First structural characterization of *Burkholderia vietnamiensis* lipooligosaccharide from cystic fibrosis-associated lung transplantation strains

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This is the first structural elucidation of the lipooligosaccharide (LOS) endotoxin isolated from *Burkholderia vietnamiensis*, a clinically important member of *Burkholderia cepacia* complex, a group of over 10 opportunistic species that are highly problematic in cystic fibrosis. We have characterized a novel LOS structure extracted from two clonal strains of *B. vietnamiensis* isolated from a cystic fibrosis patient who underwent lung transplantation. Strains were selected from the pretransplantation and post-transplantation periods and endotoxin was extracted. Subsequent analysis interestingly revealed identical oligosaccharidic sequences, but variation in lipid A moieties. Further, both LOS fractions were tested for their immunostimulatory activity on human myelomonocytic U937 cells and for signaling on an HEK293 cell line stably expressing both TLR 4 and human MD-2. We observed an increase in lipid A acylation and a resultant increase in biological activity in bio-reporter assays of TNF-α secretion in the post-transplantation strain.

**Keywords:** Burkholderia vietnamiensis/cystic fibrosis/lipid A/ lipopolysaccharide/lung transplantation

**Introduction**

*Burkholderia vietnamiensis* is a Gram-negative bacterium initially characterized from a group of nitrogen-fixing bacteria colonizing the rhizosphere of rice cultivated in Vietnam (Gillis et al. 1995). Later this strain was found to correspond to the *genomovar* V species belonging to *Burkholderia cepacia* complex (Bcc) isolated from cystic fibrosis (CF) patients (Vandamme et al. 1997). Cystic fibrosis disease is caused by a genetic mutation that alters host pulmonary defenses, allowing colonization from a variety of opportunistic bacteria (Speer 2002). Among the major CF pathogens, *Pseudomonas aeruginosa* is one of the most common species infecting adult CF patients (Smith et al. 2006), whilst less prevalent *Burkholderia cepacia* complex is responsible for the most feared infections in CF (Mahenthiralingam et al. 2000; De Soyza, Morris et al. 2004). Infections by these strains are characterized by a high transmissibility and marked resistance to antibiotic therapies. Furthermore, there is a notorious acute and almost invariably fatal respiratory illness, named “*cepacia syndrome*,” that is unique to Bcc infections (De Soyza and Corris 2003). Interestingly, this syndrome has many features suggestive of an endotoxic shock syndrome with hypotension, fevers, and is refractory in almost all cases to antibiotics even when sensitivity testing points toward a clinically relevant antimicrobial combination. The Bcc is constituted by over 10 related species, genomovars, that can be distinguished by phenotype and/or genotypic studies. The most prevalent clinical species are *B. cepacia* and *B. multivorans* with *B. vietnamiensis* the third most prevalent Bcc genomovar (Jones et al. 2004). In patients with advanced CF lung disease, lung transplantation is the only treatment that improves both the quantity and quality of life. A key difference between the above three most prevalent Bcc genomovars is the good outcomes of lung transplantation for patients pretransplantation infections with either *B. multivorans* or *B. vietnamiensis* whilst the outcomes for *B. cepacia*-infected patients have been very poor at most transplant centers (De Soyza et al. 2001). Understanding why these divergent clinical outcomes arise may allow new therapeutic strategies to emerge to help improve transplant outcomes. Identifying differences in the biology of *B. vietnamiensis* as compared to *B. cepacia* may help this understanding to emerge. As recently reviewed, a number of virulence factors may account for these divergent clinical outcomes (Mahenthiralingam et al. 2000). The major virulence factors of Gram-negative bacteria are the glycolipid molecules located on the outer leaftlet of the outer membrane and are called lipopolysaccharides (LPSs) (De Soyza et al. 2008). These molecules constitute about 75% of the outer membrane of bacteria and are essential for Gram-negative survival. They have common structural motifs composed of a hydrophilic heteropolysaccharide (formed by a core oligosaccharide and an O-specific polysaccharide) covalently linked to a lipophilic moiety termed lipid A, which anchors these macromolecules to the membrane through electrostatic and hydrophobic interactions (Alexander and Rietschel 2001). Of these three chemically different regions coded by genetically distinct sets of genes, LPSs can only lack the *O*-chain and be distinguished in lipooligosaccharides (LOSs) or rough-type LPSs (R-LPSs). Lipid A possesses a rather conservative structure consisting of a β-(1→6)-glucosamine disaccharide backbone phosphorylated
Lipopolysaccharide structure from *Burkholderia vietnamiensis* at positions 1 and 4′ and acylated with primary 3-hydroxy fatty acids at positions 2 and 3 of both GlcN residues; the hydroxyl groups of the primary fatty acids can be further acylated by secondary acyl moieties. In the core oligosaccharide, it is possible to identify an inner and an outer moiety; the inner core links to lipid A and it is constituted by typical monosaccharides as heptopyranoses and 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo). In addition to their essential structural function, LPSs have an important role in the elicitation of host innate immune responses. LPS lipid A, together with other microbial cell wall components such as teichoic acids, peptidoglycan, and flagellin, is identified as pathogen-associated molecular patterns (PAMPs) by the pathogen recognition receptors of the host innate immune system (Alexander and Rietschel 2001; Akira et al. 2006). In particular, LPS lipid A is able to induce a marked inflammatory response utilizing MD2/TLR 4-mediated signaling pathways. LPSs extracted from the different Bcc genomovars demonstrate divergent biological activity (De Soyza et al. 2008). Thus, endotoxin structural characterization is an essential requirement to understand the structure to biological activity relationships.

