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Bordetella pertussis entry into respiratory epithelial cells and intracellular survival

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Running title*: B. pertussis* survival inside respiratory epithelial cells

ABSTRACT

B. pertussis is the causative agent of pertussis, aka whooping cough. Although generally considered an extracellular pathogen, this bacterium has been found inside respiratory-epithelial cells, which might represent a survival strategy inside the host. Relatively little is known, however, about the mechanism of internalization and the fate of *B. pertussis* inside the epithelia. We show here that *B. pertussis* is able to enter those cells by a mechanism dependent on microtubule assembly, lipid raft integrity, and the activation of a tyrosine-kinase-mediated signaling. Once inside the cell, a significant proportion of the intracellular bacteria evade phagolysosomal fusion and remain viable in nonacidic lysosome-associated-membrane-protein-1–negative compartments. In addition, intracellular *B. pertussis* was found able to repopulate the extracellular environment after complete elimination of the extracellular bacteria with polymyxin B. Taken together, these data suggest that *B. pertussis* is able to survive within respiratory-epithelial cells and by this means potentially contribute to host-immune-system evasion.

INTRODUCTION

Bordetella pertussis is a strictly human pathogen and the main causative agent of whooping cough, aka pertussis. Despite a high vaccination cover pertussis remains endemic within the world population. The persistence of pertussis in countries with highly vaccinated populations has been attributed to various causes including suboptimal vaccines, a waning immunity, and pathogen adaptation (He & Mertsola, 2008; Berbers *et al.*, 2009; Mooi *et*

al., 2013). *B. pertussis* colonizes the mucosa of the respiratory tract, where the bacterium interacts with epithelial cells and local immune-surveillance cells. Although certain potential contributors to colonization have been described (de Gouw *et al.*, 2011), the mechanisms that allow this pathogen to evade immune clearance, causing a highly contagious and prolonged respiratory disease, are still under investigation.

Although *B. pertussis* is usually regarded as a noninvasive pathogen, a number of studies suggest that the bacterium is able to enter into and eventually survive inside the cells (Higgs *et al.*, 2012). *B. pertussis* have been found within pulmonary alveolar macrophages from infants and children with confirmed *B. pertussis* pneumonia (Paddock *et al.*, 2008) and inside alveolar macrophages of HIV-infected children (Bromberg *et al.*, 1991). Animal-infection models and studies using human monocytes in culture have indicated that *B. pertussis* can enter and survive inside those cells (Friedman *et al.*, 1992; Hellwig *et al.*, 1999; Vandebriel *et al.*, 2003). We recently found that *B. pertussis* is not only able to survive inside human macrophages but also replicates in nonacidic compartments having the characteristics of early endosomes (Lamberti *et al.*, 2010). These data suggest that the pathogen has evolved mechanisms to evade phagolysosome fusion and survive intracellularly, so as to eventually increase the opportunity of spreading to new hosts.

In the present study, we investigated whether *B. pertussis* is also able to reside within epithelial respiratory cells and proliferate from there. Previous studies in culture revealed the presence of *B. pertussis* inside epithelial cells (Bassinet *et al.*, 2000; Ishibashi *et al.*, 2001; Gueirard *et al.*, 2005). Moreover, *B. pertussis* has been found inside ciliated respiratory-epithelial cells in children with pneumonia (Paddock *et al.*, 2008). Nevertheless,

the ability of this pathogen to enter, survive, and replicate within human respiratory epithelial cells remains to be conclusively demonstrated.

Previous studies from our group showed that lipid raft domains play a significant role in *B. pertussis* interaction with epithelial cells and that filamentous hemagglutinin (FHA) plays a critical role in this process (Lamberti *et al.*, 2009). Lipid rafts are defined as small, heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes (Pike, 2006). Upon certain stimuli, such as protein-ligand binding, small rafts coalesce into larger signalling platforms enabling the assembly of a variety of signalling molecules within membrane rafts, including molecules playing essential roles in cytoskeletal reorganization (Hartlova *et al.*, 2010). A wide variety of pathogens -including viruses, bacteria, protozoans, and prions- target molecules in host-cell membrane rafts in order to hijack host intracellular-trafficking pathways and invade host cells (Hartlova *et al.*, 2010). In the example of *B. pertussis*, the role of lipid rafts in the invasion process have still not been studied, but previous reports have indicated that FHA was involved in the entry of *B. pertussis* into epithelial cells (Ewanowich *et al.*, 1989; Bassinet *et al.*, 2000; Ishibashi *et al.*, 2001), thus suggesting a connection between FHA interaction with lipid rafts and bacterial internalization. The present study aimed at elucidating the mechanisms underlying the entry of *B. pertussis* into epithelial-respiratory cells and assessing the ability of the bacteria to survive intracellularly.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Bordetella pertussis strain B213, a streptomycin-resistant derivative of Tohama I, was used in this study. In some experiments, B213 transformed with plasmid pCW505 (Weingart *et*

al., 1999) (kindly supplied by Dr Alison Weiss, Cincinnati, OH) -which induces cytoplasmic expression of green fluorescent protein (GFP) without affecting growth or antigen expression (Weingart *et al.*, 1999)- was used. Bacteria were stored at -70°C and recovered by growth on Bordet Gengou agar (BGA) plates supplemented with 15% (v/v) defibrinated sheep blood (bBGA) at 35 °C for 3 days. Virulent bacteria were subsequently plated on bBGA, cultured for 20 h at 35 °C, and thereafter used in infection assays.

Antibodies

The following antibodies were used: mouse monoclonal antibody against human lysosome-associated membrane protein 1 (LAMP1) (BD Biosciences Pharmingen, San Diego, CA), FITC conjugated mouse monoclonal antibody against early endosome antigen 1 (EEA-1) (BD Biosciences Pharmingen, San Diego, CA), CY3-conjugated goat $F(ab')_2$ fragments of anti-mouse immunoglobulin G (IgG) (Molecular Probes, Eugene, OR), CY3-conjugated goat $F(ab')_2$ fragments of anti-rabbit IgG (Jackson InmunoResearch, West Grove, PA), FITC-conjugated goat $F(ab')_2$ fragments of anti-rabbit IgG (Jackson InmunoResearch, West Grove, PA). Polyclonal rabbit anti-*B. pertussis* antiserum was obtained as described elsewhere (Hellwig *et al.*, 2001).

Cells and growth conditions

The human alveolar-epithelium cell line A549 (human-lung type-II pneumocyte; American Type Culture Collection CCL185, Rockville, MD) was cultured in 10% FBS(DMEM): Dulbecco's modified Eagle's Minimal Essential Medium (DMEM) supplemented with 10% (v/v) fetal-bovine serum (FBS), 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin (Sigma, St. Louis, MO) at 37 °C in 5% (v/v) CO_2. Routine

subcultures for A549 pneumocytes were performed at 1:3 split ratios by incubation with 0.05% (w/v) trypsin plus 0.02% (w/v) ethylenediamine tetracetic acid for 5 min at 37°C. For infection assays, the epithelial cells were cultured to 70% confluence.

Infection assays

Infection assays were performed as described in Lamberti *et al.* (Lamberti *et al.*, 2009), with minor modifications. Stated in brief, A549 cells were seeded on glass coverslips in 24-well tissue-culture plates (Nunc, Rockside, Denmark) and cultured to 70% confluence. The monolayers were then washed twice with sterile phosphate-buffered saline (PBS) and infected with either *B. pertussis* or GFP-*B. pertussis* in 0.2% BSA(DMEM): DMEM plus 0.2% (w/v) bovine serum albumin (BSA; Sigma, St. Louis, MO) at a multiplicity of infection (MOI) of 150 bacteria per cell unless otherwise stated. To facilitate bacterial interaction with the epithelial cells, the plates were centrifuged for 5 min at 640 x *g*. After 2 h at 37 °C in 5% (v/v) CO_2, the infected cells were washed four times with PBS to remove nonadherent bacteria. For determination of bacterial attachment the cells were fixed with 3% (v/v) paraformaldehyde (PFA). The number of adherent GFP-bacteria per cell was then determined by microscopical examination of 20 randomly selected fields. In representative samples the viability of adherent bacteria was evaluated by means of the Live/Dead Baclight kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For invasion assays, after the washing steps the cells were incubated for another 12 h at 37 °C in 0.2% BSA(DMEM) unless otherwise stated. The number of intracellular bacteria was finally determined both by double-staining immunofluorescence microscopy and by polymyxin B-protection assays as described below.

