BACTERIAL PATHOGENICITY

Lipopolysaccharide chemotypes of Burkholderia cepacia

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Burkholderia cepacia is an important pathogen in patients with cystic fibrosis (CF) and much is now known of its epidemiology. In contrast, its virulence mechanisms are poorly understood. The lipopolysaccharide (LPS) of B. cepacia, a well-recognised virulence factor of other gram-negative bacteria, is known to be strongly endotoxic in vitro. The aim of this study was to observe if there were any links between the structure of B. cepacia LPS and virulence. This has been investigated by polyacrylamide gel electrophoresis and immunoblotting to define the chemotype and antigenic cross reactivity of B. cepacia LPS. Strains (16) belonging to different genomovars of the B. cepacia complex were selected to represent epidemic and non-epidemic clinical isolates and environmental strains. All strains belonging to genomovars I and II (the latter now renamed B. multivorans) had smooth LPS. However, isolates belonging to genomovar III, the group to which most of the epidemic CF isolates belong – including the highly transmissible strain (ET12) which has been found in both the UK and North America – were of either rough or smooth LPS chemotype. In this study, B. cepacia 52315 represents the ET 12 lineage, and has a rough chemotype. Rabbit antiserum raised to strain 52315 revealed that the LPS core of this strain was antigenically related to some but not all other genomovar III strains, but it also cross-reacted strongly with all B. multivorans (genomovar II) and most genomovar I strains. Intra-strain phenotypic variation was demonstrated between bacteria grown in broth or on solid agar with a concomitant variation in antigenic cross reactivity. There was no clear evidence to associate any particular LPS phenotype with epidemic or non-epidemic strains, but changes in phenotype in vitro may provide clues to the survival and adaptability of B. cepacia in hostile environments and possibly to its ability to produce an inflammatory response in vivo.

Introduction

Chronic infection by Burkholderia cepacia is a major cause of lung disease in patients with cystic fibrosis (CF) [1]. Manifestations of B. cepacia infections are varied and include asymptomatic carriage, a gradual decline in lung function, and 'cepacia syndrome' – a rapid fatal decline in lung function, accompanied by necrotising pneumonia and occasionally bacteraemic complications – which affects c. 20% of CF patients [2]. Other causes for concern are the inherent resistance of B. cepacia to most antibiotics [3], person-to-person spread of highly transmissible strains [4], and its persistence, despite a specific and pronounced antibody response [5, 6].

Of the few recognised virulence factors produced by B. cepacia [7, 8], lipopolysaccharide (LPS) is well known for its potent immuno-stimulatory effects on mononuclear cells, granulocytes and B-lymphocytes with the production of pro-inflammatory cytokines [9]. Whereas activation of the immune cells is necessary for the development and regulation of the host immune response, overproduction of cytokines can lead to severe complications. Grealy et al. [10] suggested that the cytokine response of CF patients with Pseudomonas aeruginosa infection contributed to airway inflammation associated with acute lung injury. Furthermore, it has been shown that LPS preparations from B. cepacia exhibited greater endotoxic activity and cytokine response in vitro than preparations from P.
aeruginosa [11, 12]. B. cepacia isolates from CF patients may express either the rough (R) or smooth (S) LPS chemotype, whereas the majority of B. cepacia isolates from other clinical conditions or from the environment generally express S-LPS [7].

Prior to 1990, the genus Pseudomonas comprised a diverse collection of several hundred saprophytic and pathogenic bacteria, divided into five RNA homology groups. Early evidence from molecular analyses of pseudomonads led to the recognition of the RNA group II as a new genus – Burkholderia – with B. cepacia as the type species [13]. Subsequently, Vandamme [14] proposed a subdivision of B. cepacia into five genomovars based on analyses of fatty acid profiles, protein content and DNA–DNA and DNA–rRNA hybridisation. Of these genomovars, the first three have generally contained non-transmissible environmental isolates. Genomovars II (recently renamed B. multivorans) [15], and III contain strains responsible for major outbreaks in CF clinics. Current evidence suggests that strains associated with the most severe clinical outcomes (including cepacia syndrome) in CF populations from various geographic locations belong to genomovar III. The highly transmissible isolate J2315, which represents the transatlantic ET12 lineage [1], belongs to genomovar III and is recognised as an index strain. Strains from genomovars IV and V have occasionally been isolated from CF patients, but their clinical importance is unclear. B. cepacia genomovar V is now recognised as a new species – B. vietnamiensis [16]. At present the group of five closely related genomovars is referred to as the B. cepacia complex.

