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Interaction of environmental *Burkholderia cenocepacia* strains with cystic fibrosis and non-cystic fibrosis bronchial epithelial cells *in vitro*

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Burkholderia cenocepacia is an important human pathogen in patients with cystic fibrosis (CF). Non-clinical reservoirs may play a role in the acquisition of infection, so it is important to evaluate the pathogenic potential of environmental *B. cenocepacia* isolates. In this study, we investigated the interactions of two environmental *B. cenocepacia* strains (Mex1 and MCII-168) with two bronchial epithelial cell lines, 16HBE14o$^-$ and CFBE41o$^-$, which have a non-CF and a CF phenotype, respectively. The environmental strains showed a significantly lower level of invasion into both CF and non-CF cells in comparison with the clinical *B. cenocepacia* LMG16656^T strain. Exposure of polarized CFBE41o$^-$ or 16HBE14o$^-$ cells to the environmental strains resulted in a significant reduction in transepithelial resistance (TER), comparable with that observed following exposure to the clinical strain. A different mechanism of tight junction disruption in CF versus non-CF epithelia was found. In the 16HBE41o$^-$ cells, the environmental strains resulted in a drop in TER without any apparent effect on tight junction proteins such as zonula occludens-1 (ZO-1). In contrast, in CF cells, the amount of ZO-1 and its localization were clearly altered by the presence of both the environmental strains, comparable with the effect of LMG16656. This study demonstrates that even if the environmental strains are significantly less invasive than the clinical strain, they have an effect on epithelial integrity comparable with that of the clinical strain. Finally, the tight junction regulatory protein ZO-1 appears to be more susceptible to the presence of environmental strains in CF cells than in cells which express a functional cystic fibrosis transmembrane regulator (CFTR).

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INTRODUCTION

Burkholderia cenocepacia belongs to the *Burkholderia cepacia* complex (BCC), which includes at least 17 phenotypically similar species (Vandamme *et al.*, 1997; Mahenthiralingam *et al.*, 2005; Vanlaere *et al.*, 2008, 2009). *B. cenocepacia* is widely distributed in the natural environment, especially in the rhizosphere of crop plants (Vandamme *et al.*, 2003; Chiarini *et al.*, 2006), and it is also an important opportunistic pathogen, causing severe respiratory infections in individuals with cystic fibrosis (CF) (Mahenthiralingam & Vandamme, 2005; Reik *et al.*, 2005). Once acquired, *B. cenocepacia* is rarely eradicated by antibiotic therapy due to its intrinsic antibiotic resistance (Magalhães *et al.*, 2002), and it can be responsible for transmissible infections in individuals with CF (Govan & Deretic, 1996). Infection with *B. cenocepacia* leads to unpredictable outcomes, ranging from asymptomatic carriage to a fulminant

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Abbreviations: BCC, *Burkholderia cepacia* complex; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; JAM-A, junctional adhesion molecule A; TER, transepithelial resistance; ZO-1, zonula occludens-1.

and fatal pneumonia and sepsis, the so-called 'cepacia syndrome' (Govan & Deretic, 1996; Mahenthiralingam *et al.*, 2002). *B. cenocepacia* adopts versatile lifestyles while infecting the host (Vial *et al.*, 2011). Several virulence determinants that may play a role in the ability of *B. cenocepacia* to infect and persist in human lung have been proposed (Mahenthiralingam *et al.*, 2005; Loutet & Valvano, 2010), even if no definite role for any of them in human disease has been established (Scordilis *et al.*, 1987; Schwab *et al.*, 2002; Speert, 2001). Elucidating the exact role of virulence factors and pathogenic mechanisms in the progression of lung disease will help to establish whether *B. cenocepacia* isolates behave as innocent colonizers or become invasive pathogens. The severity of infection has been related to the ability of *B. cenocepacia* bacteria to invade and survive within respiratory epithelial cells *in vitro* and cause sepsis, as proven by the correlation between intracellular invasion by *B. cenocepacia* and infection in an *in vivo* mouse model (Cieri *et al.*, 2002). Survival and persistence within host cells are also believed to play a key role in pathogenesis (Valvano, 2006). *B. cenocepacia* forms microcolonies in close proximity to the apical cell surface, followed by invasion and destruction of epithelial cells, which also involves disruption of the glycocalyx and rearrangements of the actin cytoskeleton (Schwab *et al.*, 2002, 2003). Unlike most other pathogens in CF, which typically remain confined to the endobronchial spaces, *B. cenocepacia* can traverse polarized respiratory epithelium to cause bacteraemia and sepsis, showing its potential to disrupt tight junctions (Duff *et al.*, 2006; Kim *et al.*, 2005). These studies suggest that *B. cenocepacia* bacteria can employ several strategies to breach the epithelial layer in the airways, and this may explain, at least in part, the different clinical outcomes of *B. cenocepacia* infection in patients with CF (McClean & Callaghan, 2009).