Herein, we define the complete structure and proinflammatory activity of the endotoxin from *B. vietnamiensis* extracted from clonal strains isolated from a cystic fibrosis patient pre- and postlung transplantation. Chemical data are a fundamental prerequisite for a complete understanding of elements driving the inflammatory process. Since the endotoxin analyzed belongs to clonal strains isolated in CF airways pre- and postlung transplantation, we can pursue the hypothesis that endotoxin structural changes through chemical modulation of LPS alters innate immunity responses, i.e., a bacterial mechanism of adaptation to different biological niches such as the end-stage CF lung as compared to the normal lung allograft.

**Results**

During our analysis, we identified all recipients who had Bcc infection prior to transplantation. Bacterial lysates were prepared as previously (De Soyza, Morris, et al. 2004) for pilot experiments to assess LPS migration patterns on silver-stained SDS gels. Paired strains known to be of clonal origin based on pulsed-field gel electrophoresis experiments demonstrating alteration in migration patterns were then prepared for LOS extraction (De Soyza, Ellis, et al. 2004). A number of strains demonstrated this behavior including the *B. vietnamiensis* strain studied herein.

Isolation, SDS electrophoresis analysis, and biological activity of *B. vietnamiensis* LOSs

LPS was isolated according to the usual hot phenol–water protocol (Westphal and Jann 1965). SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) of the *B. vietnamiensis* LPS fraction revealed that the extracted *B. vietnamiensis* lipopolysaccharide was a rough-type LPS, i.e., a lipooligosaccharide (LOS). It was tested for its proinflammatory activity in eliciting TNF-α induction from human myelomonocytic U937 cells (Figure 1A). These data demonstrated that statistically significantly higher TNF-α was induced by LOS extracted from the post-transplantation *B. vietnamiensis* strain. This is in contrast...
to our prior data with *B. multivorans* that demonstrated a clear decrease in biological activity (TNF-α induction capacity) of the post-transplantation strain (Ieranò et al. 2008). Our observation of increased TNF-α induction in the post-transplantation *B. vietnamiensis* strain was also replicated in a separate CF patients’ paired *B. vietnamiensis* strain that was not structurally characterized in the current study. In these U937 macrophage stimulation experiments, the extracted *B. vietnamiensis* LOS was generally less potent at TNF-α induction than the control *Escherichia coli* O55 LPS.

**Confirmation of LOS and lipid A biological activity by HEK 293 cell TLR4/MD2 transfection**

HEK 293 cell lines do not express Toll-like receptors but can be specifically transfected with TLRs of interest and thus used to dissect proinflammatory signaling. The HEK cells after transfection with TLR 4 and the co-signaling molecule MD2 were stimulated in quadruplicate, using doses of 10 ng and 100 ng of either LOS or lipid A. The effect of stimulation was assessed using a NF-κB reporter assay measured in relative light units (RLU). Stimulation with intact LOS elicited a greater response than with extracted lipid A suggesting a proinflammatory role for LOS core (Figure 1B and C). Importantly, the intact LOS was as strongly proinflammatory as the control *E. coli* in the HEK293 assay. The pattern of increased RLU induction (proinflammatory activity) induced by post-transplantation LOS (Figure 1B) was not clearly demonstrated for the extracted post-transplantation *B. vietnamiensis* lipid A (Figure 1C). Irrespective of this the markedly decreased RLU induction activity observed in the *B. multivorans* strains we previously studied (Ieranò et al. 2008) was not observed for either of paired *B. vietnamiensis* strains studied.

**Compositional analysis of LOSs from *B. vietnamiensis* pre- and post-transplantation**

Monosaccharide analysis yielded same results either for LOS-isolated pre- or post-transplantation. All the results obtained on the products analyzed are reported in Table I. Fatty acids analysis revealed in both cases the presence of (R)-3-hydroxyhexadecanoic (16:0 (3-OH)) in amide linkage and (R)-3-hydroxytetradecanoic (14:0 (3-OH)) acid and tetradecanoic acid (14:0) in ester linkage. The overall chemical composition of lipid A matched with the archetypal structure of *Burkholderia* lipid A (De Soyza et al. 2008).

**Isolation and structural characterization of oligosaccharide fractions from *B. vietnamiensis* pre- and post-transplantation LOS**

A mild acid hydrolysis promoted by the acetate buffer, carried out on pre- and post-transplantation LOSs molecules, yielded in both cases an oligosaccharide fraction that was purified on gel-permeation chromatography. Monosaccharide analysis of both fractions (reported in Table I) and 1D NMR analysis gave the evidence of two identical oligosaccharides’ portions isolated from the two LOSs analyzed.

