Biosynthesis and Structure of the *Burkholderia cenocepacia* K56-2 Lipopolysaccharide Core Oligosaccharide

TRUNCATION OF THE CORE OLIGOSACCHARIDE LEADS TO INCREASED BINDING AND SENSITIVITY TO POLYMYCIN B

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*Burkholderia cenocepacia* is an opportunistic pathogen that displays a remarkably high resistance to antimicrobial peptides. We hypothesize that high resistance to antimicrobial peptides in these bacteria is because of the barrier properties of the outer membrane. Here we report the identification of genes for the biosynthesis of the core oligosaccharide (OS) moiety of the *B. cenocepacia* lipopolysaccharide. We constructed a panel of isogenic mutants with truncated core OS that facilitated functional gene assignments and the elucidation of the core OS structure in the prototypic strain K56-2. The core OS structure consists of three heptoses in the inner core region, 3-deoxy-d-manno-octulosonic acid, d-glycero-d-talo-octulosonic acid, and 4-amino-4-deoxy-l-arabinose linked to d-glycero-d-talo-octulosonic acid. Also, glucose is linked to heptose I, whereas heptose II carries a second glucose and a terminal heptose, which is the site of attachment of the O antigen. We established that the level of core truncation in the mutants was proportional to their increased *in vitro* sensitivity to polymyxin B (PmB). Binding assays using fluorescent 5-dimethylaminonaphthalene-1-sulfonyl-labeled PmB demonstrated a correlation between sensitivity and increased binding of PmB to intact cells. Also, the nyl-labeled PmB demonstrated a correlation between sensitivity to antimicrobial peptides as well as antimicrobial peptides (APs)5 (1, 3).

Lipopolysaccharide (LPS) is the major surface component of *Gram*-negative bacteria and consists of lipid A, core oligosaccharide (OS), and in some bacteria O-specific polysaccharide or O antigen (4, 5). The O antigen acts as a protective barrier against desiccation, phagocytosis, and serum complement-mediated killing, whereas the core OS and the lipid A contribute to maintain the integrity of the outer membrane (4, 5). The lipid A also anchors the LPS molecule to the outer leaflet of the outer membrane and accounts for the endotoxic activity of LPS (4, 6). Lipid A is a bisphosphorylated β,1-6-linked glucosamine disaccharide substituted with fatty acids ester-linked at positions 3 and 3’ and amide-linked at positions 2 and 2’ (4). The core OS can be subdivided into the inner core and outer core. The inner core OS typically consists of one or two 3-deoxy-d-manno-octulosonic acid (Kdo) residues linked to the lipid A and three d-glycero-d-manno-heptose residues linked to the first Kdo (4). The outer core OS in enteric bacteria typically consists of 8–12 branched sugars linked to heptose II of the inner core. As a result of phosphate groups on the lipid A and core OS, the bacterial surface has a net negative charge. This plays an important role in the interaction of the bacterial surface with positively charged compounds such as cationic APs, which are cat-

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5 The abbreviations used are: AP, antimicrobial peptide; DQF-COSY, double quantum filtered correlation spectroscopy; d-Quin, d-quinovosamine; HMBC, heteronuclear multiple bond correlation; HSCQ, heteronuclear single quantum coherence; Kdo, 3-deoxy-d-manno-octulosonic acid; Ko, d-glycero-d-talo-octulosonic acid; l-Ara4N, 4-amino-4-deoxy-l-arabinose; LPS, lipopolysaccharide; l-Rha, l-rhamnose; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; NOE, nuclear Overhauser effect; OS, oligosaccharide; PmB, polymyxin B; TOCSY, total correlation spectroscopy; TOF, time-of-flight; T-ROESY, transverse rotating-frame Overhauser enhancement spectroscopy; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; dansyl, 5-dimethylaminophenyl-sulfonyl; Tricine, N-[2-hydroxyethyl]glycine; BcCV, *B. cenocepacia*-containing vacuole.
ionic amphipathic molecules that kill bacteria by membrane permeabilization. In response to a series of environmental conditions such as low magnesium or high iron, bacteria can express modified LPS molecules that result in a less negative surface. This reduces the binding of APs and promotes resistance to these compounds. Previous studies have shown that Burkholderia LPS molecules possess unique properties. For example, Kdo cannot be detected by classic colorimetric methods in LPS from Burkholderia pseudomallei and Burkholderia cepacia, and the covalent linkage between Kdo and lipid A is more resistant to acid hydrolysis than in conventional LPS molecules (7). In B. cepacia, 4-amino-4-deoxy-L-arabinose (1-Ara4N) is bound to the lipid A by a phosphodiester linkage at position 4 of the nonreducing glucosamine (GlcN II) (8) and is also present as a component of the core OS. Also, instead of two Kdo molecules, the B. cepacia core OS has only one Kdo and the unusual Kdo analog, d-glycero-d-talo- octulosonic acid (Ko), which is nonstoichiometrically substituted with 1-Ara4N forming a 1→8 linkage with α-Ko (7, 9). Although this is also the case for the inner core OS of B. cepacia J2315 (10), it is not a common feature for the core OS in all Burkholderia. For example, the inner core of Burkholderia caryophylli consists of two Kdo residues and does not possess 1-Ara4N (11).

Burkholderia species, including B. cepacia, are intrinsically resistant to human and non-human APs such as those produced by airway epithelial cells (12, 13), human β-defensin 3 (14), human neutrophil peptides (15), and polymyxin B (PmB) (15, 16). The minimum inhibitory concentration determined for some of these peptides in several Burkholderia species is greater than 500 μg/ml, which could aid these microorganisms during colonization of the respiratory epithelia (13). It has been proposed that the resistance of B. cepacia to cationic APs stems from ineffective binding to the outer membrane, as a consequence of the low number of phosphate and carboxylate groups in the lipopolysaccharide (17), but a systematic analysis of the molecular basis of AP resistance in B. cepacia and other Burkholderia is lacking. We have previously reported that a heptoseless B. cepacia mutant (SAL1) is significantly more sensitive than the parental clinical strain K56-2 to APs (15). This mutant has a truncated inner core and lacks the outer core, suggesting that a complete core OS is required for resistance of B. cepacia to APs.

Apart from heptoses, the role of other sugar moieties of the B. cepacia core OS in AP resistance is not known. In this study, we report the structure of the core OS for B. cepacia strain K56-2 and its isogenic mutants XOAX3, XOAX7, and XOAX8, which carry various core OS truncations. The structural analysis, combined with mutagenesis, allowed us to assign function to the majority of the genes involved in core OS biosynthesis and ligation of the O antigen and to establish that the degree of truncation of the core OS correlates with increased binding and bacterial sensitivity to PmB in vitro and reduced bacterial intracellular survival in macrophages.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Strains and plasmids used in this study are listed in Table 1. B. cepacia strain K56-2 was grown at 37 °C in LB medium supplemented, as required, with 100 μg/ml trimethoprim and 50 μg/ml gentamicin. This strain is a clinical isolate that belongs to the same clonal group as the type strain J2315 (18). Escherichia coli strains were grown at 37 °C in LB medium supplemented with trimethoprim (50 μg/ml), kanamycin (40 μg/ml), or chloramphenicol (30 μg/ml), as required. Conditional mutants were grown at 37 °C in M9 medium supplemented with 5 mg/ml yeast extract and 0.5% (w/v) rhamnose (permissive conditions) or 0.5% (w/v) glucose (nonpermissive conditions) as described previously (19).