Potentially pathogenic strains are present in the environment, as proven by the genetic identity between environmental and clinical isolates. In fact, an isolate of *B. cenocepacia* which was indistinguishable from the PHDC epidemic clonal lineage, using standard typing methods, was detected in an agricultural soil sample (LiPuma *et al.*, 2002; Baldwin *et al.*, 2007). Subsequently, MLST revealed the existence of three distinct genotypes shared by clinical and environmental *B. cenocepacia* isolates (Baldwin *et al.*, 2007). Infection control measures, including patient segregation, have reduced but not eliminated new infections, and CF patients may occasionally become infected by isolates that show novel fingerprint types (Speert *et al.*, 2002; Mahenthiralingam *et al.*, 2008). The appearance of unique clones in individual patients suggests that acquisition of pathogenic strains likely occurs directly from the natural environment, especially the rhizosphere (Berg *et al.*, 2005). Currently, there is no clear distinction between isolates from environmental or clinical origins, and it is widely accepted that the natural environment is a potential source of BCC acquisition in patients with CF (Mahenthiralingam *et al.*, 2008). It has been found that some phenotypic traits (i.e. biofilm formation, antibiotic susceptibility, exopolysaccharide production) and genetic markers associated with virulence, persistence and transmissibility are also spread among environmental *B. cenocepacia* isolates (Bevivino *et al.*, 2002; Chiarini *et al.*, 2002, 2004; Baldwin *et al.*, 2004; Pirone *et al.*, 2008), as is the ability to colonize murine lung tissue, by persisting in the lungs of infected mice (Pirone *et al.*, 2008). It has been speculated that *B. cenocepacia* can colonize both human lung epithelial and plant root cells through similar mechanisms responsible for recognition and adherence to host cells (Cao *et al.*, 2001; Vial *et al.*, 2011). Indeed, environmental *B. cenocepacia* strains, as well as environmental strains belonging to other BCC species, display an attenuated ability to invade or replicate in cellular models, in comparison with their clinical counterparts (Martin & Mohr, 2000; Keig *et al.*, 2002; Pirone *et al.*, 2008; Zelazny *et al.*, 2009; Vial *et al.*, 2010), but to date, the relationships between environmental bacteria and CF host cells have not been addressed. As suggested by Vial *et al.* (2011), interactions with abnormal cells may trigger the pathogenic behaviour of *B. cenocepacia* strains in patients with genetic or immune deficiencies, and this could explain why opportunistic pathogens such as certain *B. cenocepacia* strains become pathogens in CF disease.

In the present study, we aimed at elucidating the ways by which environmental *B. cenocepacia* strains express their pathogenic potential by addressing the following questions. Are environmental *B. cenocepacia* strains able to invade CF bronchial epithelial cells? Can these strains penetrate through the CF epithelium? Do they utilize distinct mechanisms of internalization into host cells in comparison with clinical strains? To address these objectives, we focused our attention on two well-characterized *B. cenocepacia* strains, isolated from the maize rhizosphere, that have already shown pathogenic potential in both *in vitro* and *in vivo* models (Pirone *et al.*, 2008) and which are capable of forming strong biofilms at a level comparable with the clinical *B. cenocepacia* strain LMG16656 (our unpublished results).

METHODS

Bacterial strains. Two *B. cenocepacia* strains of environmental origin and the clinical *B. cenocepacia* strain LMG16656 were used in this study. The environmental strain Mex1, belonging to *B. cenocepacia* IIIA, kindly supplied by Jesús Caballero-Mellado (Universidad Nacional Autonoma de Mexico, Cuernavaca), was collected from the rhizosphere of maize cultivated in a field in Mexico (Pirone *et al.*, 2008). The environmental strain MCII-168, belonging to *recA* lineage IIIB, was isolated from the rhizosphere of maize plants cultivated in an experimental field located at S. Maria di Galeria, Rome, Italy (Di Cello *et al.*, 1997). A *B. cenocepacia* strain of clinical origin [the epidemic strain LMG 16656^T (*recA* lineage IIIA)], obtained from the Laboratorium voor Microbiologie collection (LMG, Ghent) and *Escherichia coli* DH5α were used as positive and negative controls, respectively. All strains were cryopreserved at −80 °C in 30 % (v/v) glycerol. Prior to any assay, bacteria were streaked from frozen stock preparations onto Nutrient Agar (NA; Difco) plates and incubated at 30 °C for 24–48 h.

Reagents. All cell cultures and electrophoresis materials were purchased from Sigma, with the following exceptions: Ultroser G (Invitrogen), Vitrogen (Nutacon). Mouse anti zonula occludens-1 (ZO-1) was purchased from Invitrogen, while rabbit anti-claudin-1, rabbit anti-occludin, and rabbit anti-junctional adhesion molecule A (JAM-A) antibodies were purchased from Zymed Laboratories. Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Millipore. Horseradish peroxidase (HRP)-conjugated anti-mouse antibody was purchased from Pierce (Fisher Scientific). HRP-conjugated anti-rabbit antibody was purchased from BD Pharmingen. FITC-conjugated goat anti-mouse and anti-rabbit antibodies were purchased from Jackson ImmunoResearch.

