 200 μg gentamicin ml^{-1} to kill extracellular bacteria. Treatment with gentamicin (200 μg ml^{-1}) did not induce any cytotoxic effect, and this was verified by measuring the release of lactate dehydrogenase (LDH) and immunofluorescence microscopy (data not shown). Cells were washed three times with PBS and lysed as described above. Results are expressed as c.f.u. per well. For time-course experiments, culture medium was replaced at 1 h post-gentamicin by RPMI 1640 containing 10% FCS, HEPES and 11 μg gentamicin ml^{-1} , and processed when required.

When indicated, cells were pre-incubated for 1 h with 10 μM nocodazole, 30 μM colchicine, 1 mM methyl- β -cyclodextrin (M β CD), 25 μg nystatin ml^{-1} , 5 μg filipin ml^{-1} or 10 μM LY294002 hydrochloride, for 30 min with 5 μg cytochalasin D ml^{-1} or 5 μM wortmannin, or for 3 h with 1 μg latrunculin B ml^{-1} , before carrying out infections. All drugs were purchased from Sigma. In all cases, infection experiments were carried out in triplicate and independently at least three times ($n=9$).

Generation of a rabbit polyclonal anti-NTHi serum. An overnight-grown culture of NTHi was centrifuged, resuspended in acetone to half of the original volume and agitated for 24 h. Acetone-killed bacteria were centrifuged, resuspended in one-tenth of the original acetone volume and agitated for 1 h. The suspension of acetone-killed bacteria was dried with a vacuum desiccator. This process was performed separately for NTHi strains 398, 375 and 2019, and whole-cell bacterial antigens were mixed in equal proportions. A rabbit anti-NTHi serum was obtained by repeated immunization of one rabbit (Charles River Laboratories) with the mixture of acetone-killed bacteria.

Immunofluorescence and TEM. Cells were seeded on 12 mm circular coverslips in 24-well tissue culture plates. Infections were performed as described above. When indicated, cells were washed three times with PBS and fixed with 3.7% paraformaldehyde (PFA) in PBS, pH 7.4, for 15 min at room temperature, or with cold absolute methanol for 5 min at -20 °C. Methanol fixation was used for cathepsin D and tubulin staining. For early endosome antigen 1 (EEA1) staining, cells were fixed with 2.5% PFA for 10 min at room temperature followed by 1% PFA + 80% methanol at -20 °C for 5 min.

NTHi was stained with rabbit anti-NTHi serum diluted 1:800. *S. typhimurium* was stained with rabbit anti-LPS diluted 1:800 (a gift from Dr G. Frankel, Imperial College London, UK). Actin cytoskeleton was stained with rhodamine-phalloidin (Invitrogen) diluted 1:200; microtubule cytoskeleton was stained with mouse anti-tubulin antibody (Sigma) diluted 1:150. Early endosomes were stained with goat anti-EEA1 (N-19) antibody (Santa Cruz Biotechnology) diluted

1:50. Late endosomes were stained with mouse monoclonal anti-human lamp-1 (H4A3), mouse monoclonal anti-human lamp-2 (H4B4) or mouse anti-human CD63 (H5C6) antibodies (Developmental Studies Hybridoma Bank) diluted 1:150. DNA was stained with Hoechst 33342 (Invitrogen) diluted 1:2500. Lysosomes were labelled with goat anti-human cathepsin D (G19) or rabbit anti-human cathepsin D (H-75) antibodies (Santa Cruz Biotechnology) diluted 1:100. The Golgi apparatus was stained with mouse anti-GM130 antibodies (BD Laboratories) diluted 1:300. Donkey anti-rabbit, donkey anti-mouse and donkey anti-goat secondary antibodies conjugated to rhodamine or Cy2 were purchased from Jackson Immunological and diluted 1:200. Fixable dextran 70 000 (molecular mass) labelled with Texas red (TR-dextran) at 25 μg ml^{-1} (Molecular Probes) was used in two types of assays: (i) TR-dextran was used to analyse the uptake of bacteria by endocytosis, by addition of this fluid endocytic marker into the medium at the onset of infection; (ii) given that endocytosed TR-dextran is delivered to and retained by endocytic compartments, it was independently used to label lysosomes in a pulse-chase assay. Briefly, epithelial cells seeded on glass coverslips were labelled by pulsing with 25 μg TR-dextran ml^{-1} for 2 h at 37 °C in 5% CO₂ in EBSS medium. To allow the TR-dextran to accumulate in lysosomes, medium was removed, and cells were washed three times with PBS and incubated for 30 min (NTHi) or 2 h (*Salmonella*) in dye-free medium (chase). After the chase period, cells were infected with NTHi or *S. typhimurium*, as described above. Different chase times were applied depending on the pathogen due to the use of different infection protocols for each one, i.e. 2 h for NTHi and 30 min for *S. typhimurium*, thus allowing the same chase time (2 h 30 min) for both pathogens before gentamicin addition. Acidic compartments were loaded with 0.5 μM LysoTracker Red DN99 (Invitrogen) 45 min before fixation. At the end of the infection period, the residual fluid marker was removed by washing the cells three times with PBS, followed by fixation.

Staining was carried out in 10% horse serum, 0.1% saponin in PBS. Coverslips were washed twice in PBS containing 0.1% saponin and once in PBS, and incubated for 30 min with primary antibodies. Coverslips were then washed twice in 0.1% saponin in PBS and once in PBS, and incubated for 30 min with secondary antibodies. Finally, coverslips were washed twice in 0.1% saponin in PBS, once in PBS and once in H₂O, and mounted on glass coverslips using ProLong Gold antifade mounting gel (Invitrogen).