For immunofluorescence staining of intracellular and extracellular bacteria, surface-bound bacteria were detected by incubation of the A549 cells with rabbit anti-*B. pertussis* serum for 1 h at 4 °C, followed by incubation with CY3-conjugated goat $F(ab')_2$ fragments of anti-rabbit IgG for another 1 h at 4 °C. In order to determine the number of intracellular bacteria, after two washes of the cultures with 0.2% BSA(PBS) the cells were permeabilized by incubation with PBS containing 0.1% (w/v) saponin (Sigma) and 0.2% (w/v) BSA for 30 min, followed by a further incubation for 1 h at 4 °C with rabbit anti-*B. pertussis* serum in the presence of 0.1% (w/v) saponin and 0.2% (w/v) BSA. After three further washes, the cells were incubated with FITC-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG for 1 h at 4 °C. The labelling of intracellular bacteria with FITC-conjugated antibodies was performed to minimize the loss of read-out sensitivity resulting from the quenching of GFP fluorescence after internalization. After washing, samples were analyzed by fluorescence microscopy under a confocal laser scanning microscope (model TCS SP5; Leica, Germany). The number of extracellular (red and green) and intracellular (green) bacteria were evaluated by examination of at least 100 eukaryotic cells.

For the polymyxin B-protection assays, cells were incubated for 1 h at 37 °C in 10% FBS(DMEM) supplemented with 100 µg mL^{-1} of polymyxin B sulfate (Sigma) -an antibiotic that cannot penetrate mammalian cells (Lee *et al.*, 1990)- to kill the remaining extracellular bacteria. After three washing steps, infected monolayers of the A549 cells were incubated with trypsin to detach the cells from the well and the number of viable eukaryotic cells determined by trypan-blue dye exclusion. Next, the cells were lysed with 0.1% (w/v) saponin in sterile water, and serial dilutions of the lysates were rapidly plated onto bBGA plates to enumerate colony-forming units (CFUs). Viable intracellular bacteria

were expressed as the number of viable bacteria per A549 cell. Control experiments to assess the efficacy of antibiotic bactericidal activity were performed in parallel, as stated here in brief: Samples of 5 x 10^8 bacteria were incubated with polymyxin B in 10% FBS(DMEM) for 1 h at 37 °C and then plated on bBGA. This antibiotic exposure resulted in a 99.999% decrease in CFUs.

When indicated, cells were preincubated for 1 h with cytochalasin D (5 µg mL^{-1}), nocodazole (10 µM) or genistein (100 µM). The drugs (purchased from Sigma) were maintained throughout the experiment. In separate experiments, cells were pretreated with 10 mM methyl-ß-cyclodextrin (MßCD; Sigma) plus 5 µg mL^{-1} of lovastatin (Sigma) for 15 min at 37 °C as described previously (Lamberti *et al.*, 2009) before infection with *B. pertussis*. In these experiments lovastatin (5 µg mL^{-1}) was maintained throughout the infection period. At the concentrations used, none of the treatments affected epithelial-cell viability as determined by trypan-blue dye exclusion. Moreover, the drugs did not affect bacterial viability or growth rate under the conditions tested.

In order to determine intracellular viability over time, in selected experiments after polymyxin B (100 µg mL^{-1}) treatment, the cells were washed to remove the antibiotic and further incubated with 10% FBS(DMEM), 1% FBS(DMEM), or 0.2% BSA(DMEM), with or without the addition of polymyxin B (5 µg mL^{-1}), for a further 0, 24, and 48 h. The viable intracellular bacteria per epithelial cell were then determined by CFU counts as described above. Informative control experiments demonstrated that polymyxin B treatment did not affect A549 cell viability over the time of the experiment, as determined by trypan-blue dye exclusion. Likewise, additional controls demonstrated that bacterial infection did

not induce A549 cell lysis as compared to uninfected cells during the experiment, as determined by trypan-blue dye exclusion.

Confocal laser scanning microscopy analysis

A549 cells were seeded onto glass coverslips. For studies of bacterial colocalization with filamentous actin, the cells were infected for 2 h with *B. pertussis* (MOI 150), washed, and incubated for a further 12 h at 37 °C. For F-actin labelling, the cells were then fixed with 3% PFA at room temperature for 15 min and incubated with FITC-conjugated phalloidin (Sigma) diluted 1:100. In order to label *B. pertussis* the cells were first permeabilized by incubation with PBS containing 0.1% saponin and 0.2% BSA for 30 min, and then incubated for 1 h with rabbit anti-*B. pertussis* serum in the presence of 0.1% saponin and 0.2% BSA. After three washes, the cells were incubated for 1 h at 4 ºC with CY3-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG in the presence of 0.1% saponin and 0.2% BSA, washed, and analyzed by confocal microscopy as described below.

For colocalization studies of bacteria with the LAMP1, the EEA1, or the LysoTracker™ probe, A549 cells were infected for 2 h with *B. pertussis* at an MOI of 150 unless otherwise stated, washed, and incubated for a further 12 h at 37 °C. Extracellular bacteria were then killed with polymyxin B (100 µg mL^{-1}) as described above and the cells further incubated in 10% FBS(DMEM) medium containing 5 µg mL^{-1} of polymyxin B for 0, 24, and 48 h. The cells were finally incubated with or without 200 nM LysoTracker™ DND-99 (Molecular Probes, Eugene, OR) for 5 min at 37 °C before fixation with PFA. Those samples that were not incubated with the LysoTracker™ stain were washed twice with PBS and incubated for 10 min at room temperature with PBS containing 50 mM

NH_4Cl. After two washes, cells were incubated for 30 min with PBS containing 0.1% saponin and 0.2% BSA. Next, the cells were incubated for 1 h at 4 °C with either mouse anti-human LAMP-1 monoclonal antibodies plus rabbit anti-*B. pertussis* serum or FITC-conjugated mouse anti-human EEA1 monoclonal antibodies plus rabbit anti-*B. pertussis* serum in the presence of 0.1% saponin and 0.2% BSA. The cells were then washed three times with 0.1% saponin and 0.2% BSA. For colocalization studies with LAMP1, the cells were incubated (1 h) with FITC-conjugated $F(ab´)_2$ fragments of goat anti-rabbit IgG and CY3-conjugated $F(ab´)_2$ fragments of goat anti-mouse IgG (1 h at 4ºC). For colocalization studies with EEA1, the cells were incubated with CY3-conjugated $F(ab´)_2$ fragments of goat anti-rabbit IgG. Isotype controls were run in parallel. To avoid cytophilic binding of antibodies to FcγR, all incubations were done in the presence of 25% (v/v) heat-inactivated human serum.

Microscopical analyses were performed with a confocal-laser scanning microscope (model TCS SP5; Leica, Germany). In all experiments, a minimum of 100 eukaryotic cells were examined per sample.

Statistical analysis

The Student t test (confidence level, 95%) or ANOVA was used for statistical evaluation of the data. The significance of the differences between the mean values of the data as evaluated by ANOVA was determined by means of the least-significant-difference test at a confidence level of 95%. The results are shown as means ± SD.

