The ionic interaction of *Klebsiella pneumoniae* K2 capsule and core lipopolysaccharide

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The complete structures of LPS core types 1 and 2 from *Klebsiella pneumoniae* have been described by other authors. They are characterized by a lack of phosphoryl residues, but they contain galacturonic acid (GalA) residues, which contribute to the necessary negative charges. The presence of a capsule was determined in core-LPS non-polar mutants from strains 52145 (O1 : K2), DL1 (O1 : K1) and C3 (O8 : K66). O-antigen ligase (*waaL*) mutants produced a capsule. Core mutants containing the GalA residues were capsulated, while those lacking the residues were non capsulated. Since the proteins involved in the transfer of GalA (WabG) and glucosamine residues (WabH) are known, the chemical basis of the capsular-K2–cell-surface association was studied. Phenol/water extracts from *K. pneumoniae* 52145 Δ *wabH waaL* and 52145 Δ *waaL* mutants, but not those from from *K. pneumoniae* 52145 Δ *wabG waaL* mutant, contained both LPS and capsular polysaccharide, even after hydrophobic chromatography. The two polysaccharides were dissociated by gel-filtration chromatography, eluting with detergent and metal-ion chelators. From these results, it is concluded that the K2 capsular polysaccharide is associated by an ionic interaction to the LPS through the negative charge provided by the carboxyl groups of the GalA residues.

Received 20 October 2005

Revised 5 February 2006

Accepted 6 February 2006

INTRODUCTION

Klebsiella spp. strains, particularly *Klebsiella pneumoniae*, are important causes of nosocomial infections (Emori & Gaynes, 1993). *K. pneumoniae* infections can occur in almost all body sites, but the highest incidence is in the urinary and the respiratory tracts. The main populations at risk are neonates, immunocompromised hosts, and patients predisposed by surgery, diabetes, malignancy, etc. (Emori & Gaynes, 1993; Hansen *et al.*, 1999; Hervás *et al.*, 1993). The existence of multiple antibiotic-resistant *K. pneumoniae* strains is well known, and they have complicated therapy. Mortality rates of up to 50 % have been found in respiratory

tract infections. As an alternative to antibiotic treatment, prevention and/or treatment of *K. pneumoniae* infections by immunotherapy have received increased attention in recent years.

K. pneumoniae strains typically express smooth LPS, LPS with O-antigen polysaccharide (O-PS), and antigenic capsular polysaccharide (K-PS) on their surface; both O-PS and K-PS contribute to the pathogenesis of the species. The *K. pneumoniae* K-PS plays a critical role in the ability of the organism to resist complement-mediated opsonophagocytic killing (Williams & Tomás, 1990). Furthermore, an extra-cellular complex containing LPS and capsule has been suggested to be involved in lung damage (Straus *et al.*, 1985). More than 90 different capsular types (K-antigens) have been described in *Klebsiella*, but only the gene clusters involved in K2 (*wca*_{K2}) and K57 (*wca*_{K57}) antigens have been described (Arakawa *et al.*, 1995). The two known *wca* gene clusters contain genes involved in K-PS residue biosynthesis, linkage, polymerization and export. In addition, the production of the K2 requires a *wca*_{K2} plasmid-borne capsular

Abbreviations: DOC, deoxycholate; EM, electron microscopy; GalA, galacturonic acid; Glc, D-glucopyranose; GlcN, D-glucosamine; Hep, L-glycero-D-manno-heptopyranose; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; K-PS, capsular polysaccharide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NaOAc, sodium acetate; OM, outer membrane; OMP, outer-membrane protein; O-PS, O-antigen polysaccharide; OS, oligosaccharide(s); PCP, phenol/chloroform/light petroleum ether; PW, phenol/water.

gene transcriptional activator (Arakawa *et al.*, 1995; Lai *et al.*, 2003).

In a few Gram-negative K-PS-containing bacteria, the basis of K-antigen attachment to the cell surface involves an interaction with a lipid moiety, either the LPS domain lipid A core (Jann *et al.*, 1992), or a glycerolipid (Gotschlich *et al.*, 1981; Kuo *et al.*, 1985). However, despite its importance as a pathogenic factor, little is known about the linkage of the *K. pneumoniae* K-PS to the cell surface. Knowledge of the chemical structures of the O1-antigen, core LPS, lipid A (Vinogradov *et al.*, 2002; Süsskind *et al.* 1995; Vinogradov & Perry, 2001, Helander *et al.*, 1996), and K2 (Corsaro *et al.*,

2005) antigens from *K. pneumoniae*, as well as the genes involved in their biosynthesis (Arakawa *et al.*, 1995; Kelly *et al.*, 1993; Regué *et al.*, 2001, 2005a), have enabled us to study the basis of the K2-capsule–cell-surface interaction.

METHODS

Bacterial strains, bacteriophages, plasmids and growth conditions. *Klebsiella pneumoniae* strains and plasmids used in this study are shown in Table 1. Bacterial strains were grown in LB broth and on LB agar (Miller, 1972). 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.

