Cellular Microbiology (2006) **8**(9), 1456–1466
doi:10.1111/j.1462-5822.2006.00724.x
First published online 13 April 2006

Intracellular trafficking and replication of *Burkholderia cenocepacia* in human cystic fibrosis airway epithelial cells

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Summary

We investigated the trafficking of *Burkholderia cenocepacia*, an opportunistic respiratory pathogen of persons with cystic fibrosis (CF), in immortalized CF airway epithelial cells *in vitro*. Our results indicate that bacteria enter cells in a process involving actin rearrangement. Whereas both live and heat-killed bacteria reside transiently in early endosomes, only live bacteria escape from late endosomes to colocalize in vesicles positive for lysosomal membrane marker LAMP1, endoplasmic reticulum (ER) membrane marker calnexin, and autophagosome marker monodansylcadavarine (MDC). Twenty-four hours after infection, microcolonies of live bacteria were observed in the perinuclear area colocalizing with calnexin. In contrast, after ingestion, dead bacteria colocalized with late endosome marker Rab7, and lysosome markers LAMP1 and cathepsin D, but not with calnexin or MDC. Six to eight hours after ingestion of dead bacteria, degraded bacterial particles were observed in the cytoplasm and in vesicles positive for cathepsin D. These results indicate that live *B. cenocepacia* gain entry into human CF airway cells by endocytosis, escape from late endosomes to enter autophagosomes that fail to fuse with mature lysosomes, and undergo replication in the ER. This survival and replication strategy may contribute to the capacity of *B. cenocepacia* to persist in the lungs of infected CF patients.

Introduction

Pulmonary infection-related inflammation is a major cause of morbidity and mortality in cystic fibrosis (CF) patients. Although *Pseudomonas aeruginosa* is the most common opportunistic bacterial pathogen in CF, species of the *Burkholderia cepacia* complex (Bcc) also have emerged as important respiratory pathogens in this population (LiPuma, 1998). Patients infected with Bcc exhibit an unpredictable and variable clinical course ranging from asymptomatic carriage to fatal acute necrotizing pneumonia and septicaemia (referred to as 'cepacia syndrome'), which occurs in as many as 20% of infected patients (Isles *et al*., 1984; Liou *et al*., 2001). This type of fatal acute syndrome is rarely seen with other CF pathogens, including *P. aeruginosa* (Govan and Deretic, 1996). The inherent resistance of Bcc strains to multiple antibiotics severely limits treatment options and infection is typically impossible to eradicate.

The Bcc consists of at least nine genetically distinct species (Coyene *et al*., 2001; Vandamme *et al*., 2003). Although all nine species have been recovered from CF patients, *B. cenocepacia* and *B. multivorans* account for the great majority (~85%) of infection in patients in North America and Europe (Agodi *et al*., 2001; LiPuma *et al*., 2001; Speert *et al*., 2002). Genotyping studies have also identified several so-called epidemic strains that are common to multiple CF patients. Most epidemic strains identified to date, including strain ET12 (common among infected patients in Canada and UK) (Johnson *et al*., 1994; Pitt *et al*., 1996; Mahenthiralingam *et al*., 2002) and strain PHDC (widespread in the United States) (Chen *et al*., 2001; Reik *et al*., 2005) are members of the *B. cenocepacia* species.

Although the pathogenic mechanisms involved in human infection by *B. cenocepacia* are largely unknown, several putative virulence factors have been described (Mahenthiralingam *et al*., 2005). These include, cable pili and the associated adhesin (Sajjan *et al*., 2002; Urban *et al*., 2005), flagella (Tomich *et al*., 2002; Urban *et al*., 2004), lipopolysaccharide and surface exopolysaccharides (Shaw *et al*., 1995; Chung *et al*., 2003), type III secretion system (Tomich *et al*., 2003), production of siderophores (Visser *et al*., 2004), catalase and superoxide dismutase (Lefebre and Valvano, 2001), proteases and lipases (McKevitt *et al*., 1989) and quorum sensing system (Sokol *et al*., 2003). To date the role of these factors in contributing to lung disease has not been clearly elucidated.

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Growing evidence suggests that the capacity of *B. cenocepacia* to enter, survive and replicate intracellularly in human cells contributes significantly to pathogenesis. Indeed, *B. cenocepacia* has been detected inside airway and alveolar epithelial cells, and macrophages in the lungs of CF patients (Sajjan *et al*., 2001a). Intracellular replication also has been observed in the mouse model of infection (Chiu *et al*., 2001; Sajjan *et al*., 2001b), and *in vitro* studies have demonstrated that *B. cenocepacia* enter and replicate in both macrophages and epithelial cells (Burns *et al*., 1996; Saini *et al*., 1999; Martin and Mohr, 2000). Recently, we found that *B. cenocepacia* of the ET12 lineage preferentially enter CF airway epithelial cells (as compared with normal airway epithelial cells) and replicate efficiently (Sajjan *et al*., 2004). However, the processes involved in this and the mechanism(s) by which *B. cenocepacia* escape host defences are not well understood.

In this study, we demonstrate that *B. cenocepacia* readily enters CF airway epithelial cells by endocytosis, escapes from the classical endocytic pathway and replicates in the endoplasmic reticulum (ER). In contrast, heat-killed bacteria enter epithelial cells to a much lesser extent compared with live bacteria, enter the classical endocytic pathway and are degraded in lysosomes.

Results

Uptake of B. cenocepacia *by immortalized CF airway epithelial cells*

IB3 cells were infected at a multiplicity of infection (moi) of 1, 10 or 100. After 2 h cells were washed, extracellular bacteria were killed and intracellular bacteria were quantified by plating serial dilutions of cell lysates. The level of *B. cenocepacia* uptake was inoculum dependent (Fig. 1).

