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The *Klebsiella pneumoniae wabG* Gene: Role in Biosynthesis of the Core Lipopolysaccharide and Virulence

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To determine the function of the *wabG* gene in the biosynthesis of the core lipopolysaccharide (LPS) of *Klebsiella pneumoniae*, we constructed *wabG* nonpolar mutants. Data obtained from the comparative chemical and structural analysis of LPS samples obtained from the wild type, the mutant strain, and the complemented mutant demonstrated that the *wabG* gene is involved in attachment to α-L-glycero-D-manno-heptopyranose II (L,D-Hep*p*II) at the O-3 position of an α-D-galactopyranosyluronic acid (α-D-GalA*p*) residue. *K. pneumoniae* nonpolar *wabG* mutants were devoid of the cell-attached capsular polysaccharide but were still able to produce capsular polysaccharide. Similar results were obtained with *K. pneumoniae* nonpolar *waaC* and *waaF* mutants, which produce shorter LPS core molecules than do *wabG* mutants. Other outer core *K. pneumoniae* nonpolar mutants in the *waa* gene cluster were encapsulated. *K. pneumoniae waaC*, *waaF*, and *wabG* mutants were avirulent when tested in different animal models. Furthermore, these mutants were more sensitive to some hydrophobic compounds than the wild-type strains. All these characteristics were rescued by reintroduction of the *waaC*, *waaF*, and *wabG* genes from *K. pneumoniae*.

In gram-negative bacteria the lipopolysaccharide (LPS) is one of the major structural and immunodominant molecules of the outer membrane. LPS consists of three domains: lipid A, core oligosaccharide, and O-specific antigen or O side chain. In smooth LPS, the core region is conceptually divided into two regions: a lipid A proximal inner core and an outer core that provides the attachment site for the O antigen (21). Comparison of the known core LPS structures from *Enterobacteriaceae* organisms reveals that the first outer core residue might be either glucose (Glc) or a galacturonic acid (GalA) residue. In the four known *Escherichia coli* core types and in *Salmonella enterica*, a substitution of the L-glycero-D-manno-heptopyranose II (L,D-Hep*p*II) at the O-3 position for a Glc*p* residue was found (12). For *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Yersinia enterocolitica*, a substitution of the L,D-Hep*p*II at the O-3 position for an α-D-galacturonic acid residue (α-D-Gal*p*A) residue has been described (20, 29, 30). On the other hand, in most of the *Enterobacteriaceae* studied, the core LPS contains inner core phosphoryl modifications (21), but *K. pneumoniae* core LPS is devoid of such modifications (29) (Fig. 1).

Important roles in outer membrane permeability and in pathogenesis have been shown for the outer core and for the negative charges contributed by phosphoryl inner core modification in *E. coli* and/or *S. enterica* serovar Typhimurium (32, 33, 34). In view of the peculiarities of the *K. pneumoniae* core LPS, we sought in this work to determine the importance of the outer core LPS in *K. pneumoniae* outer membrane permeability and in pathogenesis. The previous knowledge of the *K. pneumoniae waa* gene cluster (the nomenclature proposed by Reeves et al. [23] for proteins and genes involved in core LPS biosynthesis is used in this work) and the elucidation of the genes involved in its inner core biosynthesis (14, 24) (Fig. 1) facilitated the identification of the gene involved in the transfer of the first outer core residue to construct and characterize *K. pneumoniae* mutants devoid of the outer core LPS.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *K. pneumoniae* strains 889 (serovar O8:K69) (11), 52145 (O1:K2) (17), NC20 (*waaL* mutant) (24), and *Serratia marcescens* N28b (10) were used in this study. Bacterial strains were grown in Luria-Bertani (LB) broth and LB agar (16). LB media were supplemented with kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), and tetracycline (25 μg ml^{-1}) when needed. The plasmids used in this study and their characteristics are shown in Table 1.

General DNA methods. General DNA manipulations were done essentially as described previously (26). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the respective manufacturers.

LPS isolation and electrophoresis. Cultures for analysis of LPS were grown in tryptic soy broth at 37°C. LPS was purified by the *Pneumocystis carinii* pneumonia method (7), resulting in a yield of 2.3%. For screening purposes, LPS was obtained after proteinase K digestion of whole cells (13). LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or SDS–tricine-PAGE and visualized by silver staining as previously described (19, 28).

Isolation of oligosaccharides. LPS (20 mg) was hydrolyzed with 1% AcOH (100°C for 1 h). The resulting precipitate (8 mg) was removed by centrifugation, and the supernatant (10 mg) was analyzed by mass spectrometry. Another sample of LPS (40 mg) was deacylated and purified as described previously (3), yielding 6 mg of alditol oligosaccharide mixture.

LPS chemical analysis. For chemical analysis, either purified LPS or core LPS oligosaccharide samples were hydrolyzed with 1 N trifluoroacetic acid for 4 h at 100°C. Alditol acetates and methyl glycoside acetates were analyzed on an Agilent Technologies model 5973N mass spectrometry (MS) instrument equipped with a model 6850A gas chromatography and an RTX-5 capillary column (Restek; 30 m × 0.25 mm inside diameter, flow rate 1 ml min^{-1}, He used

