The Incorporation of Glucosamine into Enterobacterial Core Lipopolysaccharide

TWO ENZYMATIC STEPS ARE REQUIRED*

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The core lipopolysaccharide (LPS) of Klebsiella pneumoniae is characterized by the presence of disaccharide αGlcN-(1,4)-αGalA attached by an α1,3 linkage to L-glycero-d-manno-heptopyranose II (LD-HeppII). Previously it has been shown that the WabH enzyme catalyzes the incorporation of GlcNAc from UDP-GlcNAc to outer core LPS. The presence of GlcNAc instead of GlcN and the lack of UDP-GlcN in bacteria indicate that an additional enzymatic step is required. In this work we identified a new gene (wabN) in the K. pneumoniae core LPS biosynthetic cluster. Chemical and structural analysis of K. pneumoniae non-polar wabN mutants showed truncated core LPS with GlcNAc instead of GlcN. In vitro assays using LPS truncated at the level of d-galacturonic acid (GalA) and cell-free extract containing WabH and WabN together led to the incorporation of GlcN, whereas none of them alone were able to do it. This result suggests that the later enzyme (WabN) catalyzes the deacetylation of the core LPS containing the GlcNAc residue. Thus, the incorporation of the GlcN residue to core LPS in K. pneumoniae requires two distinct enzymatic steps. WabN homologues are found in Serratia marcescens and some Proteus strains that show the same disaccharide αGlcN-(1,4)-αGalA attached by an α1,3 linkage to LD-HeppII.

Klebsiella pneumoniae is an important nosocomial pathogen (1) causing infections that may occur at almost all body sites with highest incidence in the urinary and the respiratory tracts. The main populations at risk are neonates, immunocompromised hosts, and patients predisposed by surgery, diabetes, malignancy, etc. (1–4). K. pneumoniae typically express both smooth lipopolysaccharide (LPS)3 and capsule polysaccharide (K-antigen) on its surface, and both antigens (LPS and capsule) contribute to the pathogenesis of this species.

As in other Enterobacteriaceae in the K. pneumoniae LPS three domains are recognized: the highly conserved and hydrophobic lipid A, the hydrophilic and highly variable O-antigen polysaccharide, and the core oligosaccharide (OS) connecting lipid A and O-antigen. The core domain is usually divided into inner and outer core on the basis of sugar composition.

The K. pneumoniae core LPS structure has been determined for several O-serotypes. Although the inner core is highly conserved within the Enterobacteriaceae the K. pneumoniae inner core differs from those of Escherichia coli and Salmonella by the lack of L-glycero-d-manno-heptopyranose I and II (LD-HeppI and -II) phosphoryl modifications and by the presence of a D-glucose (Glc) residue linked by a β1,4 bond to LD-HeppI (5–7). Two outer core types (1 and 2) were described in K. pneumoniae, both containing the disaccharide αGlcN-(1,4)-αGalA attached by an α1,3 linkage to LD-HeppII (5–7). Type 1 core contains a 3-deoxy-D-manno-oct-2ulosonic acid (Kdo) residue linked by an α2,6 bond to the GlcNAc residue (5, 6), whereas type 2 core contains the disaccharide βGlc-(1,6)-αGlc linked by an α1,4 bond to the GlcN residue (7) (Fig. 1).

The genes involved in the K. pneumoniae core LPS biosynthesis are clustered in a region (wa) of the K. pneumoniae chromosome, and two different clusters have been identified to be responsible for type 1 and type 2 core biosynthesis (7, 8) (Fig. 1). Furthermore the functions in both inner and outer core biosynthesis of most of the transferases encoded by the wa gene products have been elucidated (7–12) (Fig. 1). Nevertheless the mechanism leading to the incorporation of the outer core GlcN residue remains unknown. In searching for a candidate glucosaminyltransferase the wabH gene product was characterized (12). The WabH protein catalyzes the incorporation of GlcNAc from UDP-GlcNAc into outer core LPS. This transferase activity is dependent on the presence of the outer core d-galacturonic acid (GalA) residue in the acceptor core LPS (12). But because GlcN instead of GlcNAc is found in the K. pneumoniae outer core either an unknown glucosaminyltransferase remains to be identified or a mechanism to deacetylate the WabH-transferred GlcNAc residue should be involved. In this work we report the identification and characterization of such a core LPS-GlcNAc deacetylase activity in K. pneumoniae and Serratia marcescens sharing the outer core disaccharide αGlcN-(1,4)-αGalA.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—Bacterial strains and plasmids used in this study are shown in TABLE ONE. Bacterial strains were grown in LB broth and LB agar (13). LB medium was supplemented with kanamycin (50 μg/ml), ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), and tetracycline (25 μg/ml) when needed. General DNA Methods—Standard DNA manipulations were done essentially as described previously (14). DNA restriction endonucleases,
T4 DNA ligase, E. coli DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

**Mutant Construction—** *K. pneumoniae* 52145 and C3 individual genes were mutated by creating *in vitro* in-frame deletions of each gene (15). Each mutated gene was transferred to the chromosome by homologous recombination using the temperature-sensitive suicide plasmid pKO3 containing the counterselectable marker sacB (15). Mutations were made in *wabN* and *wabH*. The plasmids containing the engineered in-frame deletions (pKO3Δ*wabH*<sub>C3</sub>, pKO3Δ*wabH*<sub>52145</sub>, pKO3Δ*wabH*<sub>C3</sub> and pKO3Δ*wabH*<sub>52145</sub>) were transformed into *K. pneumoniae* 52145, C3, 52145Δ*wabH*, and C3Δ*wabH* by electroporation. Mutants were selected based on growth in LB agar containing 10% sucrose and loss of the chloramphenicol resistance marker of vector pKO3. The mutations were confirmed by sequencing of the whole constructs in amplified PCR products.

