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Cloning and Sequencing of the *Klebsiella pneumoniae* O5 *wb* Gene Cluster and Its Role in Pathogenesis

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One representative recombinant clone encoding *Klebsiella pneumoniae* O5-antigen lipopolysaccharide (LPS) was found upon screening for serum resistance in a cosmid-based genomic library of *K. pneumoniae* KT769 (O5:K57) introduced into *Escherichia coli* DH5α. A total of eight open reading frames (wb_{O5} gene cluster) were necessary to produce *K. pneumoniae* O5-antigen LPS in *E. coli* K-12. The enzymatic activities proposed for the wb_{O5} gene cluster are in agreement with the activities proposed for the biosynthesis of *K. pneumoniae* O5-antigen LPS. Using the complete DNA sequence of the *K. pneumoniae* wb_{O5} gene cluster, we obtained (by single or double recombination) genetically well-characterized mutants devoid only of this O5-antigen LPS. Finally, using these $O5^-$ mutants and the corresponding wild-type strains or complemented mutants with the wb_{O5} gene cluster ($O5^+$ strains), we found that the presence of *K. pneumoniae* O5-antigen LPS is essential for some pathogenic features like serum resistance, adhesion to uroepithelial cells, and colonization (experimental infections) of the urinary tract in rats.

The O antigen is the most external component of lipopolysaccharide (LPS) and consists of a polymer of oligosaccharide repeating units. Another interesting feature is the high chemical variability shown by the O antigen, leading to a similar genetic variation in the genes involved in O-antigen biosynthesis, the so called *wb* (*rfb*) cluster (for reviews, see references 35 and 45). The genetics of O-antigen biosynthesis have been intensively studied in the family *Enterobacteriaceae*, and it has been shown that the *wb* clusters usually contain genes involved in biosynthesis of activated sugars, glycosyl transferases, O-antigen polymerases, and O-antigen export (35, 45).

Escherichia coli DH5α and other K-12-derived strains are rough, unable to produce O-antigen LPS (O^-) and serum sensitive. As we and other authors have previously shown for different gram-negative bacteria (10, 24), the presence of O-antigen LPS (smooth phenotype) is a determinant for serum resistance. We used this characteristic to clone O-antigen LPSs from different bacteria in *E. coli* DH5α.

In a recent study of the prevalence of the O serogroups among clinical *Klebsiella* isolates from different sources and countries, serogroup O5 represented 9% of the isolates (13). The chemical structure of the *Klebsiella* O5-antigen LPS was reported (20) to be a homopolymer of mannose: → 3-D-Man*p*1α → 2-D-Man*p*1α → 3-D-Man*p*1α → 2-D-Man*p*1α → 2-D-Man*p*1α →. Despite the similarity in chemical composition to the *Klebsiella* O3-antigen LPS, also a homopolymer of mannose (5), no cross-reactivity was observed for O5- and O3-antigen LPS with specific antibodies (13), in spite of the high heterogeneity of the O3 serogroup strains of this bacterium (13).

In this work, we cloned and sequenced the wb_{O5} gene cluster of *Klebsiella pneumoniae* to obtain genetically well-characterized mutants devoid of this O5-antigen LPS. Finally, using these $O5^-$ mutants and their corresponding wild-type strains or complemented mutants with the wb_{O5} gene cluster ($O5^+$ strains), we studied some pathogenic features of the *K. pneumoniae* O5-antigen LPS.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains, cosmids, and plasmids used are listed in Table 1. Bacteria were grown in Luria-Bertani (LB)-Miller broth and LB-Miller agar (26). The LB media were supplemented with ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (30 μg/ml), tetracycline (20 μg/ml), or rifampin (100 μg/ml) when needed.

General DNA methods. DNA manipulations were carried out essentially as previously described (33). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers. Recombinant clones were selected on LB-Miller agar plates containing the appropriate antibiotics.

Construction of a *K. pneumoniae* KT769 genomic library. *K. pneumoniae* KT769 genomic DNA was isolated and partially digested with *Sau*3A as described by Sambrook et al. (33). Cosmid pLA2917 (1) was digested with *Bgl*II, dephosphorylated, and ligated to *Sau*3A genomic DNA fragments. DNA packaging by using Gigapack Gold III (Stratagene) and infection of *E. coli* DH5α were carried out as previously described (11). Recombinant clones were selected on LB-Miller agar plates supplemented with tetracycline (20 μg/ml).