For extra-/intracellular differential staining, PFA-fixed cells were incubated for 10 min with PBS containing 10% horse serum, Hoechst 33342 and mouse anti-PCho TEPC-15 antibody diluted 1:200. Coverslips were washed three times with PBS and stained as described above with donkey anti-mouse secondary antibody conjugated to rhodamine. Coverslips were washed three times in PBS and once in distilled water before mounting onto glass slides using ProLong Gold antifade mounting gel. Immunofluorescence was analysed with a Leica CTR6000 fluorescence microscope. Images were taken with a Leica DFC350FX monochrome camera. Confocal microscopy was carried out with a Leica TCS SP5 confocal microscope. Depending on the marker, an NTHi-containing vacuole (NTHi-CV) was considered positive when it fulfilled these criteria: (i) the marker was detected throughout the area occupied by the bacterium; (ii) the marker was detected around/enclosing the bacterium; and (iii) the marker was concentrated in this area, compared with the immediate surroundings. To determine the percentage of bacteria that co-localized with each marker, all bacteria located inside a minimum of 100 infected cells were analysed in each experiment. Results were calculated from two independent experiments and are presented as the mean percentage of association $\pm$ SEM. Experimental details for the kinetics shown in Fig. 3(b) are specified in Results. For TEM, cells were seeded in 24-well tissue culture plates. Infections were carried out as described above, and cells were fixed

with glutaraldehyde and processed for TEM as described previously (Kruskal *et al.*, 1992).

Fluorescent *in situ* hybridization (FISH). We carried out hybridization of PFA-fixed infected cells with fluorescently labelled oligonucleotides, as described elsewhere (Neef *et al.*, 1996). Cy3- or Alexa 488-conjugated DNA probes EUB338 (5'-GCTGCCTCCCG-TAGGAGT-3') and GAM42a (5'-GCCTTCCCACATCGTTT-3') are designed for specific labelling of the rRNA of eubacteria and the gamma subclass of the proteobacteria, respectively (Manz *et al.*, 1993). A non-EUB338 DNA probe, complementary to EUB338, was used as a negative control. The detectability of bacteria using such oligonucleotide probes is dependent on the presence of sufficient ribosomes per cell, hence providing qualitative information on the physiological state of the bacteria on the basis of the number of ribosomes per cell. These probes were used together to obtain a stronger signal, and were added to a final concentration of 5 nM each in the hybridization buffer. The hybridization buffer contained 0.9 M NaCl, 20 mM Tris/HCl, pH 7.4, 0.01 % SDS and 35 % formamide. Coverslips were first washed with deionized water. Hybridization was carried out for 1.5 h at 46 °C in a humid chamber, followed by a 30 min wash at 48 °C. Washing buffer contained 80 mM NaCl, 20 mM Tris/HCl, pH 7.4, 0.01 % SDS and 5 mM EDTA (pH 8). After washing, DNA staining for total bacteria was carried out by incubating the coverslips in PBS containing Hoechst 33342 for 20 min. Coverslips were then washed three times in PBS and once in distilled water before mounting onto glass slides using ProLong Gold antifade mounting gel. As a control, FISH labelling of bacteria located extracellularly in gentamicin-treated wells did not give any detectable signal.

Plasmids and transient transfections. For transient transfections with GFP-Rab7Q67L, GFP-TGN46 or GFP-LC3 plasmids, A549 cells were grown on glass coverslips to 40 % confluency (4×10^4 cells per well), maintained in RPMI 1640 medium containing 10 % FCS, and transfected with 1.2 µg DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were infected with NTHi. To induce autophagy in GFP-LC3-transfected cells, cells were pre-treated with 500 nM rapamycin 1 h before infection and the concentration of rapamycin was maintained throughout the experiment. Cells were then infected. In all cases, samples were fixed, stained and analysed by immunofluorescence microscopy.

Detection of Akt phosphorylation by Western blotting. Cells were seeded on six-well tissue culture plates at 10^6 cells per well. Cells were infected with NTHi at m.o.i. 100:1, 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 %, w/v, glycerol, 50 mM DTT, 0.01 %, w/v, bromophenol blue) on ice. Samples were sonicated, boiled at 100 °C for 10 min and cooled on ice before PAGE and Western blotting. Akt phosphorylation was detected with primary rabbit anti-phosphoSer473 Akt antibody (Cell Signalling Technology) diluted 1:1000 and secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Thermo Scientific) diluted 1:10000. Total Akt was detected with primary rabbit Akt (Cell Signalling Technology) antibody diluted 1:1000 and secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Thermo Scientific) diluted 1:10000. Tubulin was detected with primary mouse anti-tubulin antibody (Sigma) diluted 1:3000 and secondary goat anti-mouse antibody (Pierce) conjugated to horseradish peroxidase diluted 1:1000. When necessary, the membrane was washed twice for 15 min with 0.5 % Tween-20 in PBS, incubated for 30 min in Restore Western blot Stripping Buffer (Thermo Scientific) at 37 °C, and washed twice for 15 min with 0.5 % Tween-20 in PBS. Images were recorded with a GeneGnome HR imaging system (Syngene).

Statistical methods. Statistical analysis was performed using analysis of variance, the two-sample *t* test with two tails, followed by the Mann-Whitney *U* test. A *P* value of <0.05 was considered statistically significant. Error bars in the graphs in all figures were calculated as SEM. The analysis was performed using Prism 4 for PCs (GraphPad Software).

RESULTS

NTHi infection of airway epithelial cells: invasion requires host microtubules, lipid rafts integrity and activation of phosphatidylinositol 3-kinase (PI3K) signalling

A549 cell infection with stationary phase-grown NTHi 375, at m.o.i. 100:1 for 1 and 2 h, showed that bacteria adhered to cells in a time-dependent manner (Supplementary Fig. S1A), that they associated to cells, forming microcolonies on the cell surface (Supplementary Fig. S1B), that approximately 80 % of cells were infected after 2 h, and that cell density and overall morphology remained unaltered when infection was carried out for up to 2 h. An m.o.i. of 100:1 for 2 h was the condition chosen for subsequent experiments. NTHi strains 398, 375 and 2019, displaying high, medium and low PCho levels on their surfaces, respectively (Supplementary Fig. S2A), showed comparable adhesion and formation of adherent bacterial aggregates (Supplementary Fig. S1A, B and data not shown). NTHi adhesion was also observed by TEM, showing cell extensions at the site of attachment (Supplementary Fig. S1C). NTHi internalization into A549 cells, addressed by infection, incubation with medium containing gentamicin, cell lysis and plating of bacteria, showed bacterial recovery and no significant differences among strains (Supplementary Fig. S1D). Bacterial intracellular localization was visualized by confocal microscopy and by a double-fluorescence immunolabelling method (extra-/intracellular differential staining) (Supplementary Fig. S1E and F, respectively). The formation of microcolonies seemed to be relevant for bacterial invasion because the entry rate decreased to almost undetectable levels by a reduction of m.o.i. or by lowering the infection time, which in turn limited the formation of microcolonies (data not shown). These experiments, together with earlier observations by other authors (Ahrén *et al.*, 2001; Bandi *et al.*, 2001; Fink *et al.*, 2003; Forsgren *et al.*, 1994; Hendrixson & St Geme, 1998; Holmes & Bakaletz, 1997; Kenjale *et al.*, 2009; Ketterer *et al.*, 1999; Rao *et al.*, 1999; St Geme, 2002; St Geme & Falkow, 1990; Swords *et al.*, 2000; Virji *et al.*, 1991), confirm that NTHi adheres to and can invade epithelial cells.