The 52145Δ*wabN* mutant was constructed by asymmetric PCR amplifications using 52145 chromosomal DNA and primers NA52145 (5'-GGAAGATCTTCGCGCCGACATTAAAAAGAC-3'), NB52145 (5'-CCCATCCATAACTTAACAAAGCCTCTACGGGCAATAC-3'), NC52145 (5'-CCCATCCATAACTTAACAAAGCCTCTACGGGCAATAC-3'), and ND52145 (5'-GGAAGATCTTCGCGCCGACATTAAAAAGAC-3'). The primers include BglII sites (underlined). DNA fragments of 743 (NA52145-NB52145), and 628 (NC52145-ND52145) bp were obtained, respectively. DNA fragment NA52145-NB52145 contains from nucleotide 7457, corresponding to the third base of the 9th codon of *wabN*, to nucleotide 8199 inside *waaQ*. DNA fragment NC52145-ND52145 contains from nucleotide 5887, inside *waaM*, to nucleotide 6514 including the last *wabN* five codons. DNA fragments NA52145-NB52145 and NC52145-ND52145 were annealed at their overlapping region (double underlined letters in primers NB52145 and NC52145) and amplified by PCR as a single fragment using primers NA52145 and ND52145. The fusion product was purified, BglII-digested, ligated into BamHI-digested and phosphorylation-treated pKO3 vector, electroporated into *E. coli* DH5α, and plated on chloramphenicol LB agar plates at 30°C to obtain plasmid pKO3Δ*wabN*<sub>52145</sub>.

The C3Δ*wabN* mutant was constructed by asymmetric PCR amplifications using C3 chromosomal DNA and primers NA*C3* (5'-GAAGATCTTCGCGCCGACATTAAAAAGAC-3'), NB*C3* (5'-CCCATCCATAACTTAACAAAGCCTCTACGGGCAATAC-3'), NC*C3* (5'-CCCATCCATAACTTAACAAAGCCTCTACGGGCAATAC-3'), and ND*C3* (5'-GAAGATCTTCGCGCCGACATTAAAAAGAC-3'). The primers include BglII sites (underlined). DNA fragments of 745 (NA*C3*-NB*C3*), and 623 (NC*C3*-ND*C3*) bp were obtained, respectively. DNA fragment NA*C3*-NB*C3* contains from nucleotide 7432, corresponding to the third base of the fourth codon of *wabN*, to nucleotide 8176 inside *waaQ*. DNA fragment NC*C3*-ND*C3* contains from nucleotide 5870, inside *waaM*, to nucleotide 6492 including the last *wabN* eight codons. DNA fragments NA*C3*-NB*C3* and NC*C3*-ND*C3* were annealed at their overlapping region (double underlined letters in primers NB*C3* and NC*C3*) and amplified by PCR as a single fragment using primers NA*C3* and ND*C3*. The fusion product was purified, BglII-digested, ligated into BamHI-digested and phosphorylation-treated pKO3 vector, electroporated into *E. coli* DH5α, and plated on chloramphenicol LB agar plates at 30°C to obtain plasmid pKO3Δ*wabN*<sub>*C3*</sub>.

**TABLE ONE**

**Bacterial strains and plasmids used**

| Strain or plasmid | Relevant characteristics | Ref. or source |
|-------------------|--------------------------|---------------|
| *K. pneumoniae* |
| 52145 | Serovar O1:K2 (core type 2) | 33 |
| 52145Δ*waaL* | Non-polar *waaL* mutant | 11 |
| 52145Δ*wabN* | Non-polar *wabN* mutant | This study |
| 52145Δ*wabH* | Non-polar *wabH* mutant | This study |
| 52145Δ*waaL, wabN* | Double non-polar *waaL, wabN* mutant | This study |
| 52145Δ*waaL, wabH* | Double non-polar *waaL, wabH* mutant | This study |
| C3 | Serovar O3:K66 (core type 1) | 34 |
| C3Δ*waaL* | Non-polar *waaL* mutant | 8 |
| C3Δ*wabN* | Non-polar *wabN* mutant | This study |
| C3Δ*wabH* | Non-polar *wabH* mutant | This study |
| C3Δ*waaL, wabN* | Double non-polar *waaL, wabN* mutant | This study |
| C3Δ*waaL, wabH* | Double non-polar *waaL, wabH* mutant | This study |
| *E. coli* |
| DH5α | F<sup>−</sup> endA hsdR17 (r<sub>K</sub> m<sub>K</sub> ) supE44 thi-1 recA1 gyr-A96 d80lacZ | 35 |
| **Plasmid** |
| pKO3 | Cm<sup>−</sup> temperature-sensitive replication sacB-containing suicide plasmid | 15 |
| pKO3Δ*wabN*<sub>52145</sub> | Contains an in-frame internal *wabN* deletion from strain 52145 | This study |
| pKO3Δ*wabN*<sub>C3</sub> | Contains an in-frame internal *wabN* deletion from strain C3 | This study |
| pKO3Δ*wabH*<sub>52145</sub> | Contains an in-frame internal *wabH* deletion from strain 52145 | This study |
| pKO3Δ*wabH*<sub>C3</sub> | Contains an in-frame internal *wabH* deletion from strain C3 | This study |
| pGEM-T Easy | PCR-generated DNA fragment cloning vector Amp<sup>R</sup> | Promega |
| pGEM-T-waaN*<sub>52145</sub> | pGEM-T containing the PCR-amplified *waaN*<sub>52145</sub> gene | This study |
| pGEM-T-waaN*<sub>C3</sub> | pGEM-T containing the PCR-amplified *waaN*<sub>C3</sub> gene | This study |
| pGEM-T-waaNC3 | pGEM-T containing the PCR-amplified *waaN*<sub>C3</sub> gene | This study |
| pBAD18-Cm | Arabinoose-inducible expression vector | 16 |
| pBAD18-Cm-wabN*<sub>52145</sub> | pBAD18-Cm derivative expressing WabN from strain 52145 | This study |
| pBAD18-Cm-wabN*<sub>C3</sub> | pBAD18-Cm derivative expressing WabN from strain C3 | This study |
| pBAD18-Cm-wabH<sub>52145</sub> | pBAD18-Cm derivative expressing WabH from strain 52145 | This study |
| pBAD18-Cm-wabH<sub>C3</sub> | pBAD18-Cm derivative expressing WabH from strain C3 | This study |
The Two Enzymatic Steps for GlcN Incorporation into Core LPS