Construction of mutant strains KT769-1 (*wbdC*) and KT769-6 (*wzm-wzt*). Two different mutant strains of *K. pneumoniae* KT769 were constructed. To obtain mutant KT769-1 (insertion in the *wbdC* gene), a method based on suicide plasmid pFS100 was used which renders two incomplete copies of the gene (30). A *wbdC* internal DNA fragment (697 bp) was amplified from CosKT4 using oligonucleotides 5′-CACTCGGATATTGTGAAAC-3′ and 5′-TCTTCAAAAC GACGACGC-3′; isolated; ligated to *Eco*RV-digested, blunt-ended, and dephosphorylated pSF100; and transformed into *E. coli* MC1961(*pir*) to generate plasmid pJT80. Plasmid pJT80 was isolated, transformed into *E. coli* SM10(*pir*), and transferred by conjugation to a KT769 rifampin-resistant (Rif^r) mutant (from our laboratory collection) as previously described (30).

To obtain mutant KT769-6, the method of Link et al. (21) was used to create an in frame deletion encompassing both the *wzm* and *wzt* genes. Briefly, CosKT4 and primer pairs A (5′-CGCGGATCCCAGGAAGACGCCATTTACGG-3′) plus B (5′-TGTTTAAGTTTAGTGGATGGGTGTAAAACGAGCCATAACG CG-3′) and C (5′-CCCATCCACTAAACTTAAACAGTCGTTAACACGGAA CAACAAG-3′) plus D(5′-CGCGGATCCAGGTCCCGACGCTTACATTC-3′) were used in two sets of asymmetric PCRs to amplify DNA fragments of 567 bp (AB) and 555 bp (CD). DNA fragments AB and CD were annealed at their overlapping region and amplified by PCR as a single fragment, using primers A and D (1,101 bp). The fusion product was purified, *Bam*HI digested, ligated into *Bam*HI-digested and phosphatase-treated pKO3 vector (21), electroporated into *E. coli* DH5, and plated on chloramphenicol-containing plates at 30°C to obtain plasmid pJT86. The PCR amplification procedures and mutant construction by gene replacement, using plasmid pJT86, were exactly as described by Link et al. (21).

DNA sequencing. Double-stranded DNA sequencing was performed by using the Sanger dideoxy-chain termination method (34) with the ABI Prism dye

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TABLE 1. Bacterial strains, cosmids and plasmids used in this study

Strain, cosmid, or plasmid	Relevant characteristics	Source or reference
E. coli strains		
DH5α	F$^-$ *endA hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyr-A96* ϕ80*lacZ*	12
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F′ *proAB lacI*^{q}*Z* ΔM15 Tn*10*)	Stratagene
MC1061	*thi thr1 leu6 proA2 his4 argE2 lacY1 galK2 ara14 xyl5 supE44*, λ *pir*	30
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu, Km^r, λ*pir*	30
CLM4	*lacZ trp* Δ(*sbcB-rfb*) *upp rel rpsL* Δ*recA*	22
K. pneumoniae strains		
KT769	Wild type, serotype O5:K57	I. Ørskov
KT769 Rif^r	Rif^r mutant derived from KT769	This work
KT769-1	*wbdC* insertion KT769 Rif^r mutant obtained with pJT80	This work
KT769-6	*wzm wzt* deletion KT769 Rif^r mutant obtained with pJT86	This work
KT769-1C	KT769-1 mutant complemented with CosKT4	This work
KT769-6C	KT769-6 mutant complemented with CosKT4	This work
Plasmids		
pLA2917	Tc^r Km^r	1
CosKT4	pLA2917 with 20-kb chromosomal KT679 *Sau*3A insert	This work
pFS100	pGP704 suicide plasmid, λ*pir* dependent, Km^r	30
pJT80	pFS100 with an internal fragment (697 bp) of *wbdC*	This work
pKO3	Cm^r, temperature sensitive for replication; *sacB*	21
pJT86	pKO3 with an internal fragment (1,101 bp) of *wzm wzt*	This work

terminator cycle-sequencing kit (Perkin-Elmer). Primers used for DNA sequencing were purchased from Pharmacia LKB Biotechnology. Primers 5′-GACTGG GCGGTTTTATGG-3′ and 5′-CCATCTTGTTCAATCATGCA-3′, designed by us from the known sequence in our laboratory of cosmid pLA2917, were used to sequence the inserts in the *Bgl*II restriction site on pLA2917.