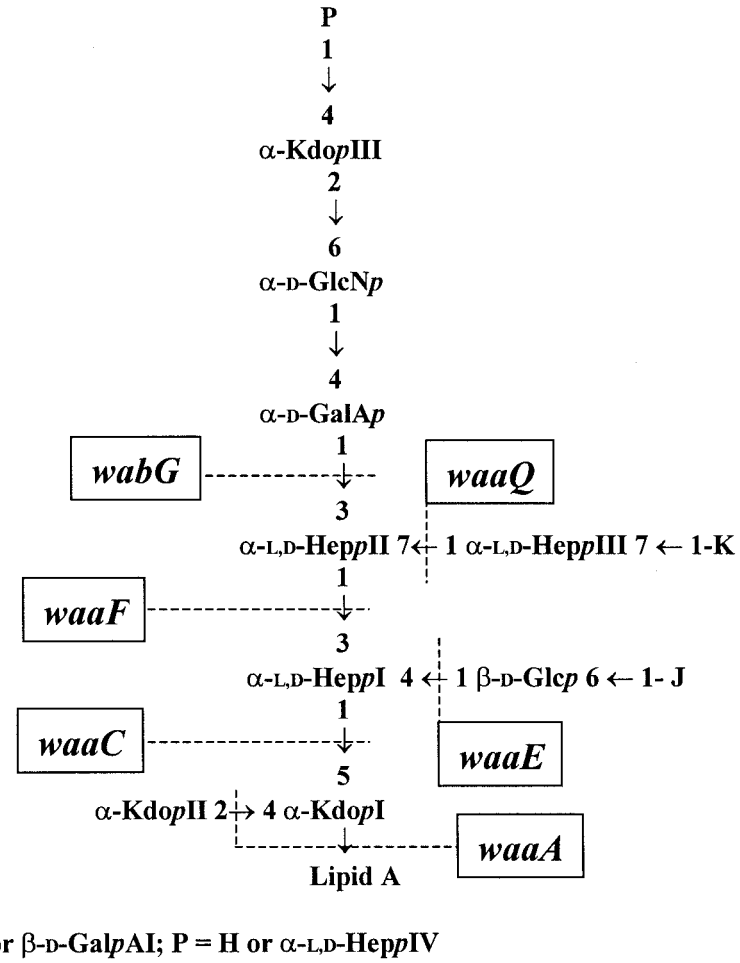

FIG. 1. Conserved region in the core LPS structure of *K. pneumoniae* (29) and genes involved in inner core biosynthesis (24, 14). Kdo*p*, 3-deoxy-D-manno-octulopyranosonic acid; Glc*p*, D-glucopyranose; GlcN*p*, glucosamine; GalA*p*, galacturonic acid. Depending on the *K. pneumoniae* strain, residues J and K could be H or GalA, and residue P could be H or Hep (29).

as carrier gas). Acetylated methyl glycoside analysis was performed with the following temperature program: 150°C for 5 min, 150 to 250°C at 3°C min^{-1}, and 250°C for 10 min. Acetylated methyl ester lipid analysis was performed as follows: 150°C for 3 min, 150 to 280°C at 10°C min^{-1}, and 280°C for 15 min. The alditol acetate mixture was analyzed with the following temperature program: 150°C for 5 min and 150 to 300°C at 3°C min^{-1}. For partially methylated alditol acetates, the temperature program was 90°C for 1 min, 90 to 140°C at 25°C min^{-1}, 140 to 200°C at 5°C min^{-1}, 200 to 280°C at 10°C min^{-1}, and 280°C for 10 min.

Glycosyl and lipid analysis. A sample (1 mg) of LPS was dried over P_2O_5 overnight and was then treated with 1 M HCl-CH_3OH (1 ml) at 80°C for 20 h to analyze both glycosyl and fatty acid composition. The crude reaction was extracted twice with hexane, and the two extracts were pooled, dried under a stream of air, and treated with acetic anhydride (100 μl) at 100°C for 15 min. The

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic[a]	Source or reference
pKO3	Cm^r *sacB* temperature-sensitive replication	15
pKO3Δ*waaC*	Contains the *K. pneumoniae* engineered *waaC* deletion	This work
pKO3Δ*waaF*	Contains the *K. pneumoniae* engineered *waaF* deletion	This work
pKO3Δ*waaL*	Contains the *K. pneumoniae* engineered *waaL* deletion	This work
pKO3Δ*wabG*	Contains the *K. pneumoniae* engineered *waabG* deletion	This work
pGEMT	Ap^r plasmid vector	Promega
pGEMT-WaaC	*waaC* gene from *K. pneumoniae* in pGEMT	This work
pGEMT-WaaF	*waaE* gene from *K. pneumoniae* in pGEMT	This work
pGEMT-WaaL	*waaL* gene from *K. pneumoniae* in pGEMT	This work
pGEMT-WabG	*wabG* gene from *K. pneumoniae* in pGEMT	This work
pGEMT-$Orf9_{Sm}$	*orf9* gene from *S. marcescens* in pGEMT	This work

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

methanol layer was neutralized with Ag_2CO_3, dried, and acetylated. Both samples were subjected to gas chromatography-MS. Another sample of LPS (1 mg) was hydrolyzed with 4 M trifluoroacetic acid for 1 h at 100°C, reduced with deuterated sodium tetrahydridoborate ($NaBD_4$), acetylated, and analyzed by gas chromatography-MS.

MS studies. Electrospray MS was performed on a Micromass ZQ instrument (Waters). The sample (100 pmol) was deionized on Dowex H+ resin (Fluka) and dissolved in 2% triethylamine in 50% acetonitrile and injected into the ion source at a flow rate of 5 μl min^{-1}. The spectrum was acquired in negative mode. Positive-ion reflectron matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectra were acquired on a Voyager DE-PRO instrument (Applied Biosystems) equipped with a delayed-extraction ion source. Ion acceleration voltage was 20 kV, grid voltage was 14 kV, mirror/voltage ratio was 1/12, and the delay time was 100 ns. Samples were irradiated at a frequency of 5 Hz by 337-nm photons from a pulsed-nitrogen laser. Postsource decay (PSD) was performed using an acceleration voltage of 20 kV (27). The reflectron voltage was decreased in 10 successive 25% steps. Mass calibration was obtained with a malto-oligosaccharide mixture from corn syrup (Sigma). A solution of 2,5-dihydroxybenzoic acid in 20% CH_3CN in water at a concentration of 25 mg/ml was used as the MALDI matrix. One microliter of matrix solution and 1 μl of the sample were premixed and then deposited on the target. The droplet was allowed to dry at ambient temperature. Spectra were calibrated and processed under computer control by using the Applied Biosystems Data Explorer software.

Methylation analysis. The alditol oligosaccharide mixture was *N*-acetylated by dissolving a sample (2 mg) in dry methanol and treating it with 50 μl of acetic anhydride for 16 h. After evaporation of the solvents, the sample was methylated as previously reported (2). Linkage analysis was performed as follows: the methylated sample was carboxymethyl reduced with lithium triethylborohydride (Aldrich), mildly hydrolyzed to cleave ketosidic linkage, reduced by means of $NaBD_4$, and was then totally hydrolyzed, reduced with $NaBD_4$, and finally acetylated as described previously (6).