RESULTS

Entry of *B. pertussis* into A549 cells

The ability of *B. pertussis* to invade A549 cells and survive intracellularly was evaluated through the use of polymyxin B-protection assays. To that end, *B. pertussis* was incubated with the epithelial cells at an MOI of 150 for 2 h at 37 °C, washed to remove nonadherent bacteria, and further incubated for 0, 3, 12, or 24 h before polymyxin B treatment. The number of viable intracellular bacteria was quantified by CFU counts. Figure 1 shows an increase in the number of viable intracellular bacteria as the incubation time progressed, indicating that after initial attachment the entry of the bacteria proceeded for at least 24 h. Double-fluorescence staining of intracellular and extracellular bacteria further revealed that more than 90% of the epithelial cells were infected (data not shown). The invasion rates at lower MOIs (100, 50, and 10 bacteria per cell) were also tested. As expected, at lower MOIs a decrease in the initial bacterial attachment occurred along with a concomitant reduction in the number of viable intracellular *B. pertussis*. At an MOI as low as 10, however, after 2 h of infection, a significant number of viable bacteria were already detected in the cells (10 ± 4 bacteria per 100 cells). In order to have a number of intracellular bacteria that enabled a proper read out, subsequent studies were carried out at an MOI of 150 with the infection proceeding for 12 h at 37 ºC unless otherwise stated.

Involvement of host microtubular network, lipid raft domains, and tyrosine-kinase activity in the entry of *B. pertussis* into A549 cells

Bacterial entry usually requires host-cytoskeleton rearrangements (Alonso & Garcia-del Portillo, 2004) and normally involves either microfilaments (Dramsi & Cossart, 1998; Chu & Lu, 2005) or microtubules alone (Oelschlaeger *et al.*, 1993; Morey *et al.*,

2011) or both microfilaments and microtubules (Richardson & Sadoff, 1988; Tsuda *et al.*, 2005; Dhakal & Mulvey, 2009). To determine the contribution of microfilaments during *B. pertussis* invasion, the spatial association of *B. pertussis* with the actin of the host cytoskeleton was analyzed by confocal microscopy after 12 h of infection. Figure 2 shows F-actin-rich structures colocalizing with adherent bacteria (Fig. 2, surface focal plane). In contrast to the adherence-associated rearrangement of actin, intracellular bacteria were not associated with the host filamentous actin (Fig. 2, middle focal plane). To further evaluate the role of actin polymerization in *B. pertussis* entry, we preincubated A549 cells with cytochalasin D, an inhibitor of actin polymerization that disrupts microfilaments, and then determined bacterial internalization by the polymyxin B-protection assay. Treatment of A549 cells with cytochalasin D did not decrease the number of intracellular bacteria (Fig. 3), indicating that the observed mobilization of F-actin around adhering *B. pertussis* is not implicated in the bacterial entry into A549 cells. We then evaluated whether microtubular polymerization might be involved in *B. pertussis* invasion. To this end, before infection the A549 cells were treated with nocodazole, a microtubule depolymerizing agent, and upon infection bacterial invasion was once again determined by the polymyxin B-protection assay. *B. pertussis* entry decreased by approximately 62% upon the addition of nocodazole (Fig. 3), suggesting that *B. pertussis* entry involves the assembly of the host microtubular network. That nocodazole affected neither bacterial attachment to A549 cells nor cell viability, as determined by trypan-blue staining (data not shown), ruled out those possible trivial explanations.

A previous report had shown that lipid rafts localized around adhering *B. pertussis* during attachment (Lamberti *et al.*, 2009). These domains were later found to be involved in the invasion by and intracellular survival of other bacterial pathogens (Hartlova *et al.*,

2010). In order to investigate more precisely the role of lipid rafts in the entry of *B. pertussis* into epithelial cells, A549 cells were incubated with MßCD -a compound that disrupts cholesterol-rich domains by extracting the sterol- and then examined for bacterial attachment at 2 h and bacterial invasion at 12 h postinfection. The experiments were performed in the presence of lovastatin to inhibit *de-novo* cholesterol synthesis. The number of intracellular bacteria was determined by fluorescent staining of extra- and intracellular bacteria in combination with the polymyxin B-protection assay. Figure 4A shows representative images of the epithelial cells incubated with or without MßCD before infection. In agreement with previous results (Lamberti *et al.*, 2009), the attachment of *B. pertussis* to the epithelial cells was significantly diminished by MßCD treatment of the cells: A 60 ± 4% reduction in bacterial adherence was observed in MßCD-treated cells as compared with untreated controls. In order to evaluate the effect of MßCD on *B. pertussis* entry independently of the reduction in bacterial attachment, the extent of bacterial internalization was calculated as a percentage of the number of surface-associated bacteria determined at 2 h postinfection. Figure 4B shows that cholesterol depletion led to a significant decrease in bacterial entry, indicating that apart from bacterial attachment lipid rafts are involved in the entry of *B. pertussis*. A similar reduction in the number of viable intracellular bacteria was observed in the polymyxin B-protection assays (data not shown).

We next studied the contribution of tyrosine kinases to *B. pertussis* entry into the pulmonary epithelial cells. Tyrosine kinases had been found to be crucial for the lipid raft-dependent invasion processes of other pathogens (Zaas *et al.*, 2005b; Schulz *et al.*, 2012). Accordingly, A549 cells were treated with genistein -a tyrosine-kinase inhibitor generally recognized as a selective blocker of raft-dependent endocytic pathways (Lajoie & Nabi,

2007)- and *B. pertussis* invasion subsequently determined by the polymyxin B-protection assay. Treatment of A549 cells with this drug did not affect the bacterial-attachment level (data not shown) but significantly decreased the number of intracellular *B. pertussis* (Fig. 5), suggesting that tyrosine-kinase activity might participate in the dependence of bacterial entry on lipid rafts.

B. pertussis survival and trafficking inside A549 cells

Host-cell invasion and intracellular survival is an effective immune-evasion strategy for many pathogenic bacteria (Haglund & Welch, 2011). We therefore evaluated the intracellular fate of *B. pertussis* after entering the respiratory-epithelial cells. To this end, A549 cells were infected with *B. pertussis* for 12 h and further treated with Polymyxin B to kill extracellular bacteria. The cells were incubated for an additional 0, 24, or 48 h in the presence of polymyxin B (5 μg mL^{-1}). The number of viable intracellular bacteria was evaluated at different times by CFU counts. In order to estimate the percentage of intracellular *B. pertussis* that were able to survive, the number of bacteria inside the cells was determined in parallel by two-color fluorescence microscopy, which enabled the discrimination between extra- and intracellular bacteria. Figure 6A shows that immediately after polymyxin B treatment (time = 0 h) 3.82 ± 0.55 bacteria per cell were alive (48 ± 9% of the intracellular bacteria). Importantly, about 100% of the bacteria associated with the epithelial cells had been alive before polymyxin B treatment, as determined by live-dead differential staining (data not shown). In order to investigate the subcellular localization of the internalized *B. pertussis*, A549 cells were stained with the late endosomal/lysosomal marker LAMP1. Fig. 6B shows that 54 ± 7% of the intracellular bacteria were found in LAMP1-negative phagosomes immediately after polymyxin B treatment (Fig. 6B, 0 h). As

time progressed, the number of viable intracellular bacteria decreased (Fig. 6A) while the number of *B. pertussis*-containing phagosomes positive for LAMP1 increased (Fig. 6B and 6C). At 24 h after polymyxin B treatment, *ca*. 28% of the intracellular bacteria (1.57 ± 0.36 out of 5.63 ± 0.6 bacteria) were found not colocalized with LAMP1 label, and at that time the CFU counts indicated a total of 1.4 ± 0.18 viable bacteria per cell. A similar correspondence between the number of LAMP1-negative bacteria and CFU counts was observed in samples taken at 48 h (Fig 6). Colocalization studies with LysoTracker™, an acidotropic fluorescent dye that accumulates in acidic organelles, showed similar results. A lack of colocalization of *B. pertussis* with LysoTracker™ was observed in 3.6 ± 0.6, 1.5 ± 0.4, and 0.7 ± 0.3 bacteria per cell at 0, 24, and 48 h, respectively, after polymyxin B treatment. To further investigate the intracellular localization of the bacteria that were not found within late-endosomal or lysosomal compartments, A549 cells were stained with the early-endosomal marker EEA1. Colocalization studies indicated that about 20% of the intracellular *B. pertussis* were located in EEA1-positive compartments 48 h after polymyxin B treatment (Fig. 7).

