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Structural Elucidation of a Novel *B. cenocepacia* ET-12 Lipooligosaccharide Isolated from a Cystic Fibrosis Patient after Lung Transplantation

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In this work, we carried out the elucidation of a novel lipooligosaccharide (LOS) from a clinical strain of the most virulent strain of the *Burkholderia cepacia* complex, which was of *B. cenocepacia* ET-12 epidemic strain lineage. The strain was isolated from a cystic fibrosis patient who had successfully undergone lung transplantation. This molecule presents some specific chemical differences when compared to the LOS reported so far from other *B. cenocepacia* ET-12 clones. The new endotoxin was also tested as a cytokine enhancer on human myelomonocytic U937 cells. Lipopolysaccharides are the main virulence factors of Gram-negative bacteria, and since they are exposed to the extracellular space, they are deeply involved in bacterial adaptation to the host environment. The characterisation of structural motifs present in these molecules is essential to the comprehension of the persistence/survival mechanisms and inflammatory potential of these bacteria.

Introduction

Burkholderia cenocepacia is a Gram-negative bacterium that belongs to the *Burkholderia cepacia* complex (Bcc), a group of species ubiquitous in nature, found in the soil rhizosphere, marine water, plants and humans.[1] Previously, *Burkholderia* species were proposed for biotechnological applications until their opportunistic pathogenic traits were recognised; Bcc can cause severe infections in immunocompromised individuals affected by cystic fibrosis (CF) or chronic granulomatous disease. Among Bcc members (identified as genomovars) *B. cenocepacia* is the most virulent species, and it is one of the most frequently isolated Bcc genomovars recovered from CF-patient sputum in Canada and the United States.[2,3] Genotyping studies showed that *B. cenocepacia* ET-12 clonal lineages are associated with the highest rates of morbidity and mortality in CF patients and are the main cause of poor outcomes in lung transplantation since they are responsible for "cepacia syndrome", a deadly exacerbation of infection.[4] The severity of Bcc infections is related to their capacity of overcoming antibiotic therapies and antimicrobial compounds of the innate immune system of the host. Moreover, some Bcc strains are able to resist phagocytosis by intracellular survival within macrophages. The acute toxicity of Bcc members is correlated with a variety of virulence factors such as cable pili, flagella, exopolysaccharides, proteases and other enzymes.[5] Among these, lipopolysaccharides (LPSs) have a major relevance, as they are constitutively expressed by Gram-negative bacteria and represent the major cell-surface component essential for the survival of this class of microorganisms. In addition to the role of a protective barrier against the external environment, LPSs also play a structural function, maintaining the integrity of the outer membrane.[6] They share a common structural motif characterised by three moieties coded by different genetic regions. Lipid A, the glycolipid portion of LPS, anchors these molecules to the external leaflet of the outer membrane. This is connected to a core oligosaccharide and, in some bacterial species, the O-specific polysaccharide or O-antigen is also present. When LPS lacks the O-chain, it is called lipooligosaccharide (LOS) or rough-type LPS (R-LPS). Lipid A, the most conserved region of LPS, consists of a β-(1→6)-linked glucosamine disaccharide, phosphorylated at positions 1 and 4′ and substituted with fatty-acid esters linked at positions 3 and 3′ and amide-linked at positions 2 and 2′; the hydroxy groups of the primary fatty acids can be further acylated by secondary acyl moieties. The core oligosaccharide can be divided into an inner core and outer core, where the inner core typically consists of 3-deoxy-D-*manno*-oct-2-

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ulosonic acid (Kdo) residues linked to the lipid A and L-*glycero*-D-*manno*-heptose (L,D-Hep) moieties. As a result of the presence of phosphate and carboxyl groups on the LPS, the bacterial surface has a net negative charge, which plays an important role in membrane permeability and in the interaction of the cellular surface with cationic, antimicrobial compounds. LPSs are also identified as bacterial endotoxins and as Pathogen Associated Molecular Patterns (PAMPs) by the host innate immune system since they are able to trigger a marked inflammatory response utilising TLR4-mediated signalling pathways.[7]

Within this context, we defined the complete structure of the endotoxin isolated from CF patients affected by a *B. cenocepacia* ET-12 strain infection who underwent lung transplantation and survived for >3 years. The strain was recovered after lung transplantation, and the patient remained culture-positive for many months with this strain. Endotoxin structural characterisation revealed a novel structure, different from that belonging to another virulent clonal lineage of *B. cenocepacia* ET-12 (LMG 16656), which we previously analysed.[8] The structural analysis of these biological compounds is required in order to better understand the PAMP molecular structures that are able to enhance the inflammatory response.

Results

Strain Selection, Extraction and SDS Electrophoresis Analysis of *B. cenocepacia* LOS

We selected *B. cenocepacia* from our repository of strains collected from patients undergoing lung transplantation at one of the most active centres in the U.K. We selected for analysis a post-transplant strain from a patient who survived lung transplantation but had a pre- and post-operative infection with the *B. cenocepacia* ET-12 clonal strain. We identified the isolate on numerous occasions following lung transplantation in bronchial lavage and/or sputum. The strain has previously been demonstrated to be of the ET-12 lineage by using pulsed-field gel electrophoresis (PFGE).[9] We extracted LOS, after growing the strain in nutrient broth, with a modified, hot-phenol extraction process and then subjected it to SDS electrophoresis. The electrophoresis migration pattern after silver staining (not shown herein), suggested the presence of LOS, since it lacked the characteristic ladder pattern typically associated with smooth LPS.

