Burkholderia cenocepacia O antigen lipopolysaccharide prevents phagocytosis by macrophages and adhesion to epithelial cells

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Chronic respiratory infections by the *Burkholderia cepacia* complex (Bcc) are of great concern to patients with cystic fibrosis. Bcc isolates may survive intracellularly within amoebae, respiratory epithelial cells and macrophages. The molecular mechanisms facilitating colonization and pathogenesis remain unclear. Given the importance of bacterial adhesion to host surfaces in microbial pathogenesis, we investigated the role of the O antigen LPS in the interaction of *Burkholderia cenocepacia*, a member of the Bcc, with macrophages and epithelial cells. Our results demonstrated that the O antigen modulates phagocytosis but does not affect intracellular survival of *B. cenocepacia*. Internalization of strains that lack O antigen was significantly increased compared to that of their isogenic smooth counterparts. However, no differences between rough and smooth strains were found in their ability to delay phagosomal maturation. We also found that the O antigen interfered with the ability of *B. cenocepacia* to adhere to bronchial epithelial cells, suggesting that this polysaccharide may mask one or more bacterial surface adhesins.

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INTRODUCTION

Respiratory tract infections are a major cause of morbidity and mortality in patients with cystic fibrosis (CF). During the past few decades, Gram-negative bacteria from a group of closely related species referred to as the *Burkholderia cepacia* complex (Bcc) have become important opportunistic pathogens that in particular cause severe infections in patients with CF and chronic granulomatous disease (Coenye & Vandamme, 2003; Mahenthiralingam *et al.*, 2005). Due to their intrinsic resistance to most clinically relevant antimicrobial agents (Govan & Deretic, 1996), and their ability to be transmitted from patient to patient (Govan *et al.*, 1993), Bcc infections are of great concern to the CF community. The majority of the members of the Bcc have been recovered from the sputum of CF patients (Coenye & Vandamme, 2003). From these, *Burkholderia cenocepacia* is one of the most predominant Bcc species in every country where the prevalence of Bcc species has been monitored (Mahenthiralingam *et al.*, 2005). Bcc isolates can survive intracellularly within amoebae (Marolda *et al.*, 1999), respiratory epithelial cells (Burns *et al.*, 1996) and macrophages (Lamothe *et al.*, 2004; Saini *et al.*, 1999). The ability of *B. cenocepacia* to enter and survive within eukaryotic cells could provide a mechanism for evasion of the host immune response and may also be important for

transmission. Indeed, we have shown that Bcc-infected amoebae release membrane-bound vesicles containing viable bacteria that are potentially respirable and could be transported to the lower airways of patients by airflow (Marolda *et al.*, 1999). Although several putative virulence factors have been identified (reviewed by Mahenthiralingam *et al.*, 2005), the mechanisms facilitating colonization and pathogenesis of *B. cenocepacia* are not well understood. Attachment to cell surfaces is one of the key steps in bacterial pathogenesis, and requires the specific interaction of bacterial surface molecules ('adhesins') with host cell membrane molecules or extracellular matrix proteins (Beachey, 1981; Karlsson *et al.*, 1992). To date, a 22 kDa pilin-associated protein is the only well documented adhesin in Bcc species, which mediates bacterial adherence to mucin and cytokeratin 13 on epithelial cells (Sajjan & Forstner, 1992, 1993; Sajjan *et al.*, 2000). However, only a subset of Bcc isolates produces the cable pilus and its associated adhesin (LiPuma *et al.*, 2001), suggesting that other uncharacterized bacterial adhesins may exist. LPS is a complex glycolipid molecule and a major constituent of the bacterial outer membrane in Gram-negative bacteria. LPS consists of lipid A, core oligosaccharide and in some strains the O-specific polysaccharide or O antigen (Raetz & Whitfield, 2002). Preliminary studies in our laboratory using a pair of clonally related *B. cenocepacia* strains with and without O antigen revealed that expression of O antigen was

Abbreviations: Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; FBS, fetal bovine serum; TMR, tetramethylrhodamine.

associated with reduced phagocytosis. In this study, we report a systematic characterization of the role of O antigen polysaccharide in the interaction of *B. cenocepacia* with macrophages and epithelial cells.