Relevant too is the observation that this intracellular trafficking was also observed in assays performed at the lowest MOI tested (an MOI of 10; data not shown).

Release of viable intracellular *B. pertussis* from the cells into the medium

Since both cell entry and exit are critical steps in the cell cycles of intracellular pathogens (Hybiske & Stephens, 2007), we next examined whether intracellular *B. pertussis* was released in a viable state into the extracellular medium. To investigate this question, epithelial-cell infection was carried out as described above; but after the initial killing of the extracellular bacteria with polymyxin B (100 μg mL^{-1}), cells were incubated

with either 10% FBS(DMEM) plus polymyxin B (5 μg mL^{-1}) or the same medium without the antibiotic for 48 h. Control experiments verified that *B. pertussis* remained alive in 10% FBS(DMEM) but not in 10% FBS(DMEM) plus polymyxin B (5 μg mL^{-1}), indicating that the intracellular bacteria released into the medium would be able to survive only in the antibiotic-free medium. Samples were taken at 0, 24, and 48 h after initial polymyxin B treatment. The number of viable bacteria either cell-associated or free in the medium was determined under each condition tested (Fig. 8A and B, respectively). In samples taken at 24 and 48 h, a significantly higher number of viable bacteria both associated with the cell and free in the medium was found in the assays performed without antibiotics as compared with those containing antibiotics. These results suggested that intracellular *B. pertussis* was released in a viable state into the surrounding medium. Similar results were obtained in experiments carried out in 0.2% BSA(DMEM) or 1% FBS(DMEM); and since both of those media limited A549-cell growth during the 48-h infection period, the exiting from the cell by *B. pertussis* was very likely not associated with cell lysis as a consequence of culture overgrowth (data not shown).

DISCUSSION

Bacterial pathogens are often divided into the categories of intracellular and extracellular microorganisms in accordance with their capacity to invade and survive within host cells. This division, however, has become increasingly blurred as more and more pathogens referred to historically as extracellular were found to have alternative intracellular life-styles (Petersen & Krogfelt, 2003; Bower *et al.*, 2005; Morey *et al.*, 2011). In the example of *B. pertussis*, an increasing number of reports based on infection assays in cultured cells (Ewanowich *et al.*, 1989; Friedman *et al.*, 1992; Masure, 1993; Gueirard *et al.*, 2005;

Lamberti *et al.*, 2008; Lamberti *et al.*, 2010) and examination of biopsies (Bromberg *et al.*, 1991; Hellwig *et al.*, 1999; Paddock *et al.*, 2008) have suggested that these bacteria are capable of invading many cell types. Recent research has further indicated that *B. pertussis* is able to evade intracellular killing (Lamberti *et al.*, 2008; Lamberti *et al.*, 2010). In particular, the bacterium has been demonstrated to be able to survive the encounter with immune cells so as to remain viable in nonacidic compartments with access to the recycling pathways (Lamberti *et al.*, 2008; Lamberti *et al.*, 2010). Moreover, *B. pertussis* seems able to replicate inside these compartments, suggesting that the species might be considered a facultative intracellular bacterium (Lamberti *et al.*, 2010).

In the present study, we investigated the interaction of *B. pertussis* with respiratory-epithelial cells, another relevant cell type at the site of infection. Several pathogens acquired through inhalation can invade respiratory-epithelial cells, including *Mycoplasma pneumoniae* (Yavlovich *et al.*, 2004), *Burkholderia cepacia* (Martin & Mohr, 2000), and *Haemophilus influenza* (Morey *et al.*, 2011). Most of these invasive microorganisms, though not all, are also capable of intracellular replication. In the instance of *B. pertussis*, a number of studies, with both primary cell cultures and established cell lines, have suggested that this pathogen is able to invade epithelial cells (Higgs *et al.*, 2012). The molecular mechanisms underlying this process and the intracellular fate of *B. pertussis*, however, have still remained unknown. The present study shows that the entry of *B. pertussis* into respiratory-epithelial cells requires microtubule assembly, lipid raft integrity, and the activation of tyrosine kinases. Once inside the cell, a significant proportion of the bacteria evade lysosomal fusion and remain viable for several days.

We have previously shown that lipid raft domains act as platforms that cluster molecules susceptible to FHA ligation and in that manner contribute to effective *B. pertussis* binding to A549 cells (Lamberti *et al.*, 2009). These domains have been shown to be involved in cellular invasion by many pathogens (Lafont & van der Goot, 2005; Hartlova *et al.*, 2010) and appear to provide the signalling platforms required for bacterial entry (Lafont & van der Goot, 2005; Zaas *et al.*, 2005a). In the present experiments, we found that lipid rafts are involved in the entry of *B. pertussis* into respiratory-epithelial cells. Accordingly, treatment of A549 cells with the lipid raft–disrupting drug MßCD decreased *B. pertussis* invasion to a greater extent than the reduction observed in attachment, suggesting that these domains not only serve as platforms that facilitate *B. pertussis* binding to the host cell but also mediate the entry of the pathogen. Indeed, invasion by *B. pertussis* was significantly impaired in the presence of genistein, a selective inhibitor of tyrosine-kinase-mediated and lipid raft-dependent endocytic pathways (Lajoie & Nabi, 2007), suggesting that tyrosine-kinase activity is involved in *B. pertussis* internalization through lipid raft domains.

Lipid raft-dependent endocytosis may proceed through microtubule- and/or actin-dependent pathways (Lajoie & Nabi, 2007). Whereas actin microfilaments are frequently associated with the bacterial invasion process, a small number of pathogens need the polymerization of the host microtubule network to invade (Yoshida & Sasakawa, 2003). *B. pertussis* seems to belong to this latter group of pathogens. Treatment of A549 cells with nocodazole, a microtubule-depolymerizing agent, significantly reduced the number of internalized bacteria. In contrast, actin-cytoskeleton depolymerization by cytochalasin D

did not affect invasion by *B. pertussis*. Accordingly, intracellular *B. pertussis* was never found associated with condensed actin filaments.

B. pertussis FHA plays a major role in adherence to epithelial cells (Perez Vidakovics *et al.*, 2006). FHA has been suggested as possibly promoting the invasion of human respiratory-epithelial cells through the interaction of the bacterial arginine-glycine-aspartate (RGD) sequence with the host cell α5ß1 integrin (Ishibashi *et al.*, 2001). Like *B. pertussis*, several microbial pathogens exploit integrins and the latter's endocytotic capacity to invade nonphagocytic cells (Guignot *et al.*, 2001; Tsuda *et al.*, 2008; Krause-Gruszczynska *et al.*, 2011; López-Gómez *et al.*, 2012). The invasin of *Yersinia enterocolitica* has been demonstrated to involve the binding of the α5ß1 integrins on eukaryotic cells in order to promote internalization via a receptor-mediated mechanism that, for its part, involves protein-tyrosine-kinase signalling (Isberg *et al.*, 2000). These kinases are usually the orchestrators of a linkage between the integrins and signal transduction, with the lipid raft domains playing a key role in this interaction (Leitinger & Hogg, 2002; Mitchell *et al.*, 2002; Gagnoux-Palacios *et al.*, 2003; del Pozo *et al.*, 2004; Upla *et al.*, 2004; van Zanten *et al.*, 2009). That lipid rafts and α5ß1 integrins participate in *B. pertussis* entry into epithelial cells suggests a spatial and functional link between raft and integrins during *B. pertussis* entry.