To determine if actin and microtubule formation is involved in bacterial entry, IB3 cells were treated with either cytochalasin D or colchicine for 1 h prior to infection with bacteria at a moi of 50. Cytochalasin D, which inhibits actin rearrangement, inhibited bacterial uptake in a dose-dependent manner; maximum inhibition was observed with a dose of 2 μg ml^{-1} of cytochalasin D (Table 1). Actin rearrangement with bacterial uptake was confirmed by staining F-actin with phalloidin conjugated to Alexa Fluor-488. Uninfected IB3 cells showed an even distribution of filamentous actin throughout the cytoplasm (Fig. 2A), whereas cells infected with *B. cenocepacia* showed F-actin polymerization, resulting in the formation of actin stress fibres (Fig. 2B). Actin rearrangement was also observed in cells incubated with heat-killed bacteria (Fig. 2C), suggesting that CF cells take up viable and non-viable bacteria by a similar endocytic pathway. Colchicine, which inhibits microtubule rearrangement, partially inhibited bacterial uptake by IB3 cells (30% inhibition with 10 μg ml^{-1} colchicine; data not shown).

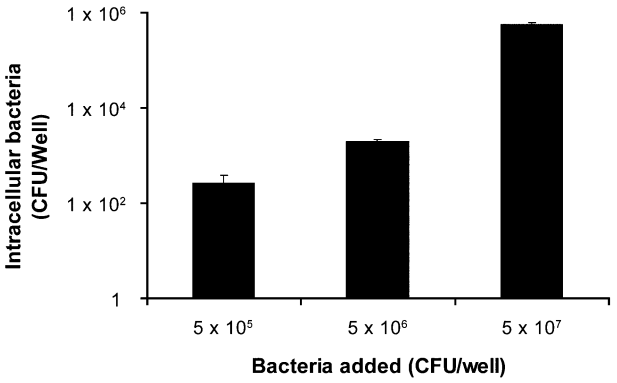

Fig. 1. Uptake of *B. cenocepacia* by IB3 cells. IB3 cells were infected with 5×10^5, 5×10^6 or 5×10^7 *B. cenocepacia* (moi of 1, 10 or 100 respectively) and incubated for 2 h. Extracellular bacteria were killed, IB3 cells were lysed, and serial dilutions of lysates were plated to determine the quantity of intracellular bacteria. Each bar represents mean (± SEM) of six to nine experiments.

Burkholderia cenocepacia *replicate intracellularly in CF epithelial cells*

To determine whether internalized *B. cenocepacia* replicate in CF airway epithelial cells, IB3 cells were infected with a moi of 1 or 10 and incubated for 2 h. Extracellular bacteria were killed by the addition of ceftazidime and gentamicin. Cells were further incubated for 24 h, 48 h or 72 h, lysed and plated to enumerate intracellular bacteria. After 24 h of incubation <1% of the infecting inoculum of viable bacteria was recovered from lysed cells (Table 2). The concentration of bacteria increased at 48 h and 72 h post infection indicating survival and replication of ingested bacteria. Up to 48 h post infection no apparent change in cell viability was observed by trypan blue uptake assay in cells infected at a moi of 1, whereas cells infected at a moi of 10 showed extensive damage by 48 h. To determine whether CF cells are inherently deficient in killing ingested live bacteria, thereby supporting survival and replication of intracellular bacteria, we exam-

Table 1. Effect of cytochalasin D on uptake of *B. cenocepacia* by IB3 cells.

Concentration of cytochalasin D (μg ml^{-1})	Intracellular bacteria (cfu well^{-1})
0	$5.02 \times 10^4 \pm 7.77 \times 10^2$
0 (vehicle control)	$5.18 \times 10^4 \pm 1.26 \times 10^3$
0.5	$1.88 \times 10^4 \pm 1.12 \times 10^2$
1.0	$6.08 \times 10^3 \pm 6.30 \times 10^{2a}$
2.0	$1.09 \times 10^3 \pm 1.15 \times 10^{2a}$
5.0	$1.895 \times 10^3 \pm 3.72 \times 10^{2a}$

a. $P < 0.05$ compared with vehicle control.
Values represent mean ± SEM of triplicate experiments.