We next elucidated the contribution of the host cell machinery to NTHi invasion of epithelial cells. Experiments were carried out in the presence of drugs which inhibit specific host cell functions. Nocodazole and colchicine significantly reduced the entry of NTHi 375 when compared with control infections, suggesting that NTHi invasion involves the assembly of the host microtubule network (Fig. 1a). Lipid rafts, specialized dynamic plasma membrane

microdomains enriched with cholesterol and sphingolipids, are structures subverted by several pathogens to gain access to their target hosts (Mañes *et al.*, 2003; Rosenberger *et al.*,

2000). M β CD, which depletes cholesterol from host cell membranes, was employed to analyse the involvement of lipid rafts in NTHi invasion. Cholesterol depletion impaired

Fig. 1. Mechanisms of NTHi entry into A549 cells. (a) NTHi 375 was used to infect cells. Wells were washed and incubated with medium containing gentamicin for 1 h. Bacterial uptake was quantified by cell lysis, serial dilution and viable counting on supplemented BHI agar plates. When required, cells were pre-treated for 1 h with nocodazole, colchicine, M β CD, nystatin, filipin or LY294002, for 30 min with wortmannin or cytochalasin D (Cyt D), or for 3 h with latrunculin B. Bacterial uptake was quantified as described above. Results are expressed in c.f.u. per well. (b) Detection of Akt-Ser473 phosphorylation by Western blotting with rabbit anti-phosphoSer473 Akt and goat anti-rabbit conjugated to horseradish peroxidase antibodies (upper panel). Extracts were prepared from cells non-infected (–) or infected for 20, 40, 60, 80, 100 and 120 min with NTHi 375. Detection of total Akt and tubulin (centre and bottom panels, respectively); LY294002 pre-treated cells (upper-right panel) were used as controls. (c) Pre-treatment with chemical inhibitors does not alter NTHi adhesion. NTHi 375 was used to infect cells for 2 h. When required, cells were pre-treated for 1 h with nocodazole, colchicine, M β CD or LY294002, for 30 min with cytochalasin D or wortmannin, or for 3 h with latrunculin B. Bacterial adhesion was quantified as described above. Data are shown as percentage adhesion compared with that of control non-treated cells.

NTHi entry into A549 cells in a dose-dependent manner (Fig. 1a and Supplementary Fig. S3B). Similar results were obtained when cells were treated with filipin and nystatin (Fig. 1a). Given that several pathogens exploit host signalling pathways to their own benefit (Pizarro-Cerdá & Cossart, 2004), we also studied the contribution of the PI3K signalling pathway to NTHi invasion by pre-treatment of cells with LY294002 or wortmannin, two specific inhibitors of PI3K activity. As shown in Fig. 1(a), these inhibitors reduced NTHi entry. Akt becomes phosphorylated upon activation of PI3K signalling (Ji & Liu, 2008). Western blot analysis revealed that NTHi infection induces the phosphorylation of Akt (Fig. 1b); a mock control is shown in Supplementary Fig. S3(A). Akt phosphorylation upon NTHi infection was abrogated by cell pre-treatment with LY294002 (Fig. 1b, upper-right panel). Akt and tubulin alone were used as controls (Fig. 1b, centre and lower panels). Cell pre-treatment with cytochalasin D or latrunculin B, two inhibitors of actin cytoskeleton polymerization, did not inhibit, but increased NTHi entry into A549 cells (Fig. 1a, right panel). These chemical inhibitors did not affect bacterial viability (Supplementary Fig. S3C) or alter bacterial adhesion (Fig. 1c).

Collectively, these results suggest that NTHi invasion of cells is a microtubule-dependent event which requires the integrity of host plasma membrane lipid rafts and the activation of the PI3K signalling pathway.

NTHi locates within airway epithelial cells inside acidic compartments

We next examined the ability of NTHi to survive within airway epithelial cells. A549 cells were infected with NTHi 375, washed, incubated with medium containing gentamicin, and lysed with 1% saponin. Intracellular bacteria were enumerated by plating at 2 h intervals up to 24 h post-gentamicin. Data revealed that bacterial numbers decreased continuously over time (data not shown). Identical infections were carried out in parallel and samples were processed for immunofluorescence microscopy. In contrast to the plating data, NTHi could be clearly observed throughout the entire course of infection by staining with the fluorescent DNA dye Hoechst 33342 (the image shown was taken at 24 h post-gentamicin, Fig. 2a, left panel). To elucidate whether those intracellular bacteria were indeed viable, FISH was carried out by using the probes EUB338 and GAM42a (see Methods). Microscopy analysis indicated the presence of metabolically active intracellular bacteria at all time points analysed (1, 4, 8, 12, 16, 24 and 28 h post-gentamicin) (Fig. 2a). The specificity of the FISH probe EUB338 was assessed by the use of the control probe non-EUB338, complementary to EUB338, which did not render any detectable signal (data not shown). Assessment of the number of bacteria metabolically active (FISH-positive) versus the total number of intracellular bacteria (Hoechst-positive) revealed that FISH-positive bacteria were 76 ± 8.3 , 77 ± 1.7 and $70 \pm 3.4\%$ of total bacteria at 4, 8, and 24 h post-gentamicin, respectively. These data showed that the efficiency of FISH labelling was

maintained throughout the infection. We did not observe changes in overall monolayer density, host cell morphology, or the appearance of actin and microtubule cytoskeleton networks at any infection time (data not shown).