METHODS

Bacterial strains, cell lines and growth conditions. Bacterial strains were grown at 37 °C in Luria–Bertani (LB) medium with shaking. *B. cenocepacia* carrying the plasmids pXO4 and pSM65 were grown in the presence of 100 µg trimethoprim ml⁻¹ and 100 µg chloramphenicol ml⁻¹, respectively. The murine macrophage cell line RAW 264.7 (ATCC TIB-71) was obtained from the American Type Culture Collection, Manassas, VA, USA, and was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (FBS). IB3 bronchial epithelial cells (Cozens *et al.*, 1994), expressing endogenous ΔF508 and W1282X CFTR mutant proteins at undetectable levels, were stably transfected with the pCEP4 expression plasmid encoding the wild-type or the ΔF508 CFTR. Mixtures of clones were selected in hygromycin B. Transfected cells were maintained in LHC-8 medium containing 5 % FBS and 100 µg hygromycin B ml⁻¹. Cells were grown at 37 °C in a humidified atmosphere of 95 and 5 % carbon dioxide.

Construction of a *wbxE* mutant in *B. cenocepacia* K56-2. An internal 300 bp fragment encompassing the 5' end of the *wbxE* gene was PCR amplified, and cloned into the *Xba*I and *Eco*RI restriction sites of the suicide vector pGPΩTp (Flannagan *et al.*, 2007). Mutagenesis plasmids were introduced into *B. cenocepacia* K56-2 by triparental mating (Craig *et al.*, 1989) and exconjugants selected on LB agar plates supplemented with 50 µg gentamicin ml⁻¹ and 100 µg trimethoprim ml⁻¹. Plasmid integration into the chromosome was confirmed by colony PCR and Southern blotting. The generated mutant was designated XOA3.

Construction of a complementing plasmid. To create the complementing plasmid pSM65, genes *wbxE*–*wbxD* were obtained by digestion of pXO4 (Ortega *et al.*, 2005) with *Nde*I and *Xba*I. The fragment was ligated into pSL6 (Loutet *et al.*, 2006), which was also cut with the same restriction enzymes. Ligation mixtures were introduced by electroporation into *E. coli* DH5α [F⁻ φ80*lacZ* Δ*M15* *endA* *recA* *hsdR*(r_K⁻m_K⁻) *supE* *thi* *gyrA* *relA* Δ(*lacZYA*–*argF*)U169] and transformants plated on LB agar plates supplemented with 30 µg chloramphenicol ml⁻¹. The correct DNA insert was verified by restriction digestion and ultimately by sequencing of the DNA insert. Plasmid pSM65 was mobilized by conjugation into the XOA3 mutant strain.

Macrophage infection assays. Macrophage infections were performed as described previously (Saldías *et al.*, 2008). Briefly, bacterial suspensions were added to RAW 264.7 cells grown on glass coverslips at an m.o.i. of 50, and incubated at 37 °C in 5 % carbon dioxide. Heat-inactivated bacteria were obtained by incubation at 60 °C for 30 min prior to infection. When needed, 0.5 µM LysoTracker Red DND-99 was added for 1 min prior to visualization. Lysosome-labelling was performed by incubating macrophages with 250 µg tetramethylrhodamine (TMR)-labelled dextran ml⁻¹ for 2 h. External TMR-dextran was removed by serial washes with PBS and chased for 1 h in DMEM with 10 % FBS before infection. Fluorescence and phase-contrast images were acquired using a Qimaging cooled charge-coupled device camera on an Axioscope 2 (Carl Zeiss) microscope. DMEM, PBS and FBS were purchased from Wisent. LysoTracker Red DND-99 and TMR-dextran were from Invitrogen. Each experiment was independently repeated at least three times.

Measurement of phagocytic index. The number of infected macrophages and the number of bacteria per macrophage was determined by visual analysis of 21 fields of view, using an Axioscope 2 microscope (Carl Zeiss) with a ×100 magnification oil immersion objective. Infections were performed as described before. Each experiment was repeated three times.