***K. pneumoniae waaC*, *waaF*, *waaL*, and *wabG* mutant construction.** To obtain *K. pneumoniae* mutant strains, a method was used to create chromosomal in-frame *waa* deletions (15). Primers for mutant construction were designed from the known *K. pneumoniae waa* gene cluster sequence. Primer pairs Fa (5′-CGCGGATCCAAATCCCGTTCCTGTACGCC-3′) and Fb (5′-CCCATCCACTAAACT TAAACACATCATCATGTCGCCCACC-3′) and Fc (5′-TGTTTAAGTTTAGTG GATGGGTTAGCGGAAAAACCGAGCAC-3′) and Fd (5′-CGCGGATCCGCAGAAACACCAGATAGGGC-3′) were used in two sets of asymmetric PCRs to amplify DNA fragments of 697 (AB) and 618 (CD) bp, respectively. DNA fragment Fa-Fb encompasses nucleotide 470, inside *gmhD*, to nucleotide 1137, corresponding to the first base of codon 16 of *waaF*. DNA fragment Fc-Fd encompasses nucleotide 2116, corresponding to the first base of the 393rd codon of *waaF*, to nucleotide 2704, which lies within the *waaC* gene. DNA fragments Fa-Fb and Fc-Fd were annealed at their overlapping region (the underlined letters in primers Fb and Fc) and amplified by PCR as a single fragment, using primers Fa and Fd. The fusion product was purified, digested with *Bam*HI (the *Bam*HI site is shown as the double-underlined letters in primers Fa and Fd), ligated into *Bam*HI-digested and phosphatase-treated pKO3 vector (15), electroporated into *E. coli* DH5α, and plated on chloramphenicol-kanamycin plates at 30°C to obtain plasmid pKO3Δ*waaF*. Primer pairs Ca (5′-CGCGGATCCGCGCTTTTAACC TGTCCTAC-3′) and Cb (5′-CCCATCCACTAAACTTAAACAAACGATCAAT ACCCGCATCC-3′) and Cc (5′-TGTTTAAGTTTAGTGGATGGGCA CACTCTAATATCTCCGACC-3′) and Cd (5′-CGCGGATCCGCTCCATGACCCTTTTTGAC-3′) were used to obtain plasmid pKO3Δ*waaC*, containing an internally deleted *waaC* gene (the first 6 codons, a 7-codon tag, and the last 26 codons). Primer pairs La (5′-CGCGCGGCCGCGGATATTGCAG GACAAAGGGC-3′) and Lb (5′-CCCATCCACTAAACTTAAACAAAGCAAACC GGCAAGGTTAAG-3′) and Lc (5′-TGTTTAAGTTTAGTGGATGGGGAT GAGAACCATGAGTGACAAG-3′) and Ld (5′-CGCGCGGCCGCATATGCCAGTGG GAACGAC-3′) were used to obtain plasmid pKO3Δ*waaL*, containing an internally deleted *waaL* gene (the first 22 codons, a 7-codon tag, and the last 5 codons of *waaL*). Primer pairs Ga (5′-CGCGGATCCCCACCCAACAGCACAACC-3′) and Gb (5′-CCCATCCACTAAACT TAAACAGACAAACCGTTCTGCGCC-3′) and Gc (5′-TGTTTAAGTTTAGTGGATGGGAGCGAGCGACTCTCAACC-3′) and Gd (5′-CGCGGATCCGCGACCGACGTGAATCAG-3′) were used to obtain plasmid pKO3Δ*wabG*, containing an internally deleted *wabG* gene (the first 23 codons, a 7-codon tag, and the last 17 codons of *wabG*). Plasmids pKO3Δ*waaC*, pKO3Δ*waaF*, pKO3Δ*waaL*, and pKO3Δ*wabG* were used to construct nonpolar mutations in the *waaC*, *waaF*, *waaL*, and *wabG* genes, respectively.

Plasmid construction. To complement the constructed mutants, the *waaC*, *waaF*, *waaL*, and $wabG_{Kp}$ genes from *K. pneumoniae* and the *S. marcescens* $wabG_{Sm}$ homologue were PCR amplified and ligated to the vector pGEMT as follows: pGEMT-WaaC (5′-GTTTAAATCGGCATTAGTCC-3′ and 5′-AAGCAAACCGGCAAGGTTAAG-3′), pGEMTWaaF (5′-TCAGCCCAGCACCTTATTC-3′ and 5′-TTTTACCGTATCCGCCAATC-3′), pGEMTWaaL (5′-TACAGGGAACGTCAGAAGC-3′ and 5′-ATGCCTTGCATCACATTAC-3′), pGEMT-$WabG_{Kp}$ (5′-CAATGGCAGCTCATTCAGAC-3′ and 5′-TGAAAGCCTTTGAACCACAC-3′), and pGEMT-$Orf9_{Sm}$ (5′-TCAAATGCTGGAGCGAAGAG-3′ and 5′-CCTGATAATCAATGCCTGAC-3′).

Urinary tract infections (UTIs) in rats. The bacterial strains used to establish infection were grown overnight in LB agar supplemented with antibiotics when needed and gently suspended in phosphate-buffered saline to the appropriate concentration. In each experiment, 12 female Wistar rats (weight, 200 to 250 g) of strain CFHB (Interfauna UK, Hungtinton, United Kingdom) were used. Ten animals were infected and two were used as controls. The infections were established and quantified as previously described (1).

Murine pneumonia model. The experiments were performed as previously described (4). Briefly, ICR-CDI mice (Harlan Ibérica, S.L.) were anesthetized and intubated intratracheally with a blunt-end needle. Approximately 10^7 CFU of exponential *K. pneumoniae* cells was suspended in 50 μl of phosphate-buffered saline and inoculated through the blunt-end needle. The mice were observed daily, and bacteremia was assessed at days 2, 4, and 6 by culturing blood obtained from the tail vein (approximately 20 μl) on LB agar plates. Lung and spleen tissues from surviving or dead animals were aseptically removed, homogenized, and plated for growth of quantitative bacterial cultures. Each experiment was performed with nine animals.

LD_{50}. Albino Swiss female mice (5 to 7 weeks old; Harlan Ibérica, S.L.) were injected intraperitoneally with 0.2 ml of the test samples. Mortality was recorded up to 7 days postinjection, and all deaths occurred within 1 to 5 days. The 50% lethal dose (LD_{50}) was calculated as previously described (22).

RESULTS

Construction of *K. pneumoniae wabG* mutants. We have previously reported the nucleotide sequence of the *K. pneumoniae waa* gene cluster and have identified the functions of the genes involved in the biosynthesis of its inner core LPS (Kdo, L,D-Hep*p*I, L,D-Hep*p*II, L,D-Hep*p*III, and branched D-Glc*p* transferases) (14, 24). Comparison of the known outer core LPS structures among *Enterobacteriaceae* revealed that the first outer core residue is either D-Glc or D-GalA, linked to the L,D-Hep*p*II residue by an α1,3 bond. Thus, it could be expected that the enzymes involved in the transfer of the first outer core residue would share some similarity. The WaaG protein has been identified as the glucosyltransferase involved in the transfer of the first outer core LPS residue in *E. coli* and *S. enterica* serovar Typhimurium. Only the *K. pneumoniae orf8*-encoded protein showed significant albeit low levels of identity (26%) and similarity (44%) to the WaaG protein from *E. coli*; therefore, this *K. pneumoniae* gene was named *wabG*. To determine the *wabG* function, nonpolar mutants were constructed in *K. pneumoniae* 889 and 52145. *K. pneumoniae* 889 (08:K69) (11) was used because its core LPS structure has been recently updated using the nonencapsulated mutant NRC6121 (Fig. 1) (29). Strain 52145 was used because it shows high virulence in different experimental animal models (17). To construct the *K. pneumoniae wabG* nonpolar mutants, an in-frame tagged deletion approach was used. Plasmid pKO3Δ*wabG*, containing the engineered deletion, was used to introduce the *wabG* deletion into *K. pneumoniae* 889 and 52145 by double recombination as previously described (15, 24). Candidate mutants were screened by PCR, and two of them, strains 889Δ*wabG* and 52145Δ*wabG*, were proved to contain the desired mutation by DNA nucleotide sequence determination. LPS from strains 889 and 52145 (wild type) and 889Δ*wabG* and 52145Δ*wabG* was extracted and analyzed by SDS–tricine-