into E. coli DH5α, and plated on chloramphenicol LB agar plates at 30 °C to obtain plasmid pKO3ΔwabN<sub>C3</sub>

The 52145ΔwabH mutant was constructed using 52145 chromosomal DNA and primers HA<sub>52145</sub> (5′-CGCGGATCCGCCGCGAGAT-TATCGAAG-3′), HB<sub>52145</sub> (5′-CCATCACTAAATTAAACAGGG-TAACCCTGTCATTAC-3′), HC<sub>52145</sub> (5′-TGTTAAGTTAGT-GATGGGTGCAGTACCGTGCACT-3′), and HD<sub>52145</sub> (5′-CGCGGATCCCTTACGGCACCGCATCAG-3′). The primers include BamHI sites (underlined). DNA fragments of 688 (HA<sub>52145</sub>-HB<sub>52145</sub>) and 715 (HC<sub>52145</sub>-HD<sub>52145</sub>) bp were obtained, respectively. DNA fragment HA<sub>52145</sub>-HB<sub>52145</sub> contains from nucleotide 9107, inside wabG, to the third base of the 10th codon of wabH<sub>52145</sub>, to nucleotide 9794. DNA fragment HC<sub>52145</sub>-HD<sub>52145</sub> contains from nucleotide 11558, inside orf<sub>10</sub>, to nucleotide 10844 including the last wabH<sub>52145</sub> 10 codons. DNA fragments HA<sub>52145</sub>-HB<sub>52145</sub> and HC<sub>52145</sub>-HD<sub>52145</sub> were annealed at their overlapping region (double underlined letters in primers HB<sub>52145</sub> and HC<sub>52145</sub>) and amplified by PCR as a single fragment using primers NA<sub>52145</sub> and ND<sub>52145</sub>. This fragment was used as template to amplify an internal 1,644 bp with primers FN<sub>52145</sub> and ND<sub>52145</sub>. This fragment was digested with BglII, blunted, and then digested with XbaI to obtain a 1,638-bp fragment. This fragment was ligated to pBAD18-Cm, HindIII-digested, blunted, and XbaI-digested. Plasmid pBAD18-Cm-wabN<sub>52145</sub> was constructed by PCR amplification of a 1,076-bp DNA fragment from plasmid pCosFGR16 (17) using primers NFW<sub>52145</sub> (5′-CAATCTAGGTCCATTCTC3′-T) and NR<sub>52145</sub> (5′-CAGAGGGCTAGCAAAAGTC-3′). This fragment was digested with NheI and HindIII and ligated into the same sites in pBAD18-Cm. For complementation studies each amplified wabN homologue was ligated to pGEM-T Easy (Promega) and transformed into E. coli DH5α.

LPS Isolation and Electrophoresis—LPS was extracted from dry cells of K. pneumoniae grown in LB. The phenol/chloroform/light petroleum ether method (18) was used for strains producing rough LPS, whereas the phenol/water procedure (19) was used for the strains producing the O-antigen domain (smooth LPS). For screening purposes LPS was obtained after protease K digestion of whole cells (20). LPS samples were separated by SDS-PAGE or Tricine-SDS-PAGE and visualized by silver staining as described previously (21, 22).

Preparation of Oligosaccharides—The LPS (20 mg) was hydrolyzed in 1% acetic acid (100 °C for 120 min), and the precipitate was removed by centrifugation (8,000 × g for 30 min) and lyophilized to give lipid A (10 mg, 50% of LPS). The supernatant was evaporated to dryness, dissolved in water, and lyophilized (6 mg, 30% of LPS).

Gas Chromatography–Mass Spectrometry (MS) Analysis—Partially methylated alditol acetates and methyl glycoside acetates were analyzed on an Agilent Technologies 5973N MS instrument equipped with a 6850A gas chromatograph and an RTX-5 capillary column (Restek, 30 m × 0.25-mm inner diameter; flow rate, 1 ml/min; carrier gas, helium). Acetylated methyl glycoside analysis was performed with the following temperature program: 150 °C for 5 min, 150 °C to 250 °C at 3 °C/min, 250 °C for 10 min. For partially methylated alditol acetates the temperature program was: 90 °C for 1 min, 90 °C to 140 °C at 25 °C/min, 140 °C to 200 °C at 5 °C/min, 200 °C to 280 °C at 10 °C/min, 280 °C for 10 min.