LPS analysis. LPS was extracted as previously described (Marolda *et al.*, 1990), resolved on 16 % polyacrylamide gels by Tricine–SDS gel electrophoresis (Lesse *et al.*, 1990; Schägger & Von Jagow, 1987) and visualized by silver staining (Marolda *et al.*, 1990).

Adhesion assays. RAW 264.7 cells (2.5 × 10⁵) in DMEM/10 % FBS or IB3 cells (8 × 10⁴) in LHC-8/5 % FBS were seeded into a 48-multiwell plate and incubated at 37 °C for 20 h in a humidified atmosphere of 95 and 5 % carbon dioxide. Overnight bacterial cultures were washed and resuspended in DMEM/10 % FBS or LHC-8/5 % FBS and added to the cells at an m.o.i. of 50, centrifuged for 2 min at 300 g and incubated for 30 min at 4 °C. Non-adherent bacteria were removed by rinsing five times with ice-cold PBS. Cells were lysed with 100 µl 0.5 % sodium deoxycholate. Serial dilutions were performed in LB and plated in duplicate. The percentage of adhesion was calculated as follows: 100 × (number of cell-associated bacteria/initial number of bacteria added). To calculate the bacterial adherence to plastic, the assays were performed as for the macrophage adhesion assays but without the eukaryotic cells. Data were calculated from at least three independent experiments performed in triplicate and are expressed as means ± SE.

Statistical analyses. The statistical significance of differences in the data were determined using the one-way analysis of variance (ANOVA) test and the Tukey post-test, provided in the Prism GraphPad software.

RESULTS AND DISCUSSION

B. cenocepacia strains of the same clonal lineage are differentially internalized by macrophages

Our laboratory commonly uses two clonally related *B. cenocepacia* clinical isolates, J2315 and K56-2, which belong to the epidemic lineage ET12 (Johnson *et al.*, 1994). Using non-opsonic macrophage infections, we noticed a higher proportion of infected macrophages in experiments with J2315 than in infections with K56-2. To determine the rates of internalization of both *B. cenocepacia* strains in the mouse macrophage-like cell line RAW 264.7, we calculated the phagocytic index by infecting macrophages with either K56-2 or J2315 at an m.o.i. of 50 for 4 h. The results showed that on average 3.5 % (±0.3 %) of macrophages were infected with *B. cenocepacia* K56-2 (Fig. 1a). This number increased up to fourfold when the infection was carried out with the J2315 strain (14.9 % ± 4.6 %, *P* < 0.01, Fig. 1a). Also, the mean number of intracellular bacteria per macrophage doubled when infected with *B. cenocepacia* J2315 as compared to K56-2 (two bacteria per macrophage for K56-2 vs four bacteria per macrophage for J2315, Fig. 1b). These results indicated that the strain K56-2 is much less internalized by RAW 264.7 macrophages than J2315.

Fig. 1. *B. cenocepacia* O antigen acts as an antiphagocytic molecule. (a) Percentage of RAW 264.7 macrophages infected with the various *B. cenocepacia* strains at an m.o.i. of 50 for 4 h. The values represent the mean and SE of 3 experiments in which 21 fields were examined at $\times 100$ magnification. (b) Mean number of intracellular bacteria per infected macrophage. The values represent the mean and SE of 3 experiments in which 21 fields were examined at $\times 100$ magnification.

B. cenocepacia O antigen acts as antiphagocytic molecule

We previously reported that *B. cenocepacia* J2315 produces a LPS that lacks a complete O antigen due to the presence of an IS402 element in the *wbxE* gene of the O antigen biosynthesis gene cluster, which encodes a predicted glycosyltransferase (Ortega *et al.*, 2005). Replacing the defective gene cloned from strain K56-2, which produces a complete LPS O antigen (Fig. 2), restores O antigen surface expression in J2315 (Ortega *et al.*, 2005). To investigate the role of the O antigen in the interaction of *B. cenocepacia* with macrophages, we constructed an isogenic K56-2 mutant strain unable to produce O antigen by the insertional inactivation of *wbxE* (mutant XOA3, see Methods). As expected, the mutant XOA3 was unable to produce a complete O antigen, producing LPS molecules that exhibited a similar banding pattern to that of J2315 LPS (Fig. 2). This LPS defect could be rescued when a complementing plasmid (pSM65, see Methods) containing both *wbxE* and *wbxD* was introduced by conjugation into XOA3 (Fig. 2). In infections carried out with the mutant XOA3 the percentage of infected macrophages was more