**Mammalian Cell Lines**—Murine macrophage (RAW 264.7; ATCC TIB-71) and human lung epithelial cell lines (A549; CCL-185) were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco’s

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**Table 1**

| Bacterial strains and plasmids used in this study |
|-----------------------------------------------|
| **Strain or plasmid** | **Genotype and relevant characteristics** | **Reference or source** |
|------------------------|------------------------------------------|------------------------|
| **B. cepacia strains** | K56-2, waaC::pGPI1Tp, TpR | This study |
| CCB1                   | ET12 clone, cystic fibrosis clinical isolate | CBCCR7 |
| XOA3                   | wbbE::pGPI1Tp, TpR | This study |
| XOA6                   | wbbP::pGPI1Tp, TpR | This study |
| XOA7                   | wbbL::pGPI1Tp, TpR | This study |
| XOA8                   | wbbO::pGPI1Tp, TpR | This study |
| XOA9                   | wbbQ::pGPI1Tp, TpR | This study |
| XOA15                  | wbbR::pGPI1Tp, TpR | This study |
| XOA17                  | waaC::pGP1ApTp, TpR | This study |
| XOA19                  | waaL::pC200, TpR | This study |
| **Plasmids**           |                                          | S. Cardona |
| pAP20                  | ori_KAW, CmR, mob*, P_pdr,             | 20 |
| pGP1TTP                | ori_KAW, IPTp, mob*                    | 21 |
| pGPAPt                 | IPTp, Ap*, mob*                        | 40 |
| pJ1R                   | PMLBAD (56) expressing the red fluorescent protein mRFP1 | |
| pRK2013                | ori_KAW, RK2 derivative, KanR, mob*, tra* | 23 |
| pSC200                 | P_pdr, IPTp, rhamnose-inducible promoter, IPTp, mob* | 19 |
| pXO13                  | pAP20, waaO under the control of P_pdr | This study |
| pXO14                  | pAP20, waaL under the control of P_pdr | This study |
| pXO20                  | pAP20, wabS under the control of P_pdr | This study |
| pXO21                  | pAP20, waaC under the control of P_pdr | This study |
| pXO22                  | pAP20, (1-Ara4N) under the control of P_pdr | This study |
|                        |                                          | *Canadian B. cepacia complex Research and Referral Repository.* |
modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and were grown at 37 °C in a humidified atmosphere with 5% CO₂. DMEM and FBS were purchased from Wisent Inc. (St. Bruno, Quebec, Canada).

Construction of Mutants in B. cenocepacia K56–2—An internal 300-bp fragment near the 5′ end of the gene targeted for mutagenesis was PCR-amplified and cloned into the XbaI and EcoRI restriction sites of the suicide vector pGPOTp to provide the homology region for recombination. Vector and recombinant plasmids were maintained in E. coli SY327 (araD Δlac-pro argE recA56 nalA apir RifR). The integration vector has a replication origin that cannot function in B. cenocepacia and also contains transcriptional and translational stops causing polar effects on genes downstream from the insertion point. Similarly, the suicide plasmid pGPAPtp (21) was used to construct nonpolar insertion mutants, and the plasmid pSC200 was used to construct conditional mutants as described (19).

These plasmids were introduced into B. cenocepacia strain K56–2 by triparental mating (22) using E. coli MM294 (endA hsdR pro) containing the helper plasmid pRK2013 (23). Exconjugants were selected on LB agar plates supplemented with 50 μg/ml gentamicin (to kill the E. coli donor and helper bacteria) and 100 μg/ml trimethoprim for selection of K56–2 exconjugants. Exconjugants were screened for plasmid integration into the chromosome by colony PCR using a primer annealing to a region of the mutagenesis vector and another primer annealing to chromosomal sequences upstream of the insertion site. Integration was confirmed by Southern blot hybridization using a probe corresponding to a DNA fragment spanning the homology region used for recombination. The sequences of the DNA primers used for mutagenesis are listed in supplemental Table S1.

Molecular Cloning for Complementation of LPS Production and PmB Sensitivity—To construct the complementing plasmids pXO13, pXO14, pXO20, pXO21, and pXO22, the corresponding genes were PCR-amplified using ProStart polymerase (Qiagen Inc., Valencia, CA). The PCR products were digested with XbaI and EcoRI and ligated into pAP20, which was also cut with the same restriction enzymes. Ligation mixtures were introduced into E. coli DH5α (F− φ80lacZ M15 endA recA hsdR[tK− mK−] supE thi gyrA relA Δ[lacZYA-argF]U169) competent cells by the calcium chloride method (24), and transformants were plated on LB agar plates supplemented with 30 μg/ml chloramphenicol. The correct DNA inserts were verified by colony PCR using primers 1630 (5′-CGCAGCGGTAGTGCACCT-3′) and 1631 (5′-ACTCTCGCATGGGGGAGACC-3′), which anneal to sequences flanking the cloning sites of pAP20. Plasmids from PCR-positive colonies were isolated, initially confirmed by restriction digestion and ultimately by DNA sequencing of the DNA insert. These plasmids were mobilized by conjugation into the corresponding B. cenocepacia mutant strains as described above.

Electrophoretic Analysis of LPS—For electrophoresis analysis, LPS samples were extracted as described previously (25). LPS was resolved by electrophoresis in 16.4% polyacrylamide gels using a Tricine-SDS system and visualized by silver staining. In the case of conditional mutants, the LPS was extracted from bacteria grown in liquid cultures at nonpermissive conditions.

Purification and Compositional Analysis of LPS—Large scale LPS preparations for structural analysis were obtained by the method of Westphal and Jann (26) from 1-liter cultures of B. cenocepacia strains XOA3, XOA7, and XOA8. The quality of the purified LPS samples was confirmed by Tricine-SDS-PAGE as described above. Fractions containing the core OS were obtained by mild acid hydrolysis in sodium acetate buffer, pH 4.4, for 3 h at 100 °C as described previously (10). The determination of sugar residues, their absolute configuration, and linkage analysis, as well as the characterization of total fatty acids content and their absolute configuration were all carried out as described previously (10).

NMR Analysis—For structural assignments of OS fractions, one-dimensional and two-dimensional 1H NMR spectra were recorded on Bruker 600 DRX equipped with a cryoprobe on a solution of 300 μl of D₂O using Shigemi tubes. T-ROESY experiments were recorded using data sets (t1 × t2) of 4096 × 256 points with mixing times between 100 and 400 ms. The spin lock field was attenuated (~ 4000 Hz) with respect to that employed for the hard pulses. No correction for Hartmann-Hahn effects was applied, because T-ROESY effectively removed most of these effects. Double quantum-filtered phase-sensitive COSY experiments were performed using data sets of 4096 × 512 points. TOCSY were performed with spin lock times from 20 to 100 ms, using data sets (t1 × t2) of 4096 × 256 points. In all homonuclear experiments the data matrix was zero-filled in both dimensions to give a matrix of 4000 × 2000 points and was resolution-enhanced in both dimensions by a cosine-bell function before Fourier transformation. Coupling constants were determined on a first order basis from high resolution one-dimensional spectra or by two-dimensional phase-sensitive DQF-COSY. HSQC and HMBC experiments were 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 were carried out in the phase-sensitive mode. A 60-ms delay was used for the evolution of long range connectivity in the HMBC experiment. In all heteronuclear experiments the data matrix was extended to 2048 × 1024 points using forward linear prediction extrapolation.

MALDI-TOF MS Analysis—MALDI-TOF mass spectra were recorded in the negative and positive polarity in linear mode on a Voyager STR from PerSeptive Biosystems (Framingham, MA) equipped with delayed extraction technology. Ions formed by a pulsed UV laser beam (nitrogen laser, λ = 337 nm) were accelerated at 24 kV. The mass spectra reported are the result of 256 laser shots. Resolution was about 1500.

Sensitivity to PmB—Bacteria were grown overnight in LB medium or LB medium supplemented with the appropriate antibiotics as required. The next day cultures were diluted to an A₅₆₀ of 0.01, and 50 μl of this suspension were added to LB medium or LB medium supplemented with 100 μg/ml trimethoprim to make a final volume of 5 ml. 500 μl of this bacterial suspension were aliquoted into Eppendorf tubes, and 10 μl of buffer or PmB (5 mg/ml stock) were added to each tube to reach a final concentration of 100 μg/ml. Cells were incubated...
with PmB at 37 °C for 22 h with constant rotation using a Barnstead Thermolyne LABQUAKE (Barnstead International, Dubuque, IA), and the A600 was recorded. To determine the MIC50 (concentration of PmB causing 50% reduction in bacterial growth), cells were incubated as described above with PmB at final concentrations of 0, 25, 50, 100, and 200 μg/ml.