Cell culture. The non-CF human bronchial epithelial cell line 16HBE14o$^-$ (Cozens *et al.*, 1994), and a CF line, CFBE41o$^-$, derived from a CF patient homozygous for the cystic fibrosis transmembrane regulator (CFTR) ΔF508 mutation (Goncz *et al.*, 1999), were a generous gift from Dr Dieter Gruenert, University of California, San Francisco. The 16HBE14o$^-$ cells (passage 2.85 to approx. 2.105) were maintained on Vitrogen/fibronectin-coated flasks in minimum essential medium (MEM) supplemented with 1 % L-glutamine, 1 % penicillin/streptomycin and 10 % FBS (Cozens *et al.*, 1994). CFBE41o$^-$ cells (passage 4.85 to 4.105) were also maintained on coated flasks in the same medium, supplemented with 1 % non-essential amino acids (Gruenert *et al.*, 2004).

Invasion of epithelial cells. Analysis of the *B. cenocepacia*–host cell interaction was performed by evaluating the ability of the bacterial cells to invade non-CF and CF epithelial cells. *In vitro* invasion assays were carried out on the bronchial epithelial cell lines using a ceftazidime–amikacin protection assay with minor modifications (Duff *et al.*, 2006). The 16HBE14o$^-$ or CFBE41o$^-$ cells were plated on 24-well coated plates (4×10^5 cells per well), cultured for 24 h at 37 °C, 5 % CO_2, in medium containing 10 % FBS without antibiotics. All bacterial strains were grown to OD_{600} 0.6 in Luria–Bertani (LB) broth and resuspended at the appropriate dilution, as determined by growth curves, in tissue culture medium without antibiotics. The *B. cenocepacia* strains were applied at an m.o.i. of 10 bacterial cells per epithelial cell (m.o.i. 10 : 1) for 2 h. The monolayers were then washed three times with PBS, and a combination of ceftazidime and amikacin (both 1 mg ml^{-1}) was added. The combination of ceftazidime (1 mg ml^{-1}) and amikacin (1 mg ml^{-1}) incubated with each *B. cenocepacia* strain for 2 h resulted in greater than 99.99 % killing (less than 5 and 10 c.f.u. were recovered from environmental and clinical strains, respectively, with an initial inoculum of $1–2 \times 10^7$ c.f.u.). After 2 h of incubation, the cell monolayers were washed and intracellular bacteria were released by lysis with 0.5 % Triton X-100, 50 mM EDTA. Invasion was quantified by plating serial dilutions of the lysates on NA (Difco) plates.

Bacterial infection of polarized lung epithelial cells. Polarized monolayers were also prepared by seeding Transwell-Clear polycarbonate filters (0.4 μm pore size) at a density of 1×10^5 cells cm^{-2} (16HBE14o$^-$ cells) or 7×10^5 cells cm^{-2} (CFBE41o$^-$ cells). The cells were incubated overnight before removal of the apical medium, grown for 6 days with an air–liquid interface and fed basolaterally on alternate days, with medium in which the FBS was replaced with 2 % Ultroser G (Kunzelmann *et al.*, 1993). Bacterial cultures were grown in LB broth to OD_{600} 0.6 and were applied at an m.o.i. of 50 : 1 apically to the monolayers as described previously (Duff *et al.*, 2006). The epithelial cells were then infected with the above three bacterial strains, and a control well was included (medium alone) and incubated for 4 h at 37 °C, 5 % CO_2. Transepithelial resistance (TER) was monitored at 0, 2 and 4 h.

Western blotting. Four hours after exposure to the bacterial strains or to medium alone (control), the polarized epithelial cell monolayers were washed and harvested using 100 μl radioimmunoprecipitaton assay (RIPA) buffer plus protease inhibitors in each Transwell filter. Protein quantification was carried out using a Nanodrop spectrophotometer at 280 nm, and proteins were applied to SDS-PAGE gels (6 % gels for ZO-1, 8 % gels for the other proteins) prior to electrophoresis at 140 V, 250 mA for 1.5–2 h. For ZO-1 analysis, the proteins were subsequently transferred to nitrocellulose at 20 V, 250 mA overnight at 4 °C. Alternatively, for the other lower-molecular-mass proteins, a semi-dry transfer system was used for 90 min. Membranes were blocked in Tris-buffered saline with 5 % non-fat dried milk, 0.1 % BSA and 0.1 % Tween 20 (claudin-1); 5 % BSA and 0.1 % Tween 20 (JAM-A, ZO-1); 10 % non-fat dried milk, 0.1 % BSA and 0.1 % Tween 20 (occludin); or 5 % non-fat dried milk and 0.1 % Tween 20 (GAPDH). Blots were incubated with primary antibody (1 : 500–5000) overnight at 4 °C. Membranes were washed and incubated with goat anti-mouse or anti-rabbit conjugated to HRP for 1 h at room temperature. Proteins were detected by chemiluminescence (Millipore). The density of each individual band was compared with the corresponding control band and normalized against GAPDH (loading control protein) by densitometry using ImageJ (http://rsb.info.nih.gov/ij/). The results were expressed as a change relative to the untreated control.