Compositional Analysis of LOS from *B. Cenocepacia*

Monosaccharide analysis on the isolated LOS molecule revealed the presence of L,D-Hep, D-galactose (D-Gal), D-glucose (D-Glc), 2-amino-2-deoxy-D-glucose (D-GlcN), 4-amino-4-deoxy-L-arabinose (L-Ara4N), Kdo and D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko). Methylation analysis showed the presence of a 6-substituted GlcN, terminal Gal, terminal Glc, 6-substituted Glc, 3,4-substituted Hep, 3,7-substituted Hep, terminal Hep, 4,5-substituted Kdo and terminal Ko. All sugar residues were in the pyranose form. Fatty-acid analysis revealed the presence of (*R*)-3-hydroxyhexadecanoic acid [16:0 (3-OH)] in an amide linkage and (*R*)-3-hydroxytetradecanoic acid [14:0 (3-OH)] and tetradecanoic acid (14:0) in ester linkages, in accordance with the typical structure of *Burkholderia* lipid A.[6,9]

Isolation and Structural Characterisation of the Oligosaccharide from the *B. Cenocepacia* LOS

We executed a mild acid hydrolysis, promoted by acetate buffer, on the intact LOS. After centrifugation and further purification by gel-permeation chromatography, we recovered an oligosaccharide fraction. Monosaccharide analysis of this product confirmed the presence of L,D-Hep, D-Gal, D-Glc and Kdo. Methylation analysis showed the presence of a terminal Gal, terminal Glc, 6-substituted Glc, 3,4-substituted Hep, 3,7-substituted Hep, terminal Hep and 5-substituted Kdo.

We analysed this fraction through homo- and heteronuclear 2D NMR experiments (DQF-COSY, TOCSY, ROESY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HSQC-TOCSY and ^{1}H-^{13}C HMBC) in order to assign all the spin systems and to define the monosaccharide sequence. The fraction analysed was constituted by a blend of oligosaccharidic species characterised by nonstoichiometric substitutions. Thus, all the NMR spectra studied present a high heterogeneity augmented by the presence of a reducing end, which in addition, could form a lactone. We identified seven major anomeric signals (spin systems **A**–**G**, Figure 1 and Table 1), and we attribute the signals at δ = 2.05/2.20 ppm to the 3-H methylene protons of the major α-pyranoside Kdo residue.

We identified spin system **A** (1-H, δ = 5.30 ppm, Table 1) as a glucose residue, as indicated by the large $^{3}J_{H,H}$ values of the ring protons (>10 Hz). A small value of $^{3}J_{H1,H2}$ (ca. 3 Hz) and the intraresidual NOE contact of 1-H with 2-H attest to an α-anomeric configuration.

We identified spin systems **B**, **C** and **F** (Table 1) as α-heptose residues, as indicated by their $^{3}J_{H1,H2}$ and $^{3}J_{H2,H3}$ values (<3 Hz) and by the intraresidual NOE of 1-H with 2-H. The ^{13}C chemical shift values of the C-6 of these heptose residues (all upfield 71 ppm) confirm these as L,D-Hep residues, in accordance with the chemical analysis.

We identified spin system **D** (1-H, at δ = 5.07 ppm) as α-D-Gal, as indicated by its $^{3}J_{H3,H4}$ and $^{3}J_{H4,H5}$ values (3 Hz and 1 Hz, respectively), diagnostic of a *galacto* configuration. The $^{3}J_{H1,H2}$ value (3.2 Hz) and the intraresidual NOE contact of 1-H with 2-H agree with an α-anomeric configuration.

We identified spin systems **E** and **G** (1-H, at δ = 5.00 and 4.62 ppm, respectively) as glucose residues, as indicated by the large $^{3}J_{H,H}$ values of the ring protons (>10 Hz). The chemical shifts of 1-H and C-1 of residue **E** (δ = 5.00 and 97.81 ppm), the $^{3}J_{H1,H2}$ value (3.2 Hz) and the intraresidual NOE contact of 1-H with 2-H all agree with an α-anomeric configuration; the chemical shifts of 1-H and C-1 of residue