**Binding of Dansyl-PmB to Whole Cells**—Dansyl/PmB was prepared from PmB and dansyl/chloride by the method of Schindler and Teuber (27) and quantified by the dinitrophenylation assay (28). For the binding assay bacteria were grown overnight in LB medium or LB medium supplemented with 100 μg/ml trimethoprim, as required. The overnight cultures were diluted in LB to an A600 of 0.1 and grown at 37 °C for an additional 4 h. Bacteria were collected by centrifugation, suspended in 1 ml of 5 mM Hepes, pH 7.4, 10 mM sodium azide buffer, and diluted to an A600 of 0.5. Aliquots (80 μl) of this suspension were added to 96-well plates (Microfluor 2 white, flat bottom 96-well microtiter plates, ThermoLabsystems, Franklin, MA) were added to 96-well plates (Microfluor 2 white, flat bottom 96-well microtiter plates, ThermoLabsystems, Franklin, MA) and incubated for 20 h in a humidified atmosphere containing 5% CO2. Overnight bacterial cultures were washed and resuspended in DMEM, 10% FBS and added to the cells at multiplicity of infection of 50, centrifuged for 2 min at 300 × g, and incubated for 30 min at 4 °C. Nonadherent bacteria were removed by rinsing five times with ice-cold phosphate-buffered saline. Cells were lysed with 100 μl of 0.5% sodium deoxycholate. Serial dilutions were performed in LB and plated in duplicate. The percentage of adhesion was calculated as follows: 100 × (number of cell-associated bacteria/initial number of bacteria added). Data were calculated from at least three independent experiments performed in triplicate and are expressed as means ± S.E.

**Statistical Analyses**—The statistical significance of differences in the data were determined using the one-way analysis of variance test and the Tukey post-test, provided in the Prism GraphPad software version 4.0.

**RESULTS**

**Organization of Core OS Synthesis Gene Loci in B. cenocepacia Strains J2315 and K56-2**—We examined the genome of strain J2315 (31) for genes predicted to encode enzymes for the synthesis of the core OS. Unlike enteric bacteria, the *B. cenocepacia* core OS genes are not found within a single cluster but rather dispersed into three different locations in chromosome 1 (Fig. 1). One of these regions is located between nucleotides 2,656,960 and 2,669,740 and contains eight genes named BCAL2401–BCAL2409 (dnaE)-BCAL3136 (apaH) and containing genes involved in Kdo transfer (waaA), core OS synthesis (waaC), and O antigen biosynthesis (34). The location of the IS402 insertion element interrupting the continuity of the wbxE gene in the type ET12 strain J2315 is indicated. However, this gene is functional in the strain K56-2 (34). A region containing BCAL0967, which was identified as a *waa* homolog, which encodes the heptosyltransferase II that is predicted to add the second heptose residue onto the core OS.

**Macrophage Infection Assays**—Macrophage infections were performed as described previously (30). Briefly, bacterial suspensions were added to RAW 264.7 cells grown on glass coverslips at a multiplicity of infection of 50 and incubated at 37 °C in 5% CO2 for 4 h. When needed, 0.5 mM Lysotracker Red DND-99 (Invitrogen) was added for 1 min prior to visualization. Fluorescence and phase contrast images were acquired using a Qimaging (Burnaby, British Columbia, Canada) cooled, charged-coupled device camera on an Axioscope 2 (Carl Zeiss) microscope. Images were digitally processed using the Northern Eclipse version 6.0 imaging analysis software (Empix Imaging, Mississauga, Ontario, Canada). Each experiment was independently repeated at least three times.

**Adhesion Assays**—Monolayers for adhesion assays were prepared by seeding 7 × 104 human lung epithelial A549 cells in DMEM, 10% FBS into a 48-multiwell plate and incubating at 37 °C for 20 h in a humidified atmosphere containing 5% CO2. Overnight bacterial cultures were washed and resuspended in DMEM, 10% FBS and added to the cells at multiplicity of infection of 50, centrifuged for 2 min at 300 × g, and incubated for 30 min at 4 °C. Nonadherent bacteria were removed by rinsing five times with ice-cold phosphate-buffered saline. Cells were lysed with 100 μl of 0.5% sodium deoxycholate. Serial dilutions were performed in LB and plated in duplicate. The percentage of adhesion was calculated as follows: 100 × (number of cell-associated bacteria/initial number of bacteria added). Data were calculated from at least three independent experiments performed in triplicate and are expressed as means ± S.E.
Structure-Function of B. cenocepacia Core Oligosaccharide

Additional loci for lipid A-core OS synthesis were previously identified in B. cenocepacia K56-2 based on the characteristics of its predicted product containing the typical topological features observed in O antigen ligases, including 12 predicted transmembrane domains and a large periplasmic loop, which contains highly conserved arginine and histidine residues (33). As expected, the inserational inactivation of waaL results in loss of O antigen surface expression in B. cenocepacia K56-2 (see below).

Additional loci for lipid A-core OS synthesis were previously reported in a 4-gene transcriptional unit next to the O antigen gene cluster (Fig. 1B) (34). They include wabC (encoding a heptosyltransferase I homolog) and waaA (encoding a Kdo transferase) genes. Also, there is a monocystic gene locus between nucleotides 1,055,340 and 1,056,400 that corresponds to waaF (heptosyltransferase II) homolog of the GT1 LPS-heptosyltransferase family, respectively. The products of wabO, wabQ, and wabS have homologies with various glycosyltransferases. BCAL2405 was assigned as waaL based on the characteristics of its predicted product containing the typical topological features observed in O antigen ligases, including 12 predicted transmembrane domains and a large periplasmic loop, which contains highly conserved arginine and histidine residues (33). As expected, the inserional inactivation of waaL results in loss of O antigen surface expression in B. cenocepacia K56-2 (see below).

Functional Assignment of Core OS Genes—To assign function and establish the order of synthesis of the core OS sugars, we constructed inserational mutants in each of the identified genes as described under “Experimental Procedures.” Also, an inserational mutation in the wbxE gene (Fig. 1B), which produces a truncated O antigen (Fig. 2, lane 1), was constructed. The LPS banding pattern profiles of parental and mutant strains were examined by gel electrophoresis and silver staining. POLymetric Oag denotes the portion of the gel where the lipid A-core containing O antigen polysaccharides of varying number of repeating units migrate. Core + Oag denotes the extra band in mutant XOA3 that corresponded to lipid A-core + an incomplete O antigen repeat. Core denotes the region of the gel corresponding to the migration of the lipid A-core. The genes in parentheses indicate that the gene that is mutated by inserional mutagenesis in the appropriate strain. Lanes 10 – 13 correspond to a different gel in which the effect of the waaF mutation on the core OS LPS was tested using the other wild type and mutant strains as controls. The gels were run under identical conditions of voltage, and the migration of the lipid A-core OS band was compared with that of the wild type strain as standard. The relative migration of the mutant lipid A-core OS bands relative to the wild type position in sugars (Table 2). As expected, all of these loci were also present with an identical gene organization in the B. cenocepacia strain XOA7 (waaL::pGPIOTp) displayed a lipid A-core OS band that migrated with the corresponding band in the parental K56-2 LPS (Fig. 2, lanes 2 and 5). This is in agreement with the functional assignment of this gene as encoding the O antigen ligase. In contrast, inserational mutations in wabR, wabS, and wabO, as well as the mutations in waaC and waaF (Fig. 2, lanes 6 – 9 and 11) resulted not only in loss of O antigen surface expression but also gave rise to progressive truncations of the lipid A-core OS, as determined by the presence of fast migrating bands of smaller mass than the band produced by XOA7 (waaL::pGPIOTp).