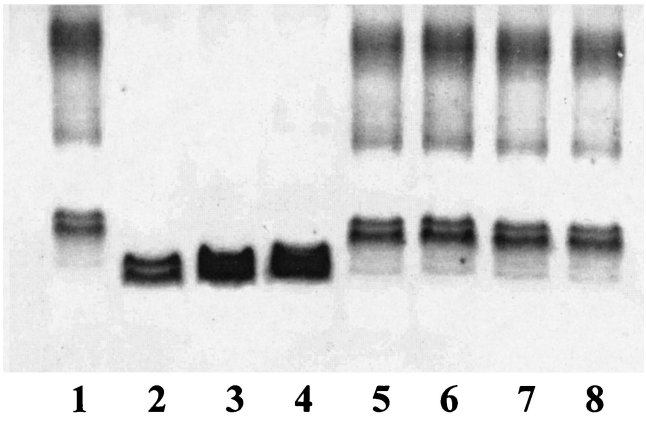

FIG. 2. SDS–tricine-PAGE analysis of LPS samples from *K. pneumoniae* 52145 (lane 1), 52145Δ*waaC* (lane 2), 52145Δ*waaF* (lane 3), 52145Δ*wabG* (lane 4), 52145Δ*waaC* plus pGEMT-WaaC (lane 5), 52145Δ*waaF* plus pGEMT-WaaF (lane 6), 52145Δ*wabG* plus pGEMT-WabG$_{Kp}$ (lane 7), and 52145Δ*wabG* plus pGEMT-ORF9$_{Sm}$ (lane 8).

PAGE. The core LPS from the mutant strains migrated faster than that from the wild-type strains, suggesting that the *wabG* mutants contain a truncated-core LPS (Fig. 2, lane 4).

To test whether the WabG and the *S. enterica* serovar Typhimurium WaaG proteins perform the same function, two complementation assays were performed. Plasmids pGEMT-WabG and pGEMT-WaaG were unable to complement *S. enterica* serovar Typhimurium SL3768 *waaG* (25) and *K. pneumoniae wabG* mutants, respectively, as determined by SDS–tricine-PAGE of LPS.

***K. pneumoniae wabG* LPS analysis.** To determine the core LPS changes produced by the mutation of *wabG*, LPS was obtained from strains 889Δ*wabG*, 52145Δ*wabG*, 889, and 52145. Comparative monosaccharide composition analysis of these LPS samples revealed major changes in LPS composition, with a complete loss of GalA and about a 30% reduction in glucosamine (GlcN) in the mutant *wabG* strains. The putative GalA residues (J and K in Fig. 1) are not present in all the *K. pneumoniae* strains studied (29). Our data suggest that the strains used in this study lack both GalA residues (J and K in Fig. 1).

To elucidate the chemical structure of the LPS core region of the *wabG* mutant (889Δ*wabG*) in more detail, LPS was hydrolyzed with 1% acetic acid, which cleaves the acid-labile ketosidic linkages between KdoI and lipid A, KdoI and KdoII, and KdoIII-GlcN as reported (29). The negative ions' Electro-Spray ionization mass spectra of core oligosaccharide (data not shown) showed several signals. One of these signals indicated the presence of a pseudomolecular ion $(M-H)^-$ at *m/z* 783.37, a value which was in agreement with the calculated average molecular weight (783.67) of the expected molecular structure, with one hexose, two heptose, and one Kdo units. The presence of a −18 signal at *m/z* 765.38 is attributable to the anhydrous form of the reducing Kdo end, and it is well documented for LPS samples that are hydrolyzed in presence of acetic acid (18).

In order to determine the oligosaccharide sequence, we analyzed the sample with the MALDI-PSD technique, which enabled us to obtain a total fragment spectrum in a single experiment. The positive ions' PSD spectra of the acetic acid product (Fig. 3) contains many fragment ions, most of them attributable to B-type ions (5). Either molecular ions or fragment ions contain sodium, as has been reported for MALDI spectra. It is also known that interpretation of a PSD spectrum of unknown sample is potentially difficult, but in this case we were able to assign almost all of the signals. Actually, other than the signals reported in the fragmentation scheme, the fragment ion at *m/z* 744.8 can be attributed to the decarboxylated anhydrous core structure (M-18-44) (9). Particularly important to define the ramified nature of the core oligosaccharide are the two signals at *m/z* 376.7 and 358.6. These signals are attributable to internal fragmentation (8), as they might arise from a loss of the terminal heptose residue from the signal at 568.9, leaving a hydroxyl group (*m/z* 376.7) or a double bond (*m/z* 358.6). In agreement with the proposed structure was the ^{1}H nuclear magnetic resonance spectrum of this sample, which mainly showed three anomeric signals at δ 5.31 and 5.09 (bs) and at δ 4.54 (doublet, $^3J_{H,H}$ 7.8 Hz) (data not shown), according to the presence of two heptose units and one Glc unit, respectively.

Similar results were obtained from the complete *O*,*N*-deacylated LPS from the *K. pneumoniae wabG* mutant. This sample was obtained after hydrazinolysis, HF treatment, $NaBH_4$ reduction, and KOH hydrolysis as already reported (3). The reflectron-positive ions' MALDI-TOF spectra of this fraction showed the presence of three clusters of signals: the first at *m/z* 1,153.87 $(M1 \text{ plus } 2Na)^+$ (calculated to be 1,154.95), corresponding to one hexose, two heptose, one Kdo, one hexosamine and one hexosaminitol; the second at *m/z* 1,374.16 $(M1 \text{ plus } 2Na)^+$ (calculated to be 1,375.08), corresponding to the same composition plus one Kdo unit; and the last at *m/z* 1,296.2 $(M2)^+$ (calculated to be 1,291.1), corresponding to one hexose, three heptose, one Kdo, one hexosamine and one hexosaminitol. Other than the two signals already found in the acetic acid hydrolysis product (*m/z* 569.0 and 789.1) in the PSD spectrum of the signal at *m/z* 1153.7 (Fig. 4), there are signals at *m/z* 365.2 and 386.7 (365.2 plus Na) which clearly indicate the presence of the two GlcN residues of the lipid A. Moreover, the intense signal at *m/z* 810.9 can be attributed to the C fragment (5), which contains one hexose residue, two heptose residues, and one Kdo residue (789.1) (26). These results strongly suggest the presence of the oligosaccharide reported in the fragmentation scheme (Fig. 4).

In order to confirm the proposed structure, a methylation analysis on the *N*-acetylated oligosaccharide alditol mixture was performed. The results obtained confirm the presence of three terminal residues (Glc*p*, Hep*p*, and Kdo*p*). The presence of a 3,4-linked Hep confirmed the branching point in the oligosaccharide structure. The detection of terminal Kdo and 5,4-linked Kdo units can be attributed to the oligosaccharide alditol having one additional Kdo (KdoII) unit linked at the O4 position of the KdoI residue. The above results strongly suggest that the WabG protein is involved in attachment of the first outer core residue (GalA*p*) to the L,D-Hep*p*II by an α1,3 linkage.

