Burkholderia cenocepacia O Antigen Lipopolysaccharide Prevents Phagocytosis by Macrophages and Adhesion to Epithelial Cells

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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
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 W128X 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 XbaI and EcoRI restriction sites of the suicide vector pGPM7p (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 XO3.

**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 NdeI and XbaI. The fragment was ligated into pSL6 (Loutet et al., 2006), which was also cut with the same restriction enzymes. Ligation mixtures were confirmed by colony PCR and Southern blotting. The generated plasmid encoding the wild-type or the Δf508 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.

**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 × 10⁵) 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.
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 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% ± 2.2% vs 14.9% ± 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% ± 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 ± 7.8 % and 12.7 ± 7.1 %, respectively, Fig. 3b), while over 80 % of the vacuoles containing heat-killed bacteria colocalized with the fluorescent probe (86.3 ± 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 µg 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 ± 1.5 % and 21.3 ± 1.2 %, respectively), while 94.7 ± 2.9 % of the vacuoles containing heat-killed bacteria colocalized with the probe. Similar results were obtained

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**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 µM 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 ΔF508 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 ΔF508 CFTR) (Fig. 4c). Although there was an apparent lower trend in the percentage of bacteria adhering to IB3 ΔCFTR 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

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**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 ± SEs are shown. In (c): black bars, IB3 WT CFTR; white bars, IB3 ΔF508 CFTR.
survival of *Brucella* species (Fernandez-Prada et al., 2003; Porte et al., 2003; Rajasheka 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 *Burkholderia 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 *Burkholderia cenocepacia* after internalization into the macrophages. Our results also showed that surface O antigen interfere with bacterial adherence 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 *Burkholderia 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 *Burkholderia cepacia* 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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