The CCB1 (waaC::pGPIOTp) mutant produced the shortest lipid A-core OS band (Fig. 2, lanes 9 and 12), which also comigrated with the lipid A-core OS of the SAL1 strain (data not shown). Because SAL1 has a defect in the synthesis of ADP-glycero-manno-heptose precursors (15), these results confirm the functional assignment of WaaC as the heptosyltransferase I enzyme. The lipid A-core OS band produced by the waaF::pSC200 mutant XOA19 migrated between the lipid A-core OS from the waaC and wabO mutants, suggesting that waaF encodes the heptosyltransferase II. LPS production in all of the mutants examined except for XOA15 (wabR::pGPIOTp) was restored to parental levels after introducing complementing plasmids carrying the corresponding gene under the control of a constitutive promoter (data not shown). In the case of XOA15 (wabR::pGPIOTp), the complementing plasmid pX022 could not be introduced by either conjugation or electroporation, suggesting this mutant has a defect preventing the stable maintenance of the complementing plasmid. Together, the mutagenesis experiments suggest that the lipid A-core OS synthesis in B. cenocepacia K56-2 requires the sequential glycosyltransferase activities of WaaC, WaaF, WabO, WabS, and WabR, although the participation of WabP and WabQ could not be deduced.

Functional Assignment of Core OS Genes—To verify the functional assignments of the core OS genes and to characterize in more detail the structure-function of the core OS in B. cenocepacia, we determined the structure of the core OS produced by strains XOA3 (wbxE::pGPIOTp), XOA7 (waaL::pGPIOTp), and XOA8 (wabO::pGPIOTp). LPS fractions from these strains were extracted by large scale purification and confirmed by SDS-PAGE. Monosaccharide and fatty acid analyses showed the same content in fatty acids (data not shown) but different composition in sugars (Table 2).

To elucidate the primary structure of the core OS of the XOA3 strain, a mild hydrolysis with sodium acetate buffer was performed to split the lipid A from the core OSXOA3 fraction that by gel permeation chromatography yielded one main frac-
The compositional analysis of the isolated OS_{XOA3} showed 3,4-di-substituted LD-Hep, 3,7-di-substituted LD-Hep, 7-substituted LD-Hep, terminal LD-Hep, 2-substituted glucose (Glc), terminal L-rhamnose (L-Rha), terminal D-Glc, 3-substituted D-quinovosamine (D-QuiN), 4,5-di-substituted Kdo, and a terminal Ko, all in the pyranose ring conformation.

The 1H NMR spectrum of OSXOA3 is shown in Fig. 3. A combination of homo- and heteronuclear two-dimensional NMR experiments, including double quantum-filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), transverse rotating-frame Overhauser enhancement spectroscopy (T-ROESY), 1H-13C HSQC, and 1H-13C HMBC were performed to assign all the spin systems of OSXOA3 and the monosaccharide sequence. In the anomeric region of the 1H NMR spectrum (Fig. 3), nine anomeric signals were identified (A–H, Table 3). Furthermore, the signals at 1.92/2.16 ppm were identified as the H-3 methylene of the Kdo residue. The NMR data indicated a mixture of two oligosaccharides with different lengths. Spin systems A, B, D, D', and F (Table 3) were all identified as α-heptose residues, as indicated by their 3J_H1,H2 and 3J_H2,H3 coupling constants (below 3 Hz) and by the intra-residual nuclear Overhauser effect (NOE) of H-1 with H-2.

### TABLE 2

| Assignment | XOAXOA3 | XOAXOA7 | XOAXOA8 |
|------------|---------|---------|---------|
| 6-Substituted D-GlcNp | x       | x       | x       |
| 5-Substituted Kdo′p  | x       | x       | x       |
| 4,5-Substituted Kdo′p | x       | x       | x       |
| 8-Substituted Kopo   | x       | x       | x       |
| Terminal Kopo        | x       | x       | x       |
| Terminal L-Ara-4N    | x       | x       | x       |
| 3,4-Substituted LD-Hep | x   | x       | -       |
| 3-Substituted LD-Hep | -       | -       | x       |
| 3,7-Substituted LD-Hep | x     | x       | -       |
| Terminal LD-Hep      | x       | x       | x       |
| 2-Substituted D-Glc  | x       | x       | -       |
| Terminal D-Glc       | x       | -       | -       |
| Terminal L-Rha       | x       | -       | -       |
| 3-Substituted D-QuiN | x       | -       | -       |
| 7-Substituted D-Hep  | x       | -       | -       |

The sugar content was obtained by performing a methanolyis (the sample was methanolyzed and acetylated) followed by analysis via gas chromatography-mass spectrometry. The ring size and the attachment points were determined by methylation analysis, and the absolute configuration of sugar residues has been determined by gas chromatography-mass spectrometry analysis of the acetylated O-(+)-Oct-2yl glycoside derivatives. In all cases, both the fragmentation pattern and a systematic comparison with standards allowed the unequivocal identification and assignment of the sugar content. The results were reproducible over the course of three repeated experiments. X is the first letter defining the name of the mutant strains (e.g. XOA3); x indicates that the sugar is present in the analysis; ~ indicates that the sugar is absent in the analysis.
These data, together with the C-6\(^{13}\)C chemical shift values (all below 70 ppm) allowed us to identify them as L-glycero-D-manno heptose, in agreement with the chemical analysis. Spin systems C and E were identified as glucose residues, as indicated by their large ring \(J_{\text{H-H}}\) coupling constants (above 10 Hz). The strong intra-residue NOE contacts of H-1 E with H-3 and
H-5 E together with the \(^3J_{\text{H}1,\text{H}2}\) coupling constant (7 Hz) were diagnostics of \(\beta\)-configuration, whereas the intra-residue NOE contact of H-1 with H-2 and the \(^3J_{\text{H}1,\text{H}2}\) coupling constant (3 Hz) were indicative of \(\alpha\)-anomeric configuration of residue C. Residue H was recognized as \(\alpha\)-Rha. Actually, in TOCSY spectrum scalar correlations of the ring protons with methyl signals in the shielded region at 1.14 ppm were visible. The \textit{manno} configuration of residue H was established from the \(^3J_{\text{H}1,\text{H}2}\) and \(^3J_{\text{H}1,\text{H}3}\) values (below 3 Hz), and the \(\alpha\)-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. Residue G was recognized as \(\beta\)-QuiN, as indicated by the ring \(^3J_{\text{H}1,\text{H}4}\) coupling constants (above 10 Hz). The \textit{intra}-residual NOE contact of H-1 with H-3 and H-5 and the \(^3J_{\text{H}1,\text{H}2}\) coupling constant were indicative of \(\beta\)-anomeric configuration. The \(^{13}\)C-\(^1\)H HSQC spectrum showed the correlation of H-2 G, at 3.76 ppm, with a nitrogen-bearing carbon signal at 55.4 ppm. The downfield shift of proton resonance of H-2 H was diagnostic of N-acetylation at this position. Because of the absence of the anomeric proton signal, spin system of Kdo K was assigned starting from the diastereotopic H-3 methylene protons, resonating in a shielded region at 1.92 and 2.16 ppm (H-3\(_{\text{ax}}\) and H-3\(_{\text{eq}}\), respectively). Because of its free reducing end, the Kdo residue was present in multiple forms. However, the signals belonging to the \(\alpha\)-reducing unit were clearly evident, whereas the \(\alpha\)-anomeric orientation at C-2 was attributed by the chemical shift values of H-3 and by the values of \(^3J_{\text{H}1,\text{H}2}\), \(^3J_{\text{H}1,\text{H}3}\) and \(^3J_{\text{H}1,\text{H}4}\) coupling constants (35, 36). Residue J of \(\alpha\)-Ko was assigned starting from its oxymethine H-3 signal at 3.74 ppm. The \textit{talo} and the anomeric configuration of Ko were assigned by the analysis of vicinal \(^3J_{\text{H}1,\text{H}4}\) coupling constants from the DQF-COSY, in particular by the existence of a \(^3J_{\text{H}1,\text{H}2}\) of typical of the W long range couplings and after the comparison with published data (35). The downfield shift of carbon resonances identified the glycosylated positions as follows: O-3 and O-4 of residue A; O-3 and O-7 of residue B; O-2 of residue C; O-7 of residue D; O-3 of residue G; O-5 of residue K; and residues D, E, F, H, and J were nonreducing terminal sugars, in full agreement with the methylation analysis. The \textit{inter}-residual NOE contacts (Fig. 3) and the long range correlations present in the HMBC spectrum yielded the OS sequence. The linkage of the heptose A to O-5 of Kdo K was proven by the NOE connectivity between H-1 of heptose A (5.21 ppm) and H-5 K (4.17 ppm). The \textit{inter}-residue NOE contacts between H-5\(_{\text{eq}}\) of Kdo and H-6 of Ko (J) were diagnostic for the \(\alpha\)-d-Ko-(2\(\rightarrow\)4)-\(\alpha\)-d-Kdo linkage (7, 35, 37) (Fig. 3). Residue A was substituted at O-3 and O-4. The NOE contact of H-4 (4.21 ppm) and H-6 A with H-1 E (4.49 ppm) evidenced that the O-4 of \(\alpha\)-heptose A was glycosylated by residue E, the \(\beta\)-glucose. Residue A was also substituted at O-3 by residue B, according to the NOE (Fig. 3) of H-3 A with H-1 B. Residue B was identified as the 3,7-disubstituted \(\alpha\)-heptose. The NOE correlation of H-3 B (3.93 ppm) with H-1 C (5.21 ppm) (Fig. 3) gave evidence of substitution of residue B at O-3 by the \(\alpha\)-glucose C. Furthermore, residue B was glycosylated at O-7 by the heptose residue F, as demonstrated by the NOE contact of H-7 B with H-1 F. Residue C was substituted at O-2 by the \(\alpha\)-heptose D, as shown by the NOE correlation of H-2 C (3.58 ppm) with H-1 D (5.00 ppm). The scalar correlation found in the HMBC spectrum confirmed the OS sequence assigned so far. Thus, methylation analyses, glycosylation shifts, NOE, and HMBC data were all in agreement to indicate an OS\(_{\text{XOA3}}\) structure as depicted in Fig. 4A.