Glycosyl and Lipid Analysis—LPS (1 mg) was dried over P<sub>2</sub>O<sub>5</sub>, overnight and treated with 1 M HCl/CH<sub>3</sub>OH (1 ml) at 80 °C for 20 h to analyze both glycosyl and fatty acid composition. The crude reaction was extracted twice with hexane; 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 methanol layer was neutralized with Ag<sub>2</sub>CO<sub>3</sub>, dried, and acetylated. Both samples were injected into the gas chromatography–MS system, and acetylated fatty acid methyl esters were recovered in the hexane phase, whereas the methylglycoside derivatives were in the methanolic phase.

NMR Spectroscopy—1H NMR spectra were recorded on a solution (0.5 ml) of K. pneumoniae 52145<sub>awal</sub> (8 mg) and 52145<sub>awal</sub> wabN (2 mg) core oligosaccharides, respectively, in D<sub>2</sub>O at 400 MHz with a Bruker DRX 400 Avance spectrometer equipped with a reverse probe in the Fourier transform mode at 303 K.

Mass Spectrometry Studies—Positive and negative ions reflectron time-of-flight mass spectra (MALDI-TOF) were acquired on a Voyager DE-PRO instrument (Applied Biosystems) equipped with a delayed extraction ion source. Ion acceleration voltage was 25 kV, grid voltage was 17 kV, mirror voltage ratio was 1.12, and delay time was 150 ns. Samples were irradiated at a frequency of 5 Hz by 337 nm photons from
The Two Enzymatic Steps for GlcN Incorporation into Core LPS

a pulsed nitrogen laser. Postsource decay was performed using an acceleration voltage of 20 kV. The reflectron voltage was decreased in 20 successive 25% steps. Mass calibration was obtained with a maltholigosaccharide mixture from corn syrup (Sigma). A solution of 2.5-dihydroxybenzoic acid in 20% CH3CN in water at a concentration of 25 mg/ml was used as the MALDI matrix. One microliter of matrix solution was deposited on the target followed by loading of 1 µl of the sample. The droplets were allowed to dry at room temperature. Spectra were calibrated and processed under computer control using the Applied Biosystems Data Explorer software.

Preparation of Cell-free Extracts Containing Core LPS Biosynthetic Enzymes—The K. pneumoniae strains 52145∆wabH(pBAD18-Cm-wabH52145) and 52145∆wabN(pBAB18Cm-wabN52145) were used to overexpress the WabH and WabN proteins, respectively. Cultures of these strains and controls (52145ΔwabH and 52145ΔwabN harboring pBAD18-Cm) were grown and arabinose-induced as described previously (24).

Preparation of Cell-free Extracts Containing Core LPS Biosynthetic Enzymes—The K. pneumoniae strains 52145∆wabH(pBAD18-Cm-wabH52145) and 52145∆wabN(pBAB18Cm-wabN52145) were used to overexpress the WabH and WabN proteins, respectively. Cultures of these strains and controls (52145ΔwabH and 52145ΔwabN harboring pBAD18-Cm) were grown and arabinose-induced as described above. The cells were harvested, washed once with 50 mM Tris-HEPES (pH 7.5), and then frozen until needed. To prepare lysates cell pellets were suspended in 50 mM Tris-HEPES (pH 7.5) and sonicated on ice (for a total of 2 min using 10-s bursts followed by 10-s cooling periods). Unbroken cells, cell debris, and the membrane fraction were removed by ultracentrifugation at 100,000 × g for 60 min. Protein expression was monitored by SDS-PAGE, and protein contents of cell-free extracts were determined using the Bio-Rad Bradford assay as directed by the manufacturer.

GlcNAc-Core LPS Deacetylase Activity of WabN—The ability of WabN to catalyze the deacetylation of GlcN-ac containing LPS was assayed by using LPS from mutant 52145∆wabH in a reaction containing both 52145ΔwabH (pBAD18-Cm-wabH52145) and 52145ΔwabN (pBAD18-Cm-wabN52145) cell-free extracts. The assay system was based on the GlcNac transferase assay described for WabH (12).

Assay reactions using UDP-GlcNac as the substrate were carried out in 0.02 ml at a final concentration of 50 mM Tris-HCl (pH 8.0) containing 10 mM MgCl2, 1 mM diithiothreitol, 1 mM UDP-GlcNac, and 0.003 mg of 52145ΔwabH mutant LPS as the acceptor. The reaction was started by addition of 0.04 mg each of the cell-free extracts from 52145ΔwabH (pBAD18-Cm-wabH52145) and 52145ΔwabN (pBAD18-Cm-wabN52145). As controls reactions containing cell-free extracts (0.04 mg) of 52145ΔwabH (pBAD18-Cm-wabH52145), 52145ΔwabH (pBAD18-Cm) and/or 52145ΔwabN (pBAD18-Cm-wabN52145) were used. The mixtures were incubated at 37 °C for 2 h, and the reactions were stopped by adding 0.08 ml of SDS-PAGE sample buffer and boiling for 10 min. Proteinase K diluted in SDS-PAGE sample buffer was then added to a final concentration of 0.8 mg/ml and incubated for 18 h at 55 °C. The reaction products were visualized by SDS-PAGE. When UDP-[14C]GlcNac was used as the substrate, assay reactions were carried out in a total of 0.1 ml at a final concentration of 50 mM Tris-HCl (pH 8.0) containing 10 mM MgCl2, 1 mM diithiothreitol, 0.3 mg of 52145ΔwabH mutant LPS, and 0.2 mg of the cell-free extracts. The reactions were started by addition of 0.25 µCi of UDP-[14C]GlcNac (specific activity of 10.2 mCi/mmol; ICN Biomedicals Inc.). Assays were performed at 37 °C for 2 h and were stopped by adding 2 volumes of 0.375 M MgCl2 in 95% ethanol and cooling at −20 °C for 2 h. The LPS was recovered by centrifugation at 12,000 × g for 15 min and resuspended in 100 µl of water. The LPS was precipitated with 2 volumes of 0.375 M MgCl2 in 95% ethanol; this step was repeated three times to eliminate the unincorporated UDP-[14C]GlcNac. The LPS was hydrolyzed by resuspension in 100 µl of 0.1 M HCl and heating to 100 °C for 48 h. The labeled residues from the hydrolyzed LPS samples were separated by TLC (Kieselgel 60, Merck) with n-butanol, methanol, 25% ammonia solution, water (5:4:2:1, v/v/v/v). The labeled residues were detected by autoradiography using as standards [14C]GlcNac and [14C]GlcN.