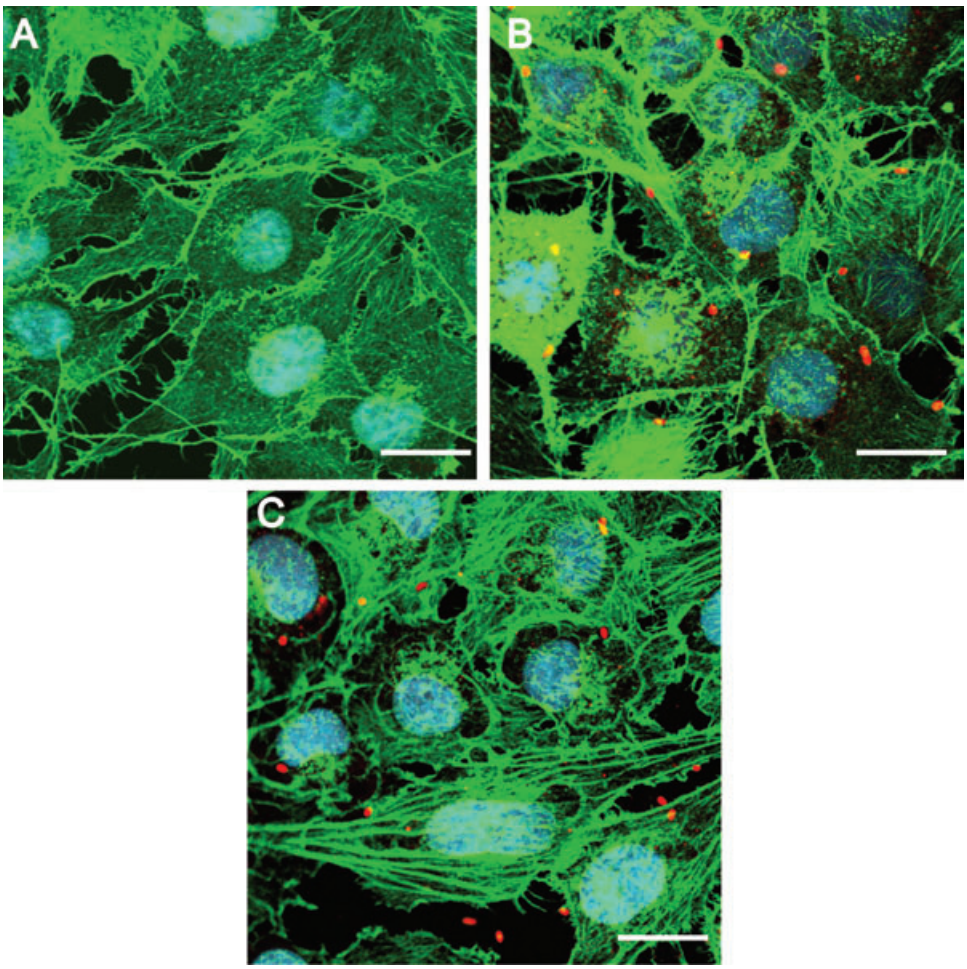

Fig. 2. Actin rearrangement in IB3 cells infected with *B. cenocepacia*. IB3 cells were grown in collagen-coated multichambered slides, serum starved overnight, infected with live or heat-killed *B. cenocepacia* at a moi of 10, and incubated for 2 h. F-actin was detected by Alexa Fluor 488-labelled phalloidin (green) and bacteria were detected by using rabbit antibody to *B. cenocepacia* and anti-rabbit IgG conjugated with Alexa Fluor 598 (red). Nuclei of epithelial cells were detected by staining with DAPI (blue). Uninfected cells and cells infected with live or heat-killed bacteria are shown in panels A, B and C respectively. Bar = 20 μm.

ined the uptake and survival of non-pathogenic *Escherichia coli* DH5α. IB3 cells were infected with *E. coli* at a moi of 10, and intracellular bacteria were quantified at 2 h and 24 h post infection. At 2 h post infection, 96 ± 23 cfu well^{-1} were recovered; this is ~1 log less bacteria compared with *B. cenocepacia*-infected cells (Fig. 1). At 24 h post infection, no bacteria were recovered, indicating that CF cells are efficient in killing ingested non-pathogenic *E. coli*.

Burkholderia cenocepacia *escapes the classical endocytic pathway and replicates in the ER*

Following internalization into non-professional phagocytes, pathogenic bacteria either escape into the cytoplasm or modify bacteria-containing vesicles to prevent fusion with lysosomes, allowing trafficking to a favourable niche for replication (Alonso and Garcia-del Protillo, 2004). To examine the intracellular trafficking and site of

Table 2. Intracellular replication of *B. cenocepacia*.

	Concentration (cfu well^{-1}) of intracellular bacteria at time indicated post infection		
Inoculum (cfu well^{-1})	24 h	48 h	72 h
5×10^5	$3.1 \times 10^3 \pm 2.2 \times 10^2$	$6.8 \times 10^4 \pm 7.5 \times 10^4$	$1.2 \times 10^7 \pm 6.8 \times 10^6$
5×10^6	$4.2 \times 10^4 \pm 9.7 \times 10^3$	$1.02 \times 10^6 \pm 8.9 \times 10^4$	$6.0 \times 10^8 \pm 3.1 \times 10^7$

Values represent mean ± SEM of triplicate experiments.

replication of *B. cenocepacia,* bacteria were colocalized with markers of early endosomes [early endosomal antigen 1 (EEA1)], late endosomes (Rab7), lysosomes (LAMP1 and cathepsin D), ER (calnexin) and autophagosomes [monodansylcadavarine (MDC)]. Heat-killed *B. cenocepacia* K56-2 was used as a negative control. IB3 cells were infected with either live or heat-killed bacteria at a moi of 100 and incubated for 30 min, 1 h, 2 h or 4 h. Both live and heat-killed bacteria were observed in EEA1 positive vesicles as early as 30 min post infection. By 2 h post infection, ~95% of live and heat-killed bacteria were found in EEA1 positive vesicles (Fig. 3A and B). At 4 h post infection, <5% of live bacteria and >80% of heat-killed bacteria colocalized with Rab7 (Fig. 3C and D). These observations suggest that whereas dead bacteria are targeted to the endocytic pathway, metabolically active bacteria either interfere with maturation of endosomes or escape from early endosomes.

Escape of intracellular bacterial pathogens from the endocytic pathway involves failure of fusion of bacteria-containing phagosomes with lysosomes (Alonso and Garcia-del Protillo, 2004). Therefore, we compared the acquisition of lysosome associated proteins, LAMP1 and cathepsin D with vacuoles containing bacteria after 4 h, 6 h and 8 h post infection. At 4 h, LAMP1 was acquired by vacuoles containing either live or heat-killed bacteria. By 6 h post infection, >90% of both live and heat-killed bacteria were localized to LAMP1 positive vesicles (Fig. 4A and B). To follow the fate of LAMP1 acquired bacterial phagosomes, we analysed the distribution of cathepsin D, a lysosomal acid hydrolase. Live bacteria rarely (<10%) colocalized with cathepsin D and remained