The archetypal CF pathogen P. aeruginosa isolated from environmental sources and from acutely infected patients has an S-LPS phenotype, whereas isolates from chronic lung infections in CF patients usually express rough phenotypes [17]. A striking feature of the highly transmissible, transatlantic B. cepacia strain lineage responsible for 50% of UK isolates, represented by the index strain isolate (J2315), is its R-LPS phenotype [10]. While it is thought that P. aeruginosa strains undergo a S → R transition after colonising the CF lung [18], there is no evidence to date to suggest that B. cepacia undergoes the same S → R change in vivo. Studies on other bacterial species have shown that phenotypic plasticity in bacteria is influenced by many factors, including nutrient availability and the fact that they grow attached to a surface rather than planktonically in a liquid medium [18].

The aims of this study were to characterise the LPS phenotype (chemotype and antigenic relatedness) of a range of strains selected from the Edinburgh collection; these included isolates from environmental sources and clinical isolates from both CF and non-CF patients, to give a broad representation of strains from several genomovars. Any differences in LPS phenotype between bacteria grown in a liquid or on a solid medium were also investigated.

**Materials and methods**

**Bacteria and media**

The bacterial strains used in this study are described in Table 1. All isolates were identified as B. cepacia by the API20NE system (bioMérieux, France), and their individual clonality confirmed by pulse-field gel electrophoresis (CHEF, BioRad Laboratories) [19]. Bacteria were grown in nutrient broth (Oxoid) supplemented with yeast extract (Difco) 0.5% (NB + YE) at 37°C in an orbital incubator at 200 rpm overnight, and harvested by centrifugation. For surface growth on solid media, strains were cultured on nutrient agar (Oxoid) supplemented with yeast extract 0.5% (NA + YE).

**Preparation of LPS**

**Aqueous phenol extraction.** LPS was extracted from whole cells with aqueous phenol (45% w/w, 50 ml/g of dry bacteria) at 70°C for 15 min, by a method slightly modified from that described by Hancock and Poxton [20]. Briefly, after extraction, the mixture was cooled in ice, centrifuged (10,000 g, 15 min, 4°C), and the aqueous phase removed. The phenolic phase was washed with pyrogen-free water and re-centrifuged. The combined aqueous phases were dialysed against running tap water and lyophilised. Before analysis by SDS-PAGE, LPS was purified enzymically with proteinase K (Sigma): LPS was solubilised in double-strength sample buffer (2.5 mg/ml, 100 μl) and incubated with proteinase K (2.5 mg/ml, 50 μl) at 60°C for 2 h.

**Rapid phenol extraction.** The method of Fomsgaard et al. [21] was used to extract LPS from small volumes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Genomovar</th>
</tr>
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<tbody>
<tr>
<td>J2535</td>
<td>Botanical</td>
<td>I</td>
</tr>
<tr>
<td>J2540</td>
<td>Soil</td>
<td>I</td>
</tr>
<tr>
<td>J2552</td>
<td>Soil</td>
<td>I</td>
</tr>
<tr>
<td>J2395</td>
<td>Botanical</td>
<td>II</td>
</tr>
<tr>
<td>C1524</td>
<td>CF sputum</td>
<td>II*</td>
</tr>
<tr>
<td>C1576</td>
<td>CF sputum</td>
<td>II*</td>
</tr>
<tr>
<td>C1579</td>
<td>CF sputum</td>
<td>II*</td>
</tr>
<tr>
<td>C1962</td>
<td>Brain abscess</td>
<td>II*</td>
</tr>
<tr>
<td>C1335</td>
<td>CF sputum</td>
<td>III</td>
</tr>
<tr>
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<td>III</td>
</tr>
<tr>
<td>C1394</td>
<td>CF sputum</td>
<td>III</td>
</tr>
<tr>
<td>C1632</td>
<td>CF sputum</td>
<td>III</td>
</tr>
<tr>
<td>C1773</td>
<td>Blood</td>
<td>III</td>
</tr>
<tr>
<td>C2374</td>
<td>CF sputum</td>
<td>III</td>
</tr>
<tr>
<td>J415</td>
<td>CF sputum</td>
<td>III</td>
</tr>
<tr>
<td>J2315</td>
<td>CF sputum</td>
<td>III</td>
</tr>
<tr>
<td>J1750</td>
<td>CDC, Atlanta, Non-CF isolate</td>
<td>IV</td>
</tr>
</tbody>
</table>

*Genomovar II strains now renamed B. multivorans.