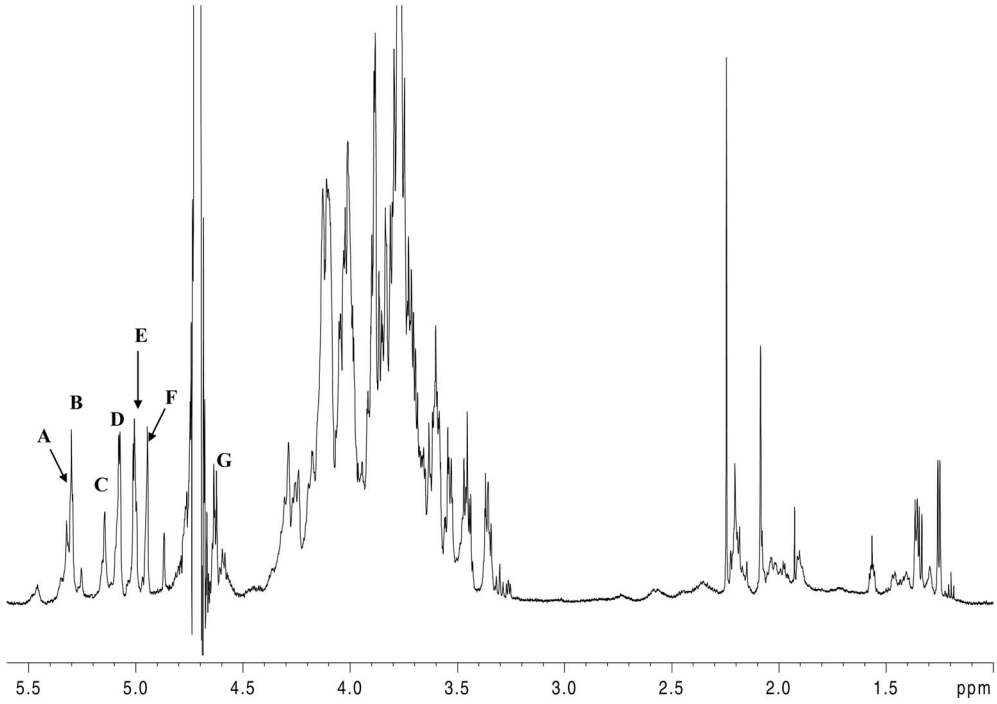


Figure 1. ^{1}H NMR spectrum of the core oligosaccharide isolated from the *B. cenocepacia* LOS. Anomeric signals of monosaccharides residues are indicated with capital letters.

Table 1. ^{1}H and ^{13}C NMR chemical shifts (ppm) of the sugar residues of the core region extracted from *B. cenocepacia* LOS (n.d. = not detected).

Entry	Spin system	Residue	Unit ($^{1}H/^{13}C$)							
			1	2	3	4	5	6	7	8
1	6-α-Glc	**A**	5.30	3.60	3.82	3.71	3.61	4.10/3.71	–	–
2			100.1	71.7	73.1	71.6	71.8	65.2	–	–
3	3,4-α-Hep	**B**	5.30	4.08	4.11	4.13	3.63	4.11	3.70/3.77	–
4			99.2	70.4	72.0	72.1	71.8	69.8	63.6	–
5	3,7-α-Hep	**C**	5.14	4.24	4.02	3.90	3.78	4.00	3.77/n.d.	–
6			100.3	69.4	79.8	69.6	73.4	70.0	69.9	–
7	t-α-Gal	**D**	5.07	3.84	3.90	4.03	4.00	3.77/3.87	–	–
8			97.8	68.5	69.4	68.9	69.9	61.1	–	–
9	t-α-Glc	**E**	5.00	3.59	3.78	3.45	3.87	3.86/3.79	–	–
10			97.8	71.6	73.2	69.7	70.6	60.4	–	–
11	t-α-Hep	**F**	4.94	4.01	3.88	3.66	3.89	4.04	3.71/3.76	–
12			100.7	70.5	70.6	71.3	70.7	69.0	63.7	–
13	6-β-Glc	**G**	4.62	3.35	3.53	3.47	3.68	3.89/3.99	–	–
14			102.1	73.5	75.8	69.7	74.5	62.9	–	–
15	5-α-Kdo	**K**	–	–	2.05/2.20	4.16	4.28	4.13	4.00	3.88/3.77
16			n.d.	n.d.	34.0	71.2	69.3	70.9	71.1	62.7

G (δ = 4.62 and 102.1 ppm respectively), the $^{3}J_{H1,H2}$ value (8.2 Hz) and the intraresidual NOE contact of 1-H with 3-H and 5-H indicate a β-anomeric configuration for this residue.

Because of its free reducing end, the Kdo residue **K** is present in multiple forms; nevertheless, the signals belonging to the α-pyranose reducing unit were clearly assignable, starting from the diastereotopic 3-H methylene proton signals, resonating at δ = 2.05 and 2.20 ppm (3-H_{ax} and 3-H_{eq}, respectively).

We established the oligosaccharide sequence by evaluating all the inter-residual NOE contacts identified in the ROESY spectrum (Figure 2) and the long-range scalar correlations present in the HMBC spectrum (not shown).