Fig. 2. Electrophoretic profiles of LPS extracted from K56-2, the mutant XOA3 (K56-2 *wbxE*::pGP Ω Tp), XOA3/pSM65 (XOA3 *wbxE*⁺), J2315 and J2315/pXO4 (J2315 *wbxE*⁺). The regions corresponding to polymeric O antigen (O Ag) and lipid A-core oligosaccharide (Os) are indicated. The loading of the LPS was standardized on the basis of the number of bacterial cells used to prepare the samples. Samples were run in 16% polyacrylamide gels in a Tricine-SDS system and developed by silver staining as described in Methods.

than threefold higher than that of infected macrophages with the parental K56-2 or the complemented strain (XOA3/pSM65), and no significant differences were observed in the phagocytic index between this strain and the O-antigen-deficient strain J2315 ($15.2\% \pm 2.2\%$ vs $14.9\% \pm 4.6\%$, respectively, $P > 0.05$, Fig. 1a). Conversely, when O antigen synthesis in J2315 was reconstituted with the plasmid pXO4 (which carries a functional *wbxE* gene) (Ortega *et al.*, 2005) we observed a threefold reduction in the phagocytic index of this strain as compared to the parental J2315 ($5.1\% \pm 0.9\%$ of infected macrophages, Fig. 1a). The presence of O antigen also affected the number of intracellular bacteria per macrophage. On average, the number of intracellular bacteria doubled when the infection was carried out with *B. cenocepacia* strains that lacked O antigen as compared to the strains expressing a complete LPS (Fig. 1b). These results suggest a role for the O antigen in preventing phagocytosis by RAW 264.7 macrophages.

O antigen expression does not affect the intracellular behaviour of *B. cenocepacia*

Previous work in our laboratory demonstrated that *B. cenocepacia* has the ability to survive intracellularly within vacuoles in amoebae and murine macrophages (Lamothe *et al.*, 2004; Marolda *et al.*, 1999; Saini *et al.*, 1999). We established that in macrophages, *B. cenocepacia*-containing vacuoles display a maturation delay that temporarily blocks fusion with lysosomes (Lamothe *et al.*, 2007). Others have shown that the LPS O antigen plays an essential role in internalization and survival of the related bacterium

Burkholderia pseudomallei in macrophages (Arjcharoen *et al.*, 2007). Therefore, to examine whether the presence or absence of O antigen affects the intracellular behaviour of *B. cenocepacia*, we infected RAW 264.7 macrophages with either K56-2 or XOA3 and assessed the colocalization of the bacteria-containing vacuoles with LysoTracker Red by single-cell analysis. LysoTracker Red is an acidotropic dye that preferentially accumulates in lysosomes (Via *et al.*, 1998). As shown in Fig. 3, the presence of O antigen did not play a role in the intracellular trafficking of *B. cenocepacia*. At 4 h post-infection, most of the vacuoles containing either smooth or rough *B. cenocepacia* K56-2 did not colocalize with LysoTracker Red (Fig. 3a; data not shown). In contrast, vacuoles containing heat-killed bacteria colocalized with the acidotropic dye 2 h post-infection (Fig. 3a). Quantitative analysis shows that less than 20 % of the vacuoles containing either K56-2 or XOA3

colocalized with LysoTracker Red ($16.7 \pm 7.8\%$ and $12.7 \pm 7.1\%$, respectively, Fig. 3b), while over 80 % of the vacuoles containing heat-killed bacteria colocalized with the fluorescent probe ($86.3 \pm 5\%$, Fig. 3b).