DNA and protein sequence analysis. The DNA sequence was translated in all six frames, and all open reading frames (ORFs) greater than 100 bp were inspected. The deduced amino acid sequences were compared with those of DNA translated in all six frames from nonredundant GenBank and EMBL databases by using the BLAST network service at the National Center for Biotechnology Information (2). Multiple sequence alignments were carried out using the Clustal W program (39). Possible terminator sequences were identified by using the Terminator program from the Genetics Computer Group (Madison, Wis.) package in a VAX 4300. Hydropathy profiles were calculated by the method of Kyte and Doolitle (17).

Cell surface isolation and analysis. Cell envelopes were prepared by lysis of whole cells in a French press at 16,000 lb/in^2. Unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min, and the envelope fraction was collected by centrifugation at 100,000 × *g* for 2 h. Cytoplasmic membranes were solubilized twice with sodium *N*-lauroyl sarcosinate, and the outer membrane (OM) fraction was collected as described above. OM proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the Laemmli procedure (18). Protein-containing gels were fixed and stained with Coomassie blue. LPS was purified by the method of Westphal and Jann (44). For screening purposes, LPS was obtained after proteinase K digestion of whole cells by the procedure of Darveau and Hancock (6). SDS-PAGE was performed, and LPS bands were detected by the silver-staining method of Tsai and Frasch (42).

Antisera. Anti-*K. pneumoniae* O5 LPS serum was obtained using purified *K. pneumoniae* O5 LPS, adsorbed by a *K. pneumoniae* rough mutant (KT141) (13), and assayed as previously described for other LPSs (24, 40).

Western immunoblotting. After SDS-PAGE, immunoblotting was carried out by transfer to polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.) at 1.3 A for 1 h in the buffer of Towbin et al. (41). The membranes were then incubated sequentially with 1% bovine serum albumin, specific anti-O antiserum (1:500), alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G, and 5-bromo-4-chloro-indolylphosphate disodium-nitroblue tetrazolium. Incubations were carried out for 1 h, and washing steps with 0.05% Tween 20 in phosphate-buffered saline (PBS) were included after each incubation step. Colony blotting was performed using *K. pneumoniae* O5 antiserum as indicated above.

Serum killing. The survival of exponential-phase bacteria in nonimmune human serum was measured as previously described in a 90% serum in PBS after 3 h of incubation at 37°C, taking samples for viable counts every 30 min (24), or by a microtiter plate-based assay for screening (43).

Cell surface hydrophobicity. Cell surface hydrophobicity was determined by two different methods. The first method used was hydrophobic interaction chromatography (HIC) on phenyl-Sepharose as previously described (14). Briefly, bacteria were resuspended in 10 mM PBS (pH 7.4) to an optical density at 470 nm (OD_{470}) of 1.0, applied to a phenyl-Sepharose column, and eluted with 4 M NaCl. The eluate was collected, and its OD_{470} was determined.

The second method used was the bacterial adherence to hydrocarbons (BATH) method, as previously described (29). Briefly, cells were washed twice in phosphate-urea-magnesium buffer (pH 7.1), suspended in the same buffer at an OD_{400} of 1.0, and vortexed with various volumes of hydrocarbon. The OD_{400} of the aqueous phase was expressed as a percentage of the OD_{400} of a standard volume of untreated cells.

Bacterial surface charge. The bacterial surface charge was determined by measurement of the zeta potential using a Zetasizer II (Malvern Instruments, Malvern, United Kingdom).

Bacterial adherence assay. The assay measuring the adherence of *K. pneumoniae* strains to uroepithelial cells (UEC) was done as described by Falkowski et al. (7) and Merino et al. (25). Briefly, samples containing bacteria and UEC (100:1) were incubated for 1 h at 37°C and filtered under vacuum through a 5-μm-pore-size filter. The filters were solubilized to lyse the UEC, and the adherent bacteria were counted by viable plate count determination. In some cases, the adherence was also examined by direct visualization of Gram-stained filters, in which a minimum of 40 UEC were examined.