To address the discrepancy observed with c.f.u. plating, infections were carried out as described above and different host cell lysis methods were used separately. The cell lysis solutions used were PBS/saponin (1, 0.5, 0.05 or 0.025 %), PBS/Triton X-100 (1, 0.5, 0.1 or 0.05 %), PBS/Nonidet P-40 (0.5, 0.1 or 0.025 %), PBS, water, PBS/0.25 % trypsin, and scraping with a cell scraper. It should be noted that significant differences in the number of intracellular bacteria recovered were obtained depending on the lysis method used (Supplementary Table S1). Even though cell lysis with PBS/0.025 % saponin gave the highest bacterial recovery (Supplementary Table S1), the c.f.u. values still underestimated the number of bacteria which could be observed by immunofluorescence microscopy, particularly at time points later than 4 h post-gentamicin (Supplementary Table S1 and data not shown).

We next analysed the link between NTHi and the host endocytic machinery by using membrane-impermeant TR-dextran. Fluorescent dextran is a valuable marker for the uptake and internal processing of exogenous materials by endocytic pathways (Swanson, 1989). Addition of this endocytic marker to the medium at the onset of bacterial infection revealed that internalized NTHi co-localized with dextran within cells at 1 h post-gentamicin (Supplementary Fig. S4A), suggesting that NTHi uses elements of the host endocytic machinery to enter epithelial cells. Similar results were obtained for NTHi strains 375, 398 and 2019 (data not shown).

We next assessed the subcellular location of NTHi during infection of A549 cells. We asked whether the subcellular compartment housing NTHi had endosomal features by staining EEA1, an archetypical early endosome marker. A time-course was carried out at 15, 30, 60, 90 and 120 min post-gentamicin. Intracellular NTHi 375 was located in subcellular compartments with early endosome features (EEA1-positive) at 15 min post-gentamicin and could be seen in this type of compartment up to 90 min post-gentamicin (quantification kinetics and an image taken at 1 h post-gentamicin are shown in Fig. 2d and b, respectively). Next, we assessed the location of the late endosome marker lamp-1. The same infection time-course was used to quantify lamp-1 bacterial co-localization (Fig. 2d). EEA1/lamp-1/bacteria triple staining showed that at those time points, bacteria were located intracellularly either in EEA1-positive or in lamp-1-positive compartments, but never in compartments simultaneously labelled with EEA1 and lamp-1. Next, a longer infection time-course was carried out. Co-localization of NTHi with lamp-1 was observed in 93.8 ± 4.86 , 93.2 ± 0.78 and $97.5 \pm 1.41\%$ of bacteria at 4, 8 and 24 h post-gentamicin, respectively (acquisition kinetics and an image taken at 8 h post-gentamicin are shown in Fig. 2e and c, respectively).

Fig. 2. NTHi survival within airway epithelial cells. (a) FISH was used to show that intracellular NTHi is metabolically active. Cells were infected with NTHi 375. Wells were washed and incubated for 1 h with medium containing gentamicin ($200 \mu\text{g ml}^{-1}$), and then with medium containing $11 \mu\text{g}$ gentamicin ml^{-1} for 23 h. Metabolically active bacteria were labelled with probes EUB338-Cy3 and GAM42a-Cy3 (red). DNA was labelled with Hoechst (blue). For a detail, see panel at right. (b) Co-localization of NTHi and the early endosomal marker EEA1. NTHi was stained with rabbit anti-NTHi and Cy2-conjugated donkey anti-rabbit (green) antibodies. EEA1 was stained with goat anti-EEA1 and rhodamine-conjugated donkey anti-goat (red) antibodies. The image was taken at 1 h post-gentamicin. For a detail, see panel at right. (c) Co-localization of NTHi and lamp-1. NTHi was stained with rabbit anti-NTHi and Cy2-conjugated donkey anti-rabbit (green) antibodies. Lamp-1 was stained with mouse anti-lamp-1 and donkey anti-mouse conjugated to rhodamine (red) antibodies. The image was taken at 8 h post-gentamicin. (d) Percentage NTHi 375 co-localization with EEA1 (▲) or with late endosome marker lamp-1 (■) over a 2 h time-course. A549 cells were infected, and coverslips were fixed and stained at the indicated times. (e) Percentage of NTHi 375 co-localization with lamp-1 over a 24 h time-course. Cells were infected, and coverslips were fixed and stained at the indicated times. Results shown in (d) and (e) represent two independent experiments each in which intracellular NTHi from infected cells was scored for EEA1 or lamp-1 co-localization. Values are given as the mean $\pm$ SEM percentage of NTHi co-localizing with the marker.

Fig. 3. NTHi remains metabolically active inside acidic endosomal compartments. (a) Location of FISH-positive NTHi inside lamp-1-positive compartments. NTHi and host cell nuclei were stained with Hoechst (blue), subcellular compartments were labelled with mouse anti-lamp-1 and secondary goat anti-mouse-Cy2 (green) antibodies, and metabolically active bacteria were labelled with the probes EUB338-Cy3 and GAM42a-Cy3 (red). Images were taken at 4 h (upper panels) and 28 h (lower panels) post-gentamicin. (b) Quantification of the number of metabolically active (FISH-positive) intracellular bacteria in lamp-1-positive compartments. Intracellular bacteria were quantified at 4, 8, 12, 16, 24 and 28 h post-gentamicin. Results are represented as number of FISH-positive, lamp-1-positive bacteria per infected cell. At least 1000 cells in two independent experiments were counted per time point. The percentage of infected cells per coverslip at each time point is shown at 4, 8, 12, 16, 24 and 28 h post-gentamicin. (c) Location of FISH-positive NTHi inside acidic compartments. NTHi and host cell nuclei were stained with Hoechst (blue), acidic subcellular compartments were loaded with LysoTracker (red), and metabolically active bacteria were labelled with the probes EUB338-Alexa 488 and GAM42a-Alexa 488 (green). The image was taken at 24 h post-gentamicin. For a detail, see panel at right.