1. Index strains: transmissible epidemic strains in UK CF centres.
Overnight bacterial cultures (NB + YE, 20 ml) were centrifuged (10 000 g, 15 min, 4°C), and the pellets were re-suspended in pyrogen free water (500 µl) in an Eppendorf tube. Bacteria were scraped from NA + YE plates and c. 0.1 g was resuspended in pyrogen free water (500 µl) in an Eppendorf tube. Aqueous phenol (90% v/v, 500 µl) was added to each sample, vortex mixed for 10 s and heated to 70°C for 10 min. The mixture was cooled in ice and centrifuged (3 000 g, 4 min). The aqueous layer was removed and stored at -20°C.

**Analysis of LPS**

SDS-PAGE was performed in acrylamide 12% w/v gels with the Laemmli buffer system, but the SDS was omitted from the stacking and separating gel buffers.

![Image of SDS-PAGE gel](image.png)

**Fig. 1.** (a) Silver-stained PAGE (12% acrylamide) of aqueous phenol preparations of *B. cepacia* LPS. Lane 1, *Escherichia coli*; 2 and 3, strain J2315; 4 and 5, strain J415; 6 and 7, strain C1340; 8 and 9, strain J2540. (b) Immunoblot of aqueous phenol-extracted preparations of *B. cepacia* LPS probed with rabbit serum raised against *B. cepacia* J2315. Lanes 1 and 2, strain J2315; 3 and 4, strain J415; 5 and 6, strain C1340; 7 and 8, strain J2540.
Gels were oxidised with periodic acid (Sigma) and silver stained as described by Hancock and Poxton [20].

Immunoblotting

Antigens separated by PAGE (as above) were transferred on to nitrocellulose membranes (0.2-μm pore size, Schleicher and Schuell, Dassel), overnight by the method of Towbin et al. [22]. Blots were developed as described by Hancock and Poxton [20]. Antigens were probed with rabbit serum raised against the index strain. The immune complexes were detected with anti-rabbit IgG (Sigma)-horseradish peroxidase conjugates.

Results

LPS phenotypes of epidemic and non-epidemic strains

Silver-stained SDS-PAGE gels of LPS extracted by aqueous phenol from duplicate samples showed that non-clonally related epidemic strains belonging to genomovar III (J2315, C1340) possessed rough LPS, whereas the non-epidemic genomovar III strain (J415), and the botanical isolate from genomovar I (J2540) possessed smooth LPS (Fig. 1a). In the immunoblotting assay (Fig. 1b), serum from a rabbit immunised with whole cells of B. cepacia J2315 demonstrated a positive reaction with the low molecular mass LPS core of J2315 and C1340, and also the core and the series of low molecular mass LPS core plus oligosaccharide units of the genomovar I botanical isolate J2540. However, this serum did not react with the LPS of the non-epidemic genomovar III isolate J415.

Comparison of epidemic strains grown in broth and on solid medium

Silver-stained PAGE gels showed no difference in the LPS structure of the transatlantic epidemic strain (J2315) when grown in the different culture conditions (Fig. 2, lanes 1 and 2). However, the expression of the LPS structure of the Newcastle epidemic strain (C1632) was markedly influenced by culture conditions; organisms grown on solid agar expressed a series of higher molecular mass LPS bands that were not apparent in broth-grown organisms. Furthermore, strain C1632 did not exhibit cross-reactivity with the serum raised against J2315 (data not shown).

Comparison of B. cepacia LPS phenotypes from genomovars I, II, III and IV

Silver-stained SDS-PAGE gels of LPS extracted by the rapid phenol method from B. cepacia grown in NB + YE, and on NA + YE (Fig. 3) show that strains from genomovars I, II and IV (lanes 3–4, 5–9 and 1, respectively) were all of smooth LPS chemotype. In contrast, strains from genomovar III (lanes 10–14) expressed either the smooth or rough chemotype. When the LPS from the strains grown in NB + YE and NA + YE were compared, there were few differences between the LPS of genomovar II strains. However, strains from genomovar I and IV expressed more lower molecular mass LPS bands when grown on solid medium (NA + YE) compared with high molecular mass bands when grown in liquid (NB + YE). The lipid A and core (bottom band) of the rough LPS of genomovar III strains are clearly expressed when grown on NA + YE, but are not as apparent when grown in NB + YE, which expressed more of the smooth type LPS.

Immunoblots of the rapid phenol extracts showed some unexpected results (Fig. 4) in that rabbit serum raised against whole cells of the genomovar III strain (J2315) cross-reacted strongly with strains from genomovar II (Fig. 4b), variably with genomovars I and IV (Fig. 4a), but only weakly with other strains from genomovar III (Fig. 4c). In all strains the major cross-reacting band (RF c. 0.27) was expressed more on solid medium than in broth.