A combination of homo- and heteronuclear 2D NMR experiments (DQF-COSY, TOCSY, ROESY, $^1$H-13C HSQC, $^1$H-13C HSQC-TOCSY, and $^1$H,13C HMBC) was executed on the most abundant product, i.e., the oligosaccharide fraction isolated postlung transplantation, in order to assign all the spin systems and to define the monosaccharide sequence. In the anomeric region of the $^1$H-NMR spectrum (Figure 2A), 10 anomeric signals were identified (A-L, Table II); the signals at 1.97/2.01 ppm were endorsed as the H-3-methylene protons of the Kdo residue. The relative intensities and the shifts of anomeric signals suggested a marked heterogeneity typical of a mixture of oligosaccharides, likely due to the presence of non-stoichiometric carbohydrate substitutions and to the presence of a reducing end.

Spin systems A, B, D, E, F (Table II) were all identified as α-heptose residues, as indicated by their $^3$J$_{H1,H2}$ and $^3$J$_{H2,H3}$ coupling constants (below 3 Hz) and by the intra-residual nuclear Overhauser effect (NOE) of H-1 with H-2. The $^{13}$C chemical shift values of C-6 of these heptose residues (all below 71 ppm) confirmed these as L-glycero-D-manno-heptose, in accordance with the chemical analysis.

Spin systems C and G (H-1 at 4.97 and 4.70 ppm, respectively) were identified as 2-deoxy-2-acetamido-galactose, as indicated by their $^3$J$_{H3,H4}$ and $^3$J$_{H4,H5}$ values (3 Hz and 1 Hz, respectively) diagnostic of a galacto-configuration. The $^1$H-$^{13}$C HSQC (heteronuclear single quantum coherence) spectrum showed the correlation of both H-2 C (4.29 ppm) and H-2 G (4.00 ppm) with nitrogen-bearing carbon signals at 47.9 ppm and at 51.38 ppm, respectively. The down-field shifts of proton resonances of both H-2 were diagnostic of N-acetylation at these positions. The chemical shifts of H-1 and C-1 of residue C (4.97 and 92.63 ppm), the $^3$J$_{H1,H2}$ coupling constant (3.2 Hz), and the intra-residual NOE contact of H-1 with H-2 were all in agreement with α-anomeric configuration of residue C; the chemical shifts of H-1 and C-1 of residue G (4.70 and 100.6 ppm, respectively), the $^3$J$_{H1,H2}$ value (8.2 Hz), and the intra-residual NOE contact of H-1 with H-3 and H-5 evidenced a β-anomeric configuration for this residue.

Spin system I (Table II) was identified as glucose, as indicated by the large $^3$J$_{H,H}$ values of ring protons (above 10 Hz). The strong intra-residue NOE contacts of H-1 with H-3 and H-5 together and the $^3$J$_{H1,H2}$ coupling constant (7.8 Hz) were diagnostic of β-anomeric configuration.

Residue L was identified as a β-galactose as attested by $^3$J$_{H3,H4}$ and $^3$J$_{H4,H5}$ low values, and by the presence of the intra-residual NOE contact of H-1 with H-3 and H-5.

Residue H (H-1 at 4.69 ppm) was recognized as an α-rhamnose residue since, in total correlation spectroscopy (TOCSY) spectrum, scalar correlations of the ring protons with methyl signals in the shielded region at 1.16 ppm were visible. The manno-configuration of these spin system was established by $^3$J$_{H1,H2}$ and $^3$J$_{H2,H3}$ values (below 3 Hz) whereas the α-configuration was assigned by the intra-residual NOE contact of H-1 with H-2 and chemical shift of its H-5 and C-5.

The spin system of Kdo K has been assigned starting from the diastereotopic H-3 methylene proton signals, resonating at 1.97 and 2.01 ppm (H-3$_{ax}$ and H-3$_{eq}$, respectively). Because of its free reducing end, the Kdo residue was present in multiple forms; nevertheless, the signals belonging to the α-reducing unit were clearly assignable. Residue J, D-glycero-D-talo-oct-2-ulosonic acid (Ko) was detected by the presence of the characteristic inter-residue NOE contact, in 2D ROESY (rotating frame Overhauser enhancement spectroscopy) spectra, between H$_{3eq}$ of Kdo moiety K and H-6 of Ko (J) that is also
diagnostic for the α-D-Ko-(2→4)-α-D-Kdo linkage (Birnbaum et al. 1987; Bock et al. 1994; Isshiki et al. 1998). Also as a consequence of Kdo heterogeneity, the close Ko residue was present as multiple spin systems and only the major one has been fully assigned.

The oligosaccharide sequence was established on the basis of the interresidual NOE contacts identified in the ROESY spectrum (Figure 2B) and the long-range scalar correlations present in the heteronuclear multiple bond correlation (HMBC) spectrum (not shown).

The linkage of the heptose B to O-5 of Kdo K was proven by the NOE connectivity between H-1 of the heptose B (5.18 ppm) and H-5 of Kdo (4.13 ppm). Given the L,D relative configuration of heptose B, the presence of further NOE contacts between H-1 of the heptose B and H-6 and H-7 of K do ultimately proved d-configuration for the Kdo residue (Bock et al. 1994).

Residue B was in turn substituted at O-3 and O-4. The NOE contacts (Figure 2B) of H-4 and H-3 B, with H-1 of residue I (4.45 ppm), evidenced that the O-4 of α-heptose B was glycosylated by residue I of β-glucose. Residue B was also substituted at O-3 by residue A of α-heptose, according to the NOEs (Figure 2B) of H-3 and H-2 B with H-1 A (5.29 ppm).