To quantify NTHi intracellular survival, the number of FISH-positive bacteria located inside lamp-1-positive compartments per infected cell was enumerated in at least 1000 cells in two independent experiments, at 4, 8, 12, 16, 24 and 28 h post-gentamicin (images taken at 4 and 28 h post-gentamicin are shown in Fig. 3a). The frequency of FISH-positive, lamp-1-positive NTHi cells per infected cell (i.e. intracellular metabolically active bacteria) was maintained for 8 h, after which a decrease occurred, reaching a plateau that was maintained until the end of the experiment (Fig. 3b). Numbers corresponding to the percentage of infected cells per well at each post-gentamicin time point are shown within Fig. 3(b).

Endosomal compartments acidify during the maturation process (Luzio *et al.*, 2007). The acidotropic probe LysoTracker Red DND-99, a weak base conjugated to a red fluorophore that freely permeates cell membranes and remains trapped in acidified organelles, is used to monitor the maturation of endocytic compartments. Immunofluorescence microscopy revealed intracellular bacteria inside acidic subcellular compartments 15 min post-gentamicin and throughout the entire assay (1, 2, 4, 8, 12, 16, 24 and 28 h post-gentamicin time points were processed for observation of LysoTracker co-localization). Similar data were obtained for NTHi strains 398, 375 and 2019 (data not shown). Triple staining of subcellular acidic compartments (red), bacteria (blue) and bacterial rRNA (green) was carried out at the time points mentioned above and further confirmed that intracellular bacteria were metabolically active and located inside acidic compartments (an image taken at 24 h post-gentamicin is shown in Fig. 3c). NTHi localization inside acidic compartments was quantified by assessing NTHi/LysoTracker co-localization at 15 min, and 2, 4, 8, 24 and 28 h post-gentamicin. Co-localization was 53.7 ± 4.9 , 63.7 ± 10.2 , 76.5 ± 4.9 , 74 ± 2.8 , 76 ± 8.4 and 76 ± 8.4 %, respectively.

Given that disturbances of this subcellular niche could affect the intracellular viability of the pathogen, we assessed the effect of inhibiting the vacuolar acidification by treating the cells with bafilomycin A1, a specific inhibitor of vacuolar type H⁺-ATPase. The percentage of infected cells containing FISH-positive, lamp-1-positive bacteria at 4 h post-gentamicin decreased from 11.5 ± 0.14 % (untreated cells) to 3.70 ± 1.84 % (bafilomycin A1-treated cells), and from 10.05 ± 0.92 to 0.4 ± 0.57 at 8 h post-gentamicin.

Together, these data show that intracellular NTHi remains metabolically active in a non-replicative state inside acidic compartments (NTHi-CVs) throughout the course of infection, displaying a persistent population of non-proliferative bacteria from approximately 12 h post-gentamicin to the end of the experiment.

Acquisition of endocytic markers by the NTHi-CV

We assessed the maturation of the NTHi-CV by analysing bacterial co-localization with late endosomal markers by immunofluorescence microscopy. Labelling of the late endosome markers lamp-2 and CD63 was found to be

positive around intracellular bacteria and was quantified at several post-gentamicin time points. Acquisition kinetics and images taken at 8 h post-gentamicin are shown in Fig. 4.

Rab7 is a small G-protein of the Rab family that controls vesicular transport to late endosomes and lysosomes in the endocytic pathway (Haas, 2007). We analysed the trafficking of Rab7 GTPase during NTHi infection of epithelial cells. A549 cells were transfected with a constitutively active Rab7 form fused to GFP (GFP-Rab7Q67L) (Berón *et al.*, 2002) and then infected with NTHi 375. Intracellular bacteria were located in Rab7-positive subcellular compartments at 2 h post-gentamicin, and co-localization could also be observed at later time points, i.e. 4 and 6 h post-gentamicin (Supplementary Fig. S4B).

NTHi may have evolved adaptations to survive within lysosomes or to modulate host cell trafficking events and modify lysosomal fusion, thereby surviving within epithelial cells. To distinguish between these possibilities, we first analysed by immunofluorescence microscopy fusion events between NTHi-CVs and lysosomes by monitoring bacterial co-localization with cathepsin D. Cathepsin D, an aspartyl protease involved in intracellular degradation of exogenous and endogenous proteins, is delivered to the lumen of late endosomes by the mannose-6-phosphate receptor (M6PR) (Kornfeld, 1986; Ludwig *et al.*, 1994; Munier-Lehmann *et al.*, 1996). Cathepsin D is one of the most abundant hydrolases that accumulates in lysosomes as they mature (Garin *et al.*, 2001), and has been used as a marker for lysosomal fusion (Garvis *et al.*, 2001). We used an antibody against cathepsin D to study interactions between this enzyme and the NTHi-CV. At 30, 60 and 90 min, and 2, 4, 6 and 8 h post-gentamicin, cells were fixed, stained and examined by microscopy. At these time-points, fewer than 10 % (time-course mean) of NTHi 375 NTHi-CVs co-localized with cathepsin D (Fig. 5a, white bars). Images shown were taken at 8 h post-gentamicin (Supplementary Fig. S5A, B).

To assess the functionality of the A549 host cell lysosomal machinery, an *S. typhimurium* $\Delta invA$ mutant strain expressing *Yersinia enterocolitica* invasin was used to infect cells (Watson & Galán, 2008). This mutant invaded epithelial cells, and the subcellular compartment in which it resided fused with lysosomes, showing co-localization with cathepsin D ranging from 32.31 ± 6.6 % at 30 min to 72.27 ± 5.2 % at 8 h post-gentamicin (Fig. 5a, black bars, and Supplementary Fig. S5B).