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
<i>K. pneumoniae</i>		
DL1	Serovar O1:K1	Tomás <i>et al.</i> (1988)
DL1Δ <i>waaL</i>	Non-polar <i>waaL</i> mutant	This study
DL1Δ <i>wabG</i>	Non-polar <i>wabG</i> mutant	This study
DL1Δ <i>wabG waaL</i>	Double non-polar <i>wabG waaL</i> mutant	This study
DL1Δ <i>wabH</i>	Non-polar <i>wabH</i> mutant	This study
DL1Δ <i>wabH waaL</i>	Double non-polar <i>wabH waaL</i> mutant	This study
52145	Serovar O1:K2	Nassif <i>et al.</i> (1989)
52145Δ <i>waaF</i>	Non-polar <i>waaF</i> mutant	Izquierdo <i>et al.</i> (2003)
52145Δ <i>waaC</i>	Non-polar <i>waaC</i> mutant	Izquierdo <i>et al.</i> (2003)
52145Δ <i>wabK</i>	Non-polar <i>wabK</i> mutant	Regué <i>et al.</i> (2005b)
52145Δ <i>waaL</i>	Non-polar <i>waaL</i> mutant	Izquierdo <i>et al.</i> (2003)
52145Δ <i>wabM</i>	Non-polar <i>wabM</i> mutant	Regué <i>et al.</i> (2005b)
52145Δ <i>wabG</i>	Non-polar <i>wabG</i> mutant	Izquierdo <i>et al.</i> (2003)
52145Δ <i>wabG waaL</i>	Double non-polar <i>wabG waaL</i> mutant	Regué <i>et al.</i> (2005a)
52145Δ <i>wabH</i>	Non-polar <i>wabH</i> mutant	Regué <i>et al.</i> (2005a)
52145Δ <i>wabH waaL</i>	Double non-polar <i>wabH waaL</i> mutant	Regué <i>et al.</i> (2005a)
52145Δ <i>waaE</i> (NC16)	Non-polar <i>waaE</i> mutant	Regué <i>et al.</i> (2001)
C3	Serovar O8:K66	Ørskov & Ørskov (1984)
C3Δ <i>waaF</i>	Non-polar <i>waaF</i> mutant	Izquierdo <i>et al.</i> (2003)
C3Δ <i>waaC</i>	Non-polar <i>waaC</i> mutant	Izquierdo <i>et al.</i> (2003)
C3Δ <i>wabI</i>	Non-polar <i>wabI</i> mutant	This study
C3Δ <i>waaL</i> (NC20)	Non-polar <i>waaL</i> mutant	Regué <i>et al.</i> (2001)
C3Δ <i>wabJ</i>	Non-polar <i>wabJ</i> mutant	This study
C3Δ <i>wabG</i>	Non-polar <i>wabG</i> mutant	Izquierdo <i>et al.</i> (2003)
C3Δ <i>wabG waaL</i>	Double non-polar <i>wabG waaL</i> mutant	Regué <i>et al.</i> (2005a)
C3Δ <i>wabH</i>	Non-polar <i>wabH</i> mutant	Regué <i>et al.</i> (2005a)
C3Δ <i>wabH waaL</i>	Double non-polar <i>wabH waaL</i> mutant	Regué <i>et al.</i> (2005a)
C3Δ <i>waaE</i>	Non-polar <i>waaE</i> mutant	Izquierdo <i>et al.</i> (2003)
Plasmid		
pKO3	Cm ^r temperature-sensitive replication <i>sacB</i> -containing suicide plasmid	Link <i>et al.</i> (1997)
pKO3Δ <i>waaC</i>	Contains an internal non-polar <i>waaC</i> deletion	Izquierdo <i>et al.</i> (2003)
pKO3Δ <i>waaF</i>	<i>waaF</i> deletion	Izquierdo <i>et al.</i> (2003)
pKO3Δ <i>wabI</i> _{C3}	Contains an internal non-polar <i>wabI</i> deletion	Regué <i>et al.</i> (2005a)
pKO3Δ <i>waaL</i> _{DL1}	Contains an internal non-polar <i>waaL</i> deletion	Regué <i>et al.</i> (2001)
pKO3Δ <i>wabJ</i> _{C3}	Contains an internal non-polar <i>wabJ</i> deletion	Regué <i>et al.</i> (2005a)
pKO3Δ <i>wabG</i>	Contains an internal non-polar <i>wabG</i> deletion	Izquierdo <i>et al.</i> (2003)
pKO3Δ <i>wabH</i>	Contains an internal non-polar <i>wabH</i> deletion	Regué <i>et al.</i> (2005a)

Bacteriophages FC3-9, $\phi 1$ and $\phi 2$, specific for capsular serovars K66, K1 and K2, respectively, have been described previously (Camprubí *et al.*, 1992; Regué *et al.*, 2004).

ELISAs. Whole cells, culture supernatants, cell-surface extracts or chromatographic fractions were studied with anti-K1-, -K2- or -K66-specific antiserum, as previously described for whole cells (Camprubí *et al.*, 1992; Regué *et al.*, 2004).

General DNA methods. General DNA manipulations were done essentially as described (Sambrook *et al.*, 1989). DNA restriction endonucleases, T4 DNA ligase, *Escherichia coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