Immunofluorescence. Monolayers on Transwell inserts were washed with PBS for 5 min prior to permeabilization with cold methanol (−20 °C) for 30 min, and blocked with PBS containing 1 %, w/v, BSA for 10 min. The cells were then immunoprobed with 10 μg ml^{-1} mouse anti ZO-1 antibody for 1 h and subsequently washed three times with PBS/1 % BSA for 5 min. The cells were incubated with FITC-conjugated goat anti-mouse antibody (20 μg ml^{-1}) for 1 h at room temperature, protected from light. The monolayers were washed five times with PBS containing 1 % BSA for 5 min, and post-fixed in PBS containing 4 %, w/v, paraformaldehyde for 10 min. Filters were then removed from the plastic support, mounted on slides with Vectashield containing DAPI, and examined by confocal microscopy.

Statistical analysis. All quantitative infection assays were performed in triplicate, on three different occasions. Dunn's multiple comparison method was used to compare the invasiveness of individual strains with that of the negative control. A P value of <0.05 was deemed to be significant in each case. Student's t tests (two-tailed) were carried out on the TER values of bacteria-treated monolayers relative to the control at individual time points. Differences were considered to be statistically significant if the P value was <0.05.

RESULTS AND DISCUSSION

Invasion of *B. cenocepacia* strains into 16HBE14o$^-$ cells and CFBE41o$^-$ cells

The two environmental *B. cenocepacia* strains MCII-168 and Mex1 were internalized by the bronchial epithelial cell lines 16HBE14o$^-$ and CFBE41o$^-$ at a level lower than that of the invasive clinical *B. cenocepacia* LMG16656 strain (Fig. 1). They were more invasive than the negative control (the non-invasive *E. coli* strain NCIB9415) but less invasive than the positive control (*B. cenocepacia* LMG16656) ($P<0.05$). The internalization of the environmental Mex1 strain was 0.167 and 0.23 % by 16HBE14o$^-$ and CFBE41o$^-$ cells, respectively, while internalization of the other environmental strain, MCII-168, was 0.23 and 0.08 % into 16HBE14o$^-$ and CFBE41o$^-$ cells, respectively. Although

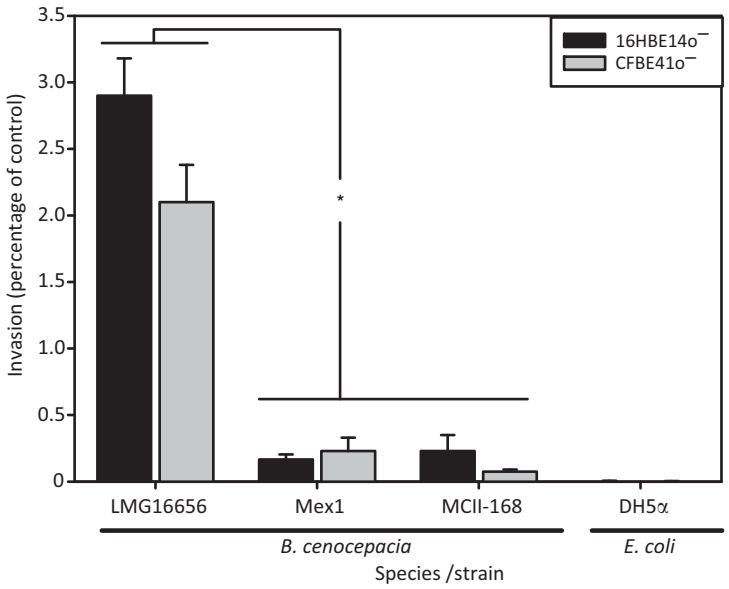

Fig. 1. Invasion by environmental *B. cenocepacia* strains in CF and non-CF bronchial epithelial cells. Two independent lung epithelial cell lines, 16HBE14o$^-$ and CFBE41o$^-$, were used. Invasion of environmental strains was compared with the positive control (clinical *B. cenocepacia* LMG16656^T strain) and with the negative control (*E. coli* strain DH5α). Bars represent percentage invasion (c.f.u. obtained after cell lysis as a percentage of c.f.u. applied) of three independent experiments. An asterisk indicates a significant difference between the clinical strain and environmental strains ($P<0.05$) in Dunn's multiple comparison test.

environmental strains were significantly less invasive than the clinical strain LMG16656, their invasiveness was independent of the CFTR status of the host cell, as found for the invasive clinical *B. cenocepacia* strain LMG16656 (Taylor *et al.*, 2010). Indeed, no statistically significant difference between the internalization of either strain by 16HBE14o$^-$ relative to CFBE41o$^-$ cells ($P=0.42$ for Mex1 and $P=0.32$ for MCII-168, respectively) was observed.