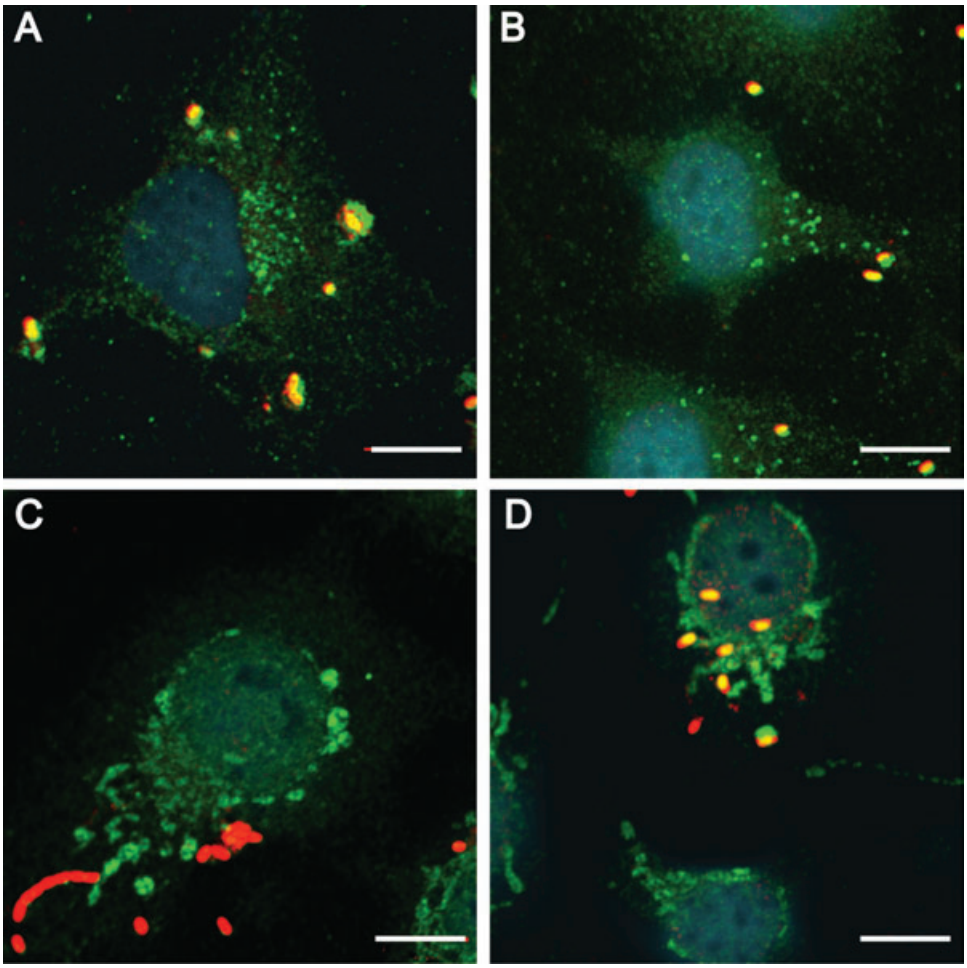

Fig. 3. Colocalization of live and heat-killed *B. cenocepacia* K56-2 with endosomal markers. IB3 cells grown in collagen-coated chamber slides were serum starved overnight, infected with live (panels A and C) or heat-killed (panels B and D) *B. cenocepacia* at a moi of 100 and incubated for 2 min (EEA1) or 4 min (Rab7). Cells were washed to remove unbound bacteria, fixed in methanol, blocked with normal donkey serum and incubated with antibodies to EEA1 (panels A and B) or Rab7 (panels C and D) and *B. cenocepacia*. Cells were washed and bound antibody was detected by anti-goat IgG conjugated to Alexa Fluor 488 (green; for detection of EEA1 or Rab7 respectively); and anti-rabbit IgG conjugated with Alexa Fluor 598 (red; for detection of bacteria). Bar = 20 μm.

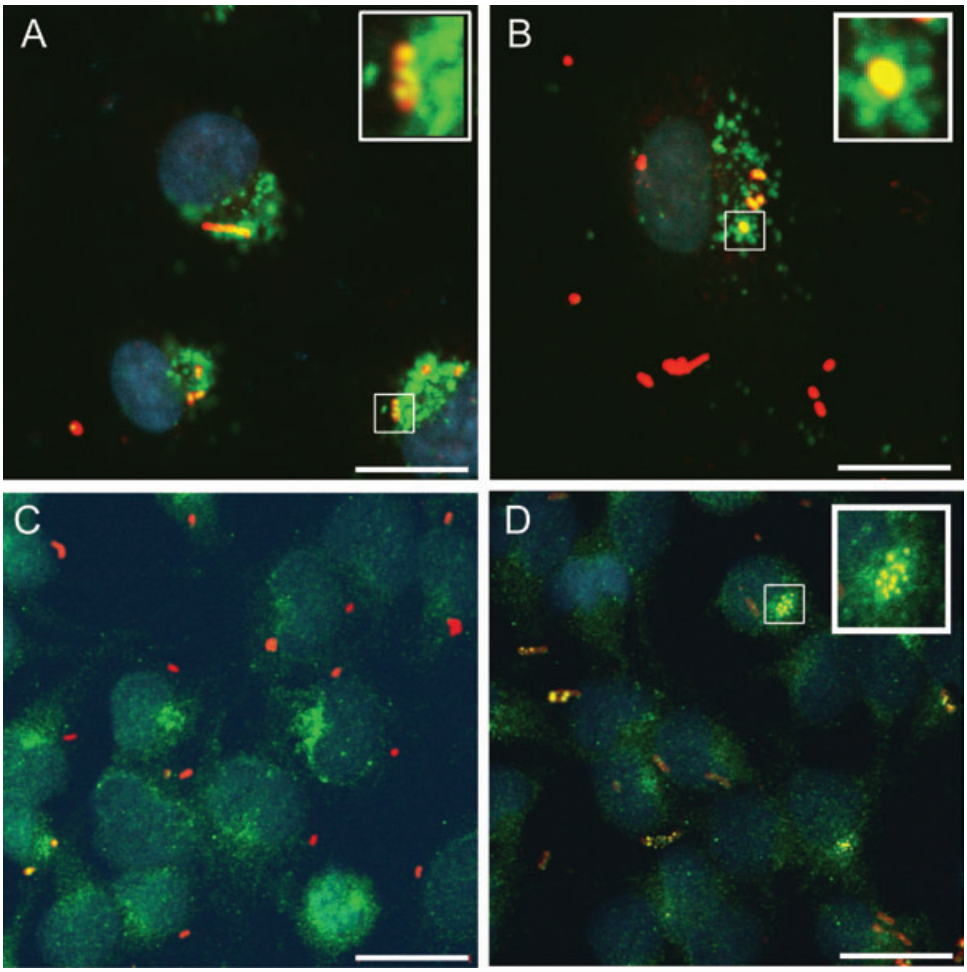

Fig. 4. Colocalization of live and heat-killed *B. cenocepacia* with lysosomal markers. IB3 cells grown in collagen-coated chamber slides were serum starved overnight, infected with live (panels A and C) or heat-killed (panels B and D) *B. cenocepacia* at a moi of 100 and incubated for 2 h. Cells were washed to remove unbound bacteria, and incubation continued for another 2 or 6 h in presence of gentamicin. Cells were fixed in methanol, blocked with normal donkey serum and incubated with antibodies to LAMP1 (panels A and B) or cathepsin D (panels C and D) and *B. cenocepacia*. Cells were washed and bound antibody was detected as described above. Green represents LAMP1 or cathepsin D and red represents bacteria. Inserts in panels A, B and D represent digitally magnified view of area marked in square to show the colocalization of bacteria with respective markers. Bar = 20 μm.

intact even after 8 h of infection, indicating that bacteria prevent maturation of lysosomes (Fig. 4C). In contrast, dead bacteria colocalized with cathepsin D as early as 4 h post infection and at 8 h post infection degraded bacterial particles were observed in the cytoplasm colocalized with cathepsin D (Fig. 4D).