Mutant construction. *K. pneumoniae* individual genes were mutated by creating *in vitro* in-frame deletions of each gene. Each mutated gene was transferred to the chromosome by homologous recombination using the temperature-sensitive suicide plasmid pKO3, containing the counterselectable marker *sacB* (Link *et al.*, 1997). Mutations were made in *waaL* and *wabI* J G and H. The plasmids containing the engineered in-frame deletions derived from DL1 (pKO3 Δ waaL_{DL1}), and C3 (pKO3 Δ wabI_{C3} and pKO3 Δ wabJ_{C3}) sequences were transformed into *K. pneumoniae* C3 and DL1, respectively, by electroporation. Mutants were selected based on growth on 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 DL1 Δ waaL mutant was constructed by asymmetric PCR amplifications using DL1 chromosomal DNA and primers LA_{DL1} (5'-CGCGCGG-CCGCGAGCGCTGAAAAACAGTAT-3'), LB_{DL1} (5'-CCCATCCAC-TAAACTTAAACAACCCTGTCTGGCGAAGTTA-3'), LC_{DL1} (5'-TGTTTAAGTTTGTAGTGGATGGGCGGAAAAACGCTAACAAA-3') and LD_{DL1} (5'-GAGCGGCGCGCTGCTATTGCGTCTGGATGA-3'). The primers include *NotI* sites (single underlined). DNA fragments of 803 (LA_{DL1}–LB_{DL1}) and 1037 (LC_{DL1}–LD_{DL1}) bp were obtained. DNA fragment LA_{DL1}–LB_{DL1} included nt 3430, inside *wabI*, to nt 4200, corresponding to the third base of the eighth codon of *waaL*. DNA fragment LC_{DL1}–LD_{DL1} included the last four codons of *waaL* (nt 5315) to nt 6320, inside *wabJ*. DNA fragments LA_{C3}–LB_{C3} and LC_{DL1}–LD_{DL1} were annealed at their overlapping region (double-underlined in primers LB_{DL1} and LC_{DL1}), and amplified by PCR as a single fragment, using primers LA_{DL1} and LD_{DL1}. The fusion product was purified, *NotI* digested, ligated into *NotI*-digested and phosphatase-treated pKO3 vector, electroporated into *E. coli* DH5 α , and plated on chloramphenicol LB agar at 30 °C to obtain plasmid pKO3 Δ waaL_{DL1}. Similarly, primers sets I [IA_{C3} (5'-CGCGCGGCGCG-AGCCTGTTTACAACCGCC-3'), IB_{C3} (5'-CCCATCCACTAAACT-TAAACACAGGGATCCCATCAGAGC-3'), IC_{C3} (5'-TGTTTAAGTT-TAGTGGATGGGATCTTCGAAAAGTTGCGTTT-3') and ID_{C3} (5'-CGCGCGGCGCGTATGGTGCCAGATGCTCAG-3')] and J [JA_{C3} (5'-CGCGCGGCGCGCACCATACGCTGGTTCTCACC-3'), JB_{C3} (5'-TGTTTAAGTTTGTAGTGGATGGGCGCGGCTTATTTCTGTATCG-3'), JC_{C3} (5'-CCCATCCACTAAACTTAAACAATGTGGCCCGTGATTA-AAGAG-3') and JD_{C3} (5'-CGCGCGGCGCGCTTATTGAATACAG-GACG-3')] were used to construct pKO3 Δ wabI_{C3} and pKO3 Δ wabJ_{C3}, respectively. Plasmid pKO3 Δ wabI_{C3} included nt 2675, inside *waaC*, to the third base of the fourth codon of *wabI* (nt 3285), an in-frame 21 bp linker, and nt 4138, inside *wabI*, to nt 4974, inside *waaL*, including the last *wabI* 14 codons. Plasmid pKO3 Δ wabJ_{C3} included nt 4581, inside *waaL*, to nt 5426, containing the last *wabJ* 17 codons, an in-frame 21 bp linker, and nt 6357, corresponding to the third base of the 19th codon of *wabJ*, to nt 7128, inside *wabN*. Due to the extensive identity among *wabI* and *wabJ* between strains C3 and DL1, the same sets of four primers and DL1 chromosomal DNA were used to construct pKO3 Δ wabI_{DL1} and pKO3 Δ wabJ_{DL1}. For the

same reason, primers used in the construction of pKO3-engineered deletions of *wabG* and *wabH* in strains C3 and 52145 were used to construct DL1 Δ wabG and DL1 Δ wabH mutants, respectively.

Plasmid constructions for mutant complementation studies.

For complementation studies, waaL_{DL1}, wabH_{DL1} and wabG_{DL1} were amplified, ligated to pGEM-T Easy (Promega), and transformed into *E. coli* DH5 α .

Cell surface extraction, and electrophoresis.

K. pneumoniae cells grown in trypticase soy broth (TSB) at 37 °C were dried (0.6 g) and extracted. The phenol/chloroform/light petroleum ether (PCP) method (Galanos *et al.*, 1969) and a modified phenol/water (PW) procedure (Westphal & Jann, 1965; Rhan *et al.*, 2003) were used. The PCP and PW methods are used for the extraction of O-PS-containing and -deficient LPS, respectively. The PW extract was processed by two different approaches. First, 100 mg PW extract was purified by hydrophobic chromatography on a Butyl Sepharose column (Pharmacia). The column (40 $\times$ 2.5 cm) was equilibrated with 200 mM sodium acetate (NaOAc), pH 4.7, and eluted initially with 150 ml of the same buffer, and then with 450 ml of a linear gradient to NaOAc:*n*-propanol (50:50, v/v) (Muck *et al.*, 1999). Fractions of 2.5 ml were collected, and monitored for carbohydrates (phenol/sulfuric acid test, A₄₉₀). Eluted fractions were pooled, dialysed and freeze-dried, yielding fraction pools 1, 2 and 3, of 11, 22 and 26 mg, respectively. Only fraction pools 1 and 3 were further analysed, because fraction pool 2 contained nucleic acid. In the second approach, the water phase obtained was ultracentrifuged (105 000 g) at 15 °C for 17 h. The supernatant was treated with DNase, RNase and proteinase K (Sigma) in order to eliminate both nucleic acid and proteins. The sample was dialysed and lyophilized (20 mg), and chromatographed on a Sephacryl S-200 column (Pharmacia). The column was equilibrated with 10 mM Tris buffer, pH 8, containing 1.25% deoxycholate (DOC), 0.2 M NaCl and 5 mM EDTA, and the eluent was monitored with a Knauer differential refractometer. On the basis of the chromatographic profile, the eluted fractions were pooled, dialysed and lyophilized. Fraction pools 1 and 2, of 7 and 3 mg, respectively, were obtained. For screening purposes, LPS was obtained after proteinase-K digestion of whole cells (Hitchcock & Brown, 1983). PCP and PW cell-surface extracts, and LPS preparations obtained by the method of Hitchcock & Brown (1983), were separated by SDS-PAGE or Tricine {N-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine} SDS-PAGE, and visualized by silver staining, as described (Pradel & Schnaitman, 1991; Tsai & Frasch, 1982).