RESULTS

Identification of a New Core LPS Gene—In the Enterobacteriaceae the genes coding for enzymes involved in core LPS biosynthesis are usually found clustered in a region (wa gene cluster) with the exception of Yersinia sp., where two different clusters exist. Inspection of the known wa gene clusters from Enterobacteriaceae shows a high level of gene density with very short spacing between the different genes. In addition, overlapping start and stop codons between adjacent genes is quite frequent. By contrast, in the K. pneumoniae C3 (type 1 core) and 52145 (type 2 core) wa gene clusters, regions of about 1,000 bp of unknown coding potential, are located upstream of the waaQ gene. The analysis of this region in strain 52145 revealed a potential 328-amino acid residue-encoding open reading frame (ORF) suggesting that a previously missed gene could play a role in type 2 core OS biosynthesis, this putative gene was named wabN. The 1,066-bp region was PCR-amplified using oligonucleotides A and B and K. pneumoniae 52145 chromosomal DNA, and the amplicon was cloned in plasmid pGEM-T and transformed into E. coli DH5α. Plasmid pGEM-T-wabN52145 was used in an in vitro transcription/translation reaction with [35S]methionine. As a control the vector pGEM-T was used. The radio-labeled proteins were resolved by SDS-PAGE, showing a specific radiolabeled polypeptide with a molecular mass of about 38 kDa (data not shown).

The 984-nucleotide wabN gene stop codon is adjacent to the wabM stop codon, and the 5′-end of wabN is located 79 nucleotides upstream from waaQ. In a similar position wabN homologues were found in K. pneumoniae C3 (type 1 core) and in S. marcescens (Fig. 1). The strain C3 wabN homologue was located 53 and 32 nucleotides upstream of genes wabI and waaQ, respectively. The S. marcescens homologue was found between ORF7 and waaQ. Additional putative protein homologues were found in Proteus mirabilis H 4320 and Photobacterium luminiscens laumontii TT01. All these WabN proteins shared similar length and high levels of amino acid identity (58.43%) and similarity (76.25%) (Fig. 2). An InterPro scan search (www.ebi.ac.uk/InterProScan/index.html) identified a domain shared by the glycoside hydrolase/deacetylase superfamily (25).

Isolation of Non-polar wabN Mutants—To determine the role of the wabN gene in core LPS biosynthesis non-polar wabN mutations were isolated by an internal in-frame deletion approach (15). The engineered in vitro wabN mutations were introduced by double recombination (see “Materials and Methods”) to wild type K. pneumoniae strains C3 and 52145 and to mutant strains C3ΔwaaL and 52145ΔwaaL. Candidate mutants were checked by PCR amplification of the mutated chromosomal region, using oligonucleotides C and D, and nucleotide sequence determination.

An initial characterization of the effect of the mutation on core LPS biosynthesis was carried out by LPS isolation and analysis by both SDS-PAGE and Tricine-SDS-PAGE. The core-lipid A region of LPS preparations from strains C3ΔwabN and 52145ΔwabN and their derived dou-
ble waaL wabN mutants migrated faster than LPS from wild type strains C3 and 52145 (Fig. 3). This suggests that the wabN mutation causes the loss of one or more sugar residues from the core. In addition LPS preparations from strains C3/H9004wabN and 52145/H9004wabN lacked O-antigen polysaccharide (Fig. 3). The K. pneumoniae O-antigen polysaccharide is linked to outer core Kdo residue in type core 1 (26) and probably to the last Glc residue in type core 2 (7), suggesting that the residues lost in the wabN mutants reside in the main core LPS branch. The cloned wabN from strain C3 (pGEM-T−wabNC3) was introduced into C3/H9004wabN and restored wild type LPS migration and O-antigen polysaccharide production (Fig. 3). Both K. pneumoniae wabN homologues (wabN KpC3 and wabN Kp52145) complemented the K. pneumoniae wabN mutations in either of the two genetic backgrounds, C3 or 52145 (Fig. 3). The S. marcescens wabN homologue in pGEM-T−wabNSm also complemented the K. pneumoniae wabN mutants (Fig. 3). These results demonstrate the presence of a new gene shared by K. pneumoniae and S. marcescens encoding an enzymatic activity necessary for core LPS synthesis.