Burkholderia cenocepacia and *B. vietnamiensis* have been shown to reside in acidic vacuoles, distinct from lysosomes, in Acanthamoebae for as long as 24 h without being degraded (Lamothe *et al*., 2004). To investigate whether bacteria reside in such vacuoles in CF cells, we stained infected cells with acridine orange, an acidotropic base and accumulates specifically in acidified vacuoles of eukaryotic cells (Howe *et al*., 2002), and examined cells with fluorescent microscopy. IB3 cells were infected with fluorescein isothiocynate (FITC)-labelled live or heat-killed *B. cenocepacia* for 4 h or 8 h, and incubated with acridine orange. Cells incubated with heat-killed bacteria showed many acridine orange positive vesicles colocalizing with bacteria or degraded bacterial particles. In contrast, cells infected with live bacteria showed relatively few acridine orange positive vesicles, which rarely colocalized with bacteria (data not shown). These observations suggest that live *B. cenocepacia* do not reside in acidified vacuoles in CF epithelial cells.

As the live bacteria containing vesicles we observed did not have features of typical phagolysosomes, we examined whether these vesicles represented autophagosomes, similar to those found with certain other intracellular pathogens such as *Brucella* and *Legionella* sp. (Pizarro-Cerda *et al*., 2000; Molofsky and Swanson, 2004). IB3 cells infected with live or heat-killed

B. cenocepacia for 4 h were incubated with MDC, an autofluorescent compound which specifically accumulates in autophagosomes, in conjunction with bacterial detection by immunostaining. In cells infected with live bacteria, >80% of bacteria-containing vacuoles acquired the autofluorescent compound (Fig. 5). These vesicles also stained positive for calnexin (a marker of ER) suggesting that autophagosomes originate from ER membrane (data not shown). In contrast, heat-killed bacteria were rarely (< 5%) found in vacuoles that stained positive with MDC. These observations suggest that while heat-killed bacteria were destroyed by lysophagosomes, live bacteria enter and reside in ER membrane-derived autophagosomes after escaping from the classical endocytic pathway.

Pathogenic bacteria that reside in autophagosomes have been demonstrated to traffic to and replicate in the ER (Pizarro-Cerda *et al*., 1998; 2000; Molofsky and Swanson, 2004). To determine whether *B. cenocepacia* residing in autophagosomes similarly traffic and replicate in the ER, we analysed cells after 24 h of infection for the presence of bacteria colocalizing with the ER markers calnexin and sec61β. Cells infected with live bacteria showed bacterial microcolonies colocalizing with calnexin primarily in the perinuclear region (Fig. 6). Sec61β also colocalized with bacteria similar to calnexin, indicating the replication of bacteria in the ER (data not shown). In contrast, cells infected with heat-killed bacteria rarely had intact bacteria.

Autophagy is an important step in B. cenocepacia *survival and replication*

To examine whether *B. cenocepacia* entry into autophagosomes is required for survival and replication, we conducted quantitative replication assays in the presence of wortmannin, which inhibits autophagocytic machinery. IB3 cells were pre-incubated with 1, 10 or 100 nM of wortman-