TR-dextran internalized by endocytosis is delivered to and retained by various compartments of the endocytic pathway, including lysosomes. Pulse-chase protocols with TR-dextran have been used to label lysosomes (Lamothe *et al.*, 2007). Prior to NTHi infection, we pulsed epithelial cells with $25 \mu\text{g TR-dextran ml}^{-1}$ for 2 h followed by a 30 min chase in dye-free medium to allow the probe to be delivered to lysosomes. Cells were then infected and TR-dextran distribution was assessed at 1, 4 and 8 h post-gentamicin,

Fig. 4. Acquisition of late endosome markers by the NTHi-CV. Co-localization of NTHi with lamp-2 (a) and CD63 (b): NTHi was stained with rabbit anti-NTHi and Cy2-conjugated donkey anti-rabbit (green) antibodies. Lamp-2 and CD63 were stained with mouse anti-lamp-2 or anti-CD63 and donkey anti-mouse conjugated to rhodamine (red) antibodies. Images were taken at 8 h post-gentamicin. (c) Percentage NTHi 375 co-localization with the late endosome markers lamp-2 (■) and CD63 (▲) over a 24 h time-course. A549 cells were infected, and coverslips were fixed and stained at the indicated times. Values are given as mean $\pm$ SEM percentage of NTHi co-localizing with the marker.

and the marker was shown to be present on average in 25 % of NTHi-CVs (Fig. 5b, white bars). In contrast, when TR-dextran pre-loaded cells were infected with *S. typhimurium* $\Delta invA$ (invasin), an average of 71.6 % of *Salmonella*-containing vacuoles (SCVs) contained the fluid marker, when the distribution was assessed at 1, 4 and 8 h post-gentamicin (Fig. 5b, black bars). Together, these data highlighted that the A549 cell lysosomal machinery was functional.

Several bacterial pathogens exploit a variety of host cell processes, such as the eukaryotic secretory pathway, in which proteins and lipids are modified and transported from the endoplasmic reticulum through the Golgi network to the plasma membrane and other cellular destinations, to establish intracellular organelles (Salcedo & Holden, 2005).

To gain insight into the location and features of the NTHi-CV in relation to host cell organelles, we investigated its position in relation to the Golgi apparatus. A549 cells were infected with NTHi and subsequently stained using an antibody directed against the cis-Golgi-resident protein GM130. Intracellular bacteria, located in acidic compartments, did not co-localize with the cis-Golgi at any time (Supplementary Fig. S6A, upper panels). In a separate set of experiments, cells were transiently transfected with a plasmid expressing GFP-conjugated trans-Golgi-resident protein TGN46, and then infected with NTHi 375. Intracellular bacteria did not co-localize with TGN46 at any time (Supplementary Fig. S6A, lower panels).

Autophagy is a tool of the intracellular host cell defence machinery that bacteria must confront upon cell invasion.

Fig. 5. Acquisition of lysosomal markers. (a) Percentage bacterial co-localization with cathepsin D over an 8 h time-course. A549 cells were infected with NTHi (white bars) or with *S. typhimurium* $\Delta invA$ (invasin) (black bars), and coverslips were fixed and stained at the indicated times. (b) Percentage bacterial co-localization with TR-dextran over an 8 h time-course. Cells were pulse-chased with TR-dextran, then infected and fixed at the indicated times. Cells were infected with NTHi (white bars) or *S. typhimurium* $\Delta invA$ (invasin) (black bars). In (a) and (b), values are given as mean $\pm$ SEM percentage of NTHi co-localizing with the marker.

Several pathogens subvert the autophagic pathway as a strategy to establish a persistent infection (Orvedahl & Levine, 2009). We asked whether NTHi-CV displays autophagy features by infecting cells transfected with GFP-tagged LC3 (light chain 3) as an autophagic marker (Guignot *et al.*, 2009) and studying bacterial co-localization with LC3. Autophagy was induced in these cells by treatment with rapamycin. We could not detect co-localization of intracellular NTHi and LC3 at any time (Supplementary Fig. S6B).

We next asked whether the observations made with A549 cells could be extended to NHBE cells. NHBE cells were

infected with NTHi 375 for 2 h, washed, incubated with medium containing gentamicin at 2 h intervals, fixed and immunostained. Staining of bacteria and host actin or microtubule cytoskeleton(s) did not reveal detectable changes in overall cell morphology upon infection (data not shown). Bacteria located inside NHBE cells co-localized transiently with early endosomal markers, being subsequently located inside subcellular acidic compartments with late endosomal features, identical to the location observed upon A549 cell infection. Transient co-localization with EEA1 was observed at 1 h post-gentamicin (Fig. 6, panels 2). Bacterial co-localization with LysoTracker, lamp-1 and lamp-2 was observed at 4, 8 and 12 h post-gentamicin. Images shown were taken at 4 h post-infection (Fig. 6).

Given its extensively demonstrated role as a virulence factor, we next assessed the involvement of PCCho in NTHi survival inside airway epithelial cells. We used a previously described NTHi 375 mutant strain lacking *lic1BC* that does not present PCCho modification in its LOS molecule (Supplementary Fig. S2A) (Martí-Llitas *et al.*, 2009). A549 cell infection using the experimental conditions described above showed comparable adhesion and invasion frequencies in wild-type and *lic1BC* mutant strains (Supplementary Fig. S2B, C). Similarly, when the biogenesis and maturation features of the NTHi-CV were determined for NTHi 375 $\Delta lic1BC$, it was found to transit progressively through subcellular acidic (LysoTracker-positive) compartments, with early (EEA1-positive) or late (lamp-1-, lamp-2- and CD63-positive) endosome features, identical to those of the NTHi wild-type strain (Supplementary Fig. S7). The general appearance of infection was indistinguishable in the two strains. To quantify NTHi $\Delta lic1BC$ intracellular survival, the number of FISH-positive bacteria located inside lamp-1-positive compartments per infected cell was enumerated as described above (see Fig. 3b and Supplementary Fig. S8). The image shown in Supplementary Fig. S8 was taken at 8 h post-gentamicin. The percentages of infected cells at this time point did not vary significantly between wild-type and $\Delta lic1BC$ mutant strains, being 10.1 ± 0.92 and 10.5 ± 0.14 %, respectively. Similar findings were observed upon NHBE cell infection with NTHi strain 375 $\Delta lic1BC$ (data not shown).