Characterization of the K. pneumoniae wabN Mutant Core Polysaccharide—To determine the core LPS changes produced by the wabN mutation, LPS was obtained from strain 52145/H9004wabN waaL by the phenol, chloroform, and petroleum ether method (18). Composition analysis by gas chromatography-MS of acetylated methyl glycosides revealed the presence of LD-Hep, GalA, D-Glc, D-GlcN, and Kdo. Acetic acid hydrolysis gave one major compound as shown by the negative ion reflectron MALDI-TOF spectrum (TABLE TWO). In this spectrum the signals at m/z 1,531.13 and 1,513.16 correspond to pseudomolecular ions \((\text{M}+\text{H})^-\) and \((\text{M}+\text{H}−18)^−\), respectively, confirming the presence of one major core OS structure. In particular, the signal at m/z 1,531.13 was in agreement with the calculated average molecular mass (1,532.30 Da) of a structure with one hexose, three heptoses, one \(\alpha\)-glucosamine and one Kdo unit. The signals at m/z 1,531.13 and 1,513.16 were accompanied by the corresponding adduct signals with sodium ions at m/z 1,553.13 and 1,535.15, respectively. From these data it can be assumed that this oligosaccharide is truncated in respect to the complete core structure (7) at the level of D-GlcN, which is \(\alpha\)-acetylated. To test this hypothesis a positive ion postsource decay MALDI-TOF experiment (TABLE THREE) was performed. This spectrum is dominated by fragment ions of B and C series (27) in addition to some double fragmentation (TABLE THREE). Starting from the pseudomolecular ion at m/z 1,554.5 the signal at m/z 1,531.13, 1,513.16 correspond to pseudomolecular ions (M − H)− and (M − H − 18)−, respectively. In this spectrum the signals at m/z 1,531.13, 1,513.16 correspond to pseudomolecular ions (M − H)− and (M − H − 18)−, respectively. In this spectrum the signals at m/z 1,531.13, 1,513.16 correspond to pseudomolecular ions (M − H)− and (M − H − 18)−, respectively. In this spectrum the signals at m/z 1,531.13, 1,513.16 correspond to pseudomolecular ions (M − H)− and (M − H − 18)−, respectively.
Hep, 3,4-linked Hep, and 3,7-linked Hep units were found. terminal GlcNAc residues confirmed the presence of the disaccharide with the proposed structure. In particular, the 4-linked GalA and the saccharide alditol mixture was performed; the results were in agreement structure in Structure 1.

To confirm this hypothesis a methylation analysis on the core oligosaccharide samples from strain 52145. These results are consistent with the core oligosaccharide

The core OS structure of the 52145ΔwabH waaL mutant was definitively confirmed by the comparison of its 1H NMR spectrum with that of the core structure of 52145ΔwaaL mutant (Fig. 4) (7). In particular the presence of an intense singlet signal at 2.11 ppm (Fig. 4), attributable to the methyl of an acetyl group, supported the N-acetylation of the GlcN residue. In addition the comparison of the anomeric region confirmed the lack of the 6-α-Glc and the terminal β-Glc residues, while the form and the chemical shifts of the other signals indicated the anomeric configuration to be the same as those of the corresponding region of 52145ΔwaaL mutant (Fig. 4).

Isolation and Characterization of Non-polar wabH Mutants—Previously a K. pneumoniae wabH mutant was constructed by insertion of a non-polar kanamycin resistance cassette in a strain producing type 1 core LPS (12). This construction results in the insertion of an 850-bp DNA fragment in the targeted gene. Although this mutagenesis approach is reported to be devoid of polar effects on downstream genes, alterations in the stability of the mRNA cannot be ruled out. Furthermore this approach results in a kanamycin-resistant mutant precluding the use of this marker to introduce additional mutations. Thus, we decided to use the internal in-frame deletion approach (see “Materials and Methods”) to construct wabH mutations in both core types 1 (C3) and 2 (52145) strains. Analysis of LPS isolated from 52145ΔwabH and C3ΔwabH confirmed that core OSs from 52145ΔwabH and C3ΔwabH were devoid of O-antigen (data not shown).

The core OS fraction obtained from 52145ΔwaaL wabH by mild acid hydrolysis was analyzed by transformed negative ion electrospray ionization-MS (TABLE TWO). The major pseudomolecular ion signal (M – H)− at m/z 1,327.8 (TABLE TWO) was in agreement with the calculated average molecular mass (1,329.11 Da) of the expected molecular structure (KdoHep,GlcGlcAβ). Thus, the wabH mutation precludes the extension of the core beyond the outer core GalA residue (depicted in Fig. 1).

Core LPS Deacetylase Assays—The core OS structure determined from strain 52145ΔwaaL wabH lacks the last two outer core residues and presents GlcNAc instead of GlcN. Interestingly the same core OS

two peaks at m/z 804.6 and 600.8, which were derived from the loss of one GalA and one GlcNAc, respectively. Another fragment sequence was generated from the B1 ion, which has lost a GalA unit (m/z 1,140.5; 1,316.6 – 176) and a GalA and a GlcNAc unit (m/z 938.0; 1,316.6 – 176 – 203). These results are consistent with the core oligosaccharide structure in Structure 1.