Isolation of oligosaccharides (OS). The LPS-containing PCP extract from *K. pneumoniae* 52145 Δ waaL *wabH* (15 mg) was hydrolysed with 1% acetic acid (100 °C for 1 h). The resulting precipitate (6 mg) was removed by centrifugation (10 000 g), and the supernatant (4 mg) was analysed by MS. Another PCP-LPS sample (15 mg) was deacylated and purified, as described (Holst *et al.*, 1991, 1993), obtaining 2 mg alditol OS. Peaks 1 and 3 from Butyl Sepharose chromatography were hydrolysed separately with 1% acetic acid, containing 0.1% SDS, at 100 °C for 3 h. After cooling, the samples were centrifuged (10 000 g) for 20 min. The supernatants were lyophilized, washed with ethanol, and analysed by MS. Samples (1 mg) of peaks 1 and 2 of gel-filtration chromatography were separately stirred in anhydrous hydrazine (0.5 ml) at 37 °C for 1.5 h (Holst *et al.*, 1991). The reaction mixtures were cooled in an ice bath, and cold acetone was added in small portions until the precipitation was complete. The samples were then centrifuged (6300 g, 4 °C, 15 min), the pellets were washed twice with cold acetone, dissolved in water for lyophilization, and analysed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS.

Glycosyl and lipid analysis. Samples (1 mg) of PCP and PW extracts were dried over P₂O₅ overnight, and treated with 1 M HCl/CH₃OH (1 ml) at 80 °C for 20 h. The crude reactions were 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, and the acetylated fatty acids methyl esters were analysed by GC-MS. The remaining methanol phase after hexane extraction was neutralized with Ag₂CO₃, dried, acetylated, and the methylglycoside derivatives were analysed by GC-MS. The same analysis was performed on relevant fractions from Butyl Sepharose and Sephacryl gel chromatography.

GC-MS analysis. Alditol acetates, methyl glycoside acetates, partially methylated alditol acetates and acetylated methyl esters lipids were analysed on a Agilent Technologies 5973N MS instrument equipped with a 6850A Gas Chromatograph and an RTX-5 capillary column (Restek; 30 m × 0.25 mm i.d., flow rate 1 ml min⁻¹, helium carrier gas), as previously described (Izquierdo *et al.*, 2002).

MS studies. Positive- and negative-ion MALDI-TOF spectra were acquired on a Voyager DE-PRO instrument (Applied Biosystems) equipped with a delayed extraction ion source, in both linear and reflector modes. Ion acceleration voltage was 20 kV, grid voltage was 14 kV, mirror voltage ratio was 1.12, and delay time was 100 ns. Samples were irradiated at a frequency of 5 Hz by 337 nm photons from a pulsed nitrogen laser. Mass calibration was obtained with a malto-oligosaccharide mixture from corn syrup (Sigma). A solution of 2,5-dihydroxybenzoic acid in 20% CH₃CN in water at a concentration of 25 mg ml⁻¹ was used as the MALDI matrix. Matrix solution (1 µl) and sample (1 µl) were premixed, and then deposited on the target. The droplet was allowed to dry at an ambient temperature. Spectra were calibrated and processed under computer control using the Applied Biosystems Data Explorer software.

Murine pneumonia model. The experiments were performed as described (Cortés *et al.*, 2002). Briefly, ICR-CDI mice (Harlan Ibérica) were anaesthetized, and intubated intratracheally with a blunt-ended needle. Approximately 10⁷ c.f.u. of exponentially growing *K. pneumoniae* cells were suspended in 50 µl PBS, and inoculated through the blunt-ended needle. The mice were observed daily, and bacteraemia was assessed on days 2, 4 and 6 by culturing blood obtained from the tail vein (approx. 20 µl) on LB agar. Lung and spleen tissues from surviving animals and dead animals were aseptically removed, homogenized, and plated for quantitative bacterial cultures. Each experiment was performed with nine animals.

RESULTS

K. pneumoniae K2 capsule association to the cell surface does not require the core LPS O-PS:lipid-A-core ligase

Two different core LPS types have been reported in *K. pneumoniae* (Regué *et al.*, 2005b; Vinogradov & Perry, 2001) (Fig. 1a, b). Knowledge of the role of *K. pneumoniae* genes involved in core LPS biosynthesis (Frirdich *et al.*, 2004; Izquierdo *et al.*, 2002, 2003; Regué *et al.*, 2001, 2005a, b) enabled the construction of a set of isogenic non-polar *waaL* mutants in *K. pneumoniae* strains producing either type 1 [C3 (O8:K66) and DL1 (O1:K1)] or type 2 [52145 (O1:K2)] core LPS. The *waaL* gene encodes the O-PS:lipid-A-core ligase, and, as expected, these *waaL* mutants were devoid of O-PS, as determined by SDS-PAGE analysis of the corresponding LPS preparations (see Fig. 2, lane 2, for 52145Δ*waaL*). Mutant strains 52145Δ*waaL*, DL1Δ*waaL* and C3Δ*waaL* were sensitive to capsule-specific phages ϕ2 (K2) (Table 2), ϕ1 (K1) and FC3-9 (K66), respectively. No difference in