Unit A was glycosylated at O-2 by the rhamnose residue H, as demonstrated by the NOE contacts of H-1 H (4.69 ppm) with H-1 and H-2 A (5.29 and 4.13 ppm). Residue A was also substituted at O-3 by the β-GalNAc G as confirmed by the strong NOE contacts between H-1 G and H-3 A. Eventually, residue A was also glycosylated at O-7 by the terminal α-heptose F, as attested by the NOE contact between H-1 F and H-7 A. An alternative spin system was identifiable for residue F, namely residue E, recognized as a 7-α-substituted heptose that was, in turn, non stoichiometrically glycosylated at position 7 by the α-heptose D, as shown by the NOE contact of H-7 E (3.61 ppm) with H-1 D (4.81 ppm).

β-GalNAc residue G was glycosylated at O-3 by residue C as attested by the NOE contact of H-3 G with H-1 of residue C. Additionally, residue C was glycosylated at position 3 by the terminal β-Gal L as proven by the NOE contact between H-1 of residue L and H-3 of residue C.

Thus, in summary, methylation analysis, MS (see below), and 2D NMR data allowed us to establish the overall oligosaccharide sequence for the post-transplantation LPS, and the sequence is reported below:

\[
\text{(L)} \quad \text{(C)} \quad \text{(G)}
\]

\[
\beta\text{-Gal-(1→3)-α-GalNAc-(1→3)-β-GalNAc}
\]

\[
\text{(D)} \quad \text{(E/F)} \quad \text{(A)} \quad \text{(B)} \quad \text{(K)}
\]

\[
\alpha\text{-Hep-(1→7)-α-Hep-(1→7)-α-Hep-(1→3)-α-Hep-(1→5)-α-Kdo}_{\text{red}}
\]

\[
\uparrow\quad 4\quad 4\quad 4
\]

\[
1\quad 1\quad 2
\]

\[
\alpha\text{-Rha} \quad \beta\text{-Glc} \quad \alpha\text{-Ko}
\]

\[
\text{(H)} \quad \text{(I)} \quad \text{(J)}
\]
GlcN II, was further substituted by a secondary 14:0 fatty acid (see below). Species \( L_2 \) at \( m/z \) 1574.5 (\( \Delta m/z \equiv 131 \) from \( L_1 \)) and \( L_3 \) at \( m/z \) 1706.4 (\( \Delta m/z \equiv 131 \) from \( L_2 \)) were tetra-acylated lipid A carrying one and two Ara4N residues. Species \( L_4 \) (\( m/z \) 1670.5), \( L_5 \) (\( m/z \) 1801.4), and \( L_6 \) (\( m/z \) 1931.5) were consistent with the penta-acylated lipid A carrying two ester-linked 14:0 (3-OH) with no, one, and or two Ara4N residues, respectively.

In analogy with other lipid A from *Burkholderia*, the position of the secondary fatty acid 14:0 on the GlcN II was identified on the GlcN II by mass spectrometry analysis of the lipid A moiety after acetate buffer hydrolysis. Briefly, the positive-ion MALDI mass spectrum (not shown) presented an in-source fragmentation due to the split of the glycosidic linkage between the two GlcN units, thus giving rise to a triacylated oxonium ion at \( m/z \) 933.1 carrying one 14:0 (3-OH), one 14:0, and one 16:0 (3-OH) residue at the nonreducing GlcN unit. The information was then completed by analyses of the compound obtained by with ammonium hydroxide hydrolysis (Silipo et al. 2002).

The assignment of the main LOS molecular ions (Figure 3B) resulted from the combination of the lipid A moieties and the core oligosaccharide species. Species LOS composed by tetra-acylated lipid A and OS were found at \( m/z \) 3353.4 (\( L_1 + OS \)), \( m/z \) 3484.5 (\( L_2 + OS \)), \( m/z \) 3615.8 (\( L_3 + OS \)), \( m/z \) 3677.3 (\( L_2 + OS + Hep \)), and \( m/z \) 3808.0 (\( L_3 + OS + Hep \)). Species constituted by penta-acylated lipid A and OS were also present at \( m/z \) 3711.5 (\( L_5 + OS \)), 3842.7 (\( L_6 + OS \)), 3903.6 (\( L_5 + OS + Hep \)), 4034.6 (\( L_6 + OS + Hep \)), and 4226.5 (matching with \( L_6 + OS + 2Hep \)).
Lipopolysaccharide structure from *Burkholderia vietnamiensis*

Table II. $^1$H and $^{13}$C NMR chemical shifts (ppm) of sugar residues of the core region of the LOS extracted from *B. vietnamiensis* strain isolated post-lung transplantation