Effect of environmental strains on TER

Bacterial host cell interactions can involve the disruption of epithelial integrity; this strategy can be used by the bacteria to invade the tissues beneath the epithelial cells. *B. cenocepacia* strains can disrupt epithelial integrity and open tight junctions of lung epithelial cells, as determined by a drop in TER (Kim *et al.*, 2005; Duff *et al.*, 2006). To investigate whether the environmental strains could also disrupt tight junction integrity, we exposed polarized CFBE41o$^-$ and 16HBE14o$^-$ cells to the *B. cenocepacia* environmental strains or the *B. cenocepacia* clinical strain for 4 h and measured TER. The mean TER at the start of the experiments was $306\pm16\ \Omega\cdot cm^2$ for CFBE41o$^-$ and $434\pm13\ \Omega\cdot cm^2$ for 16HBE14o$^-$. Exposure of both environmental strains (MCII-168 and Mex1) resulted in a significant reduction in TER ($P<0.01$) over the 4 h period, which was comparable with that observed following exposure to the clinical strain LMG16656 in both cell lines (Fig. 2). The finding that all clinical and environmental *B. cenocepacia* strains examined reduced the TER of both CF and non-CF cell lines suggests that *B. cenocepacia* strains, irrespective of their origin, can disrupt the integrity of airway epithelia, and that this effect is also CFTR-independent. These findings are in agreement with several studies in which clinical *B. cenocepacia* strains have been shown to alter epithelial permeability by the alteration of tight junction organization (Sajjan *et al.*, 2004; Kim *et al.*, 2005; Duff *et al.*, 2006).

Effect on ZO-1 levels of exposure of lung epithelial cells to environmental *B. cenocepacia* strains

Tight junctions include a complex of many different proteins, such as ZO-1, JAM-A, occludin and claudins. We have previously shown that some BCC strains have the ability to disrupt tight junctions in CFTR-expressing cells and show a reduction in expression of ZO-1 when cells are exposed to the *B. cenocepacia* strain BC-7 (Duff *et al.*, 2006). To examine whether the drop in TER following exposure to environmental strains was due to an alteration in tight junction proteins, we extracted the proteins from individual cell monolayers after exposure to the environmental or clinical strains and examined the expression of the individual tight junction proteins by Western blotting. The level of ZO-1 was consistently diminished following exposure of 16HBE14o$^-$ cells to the clinical strain LMG16656 (Fig. 3a, c). In contrast, despite a strong alteration in TER following exposure to Mex1, no clear alteration in the amount of ZO-1 was detected on Western blots from 16HBE14o$^-$ cells exposed to this environmental strain. There was also no apparent alteration in the level of ZO-1 in 16HBE14o$^-$ cells when exposed to the other environmental strain, MCII-168 (Fig. 3a).

Constitutive expression of ZO-1 in CFBE41o$^-$ cells was considerably weaker than that in 16HBE14o$^-$ cells, taking longer exposure times for development of blots, in agreement with earlier studies on CFBE41o$^-$ cells (LeSimple *et al.*, 2010) and on $\Delta F508^{-/-}$ primary cultures (Coyne *et al.*, 2002), and considerable cleavage of the protein was evident, with prominent immunoreactive bands at 110 and