Phenotypic characterization of the mutant strains. The chemical characterization of the *wabG* mutants revealed that its core LPS is devoid of the outer core region. Furthermore,

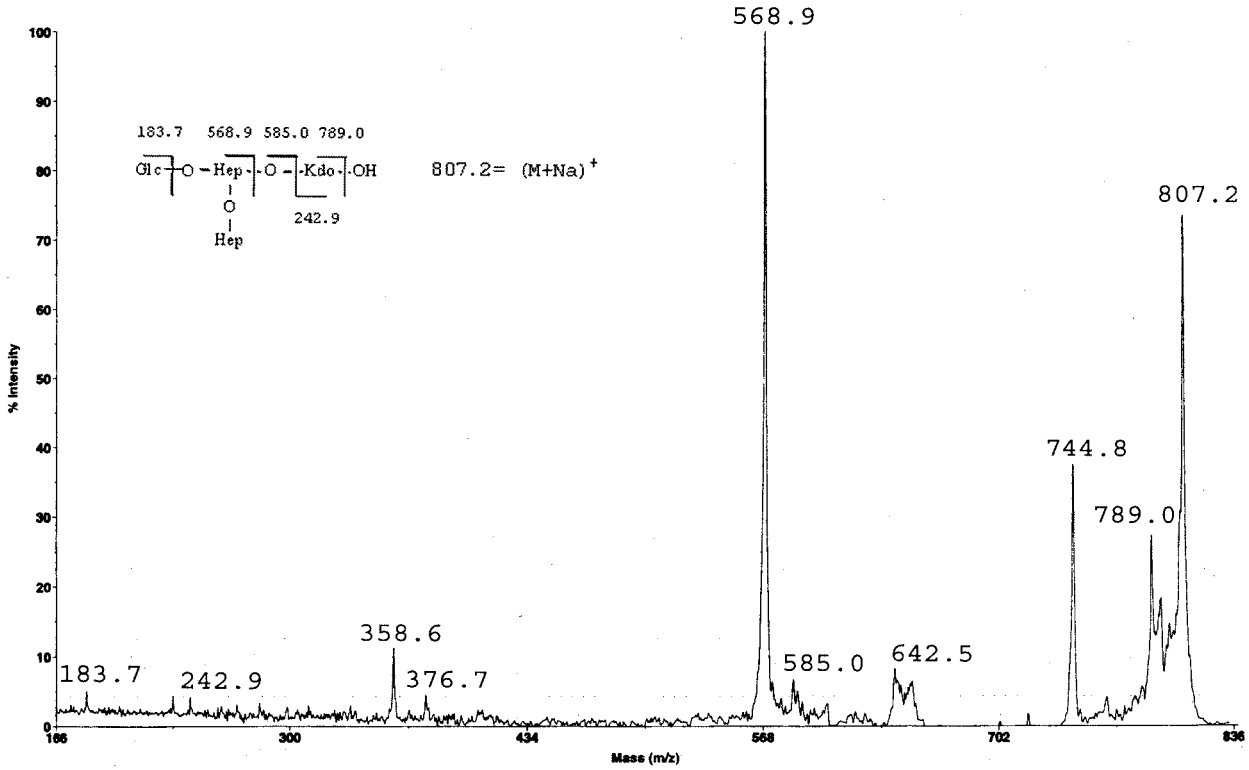

FIG. 3. PSD spectrum of *m/z* 807.2 of *K. pneumoniae* 889Δ*wabG* core oligosaccharide after acidic release of Lipid A, in the positive-ion mode. Insert shows the proposed structure and fragmentation pattern.

the nonmucoid colony morphology of the *wabG* mutants suggests that they are unencapsulated. Since it is well known that the *K. pneumoniae* capsule plays an important role in pathogenesis (31), we decided to determine if the *K. pneumoniae* capsule is linked to the core LPS. The approach was based on the analysis of several *waa* nonpolar deletion mutants, i.e., 52145Δ*waaC*, 52145Δ*waaF*, 52145Δ*wabG*, and 52145Δ*waaL*, derived from wild-type strain 52145 (O1:K2). These strains and a previously constructed *waaL* mutant (NC20) derived from wild-type strain C3 (O1:K66) (24) were analyzed for the presence of capsule by sensitivity to capsule-specific bacteriophages, by electron microscopy (EM) studies, and by enzyme immunoassay (EIA) with capsular-specific serum. The NC20 and 52145Δ*waaL* strains contained K66 and K2 capsule, respectively, as can be deduced from their sensitivity to capsule-specific bacteriophages, EM studies, and reactivity against K66- and K2-specific antibodies in EIA. In contrast, no K2 capsule was detected in the 52145Δ*waaC*, 52145Δ*waaF*, and 52145Δ*wabG* whole-cell mutants. Culture supernatants of strains 52145Δ*waaC*, 52145Δ*waaF*, and 52145Δ*wabG* reacted by EIA with K2-specific antibodies. Neither whole cells nor culture supernatants from a 52145 K2$^-$ mutant (with a mini-Tn*5* inserted in the known K2 capsular biosynthetic cluster) were unable to react by EIA with K2-specific serum. These results clearly show that in *K. pneumoniae*, the outer core LPS is somehow involved in K2 capsule's, and probably other capsular types', attachment to the cell surface.

The truncation of the core LPS in other *Enterobacteriaceae* results in profound changes in the bacterial cell behavior and permeability (reviewed in reference 21). Since these assays have been used with *Enterobacteriaceae* strains containing phosphoryl modifications in their inner core LPS (32, 33, 34), we decided to determine the behavior of LPS core-truncated mutants from *K. pneumoniae*, an *Enterobacteriaceae* organism that is naturally devoid of such inner core phosphoryl modifications. The sensitivity to hydrophobic compounds of the 52145 mutants was compared to that of the wild-type strain. For the 52145Δ*wabG* mutant, MICs of SDS, deoxycholate, and polymyxin B were found to be 0.5 mg ml^{-1} (a greater than 20-fold reduction), 10 mg ml^{-1} (a 50% reduction), and 2 μg ml^{-1} (a 60% reduction), respectively. The MICs obtained for mutants devoid of the inner core Hep region (52145Δ*waaC*) or containing only the first L,D-Hep*p*I (52145Δ*waaF*) residue were essentially similar to those for the mutants lacking the outer core region (52145Δ*wabG*). For the 52145Δ*wabG* mutant, MICs of antibiotics (nalidixic acid, erythromycin, novobiocin, and rifampin) showed about 50% reduction in comparison to those for the wild-type strain, while the MICs for the 52145Δ*waaC* and 52145Δ*waaF* mutants showed about 80 to 90% reduction when these same antibiotics were used. Similar MICs were obtained for strains 889Δ*waaC*, 889Δ*waF*, and 889Δ*wabG*.