We proved the linkage of heptose **B** to O-5 of Kdo **K** by the NOE connectivity between 1-H of heptose **B** (δ = 5.30 ppm) and 5-H of Kdo (δ = 4.28 ppm). Residue **B** is, in turn, substituted at O-3 and O-4. The NOE contacts (Figure 2) of 4-H and 3-H of **B**, with 1-H of residue **G** (δ = 4.62 ppm) show that the O-4 of α-heptose **B** is glycosylated by residue **G**. Residue **B** is also substituted at O-3 by α-heptose **C**, according to the NOE effect of 3-H of **B** with 1-H of **C** (δ = 5.14 ppm, Figure 1b). Residue **C** is also glycosylated at O-7 by the terminal α-heptose **F**, as attested by

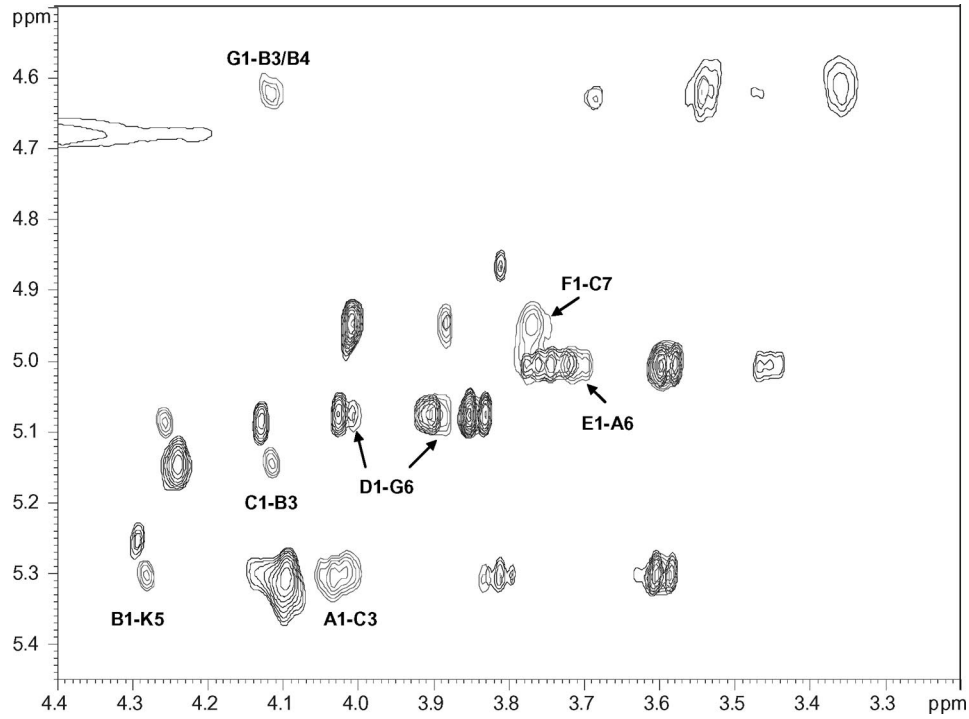
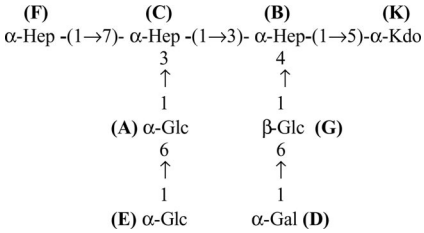


Figure 2. Zoomed-in region of overlapped ROESY (grey) and TOCSY (black) 2D NMR spectra of the core oligosaccharide isolated from *B. cenocepacia* LOS, in which inter-residue NOE contacts are indicated.

the NOE contact between 1-H of **F** and 7-H of **C**. Additionally, residue **C** is glycosylated at position 3 by the α-Glc **A**, as proven by the NOE contact between 1-H of residue **A** and 3-H of residue **C**. Residue **A** is, in turn, substituted by the terminal α-D-Glc residue **E**, as attested by the HMBC correlation between 1-H of **E** and C-6 of **A** (data not shown) and by the NOE contact between 1-H of **E** (δ = 5.00 ppm) and 6-H of **A**.

Finally β-glucoside **G** is substituted by the terminal α-Gal residue, as attested by the presence of NOE correlations between 1-H of **D** (δ = 5.00 ppm) and 6-H of **G** (δ = 3.89 and 3.99 ppm). Thus, NMR analysis and chemical data allowed us to establish the oligosaccharide sequence reported in Scheme 1.



Scheme 1.

We further investigated the oligosaccharidic product by MALDI-MS. The negative-mode mass spectrum shows a peak at *m*/*z* = 1461.5 corresponding to the oligosaccharide shown in Scheme 1, constituted by 1 Kdo, 3 heptoses and 4 hexoses (data not shown).

Structural Characterisation by MALDI-MS of the Intact LOS

We used a useful MS approach in order to gain further information on both the lipid A and core regions without any chemical manipulation, preventing the loss of labile groups typically present on LPSs such as phosphate, acetyl and others. We analysed the intact LOS molecule by MALDI-MS using a sample preparation procedure specifically designed for such amphiphilic molecules.[10] The negative-ion MALDI-MS of the intact LOS is shown in Figure 3. The spectrum presents fragments between *m*/*z* = 1400 and 2000, originating from the β-elimination cleavage of the labile glycoside bond between Kdo and the lipid A moiety, which yields either OS ions or lipid A ions.[10] In particular, the OS1 peak at *m*/*z* = 1678.8 is consistent with a nonasaccharide constituted of 3 heptoses, 4 hexoses, 1 Kdo and 1 Ko residue (i.e. the core oligosaccharide structure described above by NMR spectroscopy with the addition of a Ko residue.) Another peak at *m*/*z* = 1810.9 (OS2) is consistent with an oligosaccharide, not detected by NMR analysis, which bears an additional Ara4N moiety connected to the Ko residue, as typically found for all *Burkholderia* inner-core LPSs.[6] The same mass region also shows peaks derived from a mixture of tetra- and pentaacylated lipid A. The peak at *m*/*z* = 1443.5 matches species T_1, corresponding to a tetraacylated bis(phosphorylated) disaccharide backbone carrying one 14:0 (3-OH) acyl chain in an ester linkage and two 16:0 (3-OH) acyl chains in an amide linkage, one of which, on the GlcN II, is further substituted by a secondary 14:0 fatty acid (see below). Peaks at *m*/*z* = 1575.0 (T_2) and