An alternative glycoform of the \(\alpha\)-heptose D was found, indicated as residue D’ (H-1 4.99 ppm; Fig. 3 and Table 3), which was identified as a 7-\(\alpha\)-Hep. The NOE contact of H-7 D with H-1 of \(\beta\)-d-QuinG confirmed the glycosylation by G of residue D at position 7. Residue G was in turn substituted at O-3 by the terminal \(\alpha\)-rhamnose H, as suggested by the NOE contact of H-1 G with H-3 H. These data validated the structure depicted in Fig. 4B, differing from the one in Fig. 4A by the presence of the additional terminal disaccharide (G-(1\(\rightarrow\)3)-H-(1\(\rightarrow\)in Fig. 4B).

A MALDI-TOF mass spectrum of the OS\(_{\text{XOA3}}\) mixture OS1 confirmed the above structural hypotheses (Fig. 5A). The negative ion mass spectrum showed two major ions at \(m/z\) 1565.9 and 1899.0 (\(\Delta m/z = 333\)). Species at \(m/z\) 1565.9 was identified as the octasaccharide built up of four Hep, two Hex, one Kdo, and one Ko residues. Species at \(m/z\) 1899.0 (\(\Delta m/z = 333\)) was consistent with the decasaccharide that differed by the presence of the additional dHex-dHexNAc disaccharide.

In addition to the molecular ions in the mass range 2500–4000 Da, the negative ion MALDI mass spectrum of intact core OS\(_{\text{XOA3}}\) revealed ion peaks related to fragments arising from the very labile glycoside bond cleavage between Kdo and the lipid A moiety (Fig. 5B). Thus, at low molecular masses (Fig. 5B), the ion peak at \(m/z\) 1548.2 could be assigned to the octasaccharide described above. Ion peaks A–E were derived from the lipid A that was constituted by a mixture of tetra- and penta-acylated species differing by the phosphorylation pattern. Species A at \(m/z\) 1444.1 was identified as a tetra-acylated disaccharide backbone carrying in ester linkage one 14:0 (3-OH) chain and in amide linkage two 16:0 (3-OH) chains, one of which, on the GlcN II, was further substituted by a secondary fatty acid, a 14:0 residue. Species C at \(m/z\) 1670.8 (\(\Delta m/z = 131\)) was the corresponding penta-acylated species carrying two ester-linked 14:0 (3-OH) residues. The other species differed for the presence of one or two 1-L-Ara4N residues linked to the phosphate groups. The core OS molecular ions, in the mass range 2500–4000 Da (Fig. 3), were given by the combination of the peaks of the lipid A and the core region. Interestingly, peaks related to the presence of an additional pentosamine identified via gas chromatography-mass spectrometry as 1-L-Ara4N linked at position O-8 of the Ko residue were also present (see ions at \(m/z\) 3254.5 and 3587.1). Peaks at \(m/z\) 3455.5 and 3587.1 (\(\Delta m/z = 332\) with respect to 3123.5 and 3254.5) were consistent with the presence of the additional terminal dHex-dHexNAc disaccharide. The penta-acylated species was present in a very low amount, and peaks corresponding to penta-acylated lipid A in the core OS region were not detectable. Together, NMR and MS data validated the structure for the lipid A-core OS of strain XOA3 (\textit{wxhE}_P::\textit{gPPTt}) that is shown in Fig. 4C.

A similar approach was used to elucidate the structure of the core OS from XOA7 (\textit{waalL}::\textit{gPPTt}). The NMR fraction isolated by gel filtration chromatography following the acid treatment was identical to the OS from XOA3 (\textit{wxhE}_P::\textit{gPPTt}) but lacked the terminal \(\beta\)-d-QuinNAc-(1\(\rightarrow\)3)-\(\alpha\)-L-Rha disaccharide.
as demonstrated by the comparison of the NMR spectra of XOA3 and XOA7 (supplemental Fig. S1) and by the MALDI-MS spectrum (supplemental Fig. S2). The MALDI-MS spectrum of the intact OS (Fig. 5B) also showed, at low molecular masses, the ion peak derived from the core OS at \( m/z \) 1548.4 that was identified as an octosaccharide carrying four heptoses, one Kdo, one Ko, and two hexoses. The lipid A was constituted by a mixture of tetra- and penta-acylated species differing by the phosphorylation pattern (species A–E) and carrying from none to two L-Ara4N residues. The L-Ara4N residue on the Ko residue was present in nonstoichiometric amounts. Therefore, we concluded that the lipid A-core OS of the mutant XOA7 (\( \text{waaL}::\text{pGP} \)) has the structure indicated in Fig. 4D lacking the \( \alpha-L-\text{Rha}(1 \rightarrow 3) - \beta-D-\text{QuiNAc} \) disaccharide. This suggests that the addition of this disaccharide requires the activity of WaaL protein.

In the case of the XOA8 (\( \text{wabO}::\text{pGP\Omega Tp} \)), the ion peak derived from the core OS was found at \( m/z \) 839.4 in the MALDI-TOF MS spectrum of intact OS (Fig. 5D). This corresponded to a tetrasaccharide with two heptoses, one Kdo and one Ko. The lipid A was constituted by a mixture of tetra- and penta-acylated species differing by the phosphorylation pattern (species A–E) and carrying from none to two \( L-\text{Ara4N} \) residues. The \( L-\text{Ara4N} \) residue on the Ko residue was present in nonstoichiometric amount (see MALDI of intact core OS). Based on the results from MALDI and NMR (not shown), the core OS structure of the XOA8 (\( \text{wabO}::\text{pGP\Omega Tp} \)) contains a heptose disaccharide attached to the Kdo (Fig. 4E). The structural data, together with the rapid banding pattern of the lipid A-core OS in SDS-PAGE, support the assignment of \( \text{wabO} \) as the gene encoding the glucosyltransferase responsible for the addition of \( \beta-D-\text{Glc} \) to Hep1 (Fig. 4, B and C).