Complementation studies. The wild-type pattern of electrophoretic banding (Fig. 2, lanes 1, 5, 6, and 7), the presence of K2 capsule (as determined by sensitivity to specific capsule bacteriophage, EM studies, and reactivity against K2-specific antibodies in EIA studies), SDS, deoxycholate, and polymyxin B sensitivity were demonstrated by the mutant strains

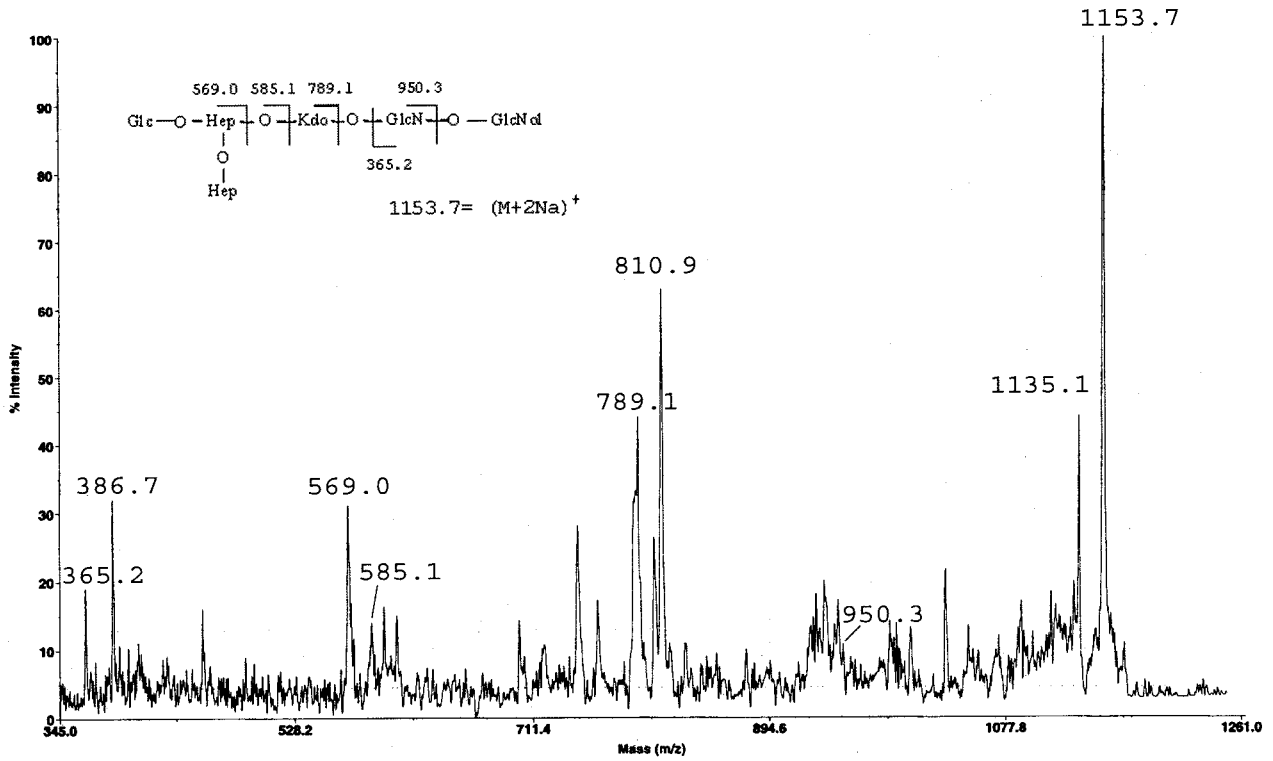

FIG. 4. PSD spectrum of *m/z* 1153.7 of *O,N*-deacylated and dephosphorylated LPS from *K. pneumoniae* 889Δ*wabG*, in the positive-ion mode. Insert shows the proposed structure and fragmentation pattern.

52145Δ*waaC*, 52145Δ*waaF*, and 52145Δ*wabG* upon complementation with plasmids pGEMT-WaaC, pGEMT-WaaF, and pGEMT-WabG, respectively. The phenotypic characteristics of the *waaC*, *waaF*, and *wabG* mutants were not changed when the plasmid vector alone (pGEMT) was introduced by transformation.

The *S. marcescens waa* gene cluster has been sequenced (GenBank accession number U52844). An open reading frame (ORF), *orf9*, has been identified as encoding a putative protein highly similar (69% identity and 81% similarity) to the *K. pneumoniae* WabG protein, suggesting that the $orf9_{Sm}$ could be a *wabG* homologue. To test this possibility, the mutant 52145Δ*wabG* was transformed with plasmid pGEMT-$Orf9_{Sm}$. The transformed strain showed full-length LPS (Fig. 2, lane 8), produced K2 capsule, and exhibited wild-type levels of sensitivity to hydrophobic compounds. This result strongly suggests that the first residue in the *S. marcescens* N28b core LPS would be an α-D-Gal*p*A residue linked to the L,D-Hep*p*II by an α1,3 linkage.

Colonization and virulence studies. As a colonization model, we used experimental UTI in rats. As shown in Table 2, 52145Δ*waaC*, 52145Δ*waaF*, and 52145Δ*wabG* mutants are unable to induce experimental UTIs in rats (unable to colonize the rat UT). However, the 52145Δ*waaL* mutant showed a reduced ability to colonize the rat UT in comparison with that of the wild-type strain, but the mutant was still able to perform some colonization. The plasmid vector harboring the corresponding wild-type gene(s) introduced by transformation restored (to the level of the wild-type strain) the ability of all the mutants to induce experimental UTIs in rats. The plasmid vector alone was unable to restore this ability when introduced in the mutant strains.

Virulence was tested in two different models: (i) a septicemia model in mice by intraperitoneal injection and recording the mortality (LD_{50}) and (ii) a murine model of pneumonia by intratracheal injection. When we measured the virulence of the strains in the septicemia model, 52145Δ*waaC*, 52145Δ*waaF*, and 52145Δ*wabG* mutants showed a strong increase (approximately 5 log) in their LD_{50}s in comparison with that of the wild-type strain (Table 3). The 52145Δ*waaL* mutant showed only a 3-log increase in its LD_{50} compared to that of the wild-type strain. When the plasmid vectors harboring the corresponding wild-type gene were introduced in the mutant strains, all of them recovered LD_{50}s similar to that of the wild-type strain in this virulence model, while no changes were observed in the LD_{50}s of the mutant strains transformed with the plasmid vector alone (Table 3).

When the virulence of the strains was assayed in the murine pneumonia model, we obtained the results showed in Table 4. The 52145Δ*waaC*, 52145Δ*waaF*, and 52145Δ*wabG* mutants were completely avirulent in this model, while the 52145Δ*waaL* mutant and the wild-type strain showed similar values. Introduction of the corresponding gene(s) in the mutants rendered them as virulent as the wild-type strain or the 52145Δ*waaL* mutant. Mutants 52145Δ*waaC*, 52145Δ*waaF*, and 52145Δ*wabG*, which were transformed with the plasmid vector (pGEMT) alone, remained avirulent in this animal model.