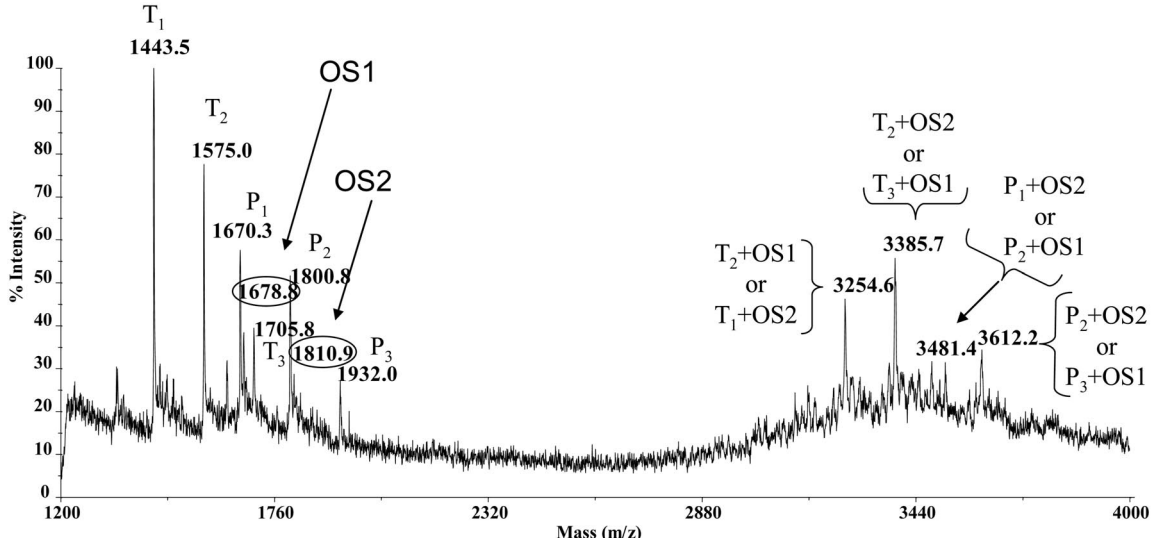


Figure 3. Negative-ion MALDI-MS of the intact LOS isolated from *B. cenocepacia* after lung transplantation. In the low-mass range (*m*/*z* = 1200–2000), lipid A and core oligosaccharides peaks are visible. Intact LOS molecule peaks populate the high-mass region (*m*/*z* = 2800–4000).

1705.6 (T_3) indicate a tetraacylated lipid A carrying one and two Ara4N residues, respectively. Peaks at *m*/*z* = 1670.3 (P_1), 1800.8 (P_2) and 1932.0 (P_3) are consistent with a pentaacylated lipid A carrying two ester-linked 14:0 (3-OH) acyl chains with 0, 1 and 2 Ara4N residues, respectively. In analogy with other lipid A from *Burkholderia*,[6,11] we identified the position of the secondary fatty acid 14:0 chain on the GlcN II by MS analysis of the lipid A moiety after acetate buffer hydrolysis. Briefly, the positive-ion MALDI-MS (not shown) presents an in-source fragmentation due to the breakage of the glycosidic linkage between the two GlcN units, 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 analysis was then completed by a similar characterisation of the compound obtained by ammonium hydroxide hydrolysis.[12]

The signals in the molecular-ion region (*m*/*z* = 3000 and 3800) of the spectrum attests to a reduced heterogeneity of LOS molecules present (Figure 3). By considering the combination of the lipid A moieties and the core oligosaccharide species, we assigned the main LOS molecular ions. The peak at *m*/*z* = 3385.7 represents an LOS species either with the tetraacylated lipid A and OS2 (T_2 + OS2) or the tetraacylated lipid A and OS1 (T_3 + OS1). Similarly, the peak at *m*/*z* = 3254.6 matches T_1 + OS2 or T_2 + OS1. Likewise, the LOS peak at *m*/*z* = 3612.2, constituted by a pentaacylated lipid A species, is consistent with P_2 + OS2 or P_3 + OS1. Finally, the species at *m*/*z* = 3481.4 could be interpreted equally well as either P_1 + OS2 or P_2 + OS1.