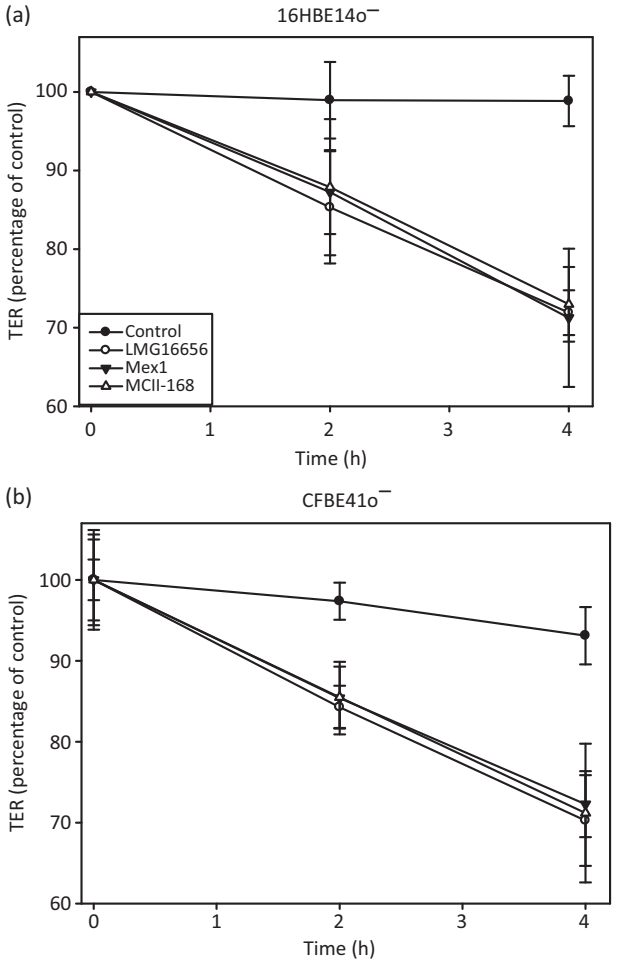

Fig. 2. Effect of environmental *B. cenocepacia* strains on TER. The TER of non-CF 16HBE14o$^-$ (a) and CF CFBE41o$^-$ bronchial epithelial cells (b) was determined at 0, 2 and 4 h post-infection. Data were compared with those obtained with the clinical *B. cenocepacia* strain LMG16656 and the negative control. The data are reported as a percentage of the control (16HBE14o$^-$: 434 ± 13 Ω•cm^2; CFBE41o$^-$: 306 ± 16 Ω•cm^2) and represent the mean of three independent experiments. Error bars, SEM.

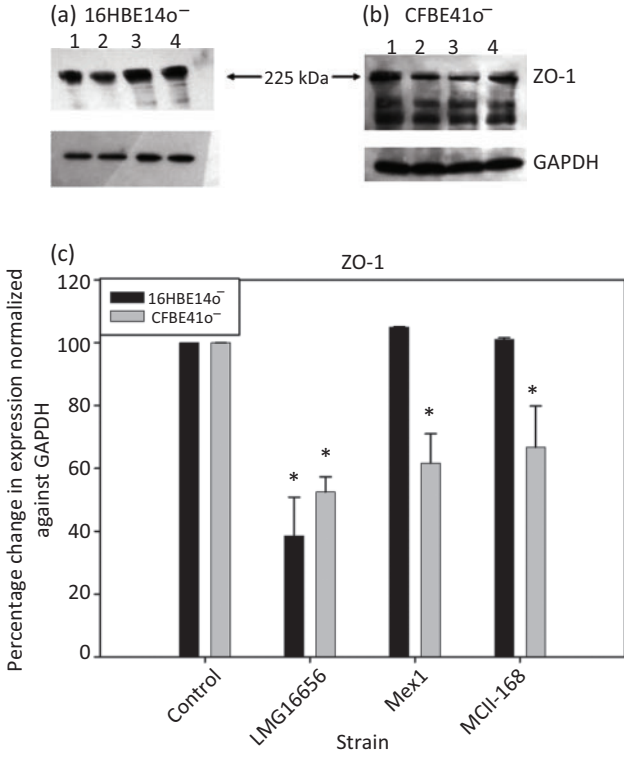

Fig. 3. ZO-1 expression in CF and non-CF bronchial epithelial cells over the course of *B. cenocepacia* infection. (a) Western blot analysis of ZO-1 expression in 16HBE14o$^-$ cells; (b) Western blot analysis of ZO-1 expression in CFBE41o$^-$ cells. Cells were treated with medium alone (control, lane 1), *B. cenocepacia* strain LMG16656^T (lane 2), Mex1 (lane 3) and MCII-168 (lane 4) for 4 h. (c) Densitometric analysis of ZO-1 expression. Band intensities for ZO-1 determined from two independent experiments were normalized against GAPDH values and are expressed as mean ± SEM percentage change relative to control uninfected cells. *P<0.05 by Student's t test.

85 kDa (Fig. 3b). When CFBE41o$^-$ cells were exposed to each of the three *B. cenocepacia* strains, a clear reduction in the detectable ZO-1 level was observed (P<0.05), suggesting that the environmental bacterial strains have a stronger effect on ZO-1 in CFBE41o$^-$ cells relative to 16HBE14o$^-$ cells (Fig. 3b, c).

Immunofluorescence analysis confirmed that the two environmental strains had a slight effect on ZO-1 following 4 h exposure of 16HBE14o$^-$ cells, in contrast to the dramatic loss of ZO-1 from the tight junctions of 16HBE14o$^-$cells when exposed to the clinical strain LMG16656 (Fig. 4a–d). Immunostaining of CFBE41o$^-$ cells showed a different pattern of expression of ZO-1 relative to the CFTR-expressing cell line. Overall, in control CFBE41o$^-$ cells, ZO-1 staining was present in the tight junctions, but it was also diffusely expressed throughout the cell cytoplasm rather than forming contiguous rings, solely at the tight junctions (Fig. 4e). When CFBE41o$^-$ cells were exposed to either of the two environmental strains (Mex1 or MCII-168) or to the clinical strain LMG16656, ZO-1-associated immunofluorescence was lost completely from the tight junctions (Fig. 4f–h). Furthermore, the ZO-1 expression and localization in the ΔF508-expressing CFBE41o$^-$ cells was more susceptible to the effects of environmental *B. cenocepacia* strains than the wild-type CFTR-expressing cells, when compared with 16HBE14o$^-$ cells.