DISCUSSION

In this study we provide evidence showing that NTHi resides within airway epithelial cells without proliferating, in a vacuolar compartment referred to as the NTHi-CV. The pathogen transits temporarily through a compartment with early endosome characteristics that later acquires late endosome features, including acidic pH and the late endosomal markers lamp-1, lamp-2, CD63 and Rab7. Most NTHi-CVs do not fuse with lysosomes. On average, fewer than 10 % of NTHi-CVs were associated with cathepsin D at 8 h after gentamicin addition. To the best of our knowledge, the current study describes for the first time the features of NTHi life inside non-phagocytic cells.

Fig. 6. Maturation of NTHi-CV inside NHBE cells. Acidic subcellular compartments were loaded with LysoTracker (red). Co-localization of NTHi and the early endosomal marker EEA1: NTHi was stained with rabbit anti-NTHi and donkey anti-rabbit conjugated to Cy2 (green) antibodies. EEA1 was stained with goat anti-EEA1 and donkey anti-goat conjugated to rhodamine (red) antibodies. Co-localization of NTHi and the late endosomal markers lamp-1 and lamp-2: NTHi was stained with rabbit anti-NTHi and donkey anti-rabbit conjugated to Cy2 (green) antibodies. Lamp-1 and lamp-2 were stained with mouse anti-lamp-1 or anti-lamp-2 and donkey anti-mouse conjugated to rhodamine (red) antibodies. For a detail of each staining, see panels at right. The EEA1 image was taken at 1 h post-gentamicin; LysoTracker, lamp-1 and lamp-2 images were taken at 4 h post-gentamicin. Images were taken with a monochrome camera and software-coloured; the last panel was kept monochrome for clarity.

NTHi is a causative agent of persistent respiratory infections. When thinking about chronic bacterial infections, distinctive features should be taken into account for a set of persistent pathogens carried asymptomatically in the nasopharynx which have the ability to cause life-threatening diseases under predisposing conditions (Kadioglu *et al.*, 2008; Monack *et al.*, 2004; Perez Vidakovich & Riesbeck, 2009; Virji, 2009). Thus, NTHi is likely to have developed strategies to be an asymptomatic colonizer of the nasopharynx, and also, under certain circumstances, to be a symptomatic colonizer of the lower respiratory tract. A recent study has analysed the events of persistent bacterial infections using game theory, where

pathogen and host are two players with a conflict of interest. This theoretical model predicts that during persistent infections, pathogenic bacteria stay in both intra- and extracellular compartments of the host, implying that the pathogen should be able to survive in both locations to cause persistent infection (Eswarappa, 2009). NTHi nicely fulfils this model by matching the data presented in the current study, in which we introduce NTHi intracellular life features inside non-phagocytic cells, with an extensive body of evidence regarding different aspects of its extracellular life, including, among others, the formation of biofilms (Erwin & Smith, 2007; Hong *et al.*, 2007a, b; Moxon *et al.*, 2008; Murphy & Kirkham, 2002).

Although the NTHi invasion rate may seem low, it should be pointed out that there are multiple examples of bacterial pathogens with a rate of invasion similar to that of NTHi whose intracellular lifestyles contribute significantly to the overall progression of the infection. Examples are, among others, *Neisseria* spp. and *Listeria monocytogenes* (Binker *et al.*, 2007; Lambotin *et al.*, 2005; Veiga & Cossart, 2006; Virji, 2009). Earlier studies have shown that NTHi invades non-phagocytic cells (Ahrén *et al.*, 2001; Ketterer *et al.*, 1999; St Geme, 2002; St Geme & Falkow, 1990; Swords *et al.*, 2000; Virji *et al.*, 1991); some mechanistic insights into the invasion process have been provided (Ketterer *et al.*, 1999; Swords *et al.*, 2001). Other data indicate that NTHi resides intracellularly within membrane-bound vacuoles (St Geme, 2002). Analysis of human adenoid tissue and bronchial human biopsies further supports the notion that NTHi may remain viable intracellularly (Bandi *et al.*, 2001; Forsgren *et al.*, 1994). In this work, we have extended these earlier observations, and our data show that NTHi internalization is an unusual microtubule-dependent endocytosis process which requires the integrity of host plasma membrane lipid rafts and the activation of PI3K signalling. Most bacterial pathogens able to invade non-phagocytic cells subvert the host actin cytoskeleton to internalize. In contrast, NTHi belongs to a small number of pathogens, including *Campylobacter jejuni*, *L. monocytogenes*, *Neisseria gonorrhoeae*, *Porphyromonas gingivalis* and *Vibrio cholerae*, which need the polymerization of the host microtubule network to invade, instead of requiring actin assembly (Yoshida & Sasakawa, 2003). In fact, actin cytoskeleton depolymerization stimulated invasion by NTHi, similar to reported data for *C. jejuni* (Oelschlaeger *et al.*, 1993). Given that currently available genomic information excludes the existence of a type III secretion system (T3SS) in the NTHi genome (Harrison *et al.*, 2005), we postulate that NTHi entry into airway epithelium could be regarded as a zipper-like (Cossart & Sansonetti, 2004), microtubule polymerization-dependent process. Future studies will aim to confirm this hypothesis and to analyse the balance between actin and microtubule cytoskeleton dynamics in the context of NTHi entry.