**PmB Sensitivity for B. cenocepacia K56-2 LPS Core Mutants**—The sensitivity of the different mutants to PmB was determined as described under “Experimental Procedures.” Except for mutants XOA9 (\( \text{wabQ} \)) and XOA6 (\( \text{wabP} \)), all the mutants with truncations in their lipid A-core OS (Fig. 2) grew significantly less (\( p < 0.001 \)) than the parental strain in the presence of 100 \( \mu g/ml \) PmB (Fig. 6A). To further investigate the role of the lipid A-core OS in the resistance of \( B. \) cenocepacia to PmB, we grew the mutants XOA7 (\( \text{waaL} \)), XOA8 (\( \text{wabO} \)), XOA15 (\( \text{wabR} \)), XOA17 (\( \text{wabS} \)), and CCB1 (\( \text{waaC} \)) in the presence of a range of PmB concentrations from 0 to 200 \( \mu g/ml \), and we determined the concentration for which the growth was reduced to 50% (MIC50) when compared with a control culture grown in the absence of PmB. Deeper truncations of the lipid A-core OS correlated with increased sensitivity to PmB in this assay (Fig. 6B). The MIC50 values for the different mutants ranged from 3.5- to 14.6-fold less than \( >512 \mu g/ml \), the MIC50 value of the parental strain K56-2 (data not shown) (15). Furthermore, the
31 and 36 µg/ml MIC50 values for XOA8 (wabO) CCB1 (waaC) were comparable with that reported for the heptoseless mutant SAL1 (15).

To determine whether the increased sensitivity to PmB correlated with increased binding of the mutants to this peptide, we performed binding assays using the fluorescence analog dansyl-PmB. This compound only fluoresces when bound to whole cells or purified LPS. As reported before for *B. cepacia* (29), *B. cenocepacia* K56-2 did not bind dansyl-PmB. In contrast, all the mutants bound dansyl-PmB with a degree of binding that was proportional to their sensitivity to PmB (supplemental Fig. S3). Thus, deeper truncations of the core lipid A-OS correlated with greater binding of the bacterial cells to dansyl-PmB.

**Loss of O Antigen Production Increases the Adherence of *B. cenocepacia* to Lung Epithelial Cells**—Airway epithelial cells play a key role in maintaining mucosal integrity, and they are the first cells to be challenged by airborne pathogens. We investigated the ability of our panel of mutants in LPS O antigen and lipid A-core OS synthesis to adhere to A549 human lung epithelial cells. Previous work has shown that *B. cenocepacia* can survive intracellularly in this cell line (38). Our results showed that XOA7 (waaL::pGP0Tp), which lost the ability to produce O antigen but produces a complete lipid A-core OS, exhibited 100-fold increased adhesion to A549 cells compared with the parental strain (31 ± 18% adhesion for XOA7 versus 0.23 ± 0.13% for K56-2). Similar results were obtained with all of the other mutants producing truncated core OS (data not shown). We conclude from these results that loss of O antigen production, but not core OS truncations, in *B. cenocepacia* correlates with increased bacterial adhesion to epithelial cells.

**Lipid A-Core OS Heptoseless Mutant CCB1 Is Defective for Intracellular Survival in Macrophages**—Previous work in our laboratory demonstrated that *B. cenocepacia* can survive intracellularly in macrophages (39). Survival occurs in *B. cenocepacia*-containing vacuoles (BcCVs) that delay normal phagosomal maturation by interfering with vacuolar acidification and the phagolysosomal fusion (40). We investigated the role of O antigen and the core OS in the intracellular behavior of *B. cenocepacia*. RAW 264.7 macrophages were infected with K56-2, XOA7 (waaL::pGP0Tp), and CCB1 (waaC::pGP0Tp) strains, and the colocalization of BcCVs with LysoTracker Red was assessed by fluorescence microscopy. LysoTracker Red is an acidotropic dye that preferentially accumulates in lysosomes.

FIGURE 5. A, negative ion MALDI mass spectrum of core OS product from XOA3 strain. B, negative ion MALDI-TOF mass spectrum of core OS from XOA3 obtained in linear mode. C, negative ion MALDI TOF mass spectrum of core OS from XOA7 obtained in linear mode. D, negative ion MALDI TOF mass spectrum of core OS from XOA8 obtained in linear mode.
disruptive experiments done in triplicate. ***, denotes wise comparisons of the % relative growth of each mutant with the growth without PmB.

Figure 6. A, growth of mutants with truncated LPS in the presence of 100 μg/ml PmB as compared with the wild type K56-2. Graph shows the % relative growth of the different strains in the presence of 100 μg/ml PmB as compared with the growth without PmB. Error bars correspond to S.E. for three independent experiments done in triplicate. ***, denotes p < 0.001 for the pairwise comparisons of the % relative growth of each mutant versus the % growth of the parental strain, as determined by the Tukey post-test. B, dose-dependent growth for mutants with truncated LPS under increasing concentrations of PmB ranging from 25 to 100 μg/ml as compared with the growth without PmB. Error bars correspond to S.E. for three independent experiments done in triplicate.

At 4 h post-infection, less than 30% of the BccVs contain H9262. Figure 6, A, demonstrates that soluble mRFP1 had leaked from the bacterial cytoplasm into the phagosomal lumen (Fig. 7, B, C). Furthermore, in contrast, over 85.4 ± 6.6% of BccVs in macrophages infected with the heptoses mutant CCB1 colocalized with the dye at 4 h (Fig. 7, A and C).

Given that intracellular CCB1 bacteria did not prevent phagolysosomal acidification, we also investigated their viability in RAW 264.7 macrophages. For these experiments we performed infections with bacteria expressing the monomeric red fluorescent protein 1 (mRFP1) encoded by pJR1 (Table 1). Using B. cenocepacia cells expressing mRFP1, we have previously demonstrated that intracellular bacteria trafficking into lysosomes rapidly lose cell envelope integrity and are destroyed, resulting in the leakage of mRFP1 into the vacuolar lumen (30, 40, 42). At 4 h post-infection, 68.9 ± 4.7% of BccVs containing CCB1(pJR1) bacteria were fluorescently labeled, suggesting that soluble mRFP1 had leaked from the bacterial cytoplasm into the phagosomal lumen (Fig. 7B). In contrast, 11 ± 5% of the BccVs containing K56-2 (pJR1) showed leakage of mRFP1 (p < 0.0001; Fig. 7D and data not shown). Furthermore, in contrast to the apparently normal bacterial morphology of intracellular K56-2 (pJR1), internalized CCB1(pJR1) exhibited a variety of abnormal morphologies such as rounding and a highly dense cytoplasm, further suggesting a compromise of their cellular envelope (data not shown). Together, these experiments indicate that the CCB1 mutant loses the ability to survive intracellularly, whereas mutants containing less severe core OS truncations or a complete core OS with no O antigen are not impaired in intracellular survival.

Discussion

We have identified gene loci responsible for the biosynthesis of the core OS moiety in B. cenocepacia K56-2. This allowed us to create a set of core OS-deficient mutants, three of which were used to determine the structure of the core OS. The mutant XOA3 has an insertion mutation in the wbxE gene that encodes a glycosyltransferase involved in O antigen synthesis, resulting in the production of lipid A-core OS and a partial O antigen unit (34). This mutation recreates the same LPS phenotype as observed in strain J2315, whose structure has been recently reported (10). In J2315, the spontaneous insertion of the IS402 element in wbxE causes the formation of a lipid A-core OS with a partial O antigen repeat that cannot be polymerized (34). The core OS structures in J2315 and XOA3 strains are identical, except for the presence of α-galactose instead of α-Glc linked to the outer core branched 3,7-disubstituted Hep. The galactose in the J2315 strain has an additional α-glucose at the O-6 position. The mutant XOA7, which has an inactivated waaL gene, lacks the terminal Rha-QuinAc disaccharide found in the outer core OS of strains XOA3 and J2315 (10). From these data, we conclude that the Rha-QuinAc disaccharide is a remnant of the interrupted O antigen in these strains. The O antigen in B. cenocepacia K56-2 is synthesized via the ABC export pathway (4, 34). This particular mode of O antigen synthesis requires an adaptor sugar bound to undecaprenyl-PP, to which the remainder of the O antigen repeating units become attached (4). Based on our structural information, combined with the mutagenesis data, we conclude that the QuinAc residue is the adaptor sugar for the O antigen synthesis in B. cenocepacia K56-2. Furthermore, our data support the conclusion that the β-D-QuinAc-(1→7)-α-L-D-Hep linkage is made by the WaaL O antigen ligase, explaining why the Rha-QuinAc disaccharide is absent in strain XOA7. The terminal rhamnose in the core OS of the XOA3 mutant, is likely the first sugar of the repeating O unit, which we have previously established as a Rha-GalNAc-GalNAc trisaccharide (34), but which cannot be completed because of the mutation in the WbxE glycosyltransferase. Therefore, our data also suggest that WbxE encodes a GalNAc transferase. Current work in our laboratories is under way to resolve the complete biosynthesis pathway of the O antigen component of the B. cenocepacia LPS.