A strategy used by several pathogens for survival in the hostile host environment is to hide within the mammalian cells. The mechanisms by which pathogens resist intracellular killing include inhibition of phagosome-lysosome fusion, escape into the cytoplasmic compartment, and resistance to the harsh environment of the lysosome (Moulder, 1985). To investigate the fate of *B. pertussis* inside respiratory cells, CFU counts

and trafficking studies were conducted in parallel. We examined the association of *B. pertussis* with acidic (LysoTracker™-positive) vacuoles and LAMP1- and EEA-1-containing vacuoles. Our overall data suggest that part of the internalized bacteria manage to avoid the lysosomal-degradation pathway. We accordingly found a correlation between the number of viable intracellular bacteria and the number of either LAMP1-negative, LysoTracker™-negative, or EEA-1 positive bacteria per cell, suggesting that those bacteria had survived through escape from the lysosomal pathway and remain in EEA-1 positive compartments. These results are in agreement with previous reports indicating that *B. pertussis* is able to survive within nonacidic compartments in human macrophages (Lamberti *et al.*, 2010).

Although we cannot rule out the possibility; under our experimental conditions, we did not detect bacterial replication inside A549 cells. A similar behavior had been found with other pathogens, such as *Campylobacter jejuni* (Watson & Galan, 2008), *B. cepacia* (Lamothe *et al.*, 2004), and *Salmonella enteric* (Cano *et al.*, 2001), all of which bacteria produce long-lasting infections inside host cells in the absence of proliferation.

Intracellular survival may provide substantial advantages for bacterial persistence within the host. Apart from protecting the pathogen from the host-immunity defenses, the intracellular location detected here might provide protection against antibacterial treatments or local inflammation during infection. Similar to what was described for other pathogens (Osterlund & Engstrand, 1995; Dubois & Boren, 2007), *B. pertussis* might reside dormant inside host cells and egress once the extracellular environment became permissive. In this study we found evidence that intracellular *B. pertussis* is able to repopulate the extracellular environment, with the implication that *B. pertussis* is released viable into the extracellular

medium. These results suggest that the intracellular survival within the respiratory epithelium might contribute to the persistence of *B. pertussis* within the host.

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FIGURE LEGENDS

FIGURE 1. Kinetics of entry of *B. pertussis* into epithelial cells. A549 cells were infected with *B. pertussis* (MOI 150) during 2 h, washed to remove non-adherent bacteria and further incubated for other 0, 3, 12 or 24 h. The number of viable intracellular *B. pertussis* at the various time points was determined by the Polymyxin B-protection assay. The data represent the mean ± SD of two independent experiments done in triplicate.

FIGURE 2. Confocal microscopy analysis of *B. pertussis* association with host actin cytoskeleton. A549 cells were infected with *B. pertussis* at an MOI of 150 for 2 h at 37°C, washed, and incubated for a further 12 h. Samples were fixed and stained for F actin (green) and *B. pertussis* (red). Images were obtained in various focal planes by scanning from the apical to the basal surfaces of the eukaryotic cell. Confocal images of a surface focal plane

and a middle focal plane of the same cell are shown. The colocalization of adherent bacteria and actin filament is indicated by arrows. The lack of colocalization of intracellular bacteria and actin filament is indicated by arrowheads. Representative panels of one out of three independent experiments are shown.

FIGURE 3. Requirement of host-cell microtubule polymerization for *B. pertussis* invasion. A549 cells treated without inhibitors (control), or with cytochalasin D (5 μg mL^{-1}), or nocodazole (10 μM) for 1 h at 37 °C were incubated with *B. pertussis* (MOI 150) for 2 h at 37 °C, washed, and incubated for a further 12 h in the presence of the respective drug. The number of live intracellular bacteria was determined by the polymyxin B-protection assay. The figure shows the mean values ± SD of two independent experiments each done in triplicate. The number of viable intracellular bacteria in nocodazole-treated cells was significantly different from the number of viable intracellular bacteria in nontreated or cytochalasin-D-treated cells (*P <0.05).

FIGURE 4. Involvement of cholesterol-rich domains in *B. pertussis* attachment to and entry into A549 cells. A549 cells incubated without (control) or with 10 mM of MβCD (15 min at 37 °C) were infected with GFP-*B. pertussis* (MOI 150) in the presence of lovastatin (5 μg mL^{-1}). After 2 h the cells were either washed, fixed, and processed for the determination of bacterial attachment or else incubated for a further 12 h in the presence of lovastatin (5 μg mL^{-1}) before determination of both surface-bound and intracellular bacteria by two-color immune fluorescence. A) Representative images showing extracellular (yellow) and intracellular (green) bacteria in control (panel a) and MβCD-treated cells (panel b) 12 h postinfection. B) Bacterial internalization. The number of intracellular bacteria 12 h after attachment was expressed as a percentage of the number of adherent bacteria at 2 h

postinfection. The data represent the mean ± SD of two independent experiments each done in triplicate. The percentage of bacteria internalized by MβCD-treated cells was significantly different from the corresponding percentage internalized by untreated cells (control) (*P<0.05).

FIGURE 5. Dependence of *B. pertussis* entry into A549 cells on tyrosine kinase activity. A549 cells incubated without (control) or with 100 μM genistein (1 h at 37 °C) were infected with GFP-*B. pertussis* (MOI 150). After 2 h the cells were washed and incubated for a further 12 h in the presence of genistein. The presence of live intracellular bacteria was determined by the polymyxin B-protection assay. The data represent the mean ± SD of two independent experiments each done in triplicate. The number of viable intracellular bacteria in genistein-treated cells was significantly different from the number of viable intracellular bacteria in the untreated controls (*P <0.05).

FIGURE 6. Survival of *B. pertussis* inside A549 cells in LAMP1-negative compartments. A) Intracellular survival. A549 cells were infected with *B. pertussis* (MOI 150) for 2 h, washed to remove nonadherent bacteria, and incubated for a further 12 h. The cells were then treated with polymyxin B (100 μg mL^{-1}) for 1 h at 37 °C, washed, and incubated for an additional 0, 24, or 48 h in the presence of polymyxin B (5 μg mL^{-1}). The number of viable intracellular bacteria was evaluated at different times by CFU counts. The data represent the mean ± SD of four independent experiments each done in triplicate. B) Colocalization of *B. pertussis* and LAMP1. A549 cells were infected with GFP-*B. pertussis* (MOI 150)) for 2 h, washed, and incubated for a further 12 h. Cells were then treated for 1 h with polymyxin B (100 μg mL^{-1}) and incubated for an additional 0, 24, or 48 h in the presence of the antibiotic. At the different time points cells were fixed and permeabilized

before incubation with antibodies against LAMP1 (red) and against *B. pertussis* (green). The bars indicate the number of LAMP1-positive (grey) or LAMP1-negative (black) phagosomes per cell. The data represent the mean of three independent experiments each done in triplicate. C) Representative confocal images showing colocalization of *B. pertussis* with LAMP1 at 48 h after polymyxin B treatment. Arrows indicate LAMP1-positive *B. pertussis* compartments.

FIGURE 7. Colocalization of *B. pertussis* with early endosomal markers. A549 cells were infected with *B. pertussis* (MOI 150) during 2 h, washed and incubated for other 12 h in 0.2% BSA(DMEM). Cells were then treated for 1 h with Polymyxin B (100 µg mL^{-1}) and incubated for other 48 h in the presence of antibiotics. At 0, 24 and 48 h the cells were fixed and permeabilized prior to incubation with antibodies against EEA1 (green) and against *B. pertussis* (red). The bars indicate percentages of EEA1-positive phagosomes. The data represent the mean ± SD of three independent experiments. Representative confocal microscopy images of the colocalization of *B. pertussis* with EEA1 48 h after polymyxin B treatment are shown. Arrows indicate EEA1-positive *B. pertussis* compartments

FIGURE 8. Release of viable intracellular bacteria into the medium. A549 cells were infected with *B. pertussis* at an MOI of 150 for 2 h at 37 °C, washed, and incubated for a further 12 h. Cells were then treated with polymyxin B (100 µg mL^{-1}) for 1 h at 37 °C, washed, and incubated in 10% FBS(DMEM) with (grey) or without (black) polymyxin B (5 µg mL^{-1}) for an additional 0, 24, or 48 h. At the indicated time points the number of viable bacteria per cell (A) and the free in the medium (B) was determined. A) Bacteria associated with A549 cells. Cells were detached by trypsin treatment and the number of bacteria per cell determined by CFU counts. The data represent the mean ± SD of three independent experiments each done in triplicate. B) Free extracellular bacteria. The number of

extracellular cell-free bacteria was determined by sampling the medium for CFU counts. The data represent the mean ± SD of three independent experiments done in triplicate.