Discussion

B. cepacia is a life-threatening human pathogen for patients with chronic granulomatous disease and,
Fig. 3. Silver-stained PAGE of rapid phenol preparations of *B. cepacia* genomovar I (lanes 3 and 4), genomovar II (lanes 5–9), genomovar III (lanes 10–14) and IV (lane 2) grown (a) in NB + YE and (b) on NA + YE. Lane 1, *E. coli*; 2, strain J1750; 3, strain J2535; 4, strain J2552; 5, strain J2395; 6, strain C1524; 7, strain C1576; 8, strain C1579; 9, strain C1962; 10, strain C1395; 11, strain C1394; 12, strain C1632; 13, strain C1773; 14, strain C2374.
especially, those with CF. However, in contrast to the large amount of information available on the antimicrobial resistance and epidemiology of *B. cepacia*, knowledge of the virulence factors and pathogenesis is scanty [7, 23]. Several putative virulence factors have been associated with clinical isolates, including exoenzymes, siderophores, and exopolysaccharides, haemolysin and LPS [7, 8, 11, 12, 23]. However, the contribution of these factors to the pathogenesis of *B. cepacia* remains unclear.

The present study demonstrated that strains belonging to genomovar III could express either a rough or smooth LPS phenotype. This is in general agreement with previous observations and confirms that the transmissible strains of *B. cepacia* express rough LPS [11]. Strain C1632, isolated during the Newcastle epidemic, expresses smooth LPS; however, the phenotypic expression of the banding pattern of the high molecular mass material is dependent on culture conditions. Larsen et al. [24] reported that *P. (B.) cepacia* demonstrated marked phenotypic variability in outer-membrane protein (OMP) profiles when strains were isolated from different sites and at different times from a single colonised patient, and suggested that such intra-isolate variability may depend on variations in the micro-environment of the respiratory tract.

It is not unreasonable to predict that host environment parameters can also influence phenotypic variation in LPS expression, but investigation of such changes in *vitro* is hampered by the practical limitations of recreating in-vivo ionic content, concentration of essential nutrients and growth state of the organism [18]. It is believed that many organisms grow in *vitro* as a biofilm, attached to a surface, encased within a glycocalyx composed of bacterial exopolymers [25]. The aim of growing *B. cepacia* on agar in this study was to observe any changes in LPS phenotype with a change in growth state. Although there was no clear pattern in the variability of LPS expression between strains and their growth rates, some strains did express a different phenotype when grown on a solid surface, and the influence of adherence to host tissue on LPS expression in *vitro* should not be overlooked.

Immunoblots, with serum raised against the genomovar III index strain (J2315), showed that there was a large degree of cross-reactivity with the LPS of strains from genomovars I, II and IV. The classification of *B. cepacia* into the various genomovars [14] is independent of LPS phenotype; the LPS varies both within genomovars and between them. All genomovars have been isolated from CF patients, but cepacia syndrome and epidemic spread are most closely associated with genomovar III. This variability in infectivity and pathogenicity might suggest that LPS itself plays no role in infection, despite being a recognised virulence factor. However, it may be that it is simply the ability to express any LPS rather than the expression of certain phenotypes of LPS that is important once the organism has colonised the host. The bioactivity/endotoxicity of LPS from *B. cepacia* appears to be independent of strain [11, 12]. It is possible that the changes in LPS expression of certain strains may reflect what occurs in *vitro*, and it may be that different forms of LPS are better, or are less prone to neutralisation by antibody, for the induction of pro-inflammatory cytokine production from human mononuclear cells.

This study has also shown that apparent differences in LPS expression may depend on the method of extraction of the product. Immunoblots of LPS extracted by the rapid phenol method showed a strong band, of intermediate mol. wt that was not necessarily present on gels or blots of LPS extracted by aqueous phenol. The major difference between the two extraction methods is the inclusion of a protein digestion step in the latter [20]. Modification of the rapid phenol method to include such a step did not remove the band from subsequent gels or blots (data not shown), suggesting that the band was not proteinaceous. Reaction to serum raised against *B. cepacia* discounts the band as an artefact of the method, and its nature may merit further investigation.

In summary, the expression and phenotype of the LPS of *B. cepacia* is not related to genomovar, although the genomovar III strains are more likely to have a rough phenotype. For this reason LPS is unlikely to be a major factor in the transmission or colonisation of the host. However, once a clone has colonised the host the LPS is likely to contribute significantly to the immunopathology of the disease.

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References