To confirm that bacterial cells were not trafficked to the lysosomes, we used TMR-dextran, which is internalized via fluid-phase endocytosis and accumulates in the lysosomes. Before bacterial infection, we incubated macrophages with $250 \mu\text{g TMR-dextran ml}^{-1}$ for 2 h followed by a 1 h chase in dye-free medium to ensure that the probe was fully delivered from early and recycling endosomes to lysosomes. As shown in Fig. 3(c), about 20 % of the vacuoles containing either K56-2 or XOA3 colocalized with dextran ($21.7 \pm 1.5\%$ and $21.3 \pm 1.2\%$, respectively), while $94.7 \pm 2.9\%$ of the vacuoles containing heat-killed bacteria colocalized with the probe. Similar results were obtained

Fig. 3. O antigen expression does not affect intracellular survival of *B. cenocepacia*. Images of RAW 264.7 macrophage cells infected for 4 h with *B. cenocepacia* K56-2 or for 2 h with heat-killed K56-2 at an m.o.i. of 50 using fluorescence and phase-contrast microscopy. (a) Macrophages were incubated with $0.5 \mu\text{M}$ LysoTracker Red prior to visualization. K56-2 bacteria are within membrane-bound vacuoles that do not colocalize with LysoTracker Red (indicated by an arrow). (b) Per cent of bacteria-containing vacuoles colocalizing with LysoTracker Red. The values correspond to the mean and SE of 3 experiments in which 21 fields were examined. White bar, 2 h post-infection; black bars, 4 h post-infection. (c) Per cent of bacteria-containing vacuoles colocalizing with TMR-dextran. The values correspond to the mean and SE of 3 experiments in which 21 fields were examined. White bar, 2 h post-infection; black bars, 4 h post-infection.

when the experiments were carried out with J2315 and J2315/pXO4 (data not shown), confirming that unlike *B. pseudomallei*, surface exposed O antigen does not affect the intracellular trafficking of *B. cenocepacia* in RAW 264.7 macrophages.

Presence of O antigen does not affect adhesion of *B. cenocepacia* to RAW 264.7 macrophages

We investigated whether the differences in phagocytic index between strains with and without O antigen were due to differences in bacterial adhesion to macrophage cells. Thus, we compared the adherence of K56-2 (O-antigen⁺), XOA3 (O-antigen⁻), XOA3/pSM65 (O-antigen⁺), J2315 (O-antigen⁻) and J2315/pXO4 (O-antigen⁺) to RAW 264.7 macrophages. Considering that macrophages do not form a confluent monolayer, we also calculated the percentage of bacterial adherence to plastic, and subtracted this number from the percentage of adherence obtained when macrophages were used in the assays. As shown in Fig. 4(a), the adherence of *B. cenocepacia* to abiotic surface was greatly affected by the presence of O antigen. Less than 1 % of the bacteria expressing O antigen adhered to plastic (0.16 ± 0.02 % for K56-2, 0.17 ± 0.01 % for XOA3/pSM65 and 0.18 ± 0.06 % for J2315/pXO4), while approximately 10 % of the rough bacteria adhered to the abiotic surface (10.95 ± 1.7 % for XOA3 and 7.9 ± 0.8 % for J2315). However, we did not find significant differences in the adherence of all of these strains to RAW 264.7 macrophages (Fig. 4b, $P > 0.05$). These results demonstrated that although the presence of O antigen affects the adherence of *B. cenocepacia* to abiotic surfaces, it does not affect the adherence of these bacteria to RAW 264.7 macrophages, indicating that there is not a direct correlation between the differences on the phagocytic index of *B. cenocepacia* with or without O antigen and a reduced bacterial adherence to macrophages.