TABLE 2. Experimental UTI in rats by different *K. pneumoniae* strains

Strain (infection dose CFU/rat)	% Of infected rats and viable counts[a]		
	Sample	% Infected[b]	Mean ± SD[c]
K. pneumoniae 51245 (O1:K2, wild type) (0.8×10^9)	Kidney	100	6.9 ± 0.6
	Bladder	100	6.7 ± 0.5
	Urine	100	8.4 ± 0.4 (6)
52145Δ*waaC* (1.1×10^9)	Kidney	10	4.3
	Bladder	10	3.8
	Urine	0	0
52145Δ*waaC* + pGEMT (1.0×10^9)	Kidney	10	4.8
	Bladder	0	0
	Urine	10	4.6
52145Δ*waaC* + pGEMT-WaaC (0.9×10^9)	Kidney	100	6.4 ± 0.6
	Bladder	100	6.1 ± 0.4
	Urine	100	8.1 ± 0.4 (6)
52145Δ*waaF* (1.1×10^9)	Kidney	10	4.8
	Bladder	10	3.5
	Urine	0	0
52145Δ*waaF* + pGEMT (0.9×10^9)	Kidney	10	4.3
	Bladder	0	0
	Urine	0	0
52145Δ*waaF* + pGEMT-WaaF (1.2×10^9)	Kidney	100	6.5 ± 0.7
	Bladder	100	6.4 ± 0.6
	Urine	100	8.2 ± 0.3 (6)
52145Δ*wabG* (1.2×10^9)	Kidney	10	4.2
	Bladder	0	0
	Urine	0	0
52145Δ*wabG* + pGEMT (1.2×10^9)	Kidney	10	4.6
	Bladder	10	3.7
	Urine	0	0
52145Δ*wabG* + pGEMT-WabG (1.1×10^9)	Kidney	100	6.2 ± 0.7
	Bladder	100	6.0 ± 0.3
	Urine	100	8.3 ± 0.8 (6)
52145Δ*waaL* (1.2×10^9)	Kidney	40	4.7 ± 0.8
	Bladder	40	4.3 ± 0.9
	Urine	30	7.2 ± 0.6
52145Δ*waaL* + pGEMT (1.3×10^9)	Kidney	40	4.9 ± 0.5
	Bladder	30	4.0 ± 0.7
	Urine	30	5.1 ± 0.3
52145Δ*waaL* + pGEMT-WaaL (1.4×10^9)	Kidney	100	6.8 ± 0.4
	Bladder	100	6.0 ± 0.3
	Urine	100	7.9 ± 0.6 (6)

[a] A total of 20 kidneys and 10 bladders were studied in each group. Numbers in parentheses represent urine samples studied.
[b] Percentage of positive cultures. The lowest number of organisms detectable by the method was 50 CFU per g (kidney or bladder) or per ml (urine).
[c] Values represent the mean $\log_{10}$ CFU per gram or per milliliter ± standard deviation of the positive cultures. All the assays were done at least in triplicate.

DISCUSSION

Structural studies of the core region of LPS from *K. pneumoniae* have revealed that all of them showed very similar core structures with minor changes among different serogroups (29). In all the studied *K. pneumoniae* strains, the LPS core structure is characterized by the substitution of L,D-Hep*p*I at the O-4 position by a Glc*p* [β-D-Glc*p*-(1→4)-α-L,D-Hep*p*I] and by the substitution of the L,D-Hep*p*II at the O-3 position by an α-Kdo-(2→6)-α-D-GlcN-(1→4)-α-D-GalA trisaccharide (29) (Fig. 1). Since the six genes involved in the biosynthesis of the *K. pneumoniae* inner core LPS (*gmhD*, *waaC*, *-F*, *-Q*, *-A*, and *-E*) have been previously identified by us (14, 24), we set up experiments to begin the identification and characterization of the genes involved in outer core LPS biosynthesis. Among the four unassigned genes in the *K. pneumoniae waa* gene cluster, the *orf4*- and *orf6*-encoded products showed similarity to the *E. coli* K-12 WaaZ and enterobacterial ADP-heptose-LPS heptosyltransferases, respectively. In addition, results of a previous analysis of a *K. pneumoniae orf10* (*yibD*) mutant suggested that it could be involved in capsule attachment (24). Thus, only *orf8* and *orf9* remained as candidates to be involved in the transfer of the first residue of the outer core LPS. In the five *E. coli* core types and *S. enterica* serovar Typhimurium, the L,D-Hep*p*II residue is substituted at the O-3 position by a D-Glc residue (Glc I), while a D-GalA residue is found in this position in *K. pneumoniae*. In addition, the *orf8*-encoded protein (WabG) showed low albeit significant similarity to the *E. coli* and *S. enterica* WaaG protein, and no cross-complementation between *wabG* and *waaG* was detected.

To study the function of the *wabG*, two nonpolar mutants were constructed and characterized by using *K. pneumoniae* wild-type strains 889 (O:8 K:69) and 52145 (O:1 K:2). SDS–tricine-PAGE analysis of LPS samples from both 889Δ*wabG* and 52145Δ*wabG* mutants suggested that these LPS are devoid of O antigen and contain a truncated-core LPS (Fig. 2). The comparative chemical and structural analyses (Fig. 3 and 4) of

TABLE 3. LD_{50}s of mice inoculated intraperitoneally with different *K. pneumoniae* strains

Strain	LD_{50}[a]
51245 (O1:K2, wild type)	$10^{2.1}$
52145Δ*waaC*	$10^{7.8}$
52145Δ*waaC* + pGEMT	$10^{7.6}$
52145Δ*waaC* + pGEMT-WaaC	$10^{2.5}$
52145Δ*waaF*	$10^{7.9}$
52145Δ*waaF* + pGEMT	$10^{7.8}$
52145Δ*waaF* + pGEMT-WaaF	$10^{2.6}$
52145Δ*wabG*	$10^{7.5}$
52145Δ*wabG* + pGEMT	$10^{7.7}$
52145Δ*wabG* + pGEMT-WabG	$10^{2.4}$
52145Δ*waaL*	$10^{5.1}$
52145Δ*waaL* + pGEMT	$10^{5.7}$
52145Δ*waaL* + pGEMT-WaaL	$10^{2.3}$

[a] LD_{50}s were calculated as previously described (22).

the LPS from wild-type and mutant strains allow us to conclude that WabG is involved in the linkage of the first outer core residue (D-GalA) to the O-3 position of the L,D-Hep*p*II residue. The complementation achieved when the mutant 52145Δ*wabG* was transformed with plasmid pGEMT-$Orf9_{Sm}$ strongly suggests that the first residue in the *S. marcescens* N28b core LPS would be an α-D-Gal*p*A residue linked to the L,D-Hep*p*II by an α1,3 linkage.