<table>
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<th>Unit</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<td>A</td>
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<td>4.13</td>
<td>3.97</td>
<td>3.88</td>
<td>3.64</td>
<td>3.96</td>
<td>3.60</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>5.18</td>
<td>3.95</td>
<td>4.03</td>
<td>4.20</td>
<td>4.11</td>
<td>4.39</td>
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</tr>
<tr>
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<td>4.29</td>
<td>3.67</td>
<td>4.16</td>
<td>3.70</td>
<td>3.84/3.72</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D</td>
<td>92.6</td>
<td>47.9</td>
<td>77.6</td>
<td>68.5</td>
<td>71.2</td>
<td>61.5</td>
<td>–</td>
<td>–</td>
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<tr>
<td>t-α-Hep</td>
<td>101.8</td>
<td>70.0</td>
<td>70.4</td>
<td>70.4</td>
<td>61.9</td>
<td>68.4</td>
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<td>E</td>
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<td>3.72</td>
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<td>3.61</td>
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<td>3.38</td>
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<td>3.63/3.70</td>
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<td>3.91</td>
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<td>3.97</td>
<td>3.88/3.68</td>
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<td>n.d.</td>
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<td>71.8</td>
<td>63.8</td>
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<td>–</td>
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<td>4.00</td>
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<td>3.92</td>
<td>3.89/3.67</td>
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<tr>
<td>4,5-α-Kdo</td>
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<td>n.d.</td>
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<td>71.6</td>
<td>68.4</td>
<td>71.5</td>
<td>71.8</td>
<td>63.7</td>
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n.d., not detected.

**Fig. 3.** Negative-ion MALDI mass spectrum of intact LOS isolated pre-lung transplantation. (A) Low mass range (1200–2500 m/z), (B) high mass range (2800–5000 m/z).

**Structural characterization by MALDI-MS of the intact LOS from *B. vietnamiensis* post-transplantation**

The same MALDI-MS analysis was carried out on intact LOS isolated after lung transplantation (Figure 4). Even in this case, ions related to fragments core and lipid A were visible in the mass range between 1400 and 2300 m/z (Figure 4A). As for pre-transplantation LOS, the same oligosaccharide species were found, with the reference OS (at m/z 1907) and other ion peaks differing by one or two additional Hep residues. Conversely, post-transplantation LOS lipid A was constituted by tetra- and penta-acylated species in a very different relative amount if compared to pre-transplantation lipid A. Predominantly, penta-acylated species were present, and in particular $L_4$ (m/z 1670.5), $L_5$ (m/z 1801.5), and $L_6$ (m/z 1932.6) bearing no, one, or two Ara4N residues. $L_1$ and $L_2$ tetra-acylated lipid A were present only in few low amount whereas $L_3$ species, with two Ara4N residues, was even not detectable.

Consequently, the MALDI-MS profile of post-transplantation LOS molecular ions (Figure 4B) showed a peak distribution largely different from that found in the pre-transplantation strains since it mainly resulted by the combination of OS with penta-acylated lipid A ($L_4$ + OS) at m/z 3580.0, $L_5$ + OS at m/z 3711.0, and $L_6$ + OS at m/z 3842.4. $L_5$ + OS + Hep at m/z 3903.2, $L_6$ + OS + Hep at m/z 4034.5, $L_5$ + OS + 2Hep at m/z 4095.8, and $L_6$ + OS + 2Hep at m/z 4226.5. The ion species due to the combination of tetra-acylated lipid A $L_2$ and OS were only present in little amount.

Summarizing, MS analysis on the intact LOSs definitely confirmed the structural hypotheses and allowed the full assignment of the LOS structures of both pre- and post-transplantation strains.

**Discussion**

This is the first report of the endotoxin structure of a clinical isolate of *B. vietnamiensis*, a member of *Burkholderia cepacia*
complex, also known as genovar V. This strain produces a rough-type LPS as it only produces a lipo polysaccharide. As the cystic fibrosis milieu appears to cause specific LPS/LOS changes (Ernst et al. 1999; Ieranò et al. 2008), we have used our lung transplantation resource to further study the host environment: bacterial adaptation paradigm. We have analyzed two LOS extracted from clonal strains of *B. vietnamiensis*: one isolated from a CF patient prior to transplantation (interacting with CF epithelium) and its clonal strain isolated post-transplantation (interacting with normal donor lung epithelium).

From our complete structural analysis results, we demonstrate that the two clonal *B. vietnamiensis* LOS possess the same core oligosaccharide structure. This oligosaccharidic sequence has characteristic and highly conserved motifs already found in other *Burkholderia* LOS (De Soyza et al. 2008) that clearly suggests a common biosynthetic pathway shared between these related but genetically distinct bacteria. The inner core is constituted by the common motif:

$$\text{Hep-(1→3)-[β-Glc-(1→4)]-α-Hep-(1→5)}$$

$$\text{-[α-Ko-(2→4)]-α-Kdo}.$$  

In this *B. vietnamiensis* strain, the latter heptose residue is glycosylated at C-2, C-3, and C-7, as previously noted for *B. multivorans* (Ieranò et al. 2008); however, the heptose carries at C-7 an additional heptose disaccharide, as found in *B. cenocepacia* ET-12 LOS (Silipo et al. 2007).

It is very likely that, as in *B. multivorans* LOS, the trisaccharide [β-D-Gal-(1→3)-α-D-GalNAc-(1→3)-β-D-GalNAc] is a reminder of the O-polysaccharide that is directly linked to the C-2 of the heptose (Ieranò et al. 2008). In fact, 2-deoxy-2-acetamido sugar functions as a primer at the nonreducing end of the core oligosaccharide region and its transfer to a lipid carrier initiates the O-antigen synthesis in O-polysaccharides that are synthesized by the Wzy-dependent pathway (Perez et al. 2008).