To confirm this hypothesis a methylation analysis on the core oligosaccharide alditol mixture was performed; the results were in agreement with the proposed structure. In particular, the 4-linked GalA and the terminal GlcNAc residues confirmed the presence of the disaccharide GlcNAc–(1→4)–GalA. Moreover terminal Gal, terminal GalA, 7-linked Hep, 3,4-linked Hep, and 3,7-linked Hep units were found.
The Two Enzymatic Steps for GlcN Incorporation into Core LPS

**TABLE TWO**
MALDI-TOF (52145ΔwaaL wabN) and electrospray ionization-MS (52145ΔwaaL wabH) negative ions of acid-released core from *K. pneumoniae* mutants

| Strain          | (M – H)+ m/z | Calculated molecular ion | Proposed composition                  |
|-----------------|--------------|--------------------------|---------------------------------------|
| 52145ΔwaaL wabN | 1,531.13     | 1,532.30                 | HexHexA\_3Hep\_3HexNAcKdo             |
|                 | 1,513.16     | 1,514.28                 | M–18\textsuperscript{a}               |
| 52145ΔwaaL wabH | 1,327.8      | 1,329.11                 | HexHexA\_3Hep\_Kdo                    |
|                 | 1,310.6      | 1,311.09                 | M–18\textsuperscript{a}               |
|                 | 1,241.0      | 1,241.05                 | M–46\textsuperscript{a}               |
|                 | 1,283.5      | 1,283.09                 | M–88\textsuperscript{a}               |

\textsuperscript{a} Signals that are 18, 46, and 88 Da, respectively, below the described pseudomolecular ion are attributable to OS with terminal Kdo containing a ring double bond (–18), a ketone at C-1 (–44), or ring fragmentation (–88). These artifacts have been described for LPS samples that are hydrolyzed in the presence of acetic acid (36).

**TABLE THREE**
Assignment of signals from postsource decay MALDI-TOF spectrum of m/z 1,554.5 ion of acid-released core OS isolated from *K. pneumoniae* 52145ΔwaaL wabN in the positive ion mode

| Signal\textsuperscript{b} | Calculated | Observed m/z | Proposed structure                        |
|---------------------------|------------|--------------|------------------------------------------|
| 1                         | 1,555.29   | 1,554.5      | M (GlcGal\_A\_3Hep\_3GlcNAc Kdo)         |
| 2                         | 1,537.28   | 1,536.8      | M – H\textsubscript{2}O                   |
| 3                         | 1,379.16   | 1,378.2      | M – GalA                                 |
| 4                         | 1,363.12   | 1,362.8      | M – H \textsubscript{3}P                  |
| 5                         | 1,317.09   | 1,316.6      | B\textsubscript{4}                       |
| 6                         | 1,159.00   | 1,158.6      | C\textsubscript{4} – GalA                 |
| 7                         | 1,140.96   | 1,140.5      | B\textsubscript{4} – GalA                 |
| 8                         | 1,113.89   | 1,113.6      | B\textsubscript{4} – GlcNAc               |
| 9                         | 980.79     | 980.5        | C\textsubscript{3}                       |
| 10                        | 962.78     | 962.6        | B\textsubscript{4}                       |
| 11                        | 937.77     | 938.0        | B\textsubscript{3} – GlcNAc-GalA          |
| 12                        | 804.66     | 904.6        | C\textsubscript{3} – GalA                 |
| 13                        | 786.65     | 786.1        | B\textsubscript{3} – GalA                 |
| 14                        | 761.64     | 761.3        | B\textsubscript{4} – GlcNAc-Gal\_A\_2     |
| 15                        | 601.46     | 600.8        | C\textsubscript{3} – GalA-GlcNAc          |
| 16                        | 583.46     | 583.5        | B\textsubscript{3} – GalA-GlcNAc          |
| 17                        | 402.28     | 402.1        | B\textsubscript{4}                       |

\textsuperscript{a} All the fragment ions contain Na\textsuperscript{+}.

\textsuperscript{b} The nomenclature used to describe the fragments is that introduced by Doman and Costello (27).

structure is predicted as the result of the WabH protein activity (12). The WabH protein catalyzes the transfer of GlcNAc to outer core OS from UDP-GlcNAc (12). The presence of a putative domain corresponding to the superfamily glycoside hydrolase/deacetylase in the WabN protein suggests that it could be responsible for the deacetylation of the GlcNAc-containing core LPS intermediate. To test this hypothesis two in vitro assays were developed. The first assay takes advantage of the differences in Tricine-SDS-PAGE migrations between LPS samples from 52145ΔwaaL wabN and 52145ΔwaaL wabK. The 52145ΔwaaL wabK mutant LPS lacks the last two outer core Glc residues, thus containing core residues up to GlcN (7). The difference in migration between these two LPS preparations is attributable to the acetyl group present in LPS from 52145ΔwaaL wabN but absent from 52145ΔwaaL wabK LPS (TABLE TWO). This assay is based in the one previously described for WabH (12) using UDP-GlcNAc as substrate and LPS from mutant 52145ΔwaaL wabH as acceptor and using as the enzyme sources cell-free extracts from *K. pneumoniae* strains 52145ΔwaaL wabH (pBAD18-Cm-wabH\textsubscript{52145}) and 52145ΔwaaL wabN (pBAD18-Cm-wabN\textsubscript{52145}) overexpressing the WabH and WabN proteins, respectively. As controls cell-free extracts from strains 52145ΔwaaL wabH (pBAD18-Cm) and 52145ΔwaaL wabN (pBAD18-Cm) were used. Tricine-SDS-PAGE analysis of reaction products formed by extract from 52145ΔwaaL wabH (pBAD18-Cm-wabH\textsubscript{52145}) showed reduced lipid A core migration in comparison to the acceptor LPS (obtained from 52145ΔwaaL wabH) as described previously (12) (Fig. 5). Product from the reaction containing both 52145ΔwaaL wabH (pBAD18-Cm-wabH\textsubscript{52145}) and 52145ΔwaaL wabN (pBAD18-Cm-wabN\textsubscript{52145}) extracts migrated to the same extent as LPS from 52145ΔwaaL wabK (Fig. 5). Reactions containing 52145ΔwaaL wabH (pBAD18-Cm) extract alone migrated as 52145ΔwaaL wabN LPS, and reactions containing 52145ΔwaaL wabN (pBAD18-Cm) extract migrated as the acceptor LPS.