capsule-specific phage e.o.p. was found between each *waaL* mutant and its isogenic parent strain, suggesting that the level of functional capsule production is independent of the O-PS ligase. To demonstrate that the *waaL* mutants and their isogenic parent strains produced the same capsular type, ELISAs using specific capsular antibodies were performed. Cells from mutant strains 52145Δ*waaL* (Table 2), DL1Δ*waaL* and C3Δ*waaL* reacted with anti-K2, -K1 and -K66 antibodies, respectively. In addition, the presence of cell-bound capsule was observed in the three *waaL* mutant strains in electron microscope (EM) studies (data not shown). Each mutant was complemented by introduction of the corresponding wild-type *waaL*. These results strongly suggest that the O-PS ligase is not involved in the association of the K1, K2 and K66 capsule polysaccharides to the cell surface in *K. pneumoniae*.

K. pneumoniae core LPS galacturonic acid (GalA) residues are essential for K2 capsule association to the cell surface

Non-polar mutants were generated in each gene predicted to be involved in core-LPS biosynthesis, with the exception of *gmhD* (ADP-Hep epimerase) and *waaA* (CMP-Kdo transferase) (Fig. 1a). These mutants were constructed in strain 52145 (type 2 core). Mutants 52145Δ*waaC*, 52145Δ*waaF* and 52145Δ*wabG* were resistant to phage ϕ2, and their cells did not react with anti-K2 capsule serum in ELISAs using capsule K2-specific antibodies. But, K2-PS-like material was detected in the culture supernatants of these mutants by ELISA (Table 2). These results suggest that these three mutants synthesize capsular polysaccharide, but that it cannot be attached to the cell surface. By contrast, mutants 52145Δ*wabH*, 52145Δ*wabK* and 52145Δ*wabM* were phage ϕ2 sensitive, and contained cell-associated K2-PS (Table 2). Comparison of the known core-OS from mutants 52145Δ*wabH* (Fig. 1c) and 52145Δ*wabG* (Fig. 1d) suggests an essential role for the core OS GalA residues in K2 interaction with the cell surface.

Capsule-K2-LPS co-elution

Cells from 52145Δ*wabH waaL* double mutant were extracted by the PCP method (Galanos *et al.*, 1969). Chemical composition of this PCP extract showed the presence of the characteristic core LPS sugars 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), L-glycero-D-manno-heptopyranose (Hep), D-glucopyranose (Glc) and GalA. The same cells were first treated with PCP, and then with a modified PW procedure (Rahn *et al.*, 2003). The PW extract contained glucuronic acid (GlcA) and mannose (Man) from the K2 capsule (Corsaro *et al.*, 2005), in addition to core LPS sugars. Protein was not detected in these preparations.

Hydrophobic chromatography of the PW extract on a Butyl Sepharose column (Pharmacia) separated this material into three major peaks (1, 2 and 3) (Fig. 3a). Peak 2 was discarded because it contained nucleic acid only. Chemical analysis of peaks 1 and 3 revealed the presence of both K2

Fig. 1. *K. pneumoniae* core OS structures. (a, b) Core type 1 and 2 structures from *K. pneumoniae* (Regué *et al.*, 2005b; Vinogradov & Perry, 2001), and genes involved in the synthesis of type 1 and 2 core LPS (Firdich *et al.*, 2004; Izquierdo *et al.*, 2002, 2003; Regué *et al.*, 2001, 2005a, b). Structures of core OS from *K. pneumoniae* 52145 *waaL wabH* (Regué *et al.*, 2005a) (c), and *waaL wabG* (Izquierdo *et al.*, 2003) (d). Depending on the *K. pneumoniae* strain, residues J and K could be hydrogen (H) or GalA, and residue Q could be H or Hep (Vinogradov & Perry, 2001). The bold broken arrow (b) denotes that the O-antigen attachment site deduced from the analysis of $\Delta wabK$ and $\Delta wabM$ mutants (Regué *et al.*, 2005b).

and core-LPS residues. The integration results of GC-MS analysis showed a GalA:GlcA ratio of 0.25 for peak 1, whereas this ratio was 3.3 for peak 3, indicating that K2-PS (GlcA) was more abundant in peak 1 while LPS (GalA) was more abundant in peak 3. These results suggest that at least K2 and core LPS co-elute in the same peaks (Fig. 3b). Material from both peaks 1 and 3 was characterized by hydrolysis with 1% acetic acid, followed by positive-ion MALDI-TOF and ^1H -NMR analyses of the recovered OS.