The structure of the core OS in the XOA8 strain revealed a major truncation, consistent with the migration pattern of the LPS in SDS-PAGE. The mutated wabO gene in XOA, encodes a putative glycosyltransferase, and based on the elucidated structure and the short lipid A-core OS band produced by the mutant strain, we predict that WabO protein is the glycosyltransferase responsible for the glucosylation of Hep1 (Fig. 4C).
Also, the structural data suggest that glucosylation of HepI may be a requirement for the glucosylation of HepII and the continuation of the extension of the lipid A-core OS. Analysis of the LPS structure of XOA17, currently in progress in our laboratories, would be required to unequivocally support this conclusion.

The composition of the lipid A moiety of B. cenocepacia K56-2 was identical to that of B. cepacia K2315 (10), and the inner core OS is also composed of the trisaccharide Kdo-Ko-1-L-Ara4N, as described before (7, 9, 10). L-Ara4N is found as a nonstoichiometric substitution in the core OS of several bacteria such as Proteus penneri (43). The presence of L-Ara4N as a component of the core OS is unusual as this positively charged sugar is commonly found as a modification of the lipid A in response to specific environmental signals (4), but it is rarely found as a component of the core OS. In a previous study, we demonstrated that the synthesis of L-Ara4N is essential for the viability of B. cenocepacia (19), and more recently we have observed the same requirement for a B. cenocepacia mutant defective in the production of UDP-glucuronic acid, a precursor for UDP-1-L-Ara4N (44), and speculated that the presence of L-Ara4N in the core OS may be critical for the completion of the synthesis or the assembly of the LPS. The presence of L-Ara4N linked to Ko is not universal to all Burkholderia, as it has been recently shown that a heptose residue is located at this position in the core OS of “B. cepacia” serotype O4 (45). This strain also has a very different core OS structure than the ones we have determined for B. cenocepacia ET12 strains. Unfortunately, given the heterogeneity of the genus Burkholderia (46), the lack of a detailed taxonomical assignment for B. cepacia serotype O4 makes it difficult to compare with other strains. The presence of the Kdo analog Ko is also unusual. Ko has also been found as a substitute for Kdo II in the LPS of Yersinia pestis and Serratia marcescens and for Kdo I in the LPS of Acinetobacter haemolyticus (5), but the function of such substitution and its biosynthesis are unknown (43). On the other hand, the LPS of other Burkholderia species such as B. caryophylli do not contain Ko (11, 47).

A heptoseless mutant of B. cepacia has been reported as a result of a mutation of the heptosyltransferase I waaC (48). The structure of the core OS for this mutant is the same as the one
we have determined for the *B. cenocepacia* K56-2 heptoseless mutant SAL1<sup>6</sup> (15). Thus, the inner core OS is highly conserved in *Burkholderia* species.

The panel of isogenic strains with gradual truncations in their lipid A-core OS allowed us to investigate the relationship between LPS and the extraordinary resistance of *B. cenocepacia* against cationic APs. Although, as it was shown previously with *B. cepacia* (49), PmB binds to *P. aeruginosa* much better than to *B. cenocepacia* K56-2, progressive truncation of the lipid A-core OS leads to increased PmB binding. These findings support the notion that the cell envelope of *B. cenocepacia* has unusual characteristics that enable it to act as a barrier against APs. Although significantly more sensitive than the wild type strain to PmB, our core mutants are still much more resistant to PmB than other organisms such as *Salmonella* and *E. coli* with intact core OS. Thus *B. cenocepacia* must possess additional mechanisms that make these bacteria extremely resistant to APs.

Taking advantage of the set of isogenic mutants in *B. cenocepacia* K56-2 that range from the formation of a full-length LPS O antigen (parental strain) to a mutant producing heptoseless lipid A-core OS (CCB1), we also investigated the biological role of LPS in adhesion to epithelial cells and intracellular survival in macrophages. Our results demonstrated that O antigen production by *B. cenocepacia* prevents bacterial adhesion to epithelial cells. This suggests that the O antigen in these bacteria masks bacterial surface molecules that can interact with epithelial cell receptors, or alternatively, the exposed core OS residues are themselves ligands for binding. We considered the latter hypothesis less likely given that all the core OS mutants with progressive truncations showed increased adhesion, suggesting that no specific sugar residue is required for adhesion, in contrast to recent observations in other bacteria (50). It has been previously shown that Bcc isolates can survive intracellularly within amoebae (51), respiratory epithelial cells (38), and macrophages (39, 40). Others have reported that the LPS O antigen plays an essential role in internalization and survival of the related bacterium *B. pseudomallei* in macrophages (52). Our data investigating the ability of the various mutants with defects in lipid A-core OS production to survive intracellularly in macrophages revealed that only the heptoseless mutant CCB1 is impaired for survival. These results are somewhat surprising and indicate that the ability of *B. cenocepacia* to survive in macrophages does not correlate with the level of truncation of the core OS. *B. cenocepacia* can resist oxidative (53, 54) and non-oxidative (55) intracellular killing mechanisms, and the latter mainly depend on APs. Therefore, the intracellular survival of the other mutants with core OS truncations, despite their increased sensitivity to PmB in vitro, suggest that either the AP concentration in BcCVs is not enough to compromise the viability of these mutants or other factors are involved. It is possible that the ability of *B. cenocepacia* to survive intracellularly is highly dependent on the stability of the outer membrane cell envelope, which may be only seriously perturbed in the presence of a drastically truncated lipid A-core OS. This is in agreement with other observations indicating that *B. cenocepacia* heptoseless mutants have defects in motility and increased permeability to other hydrophobic compounds in addition to antimicrobial peptides (15).<sup>6</sup>

In conclusion, we have identified the genes involved in the biosynthesis of the core OS in *B. cenocepacia*, performed the structural analysis of the core OS, and assigned function to most of the genes of the core OS loci. We also demonstrated that progressive truncations of core OS are associated with a dramatic reduction in the resistance to PmB, which inversely correlates with increasing binding of this peptide to the bacterial cell envelope of the mutant strains. Finally, we also show that the majority of the core OS is expendable for intracellular survival of *B. cenocepacia* in macrophages, whereas the O antigen contributes to prevent bacterial adhesion to epithelial cells. Further investigations are underway in our laboratories to better elucidate the characteristic of the outer membrane and the LPS molecules that contribute to the extraordinary resistance of *B. cenocepacia* to a wide range of antimicrobial molecules, including APs.