The composition of the *B. vietnamiensis* lipid A moiety is consistent with our prior reports of other *Burkholderia* lipid A species, which also display tetra and penta-acylated lipid A bearing Ara4N residues (De Soyza et al. 2008). The substitution of Ara4N in LPS molecules is essential for bacterial survival (Ortega et al. 2007) since it prevents the interactions with antibiotic compounds and host antimicrobial peptides. These residues are positively charged under physiological conditions and so they reduce the net charge surface on the bacterial membrane. Preventing the ionic attraction, the presence of these residues avoids membrane permeability induced by positively charged antimicrobial peptides.

Interestingly, if we compare lipid A moiety of the pretransplantation strain with the lipid A moiety of post-transplantation strain, we find that the most abundant species in each LOS analyzed have a different acylation pattern. The pre-transplantation strains’ LOS consists of a blend of tetra and penta-acylated lipid A with the same relative abundance that can carry one or two Ara4N residues. Conversely, the post-transplantation strain mainly comprises penta-acylated lipid A, bearing one or two Ara4N, with tetraacylated species present in minimal amounts. Biological tests performed on U937 myelomonocytic cells stimulated by the pure endotoxins analyzed reveal a modest but not significant difference between the proinflammatory activities of the two LOSs. We observed that LOS post-transplantation induces similar or greater levels of TNF-α secretion. This appears in contrast with our observations seen in clonal *B. multivorans* following transplantation where the post-transplantation strain was associated with a shift toward less acylated lipid A and a significant reduction in TNF-α induction capacity (Ieranò et al. 2008). The current study and our prior data correlates well with the chemical differences noted in lipid A, as it is generally found, a higher degree of lipid A acylation is related to more potent proinflammatory activity. Thus, the penta-acylated lipid A species (the most acylated species) produced by *B. vietnamiensis* is generally more strongly proinflammatory whereas the (pre-transplant) tetra-acylated species has statistically less biological activity in terms of TNF-α induction in the U937 assays. In the HEK 293 cell NF-κB bio-reporter assays, an increase in biological activity was seen for the post-transplantation strain as compared to the pre-transplantation strain when intact LOS was used as a stimulant but not when with the extracted lipid A stimulant. The reasons for this are unclear and require further studies.

The differences between the two *B. vietnamiensis* clonal strains analyzed was limited to alterations in lipid A moieties, in contrast to the changes in both lipid A and core oligosaccharide we found when comparing *B. multivorans* LOS isolated from clonal strains pre- and post-transplantation (Ieranò et al. 2008). Lipid A is directly involved in cellular interactions between bacteria and environment, and thus adaptation to allow bacteria to survive in new physiological conditions is biologically plausible.

Taken in combination the recent reports of *Burkholderia cepacia* complex lipid A from *B. cepacia* genovar I, *B. multivorans*, *B. cenocepacia* (Silipo et al. 2006, 2007; De Soyza et al. 2008; Ieranò et al. 2008), and in this study *B. vietnamiensis* reveal some important structure to biological activity
correlations. Firstly, the structure of lipid A moieties has similarities in all of the Bcc strains studied so far with shared lipid A species between most if not all reported strains (De Soyza et al. 2008). The higher relative abundance of heavily (penta)-acylated species appears most clearly seen in *B. cenocepacia* though the limited number of strains tested limits further conclusions. Nevertheless, it appears consistent from the available data that higher acylation present in genomovars or strains is generally associated with more virulence/proinflammatory activity. Furthermore, all of the Bcc strains studied so far have structural motifs, such as aminoarabinose residues and penta-acylated lipid A, that have been reported as being “unique” to cystic fibrosis-associated *P. aeruginosa* from chronically infected CF patients. These changes appear to take many years to occur (Ernst et al. 1999; Smith et al. 2006) yet a consistent feature of our recent work is that the post-transplantation environment is associated with much more rapid changes in LPS structural motifs. This rapid ability of organisms to adapt LOS may be therapeutically useful if the control mechanisms determining LOS acylation can be manipulated to cause a less inflammatory LOS structure in patients with cystic fibrosis.

Our current observation, however, do not explain why the members of the Bcc exhibit different biological characteristics following transplantation with the *B. vietnamiensis* increasing acylation (move toward a penta-acylated LOS and a generally more inflammatory phenotype) whilst *B. multivorans* decreasing LOS acylation following transplantation (less inflammatory phenotype). Understanding if these observations are simple random behavior in selected strains or truly divergent biological responses between bacteria in the face of marked changes in host environment requires more study.

Structural elucidation of bacterial virulence factors is the essential step in the comprehension of inflammatory mechanisms. The comparison between the endotoxins extracted from two clonal strains living in different habitat conditions, e.g., CF lung epithelia versus normal airway (allograft) epithelia post-lung transplant environment, should be a way to understand molecular basis that allow bacterial adaptation. It is hoped that better understanding of how bacteria adapt to their environment and how to manipulate this therapeutically may be important steps in fighting these aggressive and feared infections.

**Material and methods**

**Patient selection**

Cystic fibrosis patients were listed for transplantation according to international guidelines. Patients who had positive microbiological cultures for the *B. cepacia* complex with paired isolates pre- and post-transplantation recoverable from storage media were considered eligible for study selection.