The comparison of the MALDI-TOF spectrum of the acetic acid hydrolysis product of purified LPS with those of peaks 1 and 3 suggested the co-elution of K2 and LPS for peak 3 (Fig. 4). The assignment of core-LPS signals is summarized in Fig. 4 (insert above figure), and was obtained as previously reported (Regué *et al.*, 2005b). In the mass spectrum of peak 1 (Fig. 4), the difference (Δ) between the clusters of signals corresponded to hexose or hexuronic acid (162 or 176 Da), which are constituents of the K2

Fig. 2. Analysis of LPS by Tricine SDS-PAGE. LPS samples from wild-type *K. pneumoniae* 52145 (lane 1), and core-LPS mutants: 52145 Δ *waaL* (2), 52145 Δ *waaC* (3), 52145 Δ *waaF* (4), 52145 Δ *wabG* (5), 52145 Δ *wabH* (6), and 52145 Δ *wabK* (7). The arrow denotes the region of wild-type LPS containing a high degree of polymerized O-antigen.

capsule. In the mass spectrum of peak 3, signals of core OS were present, together with those of K2-PS (Fig. 4). By contrast, the comparison of ^1H -NMR spectra of purified K2 structure and acetic-acid-released core LPS with those of peaks 1 and 3 suggest the co-elution of the two polysaccharides in both peaks 1 and 3 (Fig. 5). The absence of core LPS signals in the mass spectrum of peak 1 could be attributable to the low abundance of LPS in this peak, and to a lower ionization ability of the core LPS than that of K2-PS.

Furthermore, the presence of K2 antigen in peaks 1 and 3 was confirmed by ELISA with specific K2 antiserum. In peaks 1 and 3, no signals corresponding to core OS covalently linked to one or more repetitive units of K2-PS were detected by the MS experiments, suggesting a non-covalent association between K2-PS and core LPS.

Fig. 3. K2-capsule-LPS co-elution. (a) Butyl Sepharose chromatogram of PW extract from strain 52145 Δ *waaL wabH*. (b) SDS-PAGE analysis of PW extract and chromatographic peaks 1, 2 and 3.

Capsule-K2-LPS ionic interaction

To determine how the K2 polysaccharide was associated to the core LPS, the 52145 Δ *wabH waaL* PW extract was treated with DNase, RNase and proteinase K, and further purified by high-speed centrifugation. This preparation was subjected to gel-filtration chromatography on a Sephacryl S-200 column (Pharmacia) using as the eluent a buffer with an increased ionic strength (0.1 M NaCl and 10 mM Tris), which contained the metal-ion chelator EDTA, and the dissociating detergent DOC (see Methods). This approach allowed the fractionation of the PW extract into two main peaks (1 and 2) (Fig. 6a). SDS-PAGE analysis showed the presence of only LPS molecules in peak 2, while in peak 1, both K2 and LPS molecules were present (Fig. 6b). Chemical analysis confirmed the presence of LPS residues in peak 2, and of both K2 and LPS residues in peak 1. Both peak 1 and 2

Table 2. Sensitivity to bacteriophage ϕ 2, and ELISAs with anti-K2 capsule serum of *K. pneumoniae* 52145 and mutant derivatives

Strain	Sensitivity to bacteriophage ϕ 2 (e.o.p.)	Reactivity (A_{405}) of anti-K2-capsule serum against:*	
		Whole cells†	Supernatant‡
52145	1	1.76 ± 0.18	0.25 ± 0.04
52145 Δ <i>waaL</i>	0.99	1.72 ± 0.14	0.19 ± 0.03
52145 Δ <i>wabK</i>	0.98	1.71 ± 0.15	0.21 ± 0.05
52145 Δ <i>wabM</i>	0.99	1.79 ± 0.13	0.22 ± 0.02
52145 Δ <i>wabH</i>	0.98	1.68 ± 0.10	0.20 ± 0.05
52145 Δ <i>wabG</i>	0	<0.1	0.87 ± 0.09
52145 Δ <i>waaF</i>	0	<0.1	0.69 ± 0.08
52145 Δ <i>waaC</i>	0	<0.1	0.74 ± 0.07

* Data are means ($\pm$ SD) derived from at least three independent experiments.

†The number of whole cells used was approximately 10^6 c.f.u.

‡Supernatants were obtained from overnight cultures that had been centrifuged and filtered to remove bacterial cells, and then concentrated tenfold.

Fig. 4. Positive-ion MALDI-TOF spectra of Butyl Sepharose chromatographic peaks. Samples were hydrolysed in 1 % acetic acid, and subjected to MALDI-TOF analysis. Spectra from core LPS and peaks 1 and 3 (see Fig. 3), separated by Butyl Sepharose chromatography. Assignment of core LPS signals is summarized in the inserted text. Δ, Difference between the clusters of signals attributable to Hex (162 Da) and/or HexA (176 Da) of K2-PS.

samples were characterized by O-delipidation and negative-ion MALDI-TOF MS analysis (Fig. 6). Comparison of mass spectra signals of peak 2 and purified O-deacylated LPS showed the presence of only O-deacylated LPS in this peak (Fig. 7). Signals corresponding to K2-PS and O-deacylated LPS were present in peak 1 (Fig. 7). In addition, ELISA analyses of peaks 1 and 2 with specific K2 antiserum showed the presence of K2 molecules in peak 1 only, thus confirming the above results. The complete separation between LPS and

K2 antigen was achieved by three successive gel-filtration chromatographs of the peak 1 material in the presence of EDTA and DOC (Fig. 8). This result indicates that the ionic strength and the amount of EDTA are crucial to completely remove cation bridges between LPS and K2 capsule.