Figure 1

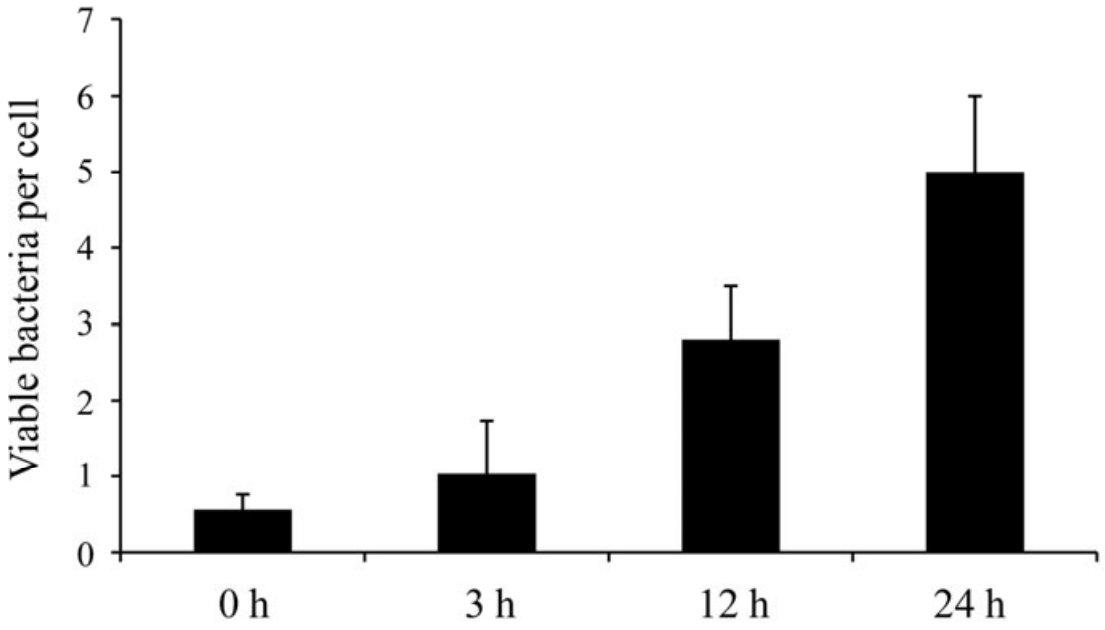

Figure 2

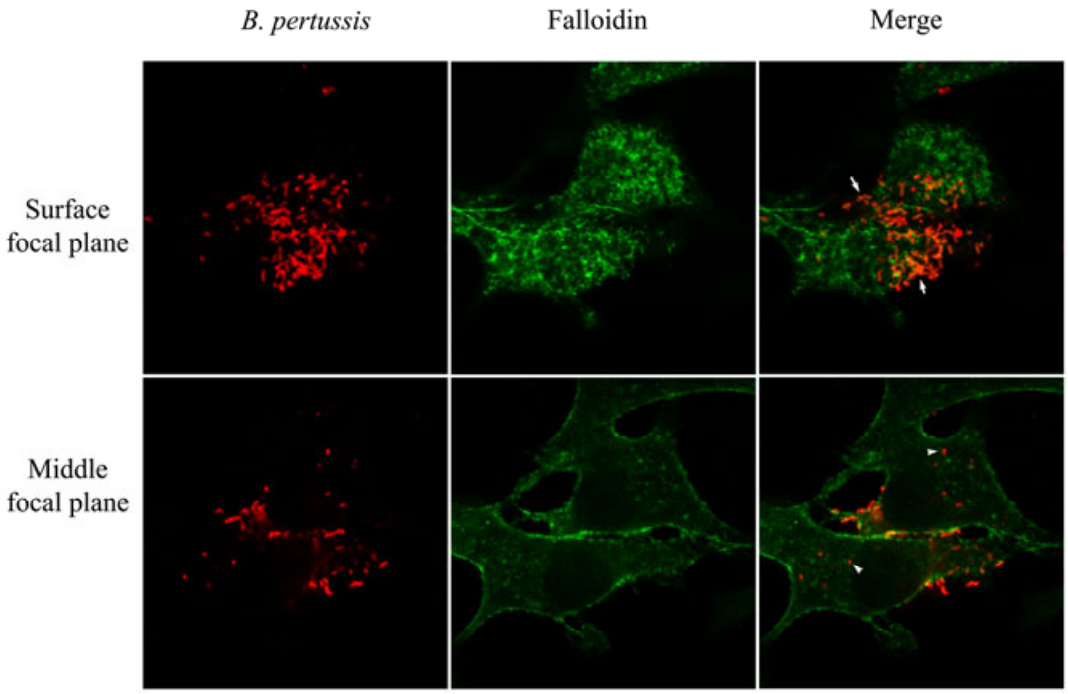

Figure 3

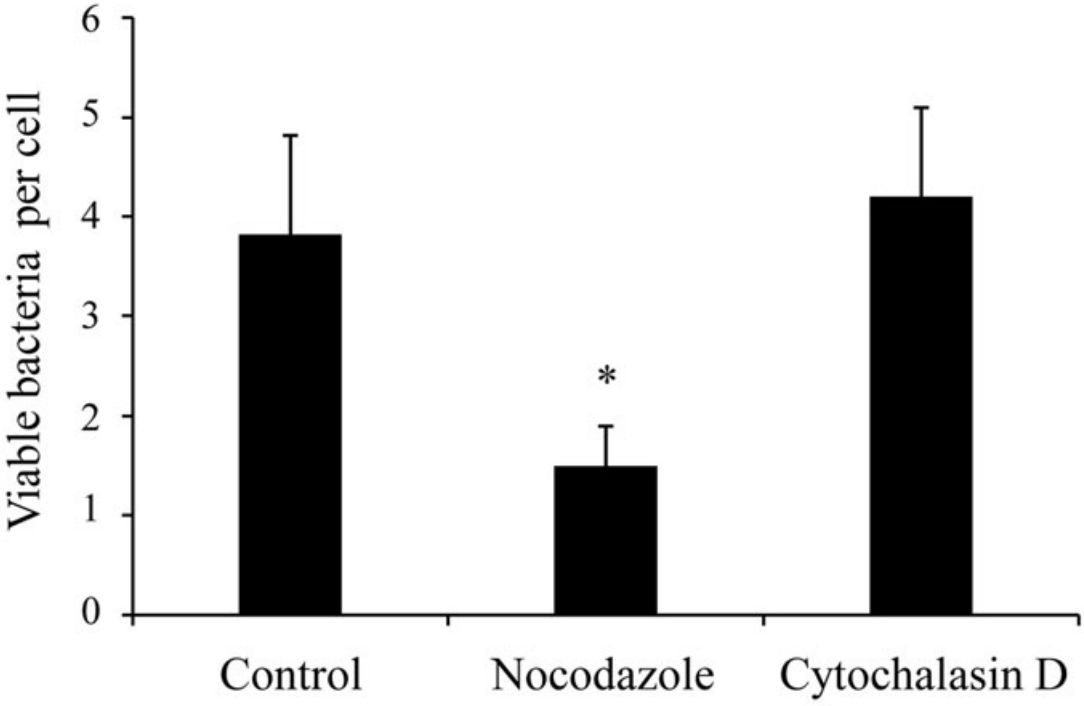

Figure 4A

A

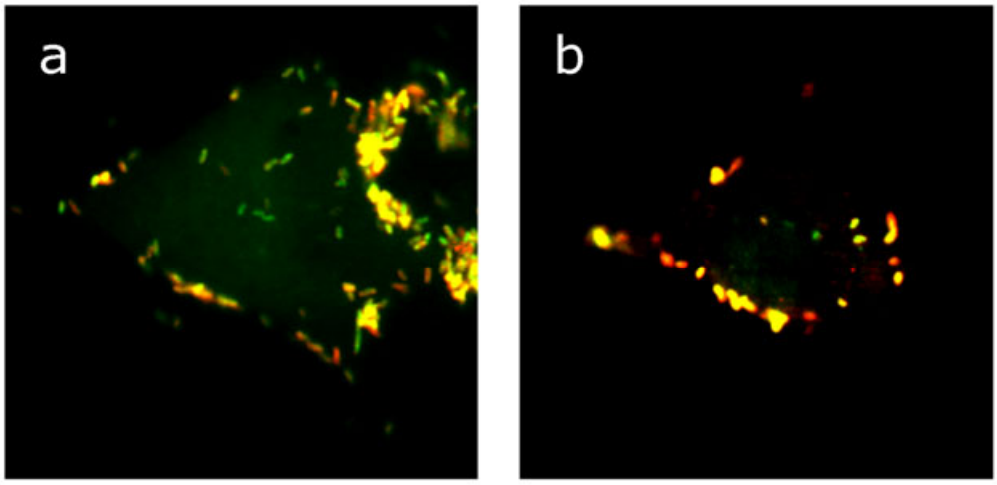