Urinary tract infections in rats. The bacterial strains used to establish infection were grown overnight in LB-Miller agar (supplemented with antibiotics when needed) and gently suspended in PBS to the appropriate concentration. In each experiment, 12 female Wistar rats (weighing 200 to 250 g) of strain CFHB (Interfauna UK, Huntington, United Kingdom) were used. Ten animals were

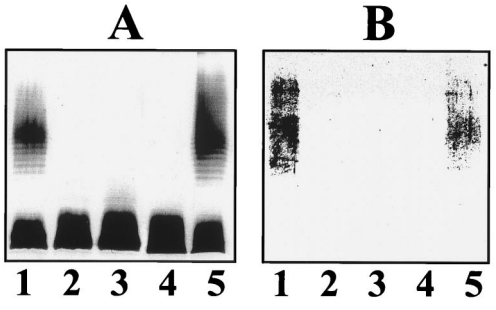

FIG. 1. Silver-stained polyacrylamide gel (A) and Western immunoblot (B) of LPS reacted with *K. pneumoniae* O5-specific antiserum. Antisera were obtained and assayed as described in Materials and Methods. LPS samples were prepared from *E. coli* strains by the method of Darveau and Hancock (6). Lanes: 1, DH5α harboring CosKT4; 2, DH5α; 3, cured strain from lane 1; 4, CLM4 (Δ*wb*); 5, CLM4 harboring CosKT4.

TABLE 2. *K. pneumoniae* KT769 (O5) *wb* gene cluster

Locus	Base positions	% G+C	Protein encoded (kDa)	pI[b]	GRAVY[c]
manC (ORF1)	1–1416	61.6	52.8	5.38	−0.273
manB (ORF2)	1436–2812	62.5	50.4	5.27	−0.236
wzm (ORF3)	2918–3607	46.3	26.0	9.17	+0.842
wzt (ORF4)	3607–4821	47.3	44.9	6.09	−0.172
wbbD (ORF5)	4821–6116	46.6	49.4	9.12	−0.310
wbdA (ORF6)	6100–9741	49.5	135.4	5.47	−0.124
wbdB (ORF7)	9809–10954	54.7	43.8	7.11	−0.212
wbdC (ORF8)	11255–12079	54.7	31.1	6.31	−0.247
hisI (ORF9)[a]	12451–12731	ND[d]	ND	ND	ND

[a] Truncated ORF.
[b] Isoelectric point of the protein, calculated using ProtParam at the Expassy server.
[c] Grand average hydropathicity of the protein, calculated using the Kyte and Doolitle method (17).
[d] ND, not determined.

infected transurethrally in the bladder after voiding urine by gentle compression of the bladder through the external abdominal wall, and two were used as controls. The infections were quantified as previously described (3).

Nucleotide sequence accession number. The nucleotide sequence of the genes described here have been assigned GenBank accession no. AF189151.

RESULTS AND DISCUSSION

Cloning of the *K. pneumoniae* (O5:K57) O5-antigen LPS genomic region that confers serum resistance to *E. coli* K-12. *K. pneumoniae* (O5:K57) strain KT769 is characterized, like other encapsulated and smooth strains, by being serum resistant (24), while *E. coli* K-12 strains like DH5α are serum sensitive. A cosmid-based genomic library of *Klebsiella* strain KT769 chromosomal DNA was constructed and introduced into *E. coli* DH5α, and recombinants were selected on LB-Miller agar plus tetracycline. Several serum-resistant clones were isolated using a microtiter plate-based assay (43); CosKT4 was one of the clones which conferred the highest serum resistance to *E. coli* DH5α (data not shown). DH5α harboring CosKT4 was characterized by analysis of the OM protein and LPS profile on SDS-PAGE. No major differences were found in the OM protein pattern, but cosmid CosKT4 conferred *K. pneumoniae* O5-antigen LPS production to *E. coli* DH5α. Of course, no antigen was detected on DH5α with or without the cosmid pLA2917 (Fig. 1A). CosKT4 was cured from the recipient strain DH5α by serial growth without antibiotics, single-colony isolation, and testing for antibiotic sensitivity and lack of the plasmid DNA. The cured strains lacked the O5-antigen LPS in gels (Fig. 1A) and became serum sensitive, like DH5α (with or without cosmid pLA2917). When CosKT4 was transferred to *E. coli* CLM4 (a strain with the *wb* cluster deleted [22]), it was able to confer O5-antigen LPS production to this strain. This result suggested that CosKT4 contains all the genetic information necessary for O5-antigen production (Fig. 1).