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**REFERENCES**

1. Mahenthiralingam, E., Urban, T. A., and Goldberg, J. B. (2005) *Nat. Rev. Microbiol.* 3, 144–156
2. Balandreau, J., Viallard, V., Cournoyer, B., Coenye, T., Laevens, S., and Vandamme, P. (2001) *Appl. Environ. Microbiol.* 67, 982–985
3. Aaron, S. D., Ferris, W., Henry, D. A., Speert, D. P., and Macdonald, N. E. (2000) *Am. J. Respir. Crit. Care Med.* 161, 1206–1212
4. Raetz, C. R., and Whitfield, C. (2002) *Annu. Rev. Biochem.* 71, 635–700
5. Caroff, M., and Karbian, D. (2003) *Carbohydr. Res.* 338, 2431–2447
6. Raetz, C. R., Reynolds, C. M., Trent, M. S., and Bishop, R. E. (2007) *Annu. Rev. Biochem.* 76, 295–329
7. Ishihiki, Y., Kawahara, K., and Zähringer, U. (1998) *Carbohydr. Res.* 313, 21–27
8. Silipo, A., Molinaro, A., Cescutti, P., Bedini, E., Rizzo, R., Parrilli, M., and Lanzetta, R. (2005) *Glycobiochemistry* 15, 561–570
9. Ishihiki, Y., Zähringer, U., and Kawahara, K. (2003) *Carbohydr. Res.* 338, 2659–2666
10. Silipo, A., Molinaro, A., Ieranò, T., De Soysa, A., Sturiale, L., Garozzo, D., Aldridge, C., Corris, P. A., Khan, C. M., Lanzetta, R., and Parrilli, M. (2007) *Chemistry* 13, 3501–3511
11. Molinaro, A., De Castro, C., Lanzetta, R., Evidente, A., Parrilli, M., and Holst, O. (2002) *J. Biol. Chem.* 277, 10058–10063
12. Baird, R. M., Brown, H., Smith, A. W., and Watson, M. L. (1999) *Immunopharmacology* 44, 267–272
13. Devine, D. A. (2003) *Mol. Immunol.* 40, 431–443
14. Sahly, H., Chubert, S., Harder, J., Rautenberg, P., Ullmann, U., Schröder, J., and Pöschchen, R. (2003) *Antimicrob. Agents Chemother.* 47, 1739–1741
15. Loutet, S. A., Flannagan, R. S., Koos, C., Sokol, P. A., and Valvano, M. A. (2006) *J. Bacteriol.* 188, 2073–2080
16. Burtner, M. N., and Woods, D. E. (1999) *Antimicrob. Agents Chemother.* 43, 2648–2656
17. Cox, A. D., and Wilkinson, S. G. (1991) *Mol. Microbiol.* 5, 641–646
18. Mahenthiralingam, E., Coenye, T., Chung, J. W., Speert, D. P., Govan, J. R., Taylor, P., and Vandamme, P. (2000) *J. Clin. Microbiol.* 38, 910–913
19. Ortega, X. P., Cardona, S. T., Brown, A. R., Loutet, S. A., Flannagan, R. S., Campopiano, D. J., Govan, J. R., and Valvano, M. A. (2007) *J. Bacteriol.* 189, 3639–3644

<sup>6</sup>S. A. Loutet and M. A. Valvano, unpublished observations.
20. Flannagan, R. S., Aubert, D., Kooi, C., Sokol, P. A., and Valvano, M. A. (2007) *Infect. Immun.* 75, 1679–1689
21. Flannagan, R. S., and Valvano, M. A. (2008) *Microbiology* 154, 643–653
22. Craig, F. F., Coote, J. G., Parton, R., Freer, J. H., and Gilmour, N. I. (1989) *J. Microbiol. Microbiol.* 135, 2885–2890
23. Figurski, D. H., and Helinski, D. R. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 1648–1652
24. Cohen, S. N., Chang, A. C., and Hsu, L. (1972) *Proc. Natl. Acad. Sci. U.S.A.* 69, 2110–2114
25. Marolda, C. L., Welsh, J., Dafoe, L., and Valvano, M. A. (1990) *J. Bacteriol.* 172, 3590–3599
26. Westphal, O., and Jann, K. (1965) in *Methods in Carbohydrate Chemistry* (Whistler, R. L., BeMiller, J. N., and Wolfrom, M. L., eds) pp. 83–91, Academic Press, New York
27. Schindler, P. R., and Teuber, M. (1975) *Antimicrob. Agents Chemother.* 8, 95–104
28. Bader, J., and Teuber, M. (1973) *Z. Naturforsch.* 28, 422–430
29. Moore, R. A., Bates, N. C., and Hancock, R. E. (1986) *Antimicrob. Agents Chemother.* 30, 923–926
30. Holden, M. T., Seth-Smith, H. M., Crossman, L. C., Sebaia, M., Bentley, S. D., Cerdeno-Tárraga, A. M., Thomson, N. R., Bason, N., Quail, M. A., Sharp, S., Cherevach, I., Churcher, C., Goodhead, I., Hauser, H., Holroyd, N., Mungall, K., Scott, P., Walker, D., White, B., Rose, H., Iversen, P., Mil-Homens, D., Rocha, E. P., Fialho, A. M., Baldwin, A., Dowson, C., Barrell, B. G., Govan, J. R., Vandamme, P., Hart, C. A., Mahenthiralingam, E., and Parkhill, J. (2009) *J. Bacteriol.* 191, 261–277
31. Reeves, P. R., Hobbs, M., Valvano, M. A., Skurnik, M., Whitfield, C., Coplin, D., Kido, N., Klena, J., Maskell, D., Raetz, C. R., and Rick, P. D. (1996) *Trends Microbiol.* 4, 495–503
32. Pérez, J. M., McGarry, M. A., Marolda, C. L., and Valvano, M. A. (2008) *Mol. Microbiol.* 70, 1424–1440
33. Ortega, X., Hunt, T. A., Loutet, S., Vinion-Dubiel, A. D., Datta, A., Choudhury, B., Goldberg, J. B., Carlson, R., and Valvano, M. A. (2005) *J. Bacteriol.* 187, 1324–1333
34. Birnbaum, G. I., Roy, R., Brisson, J. R., and Jennings, H. J. (1987) *J. Carbohydr. Chem.* 6, 17–39
35. Holst, O., Thomas-Oates, J. E., and Brade, H. (1994) *Eur. J. Biochem.* 222, 183–194
36. Bock, K., Vinogradov, E. V., Holst, O., and Brade, H. (1994) *Eur. J. Biochem.* 225, 1029–1039
37. Burns, J. L., Jonas, M., Chi, E. Y., Clark, D. K., Berger, A., and Griffith, A. (1996) *Infect. Immun.* 64, 4054–4059
38. Saini, L. S., Galsworthy, S. B., John, M. A., and Valvano, M. A. (1999) *Microbiology* 145, 3465–3475
39. Lamothe, J., Huynh, K. K., Grinstein, S., and Valvano, M. A. (2007) *Cell. Microbiol.* 9, 40–53
40. Via, L. E., Fratti, R. A., Mclalone, M., Pagan-Ramos, E., Deretic, D., and Deretic, V. (1998) *J. Cell Sci.* 111, 897–905
41. Maloney, K. E., and Valvano, M. A. (2006) *Infect. Immun.* 74, 5477–5486
42. Holst, O. (2007) *FEMS Microbiol. Lett.* 271, 3–11
43. Loutet, S. A., Bartholdson, S. J., Govan, J. R. W., and Valvano, M. A. (2009) *Microbiology* 155, 2029–2039
44. Masoud, H., Perry, M. B., Brisson, J. R., Uhrin, D., Li, J., and Richards, J. C. (2009) *Glycobiology* 19, 462–471
45. Coenye, T., and Vandamme, P. (2003) *Environ. Microbiol.* 5, 719–729
46. Molinaro, A., Lindner, B., De Castro, C., Nolting, B., Silipo, A., Lanzetta, R., Parrilli, M., and Holst, O. (2003) *Chemistry* 9, 1542–1548
47. Gronow, S., Noah, C., Blumenthal, A., Lindner, B., and Brade, H. (2003) *J. Biol. Chem.* 278, 1647–1655
48. Moore, R. A., and Hancock, R. E. (1986) *Antimicrob. Agents Chemother.* 30, 923–926
49. Hooare, A., Bittner, M., Carter, J., Alvarez, S., Zaldívar, M., Bravo, D., Valvano, M. A., and Contreras, I. (2006) *Infect. Immun.* 74, 1555–1564
50. Marolda, C. L., Hauroder, B., John, M. A., Michel, R., and Valvano, M. A. (1999) *Microbiology* 145, 1509–1517
51. Arjcharoen, S., Wikraiphat, C., Pudla, M., Limposuwan, K., Woods, D. E., Sirisinha, S., and Utasincharoen, P. (2007) *Infect. Immun.* 75, 4298–4304
52. Keith, K. E., Hynes, D. W., Sholdice, J. E., and Valvano, M. A. (2009) *Microbiology* 155, 1004–1015
53. Bylund, J., Bergess, L. A., Cescutti, P., Ernst, R. K., and Speert, D. P. (2006) *J. Biol. Chem.* 281, 2526–2532
54. Speert, D. P., Bond, M., Woodman, R. C., and Curnutte, J. T. (1994) *J. Infect. Dis.* 170, 1524–1531
55. Lefebvre, M. D., and Valvano, M. A. (2002) *Appl. Environ. Microbiol.* 68, 5956–5964