O antigen plays a role in the adherence of *B. cenocepacia* to bronchial epithelial cells

We also investigated whether the presence of O antigen influences the adhesion of *B. cenocepacia* to IB3 cells. This is an immortalized cell line from a primary culture of bronchial epithelia isolated from a patient with CF (Zeitlin *et al.*, 1991). The cell lines that we used in this study were IB3 derivatives that stably expressed either wild-type CFTR or $\Delta F508$ CFTR at higher levels. First, we compared the adhesion of *B. cenocepacia* to IB3 cells that expressed the wild-type CFTR gene (IB3 WT CFTR). The results showed that irrespective of the *B. cenocepacia* strain, the presence of O antigen reduced bacterial adhesion to the epithelial cells ~ 10 fold (Fig. 4c), indicating that the O antigen interferes with the ability of *B. cenocepacia* to adhere to bronchial epithelial cells. Similar results were obtained with IB3 cells expressing the mutated form of the CFTR protein (IB3 $\Delta F508$ CFTR) (Fig. 4c). Although there was an apparent lower trend in the percentage of bacteria adhering to IB3 $\Delta F508$ CFTR cells versus IB3 WT CFTR cells, these differences were not statistically significant. The results therefore suggest that CFTR does not have a specific role on the adhesion of *B. cenocepacia* to bronchial epithelial cells.

Concluding remarks

B. cenocepacia bacterial cells display several surface-associated molecules that could play a role in bacteria–host cell interactions. Among them, LPS is known to play an essential role in pathogenesis of many Gram-negative bacteria (Raetz & Whitfield, 2002). In particular, the O antigen contributes to intracellular survival and replication in various pathogenic bacteria. For instance, the O antigen modulates complement-mediated lysis and complement C3 deposition in *Francisella tularensis* (Clay *et al.*, 2008), and it is required for the intracellular replication of this pathogen in macrophages (Maier *et al.*, 2007). Similarly, O antigen is needed for full virulence and intracellular

Fig. 4. Adhesion of *B. cenocepacia* to abiotic and biotic surfaces: (a) adhesion to polystyrene, (b) adhesion to RAW 264.7 macrophages, (c) adhesion to IB3 bronchial epithelial cells. Adhesion assays using K56-2 (O-antigen⁺), XOA3 (O-antigen⁻), XOA3 (O-antigen⁺), J2315 (O-antigen⁻) and J2315/pXO4 (O-antigen⁺) were performed in triplicate on at least three independent experiments. Means $\pm$ SEMs are shown. In (c): black bars, IB3 WT CFTR; white bars, IB3 $\Delta F508$ CFTR.

survival of *Brucella* species (Fernandez-Prada *et al.*, 2003; Porte *et al.*, 2003; Rajashekara *et al.*, 2008), and the length of the O antigen polysaccharide molecule influences the invasion and uptake of *Salmonella enterica* by macrophages (Murray *et al.*, 2006).

The results of this study clarify the role of *B. cenocepacia* O antigen LPS in the interaction of these bacteria with eukaryotic cells. We have found that, as observed in *B. pseudomallei* (Arjcharoen *et al.*, 2007), O antigen surface expression affects the level of phagocytosis. Internalization of the O-antigen-defective strains was significantly increased compared to that of the parental counterpart. However, in contrast to *B. pseudomallei*, the intracellular survival of the O-antigen-defective mutant was not compromised. This suggests that although the surface O antigen acts as an antiphagocytic molecule it does not influence the intracellular behaviour of *B. cenocepacia* after internalization into the macrophages. Our results also showed that surface O antigen interfere with bacterial adhesion to abiotic surfaces and bronchial epithelial cells. Surface attachment is the first step in biofilm formation, and the ability to form biofilms could be advantageous for *B. cenocepacia* in the CF lung environment, possibly by protecting bacteria from antibiotics and/or mediators of host defence (Desai *et al.*, 1998). Many Bcc isolates from CF patients produce LPS O antigen, but some, including isolates of the ET12 lineage, lack the O antigen portion of LPS like BC7, C5424 and J2315 (Evans *et al.*, 1999; Ortega *et al.*, 2005). Therefore, the absence of O antigen in this group of *B. cenocepacia* strains may explain their increased invasiveness.

Given the ease of natural acquisition of antibiotic resistance genes by opportunistic pathogens, research into therapeutic alternatives to antibiotics is urgently required. A better understanding of the bacterial adherence to eukaryotic cells could open up new opportunities for overcoming the current problems associated with antibiotic resistance by using novel anti-adhesion approaches to prevent or reduce bacterial colonization.

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