Effect of environmental *B. cenocepacia* strains on claudin-1, JAM-A and occludin

To examine whether the increased susceptibility to alterations in ZO-1 following *B. cenocepacia* infection observed

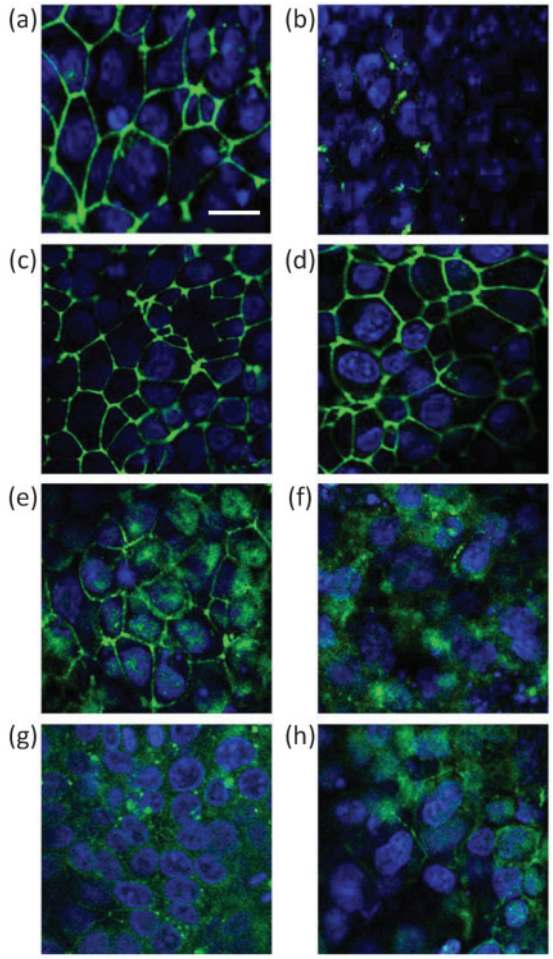

Fig. 4. Effect of exposure to environmental *B. cenocepacia* strains on expression and distribution of ZO-1 in polarized CF and non-CF bronchial epithelial cells. Immunofluorescent staining for ZO-1 in 16HBE14o$^-$ cells (a–d) and CFBE41o$^-$ cells (e–h). Clinical *B. cenocepacia* strain LMG16656^T (b, f) and environmental *B. cenocepacia* strain Mex1 (c, g) or MCII-168 (d, h) were applied for 4 h and compared with control cells treated with LB alone (a, e). The cells were immunostained with anti-ZO-1 antibody, counterstained with DAPI and examined by immunofluorescence microscopy. Magnification, ×400; scale bar, 12 μm.

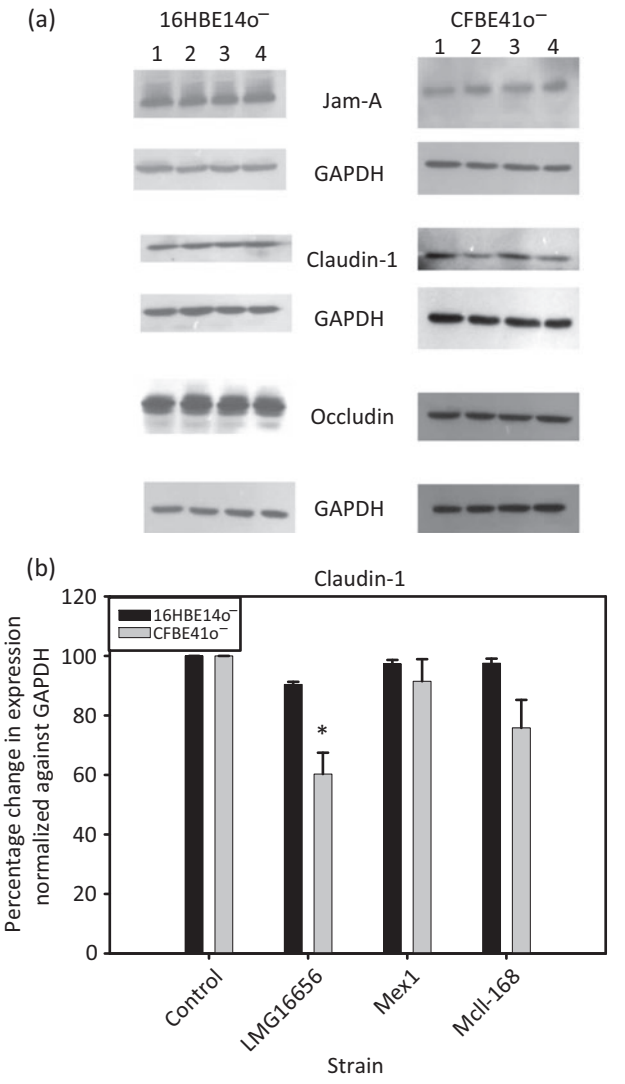

Fig. 5. Effect of environmental *B. cenocepacia* strains on JAM-A, claudin-1 and occludin expression. (a) Western blot analysis of JAM-A, claudin-1 and occludin in 16HBE14o$^-$ and CFBE41o$^-$ cells. Cells were treated with medium alone (control, lane 1), and *B. cenocepacia* strains LMG16656^T (lane 2), Mex1 (lane 3) or MCII-168 (lane 4) for 4 h. (b) Densitometric analysis of claudin-1 expression. Band intensities for claudin-1 were determined in two independent experiments. Data were normalized to GAPDH values and are expressed as mean ± SEM percentage change relative to control uninfected cells. Black bars, 16HBE14o$^-$ cells; grey bars, CFBE41o$^-$ cells. *$P<0.05$, compared with the control.