In *Enterobacteriaceae* organisms containing phosphoryl modifications in their inner core LPS, such as *E. coli* and *S. enterica* serovar Typhimurium, truncation of the outer core results in alterations in cell permeability to hydrophobic compounds (21). Mutation in the *waaG* gene of *E. coli* strain F470 results in loss of the outer core, absence of L,D-Hep*p*II phosphorylation, a 60% reduction in L,D-Hep*p*I phosphorylation, and decrease in MICs of SDS (34). An *E. coli waaP* mutant produces core LPS totally devoid of phosphoryl modifications; this mutant was found to be even more sensitive to SDS and other hydrophobic compounds than the corresponding *waaG* mutant (34). The two *K. pneumoniae wabG* mutants totally devoid of the outer core LPS obtained in this study were more sensitive to SDS and polymyxin B than the wild-type strains. In addition, for *K. pneumoniae waaC* and *waaF* mutants, MICs of these hydrophobic compounds were essentially similar to those for the *wabG* mutants; however, *K. pneumoniae waaC* and *waaF* mutants were more sensitive to deoxycholate, nalidixic acid, erythromycin, novobiocin, and rifampin. In *K. pneumoniae* there are no phosphoryl modifications of the inner core L,D-Hep*p*I and -II residues, the negative charges being contributed by the GalA residue(s) (29). Thus, the absence of the outer core GalA residue in the *wabG* mutation results in the loss of the stoichiometric core LPS negative charge and might explain why the levels of sensitivity to SDS and polymyxin B were essentially the same as those of the *waaC* and *-F* mutants but different from those of *E. coli* and *S. enterica* serovar Typhimurium (32, 33).

The fact that the *wabG* as well as *waaC* and *-F* mutants are unencapsulated but able to biosynthesize specific antiserum cross-reacting polysaccharide (K^-) may be explained in two different ways. Since deep LPS core mutants (like *waaC* and *-F*) in *Enterobacteriaceae* are altered in different outer membrane components, one of them could be the attachment site for capsule linkage. A second possibility is a direct linkage of the capsule to LPS core. If the capsular polysaccharide is linked either to the LPS core directly or to some other outer membrane molecules, the *waaC*, *-F*, and *wabG* mutants may be sufficiently altered in these outer membrane components to preclude the capsular polysaccharide attachment. Furthermore, the lack of cell-bound capsule was found in all the *Klebsiella waaC*, *-F*, and *wabG* mutants isolated belonging to different K serotypes (unpublished data). Nonpolar *K. pneumoniae* 52145 mutants have been constructed for all the nonessential genes of the *waa* cluster (our unpublished results). Only the *waaC*, *-F*, and *wabG* mutants lacked K2 capsule, and the NC18 (*yibD*) mutant showed a drastic reduction of K2 capsule, as previously described (24). All the other *K. pneumoniae* 52145 mutants showed the presence of K2 capsule.

The effects of the *wabG* mutation on colonization and virulence experiments were studied in the *K. pneumoniae* 52145 background because this strain is highly virulent and is able to colonize different surfaces. The *wabG* mutation drastically reduces the colonization ability of *K. pneumoniae* in experimental UTIs (Table 2). In addition, this mutation also results in a 5-log-fold increase in LD_{50} in mice inoculated intraperitoneally (Table 3) and is completely avirulent in an experimental model of pneumonia (Table 4). Similar levels of reduction in colonization and virulence were observed in the corresponding

TABLE 4. Experimental pneumonia induced by different *K. pneumoniae* strains[a]

Strain	Mean inoculum (range)	Mean lung wt (g)	Mean log CFU/g	No. dead/total no. (%)	No. positive culture/total no. (%)
52145	1.0×10^7 (±1.4)	0.29 ± 0.11	6.11	21/36 (58)	19/36 (53)
52145Δ*waaC*	1.8×10^7	0.16 ± 0.07	<1.0	0/9 (0)	0/9 (0)
52145Δ*waaC* + pGEMT	1.5×10^7	0.17 ± 0.08	<1.0	0/9 (0)	0/9 (0)
52145Δ*waaC* + pGEMT-WaaC	2.0×10^7 (±0.3)	0.27 ± 0.12	6.02	10/18 (56)	9/18 (50)
52145Δ*waaF*	1.6×10^7	0.15 ± 0.05	<1.0	0/9 (0)	0/9 (0)
52145Δ*waaF* + pGEMT	1.3×10^7	0.16 ± 0.04	<1.0	0/9 (0)	0/9 (0)
52145Δ*waaF* + pGEMT-WaaF	1.8×10^7 (±0.9)	0.28 ± 0.09	6.05	10/18 (56)	10/18 (56)
52145Δ*wabG*	1.9×10^7	0.18 ± 0.05	<1.0	0/9 (0)	0/9 (0)
52145Δ*wabG* + pGEMT	1.8×10^7	0.16 ± 0.07	<1.0	0/9 (0)	0/9 (0)
52145Δ*wabG* + pGEMT-WabG	2.2×10^7 (±0.5)	0.27 ± 0.12	5.98	10/18 (56)	9/18 (50)
52145Δ*waaL*	1.0×10^7 (±2.1)	0.35 ± 0.09	6.31	10/18 (56)	10/18 (56)
52145Δ*waaL* + pGEMT	1.7×10^7 (±1.6)	0.37 ± 0.05	6.42	10/18 (56)	10/18 (56)
52145Δ*waaL* + pGEMT-WaaL	1.3×10^7 (±1.1)	0.37 ± 0.11	6.09	10/18 (56)	9/18 (50)

[a] *P* was <0.01 for all comparisons (lung weight, log CFU per gram of lung, mortality, and blood or spleen positive cultures) between the strains, two-tailed *t* test.

waaC and *-F* mutants. On the other hand, a *K. pneumoniae waaL* mutant, with a full inner and outer core but devoid of O antigen, showed a smaller reduction in colonization and virulence when tested in mice inoculated intraperitoneally and showed no reduction in the pneumonia model when compared to the wild-type strain. The effect of the *waaL* mutant could be fully attributed to the O-antigen deficiency, since this mutant still contains capsule, as judged by EM, sensitivity to phage Φ2, and reaction with anti-K2-specific polyclonal serum. From these results we can conclude that the capsule is essential in the *K. pneumoniae* experimental model of pneumonia, while the colonization of the UT by *K. pneumoniae* requires a complete LPS with O antigen. The *K. pneumoniae* virulence tested as LD_{50} in mice inoculated intraperitoneally seems to be dependent on the capsule and the complete LPS (probably full-core LPS and O-antigen molecules). Finally, all the changes observed in the *K. pneumoniae waaC*, *-F*, *-L*, and *wabG* mutants are ameliorated by introduction of the corresponding single wild-type gene, while the introduction of the plasmid vector alone is unable to accomplish this.

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