Biological Activity of *B. Cenocepacia* LOS

We tested the isolated LOS for its pro-inflammatory activity of eliciting TNF-α induction in human myelomonocytic U937 cells (Figure 4). We stimulated U937 cells with 10 ng/mL of purified LPS or left them nonstimulated, collected supernatants after 24 h and analysed them by using ELISA for TNF-α induction. We also used purified LOS from strains LMG 14273 (*B. multivorans*) and LMG 12614 (*B. cenocepacia* ET-12) as comparators, as previously reported.[13] The results shows that the *B. cenocepacia* LOS isolated after lung transplantation is a stronger inducer of TNF-α than the LOS from another *B. cenocepacia* ET-12 epidemic strain clone (LMG 12614). We note that the

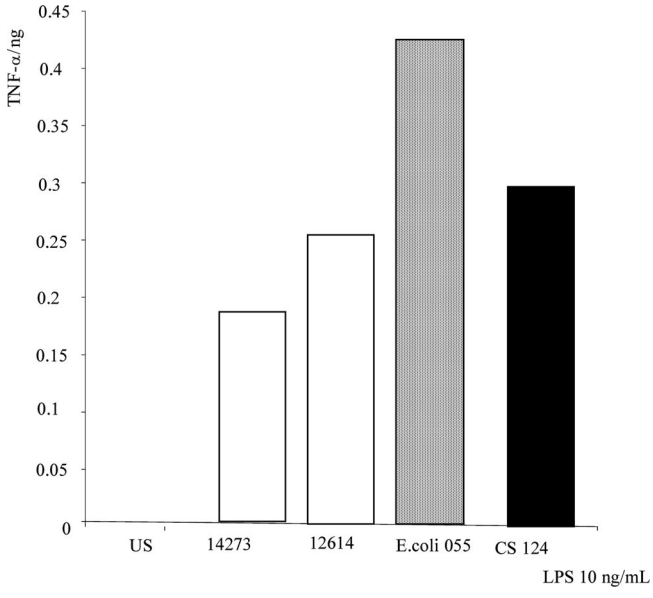


Figure 4. U937 macrophages were stimulated with 10 ng/mL of extracted LPS or unstimulated (US) for 24 h. TNF-α secretion was assayed by ELISA. The strains studied included LMG 14273 (*B. multivorans*), LMG 12614 (*B. cenocepacia* ET-12 clone) and CS 124, a post-transplant, *B. cenocepacia* ET-12 strain from a CF patient who survived lung transplantation. Commercially available *E. coli* 055 LPS was used as an internal control.

higher inflammatory potential of LMG 12614 was previously attributed to a higher degree of lipid A acylation relative to that in the *B. multivorans* strain. We found that the strongest-inducing strain is not heavily acylated. The possible role of the core oligosaccharide as a contributor to the increased biological activity of this strain remains to be elucidated.

Discussion

Lung transplantation is the only therapy for CF patients with end-stage lung disease. Typically, this treatment is contraindicated in CF patients who are affected by *B. cenocepacia* infections, since they are the most aggressive and associated with much poorer survival than are other Bcc genomovars.[13,14] This observation, therefore, allows interesting insights to be gleaned from historical, microbiological repositories from transplant centres, where clinical progress and microbial strains can be correlated. The endotoxin analysed in this work was a clinical isolate extracted from a *B. cenocepacia* ET-12 clone infecting a CF patient who underwent lung transplantation and survived. We recovered the *B. cenocepacia* strain analysed after transplantation.

The chemical structure of the endotoxin analysed shows both unique and common features compared to other *Burkholderia* LPSs.[6] The lipid A moiety is comprised of a mixture of tetra- and pentaacylated species of a GlcN bis(phosphorylated) disaccharide backbone, and consistent with previous reports of a *B. cenocepacia* ET-12 strain LOS, the tetraacylated species is the most abundant one.[8] The lipid A also carries one or two Ara4N residues, as described for other *Burkholderia*.[6] It has been reported that the biosynthesis and transfer of the Ara4N moiety is controlled by a gene cluster regulated by the *Arn* operon, described in *E. coli* and *Salmonella enterica* sv. Typhimurium, which is responsible for polymyxin resistance.[15] Our NMR and MS analyses indicate that the core oligosaccharide is formed by a short saccharidic chain, in which we distinguished the common *Burkholderia* inner-core oligosaccharide motif: Hep-(1→3)-[β-Glc-(1→4)]-α-Hep-(1→5)-α-Kdo. The MALDI-MS of the intact LOS shows two additional residues (Ko and Ara4N) in the oligosaccharide moiety. They are linked to Kdo to form the known trisaccharide β-Ara4N-(1→8)-α-Ko-(2→4)-α-Kdo, previously described for *Burkholderia*. These two monosaccharides were lost during the acidic hydrolysis, leading to the isolation of the core oligosaccharide. This result highlights the importance of MALDI-MS, which was able to analyse the intact LOS species.[16]

We have demonstrated that the presence of Ara4N residues on the LPS of *Enterobacteriaceae* is a structural modification that may occur after LPS transmigration on the cellular surface in response to irregular environmental conditions (such as low concentrations of Mg^{2+}), which require a reduction of the net negative surface charge. LPS is typically constituted of Kdo and phosphate groups, which are responsible for the net negative charge of the bacterial surface. Thus, cationic, antimicrobial peptides involved in the innate immune response against bacterial infections and positively charged antibiotics [e.g. polymyxin B (PmB)] exert their antimicrobial activity by permeating bacterial membranes with bactericidal effects. The presence on LPS of Ara4N residues that are positively charged under physiological conditions prevents bacterial killing, since these residues reduce the total net negative charge of the cellular surface. We find it intriguing that the LPSs from Bcc constitutively express Ara4N, regardless of the environmental conditions the strains have been studied under. This constitutive expression of Ara4N appears limited to a select group of bacterial species, which also includes *Proteus*. No clear evolutionary link has yet been identified to explain the narrow range of organisms constitutively expressing Ara4N.