in CFBE41o$^-$ cells was an effect which was common among tight junction proteins in these cells, additional Western blotting analysis was carried out on JAM-A, claudin-1 and occludin. Neither of the environmental *B. cenocepacia* strains nor the clinical strain had any effect on claudin-1, JAM-A or occludin in the 16HBE14o$^-$ cells (Fig. 5a). In contrast, a 40 % reduction in claudin-1 protein was observed in CFBE41o$^-$ cells in response to LMG16656 exposure. This effect was not observed in cells exposed to either of the two environmental strains, Mex1 or MCII-168 (Fig. 5a–b). ZO-1 plays an important role in claudin trafficking to the tight junction (Umeda *et al.*, 2006), and therefore the dramatic loss of ZO-1 in the CFBE41o$^-$ cells, which have relatively low basal expression of ZO-1, most likely resulted in this concomitant loss in claudin-1 level. The lack of alteration in occludin in both 16HBE14o$^-$ and CFBE41o$^-$ cells in this study in response to any of the *B. cenocepacia* strains was unexpected and in contrast with data shown by Kim *et al.* (2005), who carried out their study for longer time periods (8 and 24 h). In our experience, continuing bacterial growth to these longer

time points may result in competition between the bacteria and the polarized human cells for nutrients, and can contribute to non-specific effects.

In the 16HBE14o$^-$ cells, the environmental strains, Mex1 and MCII-168, resulted in a drop in TER without any apparent effect on ZO-1 or any other tight junction protein, whilst a significant drop in expression of ZO-1 and a dramatic alteration in its localization were observed following infection with either of the environmental strains in the CFBE41o$^-$ cells, suggesting that the mechanisms of tight junction disruption are different in CFTR-expressing epithelia versus CFTR-deficient epithelia. This is in agreement with the finding that defects in CFTR trafficking, such as those documented in ΔF508CFTR-expressing cells, cause alterations of the cytoskeleton (Favia *et al.*, 2010), plasma membrane, or membrane-interacting proteins (Guerra *et al.*, 2005) and tight junctions (LeSimple *et al.*, 2010; Nilsson *et al.*, 2010). To date, trafficking of ΔF508CFTR has been related to some CF defects such as alteration of ceramide metabolism (Becker *et al.*, 2010) and NAPDH oxidase activation (Zhang *et al.*, 2008). Herein we suggest that the observed alteration of tight junctions in CF epithelial cells may be responsible for the differential susceptibility to infection by *B. cenocepacia* strains. ZO-1 regulates tight junction function indirectly because of its anchoring to the cytoskeleton, and it has previously been shown to be altered in CFTR-expressing lung epithelial monolayers after infection with clinical *B. cenocepacia* strains (Duff *et al.*, 2006). In contrast, Kim *et al.* (2005) did not show any alteration in ZO-1 expression in 16HBE14o$^-$ monolayers infected with a different subset of *B. cenocepacia* strains. Taking these data together with our previous (Duff *et al.*, 2006) and present results, it can be suggested that ZO-1 expression may be more robust in these CFTR-expressing cells and that its disruption may sometimes be strain-dependent. It is significant that both *B. cenocepacia* strains that have been associated with altering ZO-1 expression in CFTR-expressing cells, i.e. LMG 16656 and BC-7 (Duff *et al.*, 2006), are clones of strain ET-12 (Mahenthiralingam *et al.*, 2000), which is known to be particularly virulent (Drevinek & Mahenthiralingam, 2010). The exact means by which the bacteria alter the epithelial barrier remain to be elucidated.

Conclusion

Overall, the environmental strains Mex1 and MCII-168 showed less intracellular invasion than the clinical *B. cenocepacia* strain LMG16656. In spite of this, the environmental strains do appear to have an effect on epithelial integrity similar to that of the clinical strain in both CF and non-CF cells, although the mechanism of tight junction disruption is different. A significant finding is that the tight junction regulatory protein ZO-1 is more susceptible to the presence of environmental strains in CF epithelial cells than in cells which express functional CFTR. The dramatic effect of the two environmental strains on tight junction integrity, and on the presence and distribution of the tight junction protein ZO-1 in CF epithelial monolayers, has important implications for the pathogenicity of these environmental strains in patients with CF. Since the acquisition of clinical strains likely occurs directly from the natural environment, a knowledge of the molecular mechanisms employed by environmental *B. cenocepacia* bacteria in virulence and pathogenesis is of crucial importance to identify new targets for the rational design of novel molecular strategies to fight the devastating and currently difficult-to-treat infections caused by *B. cenocepacia* strains.

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