**Strain preparation and selection**

Sputum was collected from patients immediately before surgery. Post-transplantation bronchoalveolar lavages were collected from recipients at day 7 post-op. Presumed *B. cepacia* complex bacteria were isolated by culture. Phenotypic analyses were performed using the API 20NE diagnostic test (Biomérieux, Marcy l’Etoile, France). Strains were selected for structural analysis where differences were noted in LPS migration patterns upon silver staining of 16% SDS-gels. For this study, we restricted investigations to *B. vietnamiensis* strains. The pre-transplantation strain was isolated immediately pre-transplant and the post-transplantation strains were isolated at 1 week after transplantation. Only one of the paired strains was fully structurally characterized but two patients with paired strains had LPS extracted and assessed in bio-reporter assays.

**Pulsed-field gel electrophoresis**

Confirmation of the clonal nature of paired pre- and post-transplant strains has previously been reported (De Soyza, Ellis, et al. 2004). *B. cepacia* complex strains were blinded to the investigators and genotyped by macrorestriction of whole genomic DNA with the restriction enzyme Spel (New England Biolabs, UK), followed by separation of the fragments by pulse-field gel electrophoresis (PFGE) (CHEF DRII system; Bio-Rad).

**Bacterial growth and LPS extraction**

Bacterial strains isolated from recipients were stored on microbeads. These were streaked on to LB-agar. Standard hot phenol/water LPS extraction was undertaken as previously described (Westphal and Jann 1965). For large volume, extraction strains were grown in 5 × 1L cultures of nutrient broth containing 0.5% yeast extract (DIFCO, Oxford, UK) 37°C to late log phase. This growth was harvested by centrifugation at 1000 × g and resuspended in minimal volume of distilled water and freeze-dried. The pellets were then resuspended in distilled water and sonicated on ice as previously. The resulting sonicated suspension was then subjected to DNAse II digestion (final concentration 200 μg/mL) at 37°C for 2 h. A final digest with proteinase K, final concentration 2 mg/mL, was undertaken at 60°C for 2 h prior to boiling. This was then mixed with hot phenol for 20 min at 70°C, cooled on ice then centrifuged at 800 × g. The water soluble phase was then removed and dia- lyzed against repeated changes of fresh distilled water for 72 h. Ultracentrifugation at 39,500 × g for 16 h at 13°C was then undertaken and the supernatant was subsequently removed. The remaining pellet was dissolved in a minimal volume of distilled water and then freeze-dried. Extracted LPS was reconstituted and protein contamination assessed using a BCA protein kit (Perbio Science, UK). The level of DNA contamination was assessed using the method of Warburg and Christian using absorption ratios at E280/260. Repeat DNase and proteinase K digestion followed by ultracentrifugation were conducted until satisfactory LPS purity levels of less than 5% contamination were obtained. LPS fractions were analyzed by SDS–PAGE on 16% gels, which were stained with silver nitrate.

**Biological activity of B. vietnamiensis LOSs**

*B. vietnamiensis* LOS was tested for its proinflammatory activity, in particular, for TNF-α induction in human myelomonocytic U937 cells. As previously, TNF-α induction was compared to un-stimulated controls and other LOS of interest at 24 h using ELISA (De Soyza, Morris, et al. 2004).

**Assessment of lipid A and LOS activity using a TLR 4/MD2/CD 14 reporter cell line**

An HEK293 cell line stably expressing both TLR 4 and MD-2 was generated by retroviral transduction of HEK-TLR 4 cells
with a retrovirus encoding human MD2. As this cell does not express other Toll-like receptors, it allows only TLR 4-mediated responses to be assessed. Any observed differences between pre- and post-transplantation strains would therefore relate to TLR 4-mediated signaling without the effect of any possible contaminating DNA (CpG) or other LPS-associated contaminants signaling through other non-TLR 4 receptors. Briefly the extracted LOS and lipid A were used as stimulants of the above cell line at two concentrations 10 ng and 100 ng of stimulant for 24 h and compared to control (media only) and as a positive control 10 ng of E. coli LOS. The effect of stimulation was assessed using a NF-κ reporter assay with output measured in relative light units (RLU) (Ieranò et al. 2008). The results were expressed as a percentage of the positive control. Experiments were repeated in quadruplicate.

Transfection of human embryonic kidney cells

HEK293 cells were plated out at 4 × 10^4 per well of a 96-well plate 24 h prior to transfection. Cells were transfected with Jet PI (Autogen Bioclear, UK) according to the manufacturer’s instructions, using human pcDNA3-TLR 4 (1 ng), pcDNA3-CD14 (1 ng), pEFBOS-MD2 (1 ng), pRL-TK (5 ng; Promega), and pNFkB-Luc (50 ng; Promega) per well. Forty-eight hours after transfection, cells were stimulated with ligands for 6 h. At the end of stimulation, cells were lysed with the RLT buffer (Promega) and assayed for firefly luciferase and renilla luciferase activity using the Dual Luciferase assay (Promega). Firefly luciferase activity was divided by renilla luciferase activity to yield relative light units (RLU). Fold increase in RLU over baseline was calculated between stimulated samples and control samples exposed to the culture medium alone.