Subcloning and sequencing of CosKT4. We tried to subclone the genes responsible for the biosynthesis of O5-antigen LPS from CosKT4 using different restriction enzymes and plasmid vectors commonly used in other cases, such as *K. pneumoniae* O1 or O8 antigen-LPS, but these attempts were unsuccessful. For this reason, we used oligonucleotides flanking the *Bgl*II pLA2917 cosmid site (see Materials and Methods). This sequence rendered a high degree of homology to *hisIE* genes in one of the ends, and the presence of *wb* O5 genes in CosKT4 was confirmed by using the oligonucleotide primers from the boundary region of the *K. pneumoniae* O5 *wb* (*wbdC*) and *his* genes described by Sugiyama et al. (37). Other sequence-derived oligonucleotides were used to complete the nucleotide sequence. A total of eight complete ORFs were determined, and their characteristics are shown in Table 2. Upstream of each ORF, putative ribosomal binding sequence were found. On the other hand, no Rho-independent transcription termination similar sequences were found among the eight ORFs. This feature, plus the overlap between the ORF5 stop codon and the ORF6 initial codon and the short spacing (no spacing among ORF3, ORF4, and ORF5) between the eight ORFs, strongly suggested that these ORFs are part of a transcriptional unit. The last (truncated) ORF, ORF9, was found to be similar to *hisI* from several members of the *Enterobacteriaceae* and was unrelated to the wb_{O5} cluster. In other *wb* gene clusters, a similar situation was found, with a *hisI* gene transcribed into opposite direction from the *wb* operon.

Analysis of the ORF deduced amino acid sequence. The analysis of the ORF deduced amino acid sequences showed that the ORF1 and ORF2 products are highly similar to two enzymes involved in the biosynthesis of the mannose (Table 3). Accordingly we suggest that ORF1 and ORF2 correspond respectively to the *manC* and *manB* genes, encoding GDP-mannose pyrophosphorylase or mannose-1-phosphate guanyltransferase and phosphomannomutase, respectively (36). The

TABLE 3. Percent identity and similarity of the amino acid sequences proteins encoded *K. pneumoniae* KT769 ORF1 through ORF8 to the most significant other proteins

Protein	No. of amino acids	% Similarity[a]	% Identity[a]	Accession no.
ORF1 (ManC)				
K. pneumoniae KT769 ManC	471			AF189151
K. pneumoniae O3 ManC	452	100	99	Q48462
E. coli O9	471	100	99	Q59427
ORF2 (ManB)				
K. pneumoniae KT769 ManB	458			AF189151
E. coli O9a ManB	458	99	98	Q66229
E. coli O9	460	99	98	Q59428
ORF3 (Wzm)				
K. pneumoniae KT769 Wzm	229			AF189151
E. coli O9a Wzm	264	96	92	Q66230
E. coli O9	261	78	63	Q47590
ORF4 (Wzt)				
K. pneumoniae KT769 Wzt	404			AF189151
A. salmonicida AbcA	308	65	45	Q07698
E. coli KpsT (polysialic capsule)	359	59	40	P23888
ORF5 (WbdD)				
K. pneumoniae KT769 WbdD	431			AF189151
E. coli O9a	425	96	92	O66232
ORF6 (WbdA)				
K. pneumoniae KT769 WbdA	1,213			AF189151
E. coli O9a	704	96	93	O66234
ORF7 (WbdB)				
K. pneumoniae KT769 WbdB	381			AF189151
E. coli O9	381	100	99	Q47594
ORF8 (WbdC)				
K. pneumoniae KT769 WbdC	274			AF189151
E. coli O9a	274	100	98	O24713

[a] The percentages were obtained from pairwise comparisons using the Gap program with the following settings: gap weight, 12; length weight, 12.

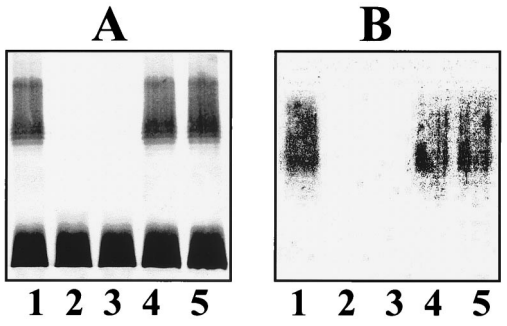

FIG. 2. Silver stained polyacrylamide gel (A) and Western immunoblot (B) of LPS reacted with *K. pneumoniae* O5-specific antiserum. LPS samples were prepared from *K. pneumoniae* strains by the method of Darveau and Hancock (6). Lanes: 1, KT769 (O5:K57, wild type); 2, KT769-1 (*wbdC* mutant); 3, KT769-6 (*wzm-wzt* mutant); 4, KT769-1C (KT769-1 mutant complemented with CosKT4); 5, KT769-6C (KT769-6 mutant complemented with CosKT4).