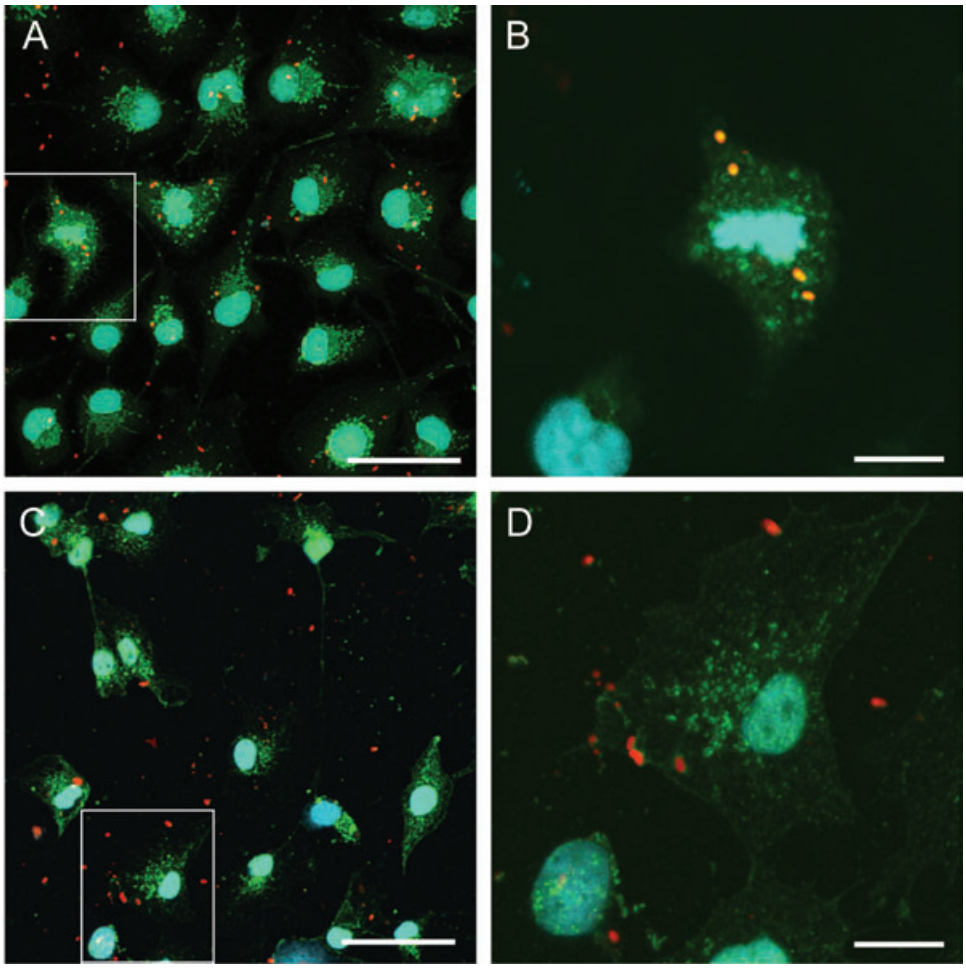

Fig. 5. Uptake of MDC by *B. cenocepacia* containing vesicles. IB3 cells were infected with live (panel A) or heat-killed (panel C) *B. cenocepacia* for 2 h. Unbound bacteria were removed and cells were incubated for another 2 h. Cells were washed, and incubated with 0.05 mM MDC for 30 min (green). Cells were then fixed in ice cold methanol for 2 min and *B. cenocepacia* (red) were detected as described in Fig. 3. Panels B and D represent magnified view of area marked in panels A and C, respectively, to demonstrate colocalization of live bacteria in MDC positive vacuoles. Bars in panels A and C =50 μm; Bars in panels B and D =20 μm.

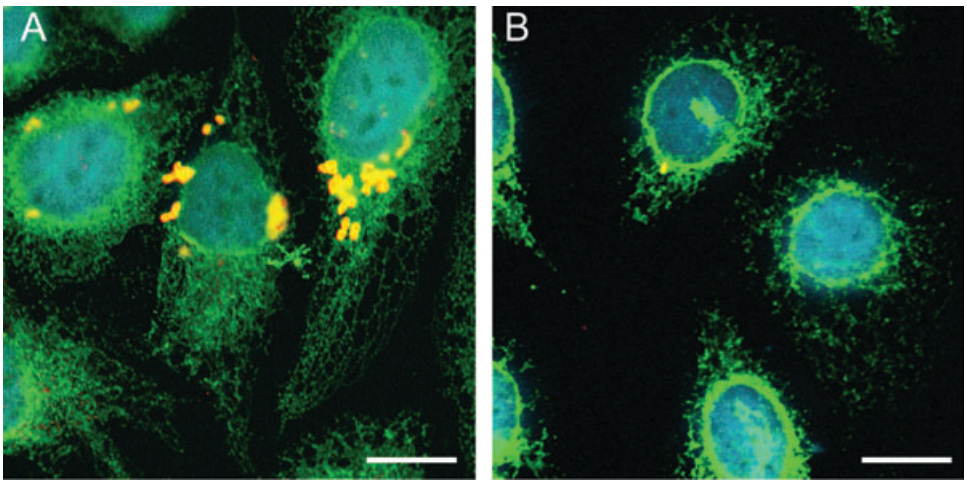

Fig. 6. Colocalization of live and heat-killed *B. cenocepacia* with ER. IB3 cells were infected with live (panel A) or heat-killed (panel B) *B. cenocepacia* at a moi of 100 and incubated for 2 h. Cells were washed to remove unbound bacteria, incubated with ceftazidime and gentamicin to kill extracellular bacteria and further incubated for 24 h in the presence of gentamicin. Cells were washed, fixed in methanol, blocked with normal donkey serum and incubated with antibodies to calnexin (green) and *B. cenocepacia* (red). Yellow represents bacteria colocalized with calnexin. Bar = 20 μm.

nin and infected with *B. cenocepacia* at a moi of 10. Extracellular bacteria were removed after incubating for 2 h and the number of intracellular bacteria was determined 24 h post infection. Wortmannin significantly inhibited intracellular replication of bacteria in a dose-dependent manner (Table 3). In a separate experiment, the growth rate of bacteria in cell culture media in the presence or absence of 100 nM wortmannin was examined to determine the effect of wortmannin on bacterial growth. *B. cenocepacia* grew equally well in the presence and absence of wortmannin (data not shown). These results indicate that the reduced recovery of bacteria from wortmannin-treated cells is due to inhibition of autophagy of *B. cenocepacia* rather than the effect of wortmannin on bacterial growth.

Discussion

Burkholderia cenocepacia is a facultative intracellular respiratory pathogen capable of residing in human macrophages and airway epithelial cells (Mahenthiralingam *et al*., 2005; Valvano *et al*., 2005). The mechanisms involved in intracellular survival and replication of this species are poorly understood. Previous work has demonstrated that *B. cenocepacia* is phagocytosed by both professional phagocytes and epithelial cells (Burns *et al*., 1996; Saini *et al*., 1999; Martin and Mohr, 2000; Schwab *et al*., 2002; Sajjan *et al*., 2004). Whereas bacteria internalized by macrophages reside in acidic vacuoles and fail to replicate (Saini *et al*., 1999), bacteria phagocytosed by epithelial cells appear to replicate efficiently (Burns *et al*., 1996; Martin and Mohr, 2000; Schwab *et al*., 2002; Sajjan *et al*., 2004). To understand how intracellular *B. cenocepacia* are capable of evading host defences, we examined the trafficking of bacteria within human airway epithelial cells *in vitro*.

Our findings indicate that unlike other intracellular pathogens such as *Legionella pneumophila*, *Salmonella typhimurium* and *Brucella abortus*, *B. cenocepacia* are internalized by epithelial cells at a low rate. Within 30 min after internalization, phagosomes containing live bacteria interact with early endosomes as evidenced by bacterial colocalization with EEA1, as is the case with certain other pathogenic bacteria (Duclos and Desjardins, 2000; Alonso and Garcia-del Protillo, 2004). Vesicles containing bacteria start loosing EEA1 after ~2 h, and by 4 h post infection they are neither positive for EEA1 nor the late endosomal marker GTPase Rab7, suggesting that internalized *B. cenocepacia* either diverts young phagosomes from their normal trafficking, similar to *S. typhimurium* and *B. abortus* (Pizarro-Cerda *et al*., 1998; Duclos and Desjardins, 2000), or exit from early endosomes to reside in the cytoplasm or enter specialized vesicles. In contrast, vesicles containing heat-killed bacteria acquired EEA1, but by 4 h post infection also acquire Rab7, indicating that heat-killed bacteria are targeted to the classical endocytic

Table 3. Effect of wortmannin on intracellular replication of *B. cenocepacia*.