We also demonstrated that the core sequence contains the additional disaccharide hexose-(1→6)-hexose, linked to the second heptose residue of the inner core, as was found for the *B. cenocepacia* ET-12-type strain J2315.[8] In contrast to the ET-12 strain already extensively characterised, the highly conserved residue of β-Glc present in the inner core is, in turn, glycosylated at O-6 by a terminal Gal residue, as reported for *B. caryophylli* and for *B. multivorans*.[11,17]

In the ET-12 clone analysed herein, a short LPS core oligosaccharide portion was biosynthesised, in contrast to the core oligosaccharide from strain J2315 *B. cenocepacia* ET-12.[8] In the known *B. cenocepacia* core, there is a α-D-Gal residue connected to the C-3- and C-7-branched heptose residue, which is substituted by the trisaccharide α-L-Rha(1→3)-β-D-QuiNAc(1→7)-α-L,D-Hep(1→) at C-2. In the core structure reported herein, an α-D-Glc residue has replaced the α-D-Gal present in the J2315 strain, as was also reported for the XOA3 mutant of *B. cenocepacia* K56-2.[18] The *B. cenocepacia* post-transplantation core is truncated at the level of the α-Glc residue; thus, it lacks the disaccharide Rha-QuiNAc, which is the remainder of the O-chain. Recent studies show that O-chain biosynthesis in *B. cenocepacia* requires an adaptor sugar, to which the remainder of the O-antigen repeating units are attached. QuiNAc was found to be the adaptor sugar, whereas the terminal Rha residue is the first sugar of the repeating O-chain unit (Rha-GalNAc-GalNAc). The ligase responsible for the β-D-QuiNAc(1→7)-α-D,L-Hep linkage is coded by *waaL*, and its inactivation produces rough LPS mutants. In addition, the identification of gene loci responsible for the biosynthesis of the core oligosaccharide moiety in *B. cenocepacia* K56-2 allowed the creation of a set of gradually truncated mutants.[18] These were utilised in the establishment of the role of the core portion in *B. cenocepacia* resistance to antimicrobial compounds, such as PmB. The progressive truncation of the core oligosaccharide led to increased sensitivity to PmB, even though *B. cenocepacia* mutants were still more resistant to PmB than were other pathogens with an intact core portion (e.g. *Salmonella* and *E. coli*.) Thus, *B. cenocepacia* must possess additional mechanisms, independent from the core length, that contribute to bacterial resistance to antimicrobial compounds. Moreover, a recent find-

ing shows that the *B. cenocepacia* truncated-core mutants do not display impaired intracellular survival within macrophages. Hence, bacterial viability is not compromised when the LOS contains even the shortest saccharidic chain.

Experimental Section

Bacterial Growth and LPS Extraction: Bacterial strains isolated from recipients were stored on microbeads. These were streaked onto LB agar. Strains were grown in 5 × 1 L cultures of nutrient broth containing 0.5% yeast extract (DIFCO, Oxford, UK) at 37 °C to late log phase. Bacteria were harvested by centrifugation at 1000 × *g* and suspended in a minimal volume of distilled water and freeze-dried. LPS was extracted by a modification of the standard hot-phenol/water extraction previously described.[19] The pellets were suspended in distilled water and sonicated on ice. The resulting sonicated suspension was then subjected to DNAse II digestion (final concentration of 200 μg/mL) at 37 °C for 2 h. A final digest with proteinase K (final concentration of 2 mg/mL) was undertaken at 60 °C for 2 h prior to boiling. The digested cellular suspension was then mixed with hot phenol at 70 °C for 20 min, cooled with ice and then centrifuged at 800 × *g*. The aqueous phase was then removed and dialysed against repeated changes of fresh, distilled water for 72 h. The dialysed material was ultracentrifuged at 39500 × *g* at 13 °C for 16 h, 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 was assessed by using a BCA protein kit (Perbio Science, UK). Repeated 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 analysed by SDS-polyacrylamide gel electrophoresis on 16% gels, which were stained with silver nitrate.

Patient and Strain Selection: CF patients were listed for transplantation according to international guidelines. Post-operative strains were selected from storage repositories, if recoverable, from patients who had a verified pre-transplant infection with *B. cenocepacia*. Post-transplant, lower-airway (bronchoalveolar) lavages were collected from recipients at day 7 post operation. Presumed *B. cepacia* complex bacteria were isolated by culturing techniques. For this study, we restricted our investigations to *B. cenocepacia* strains. Prior molecular studies had confirmed the *B. cenocepacia* strains were of the ET-12 lineage by using probes for the *B. cepacia* epidemic strain marker (BCESM) and the *cblA* gene in addition to clonality testing by comparison to a reference ET-12 strain.