There was no difference in the spectra of outer-membrane protein (OMP) bands seen in wild-type 52145, and 52145Δ-*waaL* and 52145Δ-*wabH* mutants. However, some changes in

Fig. 5. ^1H NMR spectra in D_2O at 400 MHz of (a) PCP-LPS acetic acid hydrolysis product from mutant 52145 Δ waaL wabH, (b) K2-PS, and (c) peak 1 and (d) peak 3 from the Butyl Sepharose chromatogram.

the relative intensities of the minor OMPs were observed, but the major OMPs remained unchanged in their relative intensity [OmpK36, K35, OmpA-like, OmpK17 (Albertí *et al.*, 1995; Climent *et al.*, 1997; Hernández-Allés *et al.*, 1999; Martínez-Martínez *et al.*, 1996)]. Nevertheless, truncation of the core LPS beyond the outer core GalA residue in the wabH mutant might alter the presence or the topology of other outer-membrane (OM) minor molecules, which could block capsule attachment to other(s) target(s) in the OM. To test this possibility, 52145 Δ waaL cells, containing

Fig. 6. K2-capsule-LPS ionic interaction. (a) Sephacryl gel-filtration chromatography with EDTA and DOC (see Methods) of the PW extract from strain 52145 Δ waaL wabH. (b) SDS-PAGE analysis of the PW extract, and chromatographic peaks 1 and 2.

full-length type 2 core OS, were extracted successively with PCP and PW. PW extracts from these cells also contained LPS and K2-PS co-purifying through hydrophobic chromatography. Both LPS and K2-PS were fully separated into two peaks after three consecutive gel-filtration chromatography assays in the presence of EDTA and DOC, as determined by SDS-PAGE, ELISA and chemical analysis (data not shown). This result suggests that the postulated ionic interaction between core OS and K2-PS is not due to 5245 Δ wabH mutant pleiotropic effects on the OM.

Other *Klebsiella* capsular types (K1 and K66)–core-LPS interaction

To study if the K2–core-LPS interaction is a feature shared by other *K. pneumoniae* capsular serotypes, core-LPS-deficient mutants were constructed in *K. pneumoniae* strains C3 (O8:K66) and DL1 (O1:K1). The capsular phenotypes of these mutants were determined using capsule K66- and K1-specific bacteriophages FC3-9 and ϕ 1, respectively, ELISAs with specific antiserum against K66 and K1, and EM studies. Strains with mutations in the genes waaC (C3 Δ waaC, DL1 Δ waaC), waaF (C3 Δ waaF, DL1 Δ waaF) and wabG (C3 Δ wabG, DL1 Δ wabG) were devoid of cell-surface-associated capsule, although K-PS-like material was detected in their culture supernatants by ELISA. Strains with mutations in the genes wabH (C3 Δ wabH, DL1 Δ wabH), wabI (C3 Δ wabI, DL1 Δ wabI) and wabJ (C3 Δ wabJ, DL1 Δ wabJ) were capsulated. Double-deletion mutants C3 Δ waaL wabG and DL1 Δ waaL wabG were non-capsulated, while C3 Δ waaL wabH and DL1 Δ waaL wabH contained cell-associated K66- and K1-PS, respectively. These results suggest that capsule–core-LPS interaction could be a general phenomenon in *K. pneumoniae*, because this interaction determines the presence of cell-associated capsule for the studied capsular types K1, K2 and K66. Furthermore, the capsule–core-LPS interaction appears to require the core OS GalA residues.

Fig. 7. Negative-ion MALDI-TOF spectra of Sephacryl gel-filtration chromatographic peaks. Samples were O-deacylated, and subjected to MALDI-TOF analysis (see Methods). Spectra from O-deacylated LPS, and peaks 1 and 2 separated by Sephacryl gel filtration, are shown. Assignment of O-deacylated LPS signals: a, b and c, cluster of pseudomolecular ion peaks of species consisting of diacylLipidAKdoP, diacylLipidAKdoP₃ and diacylLipidAKdo₂P₃ respectively; d, cluster of peaks of species corresponding to diacylLipidAKdo₂P₃Hep₃HexHexA₂. Δ, difference between the clusters of signals attributable to Hex (162 Da) and/or HexA (176 Da) of K2-PS.

Virulence studies

Virulence was tested in a murine model of pneumonia by intratracheal injection. When the virulence of the strains was assayed in the murine pneumonia model, we obtained the

results shown in Table 3. The 52145Δ*wabG* mutant was completely avirulent in this model, while 52145Δ*waaL* and 52145Δ*wabH* mutants, and the wild-type strain, showed similar values. Introduction of the corresponding genes in the mutants rendered them as virulent as the wild-type

Fig. 8. K2-capsule–LPS complete separation. Peak 1 from the Sephacryl gel filtration chromatography (Fig. 6), containing both K2-PS and LPS, was rechromatographed three times using the conditions described in Fig. 6 and Methods. The final two chromatographic peaks were analysed by SDS-PAGE. Lanes: 0, starting material; 1, K2-PS; 2, LPS. Chemical analysis confirmed the complete separation of K2-PS and LPS.

Table 3. Experimental pneumonia induced by *K. pneumoniae* strains

$P < 0.01$ for all comparisons [lung weight, log c.f.u. (g lung)⁻¹, mortality and blood or spleen positive cultures] between the strains (two-tailed *t* test).