Wortmannin (nm)	Intracellular bacteria (cfu well^{-1})
0	$4.8 \times 10^4 \pm 8.9 \times 10^2$
1	$4.2 \times 10^4 \pm 1.5 \times 10^3$
10	$3.6 \times 10^3 \pm 3.8 \times 10^{2a}$
100	$1.2 \times 10^3 \pm 3.9 \times 10^{2a}$

a. $P < 0.05$ compared with control (in the absence of wortmannin). Values represent mean ± SEM of triplicate experiments.

pathway. These results further suggest that live *B. cenocepacia* are required for subverting normal trafficking of young phagosomes.

By using transmission electron microscopy, we previously demonstrated that *B. cenocepacia* may be found in the cytoplasm surrounded by intermediate filaments, as well as in membrane-bound phagosomes (Sajjan *et al.*, 2002; 2004). Based on these results we speculated that bacteria exit endosomes, transiently reside in the cytoplasm and enter vesicles other than phagolysosomes. Results from the present study, which demonstrates *B. cenocepacia* in non-acidified, MDC-positive autophagosomes, support this speculation. Moreover, wortmannin, which impedes the uptake and processing of bacteria by autophagosomes by inhibiting phosphotidyl inositol 3 kinase (Blommaart *et al.*, 1997; Pizarro-Cerda *et al.*, 1998), reduces the number of intracellular *B. cenocepacia* found 24 h post infection. Maturation of autophagosomes, which are derived from rough ER and sequester large aberrant protein aggregates or invading organisms from the cytoplasm, occurs in a stepwise fashion after sequestering their cargo (Meijer and Codogno, 2004). Acquisition of lysosomal membrane proteins occurs by fusing of the autophagosome with pre-lysosomes devoid of degradative enzymes. These vacuoles are acidified and then acquire degradative enzymes by fusing with mature lysosomes. Autophagosomes that sequester *B. cenocepacia* appear to fuse with pre-lysosomes as the bacteria containing vesicles are positive for LAMP1, but fail to mature as evidenced by the lack of accumulation of acridine orange. It appears that metabolically active bacteria are required to enter and inhibit the acidification of autophagosomes, as heat-killed bacteria neither enter autophagosomes nor inhibit the acidification of vesicles containing heat-killed bacteria.

After 24 h, internalized live *B. cenocepacia* were found in the perinuclear area in the form of microcolonies that colocalize with calnexin, an ER marker, suggesting that *B. cenocepacia* traffic to and replicate in the ER. In contrast, *B. cenocepacia* or *B. vietnamiensis* ingested by *Acanthamoeba* was shown to reside primarily in acidified vacuoles that are unlikely to be autophagosomes as they neither accumulated MDC nor had double membranes characteristic of autophagosomes (Lamothe *et al.*, 2004). These observations suggest that the fate of ingested *B. cenocepacia* depends on the type of host cells or the bacterial strain used. In the present study we used strain K56-2, a representative of the clinically important ET12 lineage that has been shown previously to reside and replicate in intracellularly both *in vivo* and *in vitro* (Martin and Mohr, 2000; Schwab *et al.*, 2002; Sajjan *et al.*, 2004).

IB3 cells incubated with heat-killed bacteria processed ingested bacteria by the typical endocytic degradation pathway, indicating that the abnormal trafficking of live *B. cenocepacia* is not due to an inherent defect in the endocytic pathway in these cells. This also indicates that metabolically active bacteria are required to prevent fusion of phagosomes with lysosomes and to traffic to the ER where they replicate. Our observation that non-pathogenic *E. coli* failed to survive and replicate in these cells indicates that IB3 cells are not defective in killing all bacteria.

In general, the trafficking and replication of *B. cenocepacia* in CF airway epithelial cells resembles that found with *L. pneumophila* and *B. abortus*. In these species effector proteins secreted by Type IV secretion systems (TFSS) control these processes (Comerci *et al.*, 2001; Delrue *et al.*, 2001; Molofsky and Swanson, 2004). Recently, *B. cenocepacia* K56-2 has been found to contain two TFSSs, one plasmid-encoded system and the other encoded by chromosomal DNA (Engledow *et al.*, 2004). Although these systems do not appear to be required for invasion or persistence of *B. cenocepacia* in macrophages or Acanthamoebae (Lamothe *et al.*, 2004), ongoing work is assessing the involvement of TFSSs in *B. cenocepacia* intracellular trafficking and replication.

In summary, we have demonstrated that *B. cenocepacia* internalized by CF airway epithelial cells interact with early endosomes, but escape from late endosomes and lysosomes to enter autophagosomes and ultimately replicate in the ER. These processes enable *B. cenocepacia* to escape degradation by host cells and likely contribute to the ability of this species to cause persistent chronic respiratory infection in susceptible hosts.

Experimental procedures

Bacterial strains and growth conditions

Burkholderia cenocepacia K56-2 was isolated from a CF patient and represents the epidemic ET12 lineage. For bacterial uptake assays, K56-2 was grown on Luria–Bertani (LB) agar (FisherBiotech, Fair Lawn, NJ) for 24–48 h. A single colony was inoculated into 3 ml of LB broth and grown overnight at 37°C on an orbital shaker at 200 rpm. Bacteria were harvested by centrifugation, washed once with phosphate-buffered saline (PBS) and suspended in LHC-8 medium (Biosource International, Rockville, MD) to the desired concentration. To obtain heat-killed bacteria, a bacterial suspension of 10^9 cfu ml^{-1} was incubated at 80°C for 20 min. This procedure routinely killed 99.9% of bacteria, which retained their shape and size. Non-pathogenic *E. coli* DH5α was purchased from Invitrogen (Carlsbad, CA).

Cell culture

IB3 cells, an adeno-12-SV40 hybrid virus transformed cell line of bronchial epithelial cells originally isolated from a CF patient, were kindly provided by P. Zeitlin (Johns Hopkins University, Baltimore, MD) (Zeitlin *et al.*, 1991). IB3 cells retain the features

of bronchial epithelial cells but have abnormal chloride channel regulation, a characteristic of CF. Cells were routinely grown in LHC-8 cell culture medium with glutamine (Biosource International, Rockville, MD) amended with 5% fetal bovine serum and 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (supplemented LHC-8 media) at 37°C in 5% CO_2. For bacterial uptake assays, IB3 cells (1×10^5 cells $well^{-1}$) were seeded in 24 well tissue culture plates (BD Falcon, Franklin Lakes, NJ) or in collagen-coated 8 well chamber slides (BD BioCoat, Bedford, MA).