ORF3 and ORF4 products are similar to the ATP binding cassette 2 (ABC-2)-type transport system integral membrane and ATP binding proteins, respectively (Table 3). Exporter systems similar to the ORF3-ORF4 system are involved in export of O antigen, except for ATP binding protein AbcA, which is involved in A-protein expression (4). The putative exporter component (the ORF3 product) showed a 96% level of amino acid similarity to the corresponding Wzm protein involved in *E. coli* O9a antigen export, while the putative ATP-binding component showed 45% similarity to the ATP binding protein AbcA involved in *Aeromonas salmonicida* A-protein expression (4) and 35 to 40% similarity to several ATP binding proteins (Wzt) involved in capsule or LPS biosynthesis. Hydrophobicity analysis and identification of putative transmembrane domains of Wzm protein (amino acid residues 29 to 51, 76 to 98, 112 to 134, 145 to 167, and 197 to 218) by the method of Klein et al. (16) suggested that this protein is indeed an integral membrane protein. On the other hand, the sequence GINGAGKS (residues 58 to 65) from Wzt was found to correspond to box A, a motif present in ATP binding proteins, as well as the ABC transporter family signature YSSGMQVRL AFSVAT (residues 146 to 160). Thus, ORF3 and ORF4 have been named the *wzm* and *wzt* genes, respectively.

The ORF5 product was found to be practically identical to WbdD of *E. coli* O9a, with unknown function, and we used the same name (WbdD) for our ORF5 product. ORF6 to ORF8 encoded products with very high levels of amino acid similarity to mannosyl transferase A (α→2-D-Man*p*1), B (α→3-D-Man*p*1) and C (initial, α→3-D-Man*p*1 depending on *wecA*), respectively (15). The high levels of amino acid identity strongly suggest that these ORFs encoded the same enzymes in the *K. pneumoniae* O5 biosynthesis, which prompted to us to name the ORF6 to ORF8 products WbdA to WbdC, respectively. The enzymatic activities proposed for the wb_{O5} cluster are in agreement with those proposed for the biosynthesis of *K. pneumoniae* O5-antigen LPS.

The final truncated ORF9 deduced amino acid sequence showed a high level of similarity to the product of the *hisI* gene of *E. coli*, located at 45.2 min on the *E. coli* map (a bifunctional enzyme related to histidine biosynthesis).

Characterization of mutant strains KT769-1 (*wbdC*) and KT769-6 (*wzm wzt*). Both mutants KT769-1 and KT769-6 were devoid of the O5-antigen LPS in LPS gels and Western blots (Fig. 2), while no other major differences were observed in their other OM molecules. These mutants contained capsular polysaccharide, which reacts with K57 antiserum like the wild-type strain and the rifampin-resistant mutant. However, there are some differences among the mutants; while KT769-1 is unable to form O5-antigen LPS, KT769-6 is able to form this antigen but is unable to transport it to the OM (instead, it is accumulated in the inner membrane) (data not shown). This situation for *wzm wzt* mutants was described previously (32). When CosKT4 was transferred by mating, it was able to complement both mutants, rendering them able to biosynthesize the O5-antigen LPS, as in LPS gels and Western blots (Fig. 2).

Contribution of the O5-antigen LPS to pathogenic features. Taking advantage of the isogenic mutants obtained lacking only the O5-antigen LPS (KT769-1 and KT769-6) and the complementation of these mutants by CosKT4 (KT769-1C and KT769-6C, showing a complete O5-antigen LPS), we decide to investigate the contribution of this molecule to pathogenic features. Some of these pathogenic features have been previously described in studies of *K. pneumoniae* O1-antigen LPS using spontaneous phage resistance mutants, but they have not genetically characterized.