The second in vitro assay measured the amount of radiolabeled GlcNAc and/or GlcN incorporated into acceptor LPS from UDP-[\textsuperscript{14}C]GlcNAc after acid hydrolysis and TLC separation of the radiolabeled residues. The reaction with extract from 52145ΔwaaL wabH (pBAD18-Cm-wabH\textsubscript{52145}) allowed the detection of radiolabeled GlcNAc residue, whereas the reaction containing both 52145ΔwaaL wabH (pBAD18-Cm-wabH\textsubscript{52145}) and 52145ΔwaaL wabN (pBAD18-Cm-wabN\textsubscript{52145}) extracts showed radiolabeled GlcN (Fig. 6). In control reactions using
The Two Enzymatic Steps for GlcN Incorporation into Core LPS

52145ΔwaaL (pBAD18-Cm), 52145ΔwaaL, wabH (pBAD18-Cm), or 52145ΔwaaL, wabN (pBAD18-Cm-wabN2245) extract neither GlcNAc nor GlcN was incorporated into the acceptor LPS (Fig. 6). These results indicate that the GlcNAc residue transferred to core LPS by the WabH protein is deacetylated by the WabN enzyme. The deacetylation step is necessary for core completion by addition of the last two outer core residues.

**DISCUSSION**

We identified a novel gene involved in *K. pneumoniae* core OS biosynthesis. Analysis of LPS extracted from non-polar *wabH* mutants, constructed in *K. pneumoniae* strains producing either type 1 or type 2 core LPS, revealed that these OSs are truncated and that the non-reducing end GlcNAc replaces the wild type GlcN residue. In addition non-polar *wabH* mutants were constructed in strains producing type 1 (C3) and type 2 (52145) core LPS by internal in-frame deletion. Chemical characterization by mass spectrometry of LPS from these *wabH* mutants showed that they produce truncated core OS extending up to the GaLA residue as shown previously for a strain producing type 1 core LPS (12). It has been shown that the WabH enzyme catalyzes the in vitro incorporation of GlcNAc from UDP-GlcNAc into core LPS truncated at the level of the GaLA residue (12). These results did not explain how the GlcN residue is incorporated into outer core LPS, although the functions of the reported genes of the *K. pneumoniae* wa gene clusters were known (7, 8, 10–12).

In both the lipid A and nodulation factor biosynthesis a GlcNAc residue is first incorporated from UDP-GlcNAc and later deacetylated to GlcN (for reviews, see Refs. 28 and 29). Because the core LPS structure generated in vitro by the WabH enzyme is also found in *wabN* mutants we hypothesized a LPS-GlcNAC deacetylase function for WabN. This hypothesis was confirmed by in vitro reactions using LPS from mutant *wabH*, UDP-GlcNAc, and cell extracts containing WabH, WabN, or both enzymes.

The *K. pneumoniae* outer core disaccharide αGlcN-(1,4)-αGalA linked by an α1,3 bond to 3-deoxyheptulosonate is also found in *S. marcescens* (17); *P. mirabilis* serovars O3/S1959, R110/1959, and OXK; *Proteus vulgaris* O25; and *Proteus penneri* strains 37 and 34 (for a review, see Ref. 30). Thus, it is predicted that these strains contain *wabG*, *wabH*, and *wabN* homologues. This has been shown for *S. marcescens* N28b where these gene homologues are able to complement the corresponding *K. pneumoniae* mutants as shown in this and previous work (7, 11, 17). Interestingly these three genes are also found as predicted in *P. mirabilis* H14320 (pedant.gsf.de/cgi-bin/wwwfyli.pl?Set=Proteus_mirabilis& Page=index). In addition *P. luminosum* laumondii TT01 (31) also contains *wabG*, *wabH*, and *wabN* homologues, suggesting that it has a core LPS of the *K. pneumoniae* type.

Our work clearly resolves for the first time the complete biosynthesis of the *K. pneumoniae* core LPS and strongly suggests that the three genes *wabG* (galacturonosyltransferase), *wabH* (N-acetylgalcosaminyltransferase), and *wabN* (LPS-GlcNAC deacetylase) could be used to...
identify bacterial species containing the disaccharide αGlcN-(1,4)-αGalA linked to inner core HepII. Furthermore, the lack of reported UDP-GlcN in bacterial cells (32), the results obtained in *K. pneumoniae*, and the full complementation achieved by the *S. marcescens* wabN led us to conclude that this could be a general mechanism in the biosynthesis of GlcN-containing enterobacterial core OS (a GlcNAc residue is first incorporated and later deacetylated to GlcN). The limited similarities shown by the WabN homologues to members of the hydrolase/deacetylase superfamily (25) and thus to known deacetylase enzymes of this superfamily probably reflect the specificity of these enzymes for their natural substrates (chitin, lipid A, and core LPS). The established function for *K. pneumoniae* WabN, LPS-GlcNAc deacetylase, would be extremely useful to identify similar deacetylases in other core OSs containing GlcN in different bacteria.

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