Pulsed-Field Gel Electrophoresis (PFGE): Confirmation of the clonal nature of paired pre- and post-transplant strains has previously been reported.[8] Briefly, *B. cepacia* complex strains from the whole transplant program were anonymised and genotyped by the macrorestriction of whole genomic DNA with the restriction enzyme *SpeI* (New England Biolabs, UK), followed by the separation of the fragments by PFGE (CHEF DRII system; Bio-Rad).

Isolation of the Core Oligosaccharide from *B. cenocepacia* LOS: The core oligosaccharide fraction from *B. cenocepacia* LOS was obtained by SDS-promoted acetate buffer hydrolysis (100 mM AcONa, pH = 4.5, 0.1% SDS, 100 °C, 4 h). The SDS was removed by treating the sample with EtOH/HCl (2 M); the lipid A fraction precipitated and was isolated by centrifugation at 4 °C and 8500 × *g* for 1 h. The recovered supernatant containing oligosaccharide fractions was purified by gel-permeation chromatography by using a column (1.5 × 94 cm, total volume of 166 mL) of Biogel P-6 in H_2O (flowrate: 13 mL/h), as previously described.[7,18]

Monosaccharides and Fatty-Acid Analysis: The determination of sugar residues and their absolute configuration was carried out as described by GLC-MS analysis.[20] As for Ko and Kdo residues, their absolute configuration was carried out by comparing GLC-MS analysis results with those from other *Burkholderia* LPS.[6] Monosaccharides were identified as acetylated *O*-methyl glycoside derivatives. After methanolysis (2 M HCl/MeOH, 85 °C, 14 h) and acetylation with acetic anhydride in pyridine (85 °C, 30 min), the sample was analysed by GLC-MS. Linkage analysis was carried out by methylation of the complete core region as described.[21] Total fatty-acid content was obtained by acid hydrolysis. The LOS was first treated with aqueous HCl (4 M, 4 h, 100 °C) and then neutralised with aqueous NaOH (5 M, 30 min, 100 °C). Fatty acids were then extracted with $CHCl_3$, methylated with diazomethane and analysed by GLC-MS. The ester-bound fatty acids were selectively released by base-catalysed hydrolysis with aqueous NaOH (0.5 M in MeOH, 1:1 v/v, 85 °C, 2 h), and the product was then acidified and extracted with $CHCl_3$, methylated with diazomethane and analysed by GLC-MS. The absolute configuration of fatty acids was determined as previously described.[22]

NMR Spectroscopy: All 1D and 2D ^{1}H NMR spectra were recorded on a solution of 1 mg in 0.5 mL of D_2O at 300 K with a Bruker 600 DRX spectrometer, equipped with a cryo probe. Spectra were calibrated against acetone (δ_H = 2.225 ppm; δ_C = 31.45 ppm) as an internal standard. 2D-DQF COSY spectra were acquired with 4096 × 512 data points in both the *F*2 and *F*1 dimensions. Quadrature indirect dimensions were achieved through the States-TPPI (time proportional phase increments) method; spectra were processed by applying a Qsine function to both dimensions, and the data matrix was zero-filled by a factor of 2 before Fourier transformation. Coupling constants were determined on a first-order basis from 2D phase-sensitive DQF-COSY.[23,24] Rotating-frame Overhauser enhancement spectroscopy (ROESY) was performed by using data sets ($t_1 \times t_2$) of 4096 × 256 points and mixing times of 200–400 ms. Total-correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 100 ms by using data sets ($t_1 \times t_2$) of 4096 × 256 points. In homonuclear experiments, the data matrix was zero-filled in the *F*1 dimension to give a matrix of 4096 × 2048 points and was resolution-enhanced in both dimensions by a 90°-shifted Qsine function before Fourier transformation. Heteronuclear single-quantum coherence (HSQC) TOCSY and heteronuclear multiple-bond correlation (HMBC) experiments were performed in the ^{1}H-detected mode by single-quantum coherence with proton decoupling in the ^{13}C domain by using data sets of 2048 × 256 points. Experiments were carried out in the phase-sensitive mode according to the method of States et al.[25] ^{1}H,^{13}C HMBC was optimised for a 6 Hz coupling constant. In all heteronuclear experiments, the data matrix was extended to 2048 × 1024 points by using forward linear prediction extrapolation.

MALDI-MS: MALDI-MS of native LOSs and lipid A samples was performed in the linear mode with 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 positive-ion polarity (lipid A moieties).

Sample Preparation: The native LOSs required specific preparations as described in detail previously.[10] Briefly, a few aliquots of sample were first desalted with cation-exchange beads (Dowex, 50WX8, Sigma–Aldrich) in the ammonium form, prior to crystallisation on the MALDI plate. A thin film composed of 2,4,6-trihy-

droxyacetophenone (THAP) and nitrocellulose (trans-blot membrane, BioRad) was used as the matrix. MS analysis of lipid A species was performed by dissolving the samples obtained after acetate buffer hydrolysis in CH_3Cl/CH_3OH (50:50). Such samples were finally mixed in a 1:1 (v/v) ratio with the matrix solution [THAP, 75 mg/mL in CH_3OH/trifluoroacetic acid/CH_3CN (7:2:1)], deposited onto the MALDI plate and left to crystallise at room temperature.

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