Strain	Mean inoculum (range)	Mean lung weight (g)*	Mean log c.f.u. (g lung) ⁻¹	No. dead animals/total no. (%)	No. positive cultures/total no. (%)†
52145	1.0×10^7 ($\pm 1.4 \times 10^7$)	0.29 ± 0.11	6.11	21/36 (58)	19/36 (52)
52145ΔwaaLL	1.0×10^7 ($\pm 2.1 \times 10^7$)	0.35 ± 0.09	6.31	10/18 (55)	10/18 (55)
52145ΔwaaL + pGEMT	1.7×10^7 ($\pm 1.6 \times 10^7$)	0.37 ± 0.05	6.42	10/18 (55)	10/18 (55)
52145ΔwaaL + pGEMT-WaaL	1.3×10^7 ($\pm 1.1 \times 10^7$)	0.37 ± 0.11	6.09	10/18 (55)	9/18 (50)
52145ΔwabG	1.9×10^7	0.18 ± 0.05	<1.0	0/9 (0)	0/9 (0)
52145ΔwabG + pGEMT	1.8×10^7	0.16 ± 0.07	<1.0	0/9 (0)	0/9 (0)
52145ΔwabG + pGEMT-WabG	2.2×10^7 ($\pm 0.5 \times 10^7$)	0.27 ± 0.12	5.98	10/18 (55)	9/18 (50)
52145ΔwabH	1.6×10^7 ($\pm 1.2 \times 10^7$)	0.33 ± 0.07	6.02	9/18 (50)	9/18 (50)
52145ΔwabH + pGEMT	1.3×10^7 ($\pm 0.9 \times 10^7$)	0.31 ± 0.05	6.07	10/18 (55)	10/18 (55)
52145ΔwabH + pGEMT-WabH	1.5×10^7 ($\pm 1.0 \times 10^7$)	0.36 ± 0.09	6.14	11/18 (60)	9/18 (50)

*Values are given as mean $\pm$ SD.

†Blood or spleen cultures.

strain. However, mutant 52145ΔwabG transformed with the plasmid vector alone (pGEMT) remained avirulent in this animal model.

DISCUSSION

The constitutive cell-associated K-PS is a major pathogenic factor in *K. pneumoniae* infection, but the basis for its attachment to the cell surface is unknown. In the few studied encapsulated Gram-negative bacteria, K-PS has been proposed to be covalently linked to a lipid moiety, either core lipid A or a glycerolipid. The K-PS association to core lipid A has been suggested to involve the O-PS ligase. Results based on the analysis of three non-polar O-PS ligase (WaaL) mutants showed that in *K. pneumoniae* this activity is not required for functional K2-PS cell-surface linkage.

K. pneumoniae core-LPS mutants containing the GalA core residues contained functional K2-PS. By contrast, mutants devoid of core GalA residues were devoid of functional K2-PS. PCP extraction of waaL wabH mutant did not enable us to recover K2-PS, while PW extraction recovered both LPS and K2-PS. This is in agreement with the amphipathic and hydrophilic natures of LPS and K2-PS, respectively.

Characterization of PW extracts from *K. pneumoniae* 52145ΔwaaL wabH showed that K2 capsule and LPS co-purify after hydrophobic-interaction chromatography. MS of the chromatographic peaks agreed with a non-covalent association between both molecules. The possibility that phenol used in both extraction methods could hydrolyse the putative ester linkage between K2-PS and core LPS is unlikely since these methods allow the isolation of LPS molecules containing O-acylated residues (Knirel & Kochetkov, 1994).

Gel-filtration chromatography and elution in a buffer with EDTA and DOC enabled the isolation of a chromatographic peak with LPS only, according to chemical composition and MS analysis. The other chromatographic peak containing K2-PS associated to core LPS was fully separated on successive chromatographic assays. The same results were obtained using 52145ΔwaaL containing a full-length core OS. These results indicate that the K2-PS is associated to the cell surface by an ionic interaction between K2-PS and core OS. The role of Mg²⁺ ions in establishing ionic bridges between adjacent LPS molecules through negatively charged core residues has been established (Nikaido & Vaara, 1985). In a similar way, we suggest that the divalent ions Mg²⁺ and/or Ca²⁺ could play a role in the ionic interaction between GlcA (K2-PS) and GalA residues (core OS). This ionic interaction could also explain the formation of the extracellular toxic complex, containing both K2-PS and LPS, in *K. pneumoniae* (Straus *et al.*, 1985). Furthermore, the similar behaviour of waaL wabH and waaL wabG mutants from *K. pneumoniae* belonging to capsular serotypes K1 and K66 suggests that the capsule-core-LPS ionic interaction is a general *K. pneumoniae* phenomenon. In the three *K. pneumoniae* capsular serotypes analysed, the presence of GalA residues in core OS was necessary for capsule association to cell surface.

The effects of the wabG, wabH and waaL mutations in virulence experiments were studied in the *K. pneumoniae* 52145 background because this strain is highly virulent in the model used. The wabG mutant was completely avirulent in the experimental model of pneumonia (Table 3). A similar result was obtained using a non-encapsulated mutant of strain 52145 (Cortés *et al.*, 2002). On the other hand, a *K. pneumoniae* waaL mutant, with a full inner and outer core, but devoid of O-antigen, showed similar virulence when compared with the wild-type strain. A similar result was

obtained for the *wabH* mutant. All the changes observed in the *K. pneumoniae wabG* and *H*, and *waaL* mutants were rescued by introduction of the corresponding single wild-type gene, while the introduction of the plasmid vector alone was unable to perform the rescue. The capsule is essential in the *K. pneumoniae* experimental model of pneumonia, as previously indicated (Cortés *et al.*, 2002). Furthermore, the K2 present in *wabH* and *waaL* mutants enables them to be as virulent as the wild-type strain in the experimental model of pneumonia. These studies clearly confirmed the presence of virulent functional K2 in *waaL* and *wabH* mutants, and its absence in the *wabG* mutant.

Summarizing, *K. pneumoniae* lacks phosphoryl residues in its core, but instead contains GalA as a critical source of negative charge. These results indicate that the negative charge provided by the carboxyl groups of GalA play an essential role in capsule attachment by an ionic interaction. Further work will be necessary to obtain a detailed knowledge of this interaction, which could contribute to the design of more efficient methods of prevention and/or treatment of *K. pneumoniae* infections.

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