Figure 4B

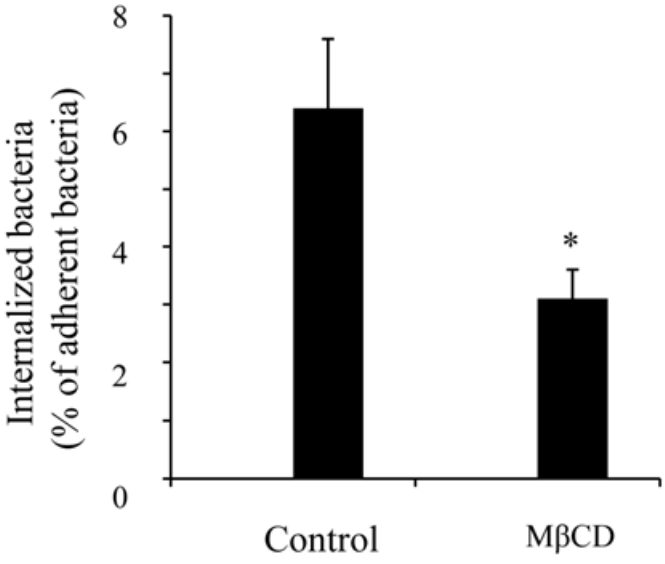

Figure 5

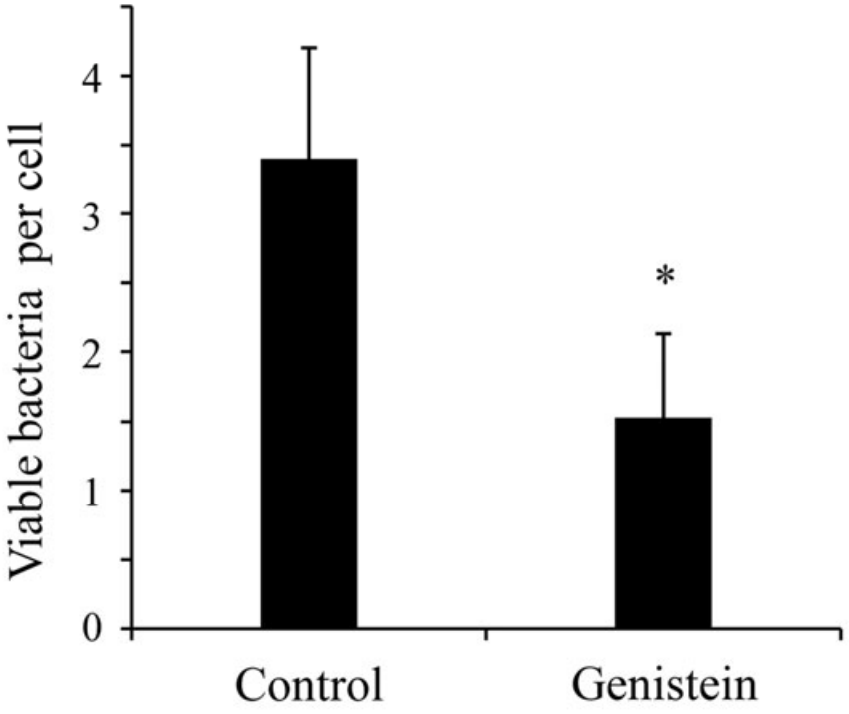

Figure 6A

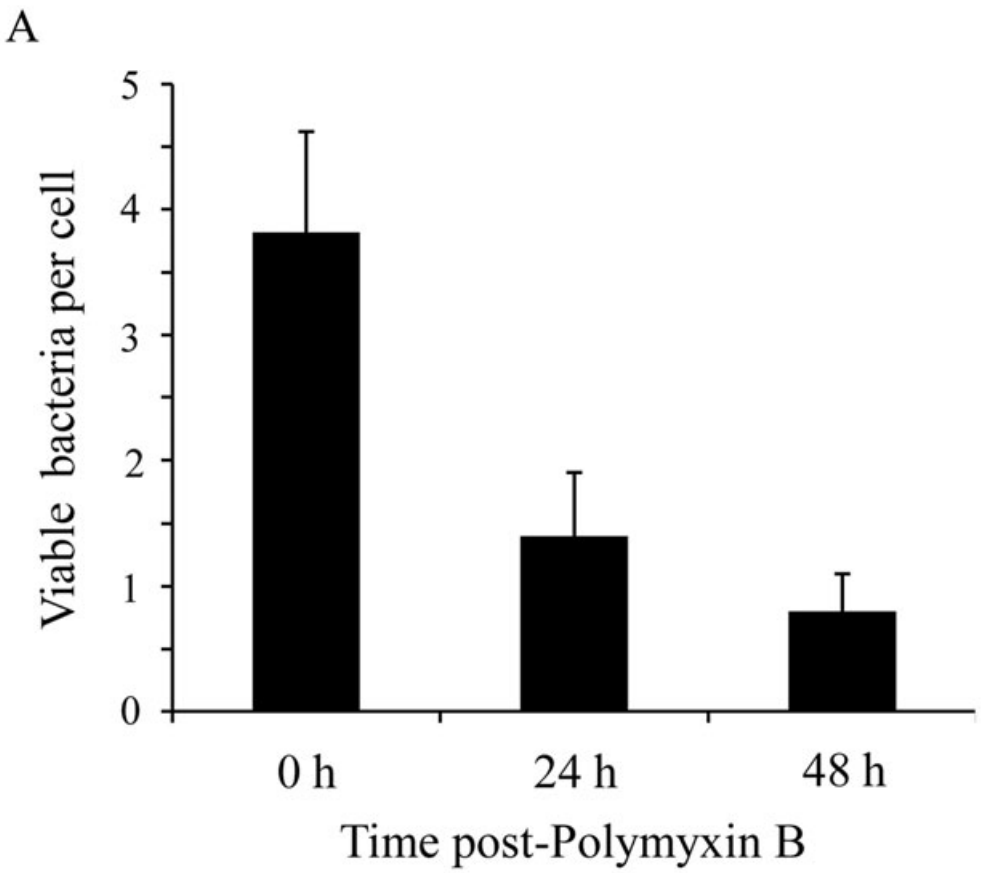

Figure 6B