Once inside the host cell, NTHi was found to be metabolically active for a long period of time. However, widely used methods for intracellular bacteria recovery and quantification using detergents (i.e. Triton X-100 or saponin at concentrations higher than 0.025 %) were not appropriate to recover intracellular NTHi, hence underestimating the extent of its intracellular survival at post-gentamicin times longer than 4 h. This is in good agreement with the fact that the NTHi outer membrane is permeable to hydrophobic agents (Martínez de Tejada & Moriyón, 1993). Therefore, special attention should be paid when this type of agent is used. It should be noted that dissection of *C. jejuni* life inside intestinal epithelial cells has shown that intracellular bacteria acquire a metabolic state that renders them unculturable under standard culture conditions (Watson & Galán, 2008). An independent study has shown that it is not possible to

obtain accurate viable cell counts of intracellular *Burkholderia cepacia* inside *Acanthamoeba polyphaga* due to difficulties with the use of membrane-impermeable antibiotics (Lamothe *et al.*, 2004). Together, these observations emphasize the fact that culture and/or recovery conditions should be optimized for each pathogen and sample origin. Moreover, bacteria plating-based methods should be used in parallel with microscopy, to reach a more comprehensive understanding of the host-pathogen interplay under study. In this work, we used microscopic single-cell analysis and assessed bacterial viability based on FISH. FISH-positive staining was taken as an index of bacterial ribosomes, and therefore as a method to address NTHi viability or metabolic activity (Amann *et al.*, 1995; Bertaux *et al.*, 2003; Christensen *et al.*, 1999). We could quantify the metabolically active intracellular bacterial load for up to 28 h post-infection. It should be noted that a 5' fluorescein-tagged DNA oligomer that recognizes *H. influenzae* 16S rRNA has been used as a probe for the *in situ* detection of this pathogen in adenoid tissue sections of human origin (Forsgren *et al.*, 1994).

The features of NTHi-CVs became evident at early post-gentamicin time points, when we could determine the late endosome signature of this subcellular compartment, which was constant to the end of the infection. The intracellular NTHi population was stable for 8 h post-gentamicin, then underwent a decrease to enter a second plateau that was maintained to the end of the assay. The acquisition of cathepsin D or of TR-dextran by intracellular NTHi was observed in a low percentage of bacteria. The host cell lysosomal machinery was shown to be functional, given that the SCV harbouring *S. typhimurium* Δ invA (invasin) increasingly co-localized with cathepsin D in a time-dependent manner, and also accumulated TR-dextran. The bacterial decrease observed at 8 h could be due to the small amount of lysosomal fusion observed, but we cannot rule out that it was due to the physiology of the pathogen itself, establishing an intracellular, metabolically active, non-proliferative bacterial population. Pathogens use a variety of strategies to survive intracellularly. Some pathogens, such as *L. monocytogenes* and *Shigella flexneri*, break and escape out of the phagocytic vacuoles and replicate within the cytosol of the infected cells (Ray *et al.*, 2009). Other pathogens, such as *Leishmania*, have evolved adaptations to survive the hostile environment of the phagolysosome, which is characterized by low oxygen tension, poor nutrient content, low pH, and microbicidal agents such as antimicrobial peptides and lysosomal enzymes (Amer & Swanson, 2002). A growing number of intracellular pathogens is now known to have developed strategies to create a unique/customized intracellular niche in which they survive by avoidance of lysosomal fusion and/or by acquisition of autophagic or secretory network-related features (García-del Portillo *et al.*, 2008; Kumar & Valdivia, 2009; Watson & Galán, 2008). The results presented here reveal that the NTHi-CV creates a niche favourable to remaining within host cells without replicating. This could be related to a

separation from the canonical endocytic pathway or to a block in the delivery of lysosomal enzymes from the biosynthetic pathway. In agreement with our observations, it has been reported that *Bartonella*, uropathogenic *E. coli*, *C. jejuni*, *B. cepacia* and *Salmonella enterica* produce long-lasting infections inside cells without proliferating, a means of intracellular colonization that protects the pathogen from host immunity (Cano *et al.*, 2001; Lamothe *et al.*, 2004; Martínez-Moya *et al.*, 1998; Mysorekar & Hultgren, 2006; Schülein *et al.*, 2001; Watson & Galán, 2008; Wright *et al.*, 2007). In turn, this implies that disturbances of this niche should affect the intracellular viability of the pathogen. Indeed, in the case of NTHi, inhibition of vacuolar compartment acidification by treatment of cells with a specific inhibitor of vacuolar type H⁺-ATPase significantly reduced NTHi intracellular survival.

Given that a wealth of evidence has demonstrated the importance of LOS decoration with PCho in several aspects of NTHi pathogenesis, we explored its involvement in NTHi intracellular life. Intracellular bacteria remained within acidic compartments with late endosome features, indistinguishable from wild-type NTHi-CVs. Significant differences between NTHi 375 wild-type and Δ lic1BC mutant strains regarding adhesion and invasion were not observed. NTHi isolates display a high variability in terms of genetic content and gene expression, likely to have an impact in bacterial–host interaction dynamics (Gilsdorf *et al.*, 2004). Differences regarding the genetic content of NTHi bacterial isolates, cell lines and the experimental procedures used for infection could explain the divergence observed between our data and the existing literature with respect to PCho involvement in NTHi adhesion and entry into epithelial cells. The data presented here should be considered as complementary to earlier information (Swords *et al.*, 2000). Future studies will attempt to elucidate bacterial factors associated with the intracellular features of NTHi.

NTHi is the most frequently isolated bacterial pathogen in the lungs of COPD patients (Foxwell *et al.*, 1998; Rao *et al.*, 1999). COPD is a respiratory disease with a 50 % prevalence among heavy smokers, characterized by a not fully reversible airflow limitation associated with an abnormal inflammatory response in the small airways, emphysema, and chronic bacterial colonization of the lower respiratory tract (Cosio *et al.*, 1978; Rennard & Vestbo, 2006; Sethi, 2000; Sethi & Murphy, 2008). There is evidence that COPD patients display elevated levels of antimicrobial peptides, metalloproteases and surfactant in their lower respiratory tract, together with neutrophil infiltration. Moreover, Th1 cell-mediated inflammation and interferon (IFN)- γ -mediated activation of macrophages and B lymphocytes contribute to COPD progression (Cosio *et al.*, 2009; Kaufmann & Schaible, 2005). NTHi overcomes humoral and cellular immune responses and, as a result, COPD patients are chronically colonized. Based on the results shown in this work, we propose that NTHi intracellular colonization may contribute to protecting the pathogen from host immunity

and from antibiotic therapies, hence facilitating the chronic colonization frequently observed in COPD patients. In conclusion, we show here a novel aspect of the interplay of NTHi with the human airway epithelium, i.e. the fact that the pathogen can survive inside epithelial cells, located within a subcellular, membrane-bound compartment. The ability of NTHi to survive both intra- and extracellularly during the infection process may be essential to its chronic persistence inside the host.

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