Isolation of oligosaccharides and lipid A from B. vietnamiensis LOS pre- and post-transplantation

Lipid A and core oligosaccharides fractions, from B. vietnamiensis pre- and post-transplantation LOS, were obtained by SDS promoted acetate buffer hydrolysis (100 mM AcONa, pH 4.5, 0.1% SDS, 100°C, 4 h). Lyophilized samples were then treated with EtOH/HCl 2M in order to remove SDS. Lipid A fractions were precipitated by adding water to dried samples and centrifuging at 4°C at 8500 × g for 1 h. Recovered supernatants containing oligosaccharides fractions were purified by gel-permeation chromatography using a column (1.5 × 94 cm, total volume 166 mL) of Biogel P-6 in H2O (flow: 13 mL/h) as described (Ieranò et al. 2008).

Monosaccharides and fatty acids analysis

Determination of sugars residues and their absolute configuration were all carried out as described by GLC-MS analysis (Leontine and Lönngren 1978; Molinaro et al. 2002). Monosaccharides were identified as acetylated and analyzed by GLC-MS. The absolute configuration of fatty acids was determined as described (Rietschel 1976).

NMR spectroscopy

All 1D and 2D 1H-NMR spectra were recorded on a solution of 1 mg in 0.5 mL of D2O, at 300 K, at pD 7 (uncorrected value) on Bruker 600 DRX equipped with a cryo probe. Spectra are calibrated with internal acetone [δH 2.225, δC 31.45]. 2D-DQF-COSY (double-quantum-filtered correlation spectroscopy) spectra were acquired with 4096 × 512 data points in both F2 and F1 dimensions. Quadrature indirect dimensions are achieved through the States-TPPI method; spectra are processed applying a Qsine function to both dimensions, and data matrix was zero-filled by factor of 2 before Fourier transformation. Coupling constants were determined on a first-order basis from 2D phase sensitive DQF-COSY (Piantini et al. 1982; Rance et al. 1983). ROESY are measured using data sets (t₁ × t₂) of 4096 × 256 points, and mixing times of 200–400 ms are used. TOCSY experiments are performed with a spinlock time of 100 ms, using data sets (t₁ × t₂) of 4096 × 256 points. In homonuclear experiments, the data matrix is zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and is resolution enhanced in both dimensions by a 90° shifted Qsine function before Fourier transformation. HSOQC, HSQC-TOCSY, and HMBC experiments are measured in the 1H-detected mode via single quantum coherence with proton decoupling in the 13C domain, using data sets of 2048 × 256 points. Experiments are carried out in the phase-sensitive mode (States et al. 1982). 1H,13C HMBC is optimized for 6 Hz coupling constant. In all heteronuclear experiments, the data matrix is extended to 2048 × 1024 points using forward linear prediction extrapolation.

MALDI-TOF mass spectrometry

MALDI mass spectra of native LOSs and Lipid A samples were performed in linear mode on a Perspective (Framingham, MA, USA) Voyager STR instrument, equipped with delayed extraction technology. Ions formed by a pulsed laser beam (nitrogen laser, λ 337 nm) were accelerated by 24 kV and detected in negative-ion (LOSs) and in positive-ion polarity (Lipid A moieties).

Sample preparation. The native LOSs required specific preparations as described in details (Sturiale et al. 2005). Briefly, a few aliquot of sample was first desalted with cation exchange beads (Dowex 50WX8, Sigma-Aldrich) in the ammonium form, prior to crystallization on the MALDI plate. A thin film composed of 2,4,6-trihydroxyacetophenone (THAP) and nitrocellulose (trans-blot membrane, BioRad) was used as a matrix. MS analyses of Lipid A species were performed by dissolving the samples obtained after acetate buffer hydrolysis in CH3Cl/CH3OH (50:50). Such samples were finally mixed in a 1:1 (v/v) ratio with the matrix solution [THAP, 75 mg/mL 4M (4 h, 100°C) and then neutralized with NaOH 5M (30 min, 100°C). Fatty acids were then extracted in CHCl3, methylated with diazomethane, and analyzed by GLC-MS. The ester bound fatty acids were selectively released by base-catalyzed hydrolysis with NaOH 0.5 M/MeOH (1:1 v/v, 85°C, 2 h), and then the product was acidified, extracted in CHCl3, methylated with diazomethane, and analyzed by GLC-MS. The absolute configuration of fatty acids was determined as described (Rietschel 1976).
in CH$_3$OH/trifluoroacetic acid/CH$_3$CN (7:2:1)], deposited onto the MALDI plate and left to crystallize at room temperature.

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**Conflict of interest statement**

None declared.

**Abbreviations**

Ara4N, 4-amino-4-deoxy-1-arabinose; Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; DQF-COSY, double-quantum-filtered correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; Kdo, 3-deoxy-D-manno-2-ulosonic acid; Ko, D-glycero-D-talo-2-ulosonic acid; LOS, lipooligosaccharide; LPS, lipopolysaccharides; MALDI, matrix-assisted laser desorption ionization; NOE, nuclear Overhauser effect; PAMP, pathogen-associated molecular pattern; ROESY, rotating frame Overhauser enhancement spectroscopy; RLU, relative light units; SDS, sodium dodecyl sulfate; TOCSY, total correlation spectroscopy.

**References**