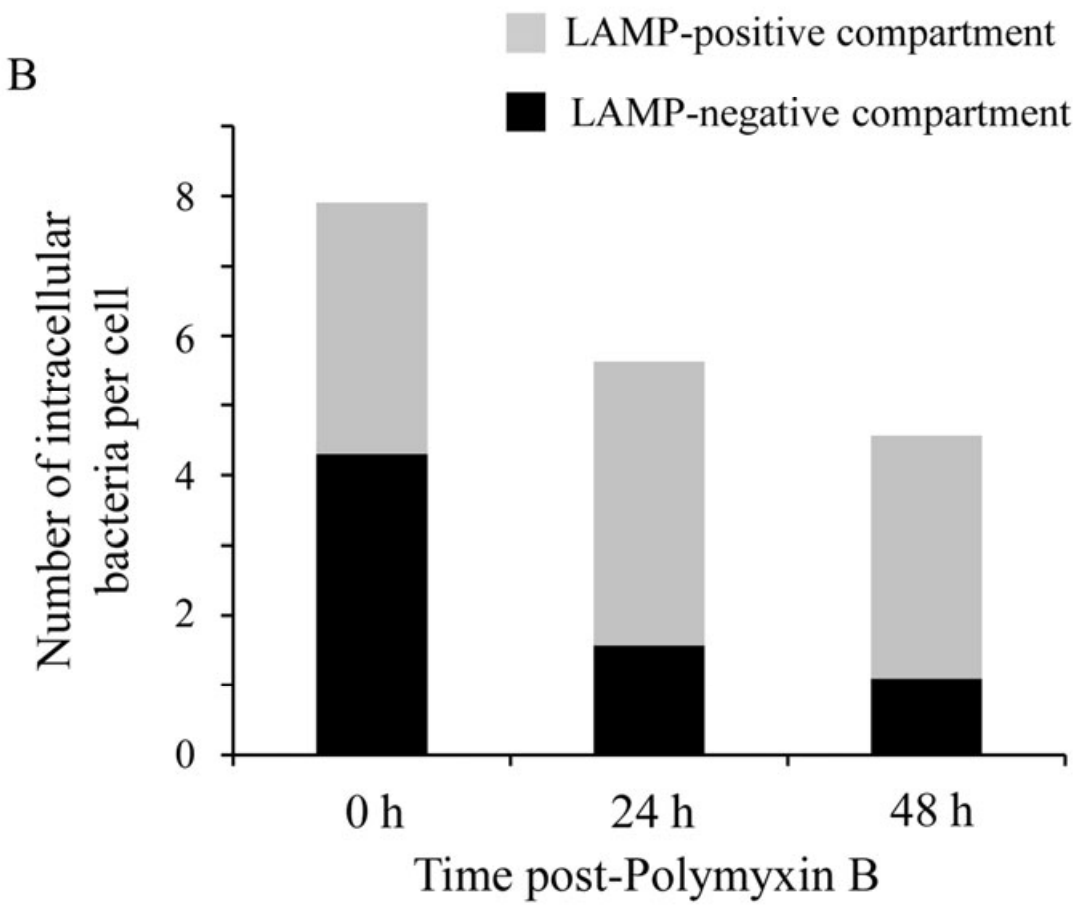

Figure 6C

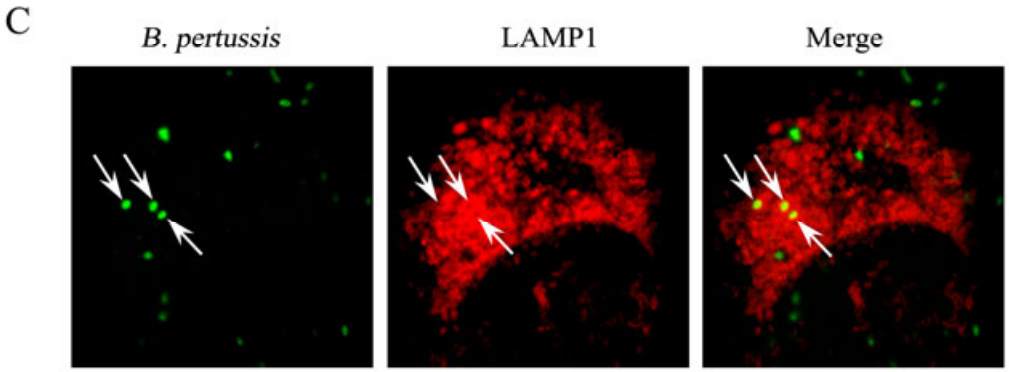

Figure 7

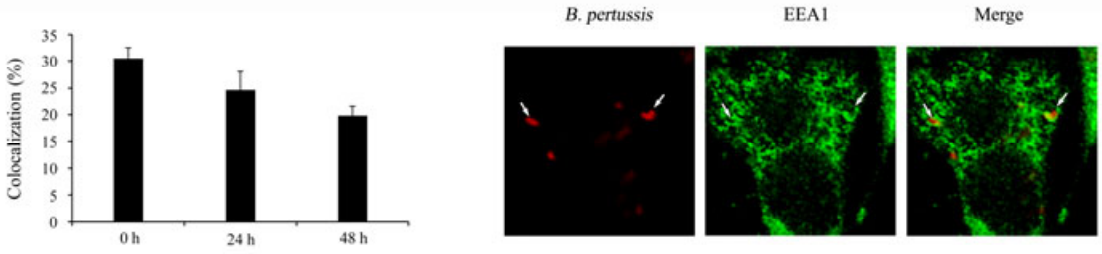

Figure 8A

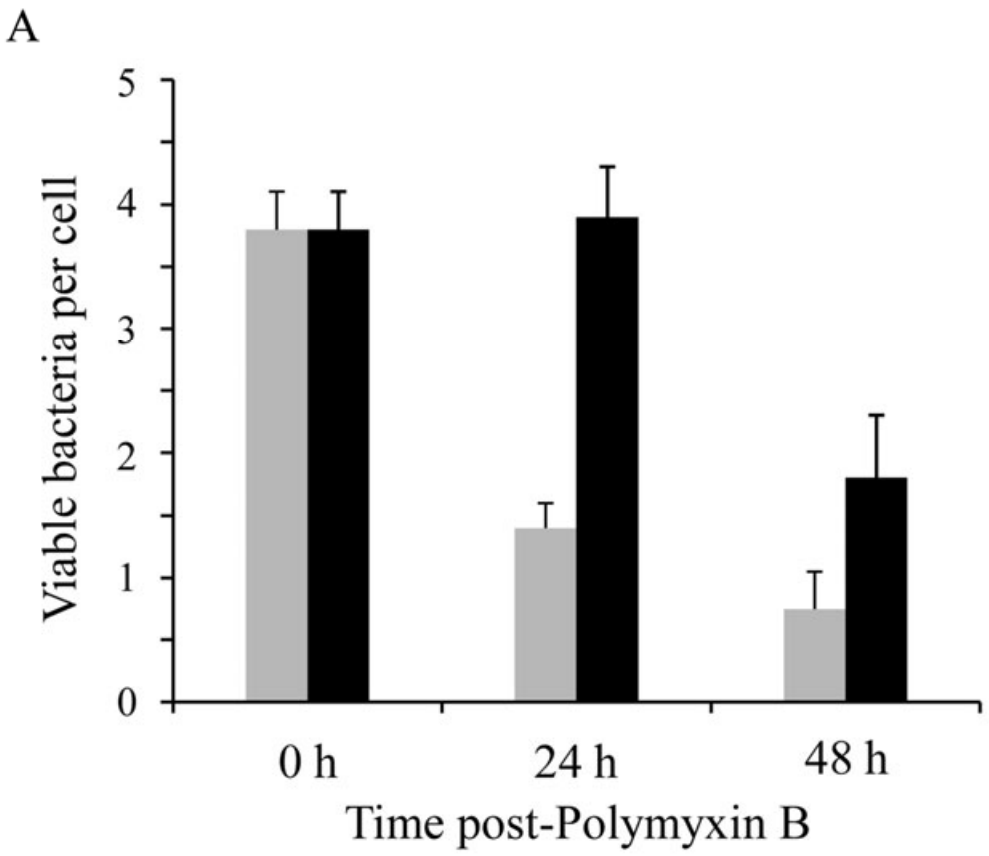

Figure 8B