Bacterial uptake assay

The modified gentamicin protection assay described previously (Burns *et al*., 1996; Martin and Mohr, 2000) was adapted to examine uptake of live *B. cenocepacia* by IB3 cells. Briefly, IB3 cells grown in collagen-coated 24 well culture plates were infected in triplicate with live K56-2 at a moi of 1, 10 and 100. After 2 h of incubation at 37°C in 5% CO_2, media from the wells were aspirated, and washed three times in Dulbecco's phosphate-buffered saline (D-PBS) to remove unbound bacteria. Cells were then incubated for 1 h with 500 μl of LHC-8 basal medium containing 500 μg ml^{-1} ceftazidime (Sigma, St Louis, MO) and 500 μg ml^{-1} gentamicin (Sigma) to kill extracellular bacteria. Preliminary studies showed that this antibiotic concentration and incubation period yielded greater than 99.99% killing (< 200 cfu ml^{-1} were recovered from an initial inoculum of 10^7 cfu ml^{-1}). The cells were washed three times in D-PBS, trypsinized and lysed with 0.1% Triton X-100. The number of bacteria recovered per well was determined by quantitative culture. For *E. coli* DH5α, the same procedure was followed except that gentamicin alone (100 μg ml^{-1}) was used to kill extracellular bacteria.

For determination of involvement of actin filaments and microtubules during uptake of bacteria, cells were pretreated with either cytochalasin D (0.5, 1, 2 or 5 μg ml^{-1}) or colchicine (2, 5 or 10 μg ml^{-1}) and bacterial uptake assays were conducted in the presence of the inhibitors for the duration of the 2 h incubation period.

Intracellular replication assay

To determine the ability of bacteria to survive and replicate intracellularly, epithelial cell cultures were infected with bacteria at a moi of 1 or 10, and incubated for 2 h. Extracellular bacteria were killed as described above and the cells were further incubated in LHC-8 medium containing 100 μg of gentamicin for 24, 48 or 72 h. Media were changed every 24 h during the post-infection period. Cells were washed with PBS, and treated with a mixture of ceftazidime and gentamicin, lysed and plated to determine the number of intracellular bacteria. To determine the effect of wortmannin on bacterial replication, cells were pre-incubated with wortmannin (1, 10 or 100 nM) for 30 min and infected with *B. cenocepacia* at a moi of 10. Extracellular bacteria were removed after 2 h incubation as described above and cells were incubated in presence of wortmannin for a further of 24 h.

Staining for actin filaments

Cells grown on collagen-coated slides (BD Biosciences, San Jose, CA) were incubated with bacteria for 2 h, washed, fixed in 4% paraformaldehyde and incubated with phalloidin conjugated with Alexa Fluor-488 (Molecular Probes, Carlsbad, CA). Cells were then fixed in ice cold methanol for 2 min, stained for bacteria as above, and counterstained with DAPI (Sigma chemicals, St Louis, MO). Slides were observed with a fluorescence microscope.

Primary and secondary antibodies

Polyclonal rabbit antibody (R418) to whole lysed *B. cenocepacia* has been described previously (Sajjan *et al*., 2001a). Monoclonal antibody to LAMP1 was purchased from the Hybridoma Facility, University of Iowa, Iowa City. Monoclonal antibodies to cathepsin D and polyclonal antibodies to EEA1, Rab7 and calnexin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated with Alexa Fluor-488 or Alexa Fluor-594 were purchased from Molecular Probes (Carlsbad, CA).

Immunofluorescence staining and microscopy

IB3 cells grown on collagen-coated slides were infected with either live or heat-killed bacteria at a concentration of 10^7 cfu ml^{-1} (moi 100) and incubated for 30 min, 1 h, 2 h or 4 h. Cells were washed three times to remove unbound bacteria, then permeabilized and fixed in cold methanol for 10 min at –20°C. Cells destined for ER staining were processed as in the 24 h replication assay described above before fixation with cold methanol. Slides were incubated at room temperature in PBS containing 5% donkey serum for 1 h to block non-specific binding and then incubated with appropriately diluted primary antibodies overnight at 4°C. Bound antibodies were detected by anti-mouse (LAMP1, and cathepsin D), anti-rabbit (R418) and anti-goat (EEA1, Rab7, calnexin and sec61β) conjugated with either Alexa Fluor-488 or Alexa Fluor-594. Cells were counterstained with DAPI, mounted and visualized under fluorescence microscopy and/or confocal laser microscopy. At least 10 fields or 500 bacteria were visualized under fluorescent microscope to semi-quantify bacterial localization with a particular cell organelle marker.

Labelling of cells with MDC or acridine orange

IB3 cells infected with live or heat-killed bacteria for 2 or 4 h were washed with PBS, and incubated with 0.05 mM MDC (Sigma) for 30 min. Cells were then washed, fixed in methanol and incubated with antibody to *B. cenocepacia* followed by antibody conjugated to Alexa Fluor-594 to detect intracellular bacteria. Bacteria were labelled with FITC (Pierce Biochemicals, Rockford, IL) as described previously (Sajjan *et al*., 2000) and incubated with IB3 cells for 4 or 8 h. Cells were washed with PBS and then incubated with 5 μg ml^{-1} acridine orange (Sigma) prepared in LHC-8 media for 15 min. Cells were washed with PBS, fresh media added and observed under fluorescence microscope.

Statistical analysis

Statistical analyses were carried out using Sigma Stat (SPSS, Chicago, IL). Unpaired *T*-test with Welch's correction was used to compare two groups and parametric one-way ANOVA with

Tukey post-test was used to compare three or more groups. Difference were considered statistically significant if P was <0.05.

Acknowledgements

This work was supported in part by a grant from the Cystic Fibrosis Foundation (to J.J.L.). U.S. was supported by the Carroll Haas Research Fund in Cystic Fibrosis.

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