As observed in Fig. 3, mutant strains KT769-1 and KT769-6 are sensitive to the bactericidal activity of nonimmune human serum, while the wild-type strain as well as the CosKT4-complemented mutants (KT769-1C and KT769-6C, respectively) are resistant to this activity. Because the mutants showed a complete LPS core, we demonstrated that the *K. pneumoniae* O5-antigen LPS, as with O1-antigen LPS from the same bacterium (24) or from other members of the *Enterobacteriaceae*, is critical for complement resistance (8, 38). We suggested that the reasons for complement resistance in *K. pneumoniae* $O5^+$ strains are the same as we described for $O1^+$ strains (24). The mutants that lacked the O5-antigen LPS also showed an increase (less electronegative) in their surface charge as measured in millivolts (-40.6 ± 0.4 mV) in comparison with the wild-type strain or the strains that recovered the O5-antigen LPS with plasmid CosKT4 (-52.8 ± 0.5 mV). This surface charge increase is explained by the loss of negative surface molecules like the O5-antigen polysaccharide chains. The change in the surface charge leads to changes in the surface hydrophobicity of the $O5^-$ mutants. As shown in Table 4, the surface hydrophobicity of $O5^-$ mutants, as measured by several methods, is increased (more hydrophobic) with respect to that of the $O5^+$ strains (wild type or COS-KT4 complemented

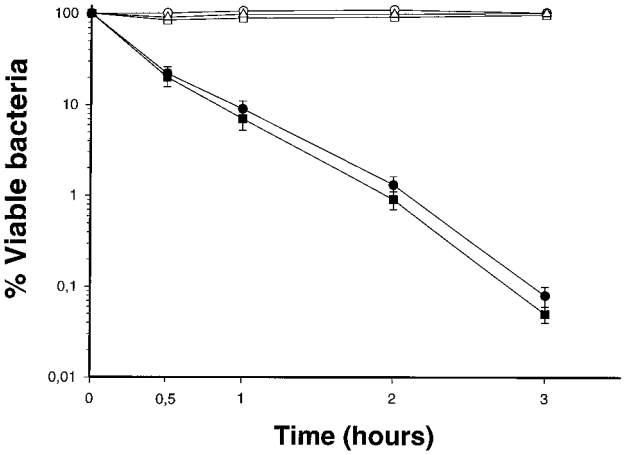

FIG. 3. Survival of *K. pneumoniae* strains in nonimmune serum. (△) KT769 (O5:K57 wild type), (●) KT769-1 (*wbdC* defined insertion mutant, $O5^-$), (■) KT769-6 (*wzm-wzt* deletion mutant, $O5^-$), (○) KT769-1C (KT769-1 mutant complemented with CosKT4, $O5^+$), (□) KT769-6C (KT769-6 mutant complemented with CosKT4, $O5^+$). The results are the averages of at least three independent experiments (values are means and standard deviations).

TABLE 4. Surface hydrophibicity of *K. pneumoniae* strains measured by different methods

Strain	% Surface hydrophobicity[a] measured by:		
	HIC[b]	BATH[c]	
		Xylene	Hexadecane
KT769 (O5:K57, wild type)	1.5 ± 0.3	74 ± 3.4	89 ± 5.1
KT769-1 (O5$^-$; *wbdC* defined insertion KT769 mutant)	28.4 ± 0.7	69 ± 2.8	78 ± 4.4
KT769-6 (O5$^-$; *wzm wzt* KT769 deletion mutant)	29.3 ± 0.6	66 ± 2.6	74 ± 4.2
KT769-1C (O5$^+$, KT769-1 complemented with CosKT4)	1.7 ± 0.2	75 ± 3.0	88 ± 4.9
KT769-6C (O5$^+$, KT769-6 complemented with CosKT4)	1.6 ± 0.3	74 ± 3.6	85 ± 2.7

[a] Mean ± standard deviation of three independent determinations.
[b] Percentage of bacteria retained in the gel.
[c] Percent absorbance of the aqueous phase after treatment with hydrocarbon (0.2 ml) relative to initial absorbance.

mutants). Also, the reason for this change is the loss of a hydrophilic molecule, as with the O5-antigen LPS. However, HIC seems to be a more sensitive technique to assay the surface hydrophobicity on these mutants than is BATH, which seems to be more useful with *n*-xylene than with hexadecane (Table 4), perhaps because the *n*-xylene is a more penetrating agent than the hexadecane and these mutants are only devoid of the O5-antigen LPS but are encapsulated (K57).

The physicochemical properties of the bacterial surface are basic to the interactions (association or adhesion) between the bacteria and the eukaryotic cells of the host tissues. When we measured the adhesion of these strains to UEC (Table 5), we found that mutants lacking the O5-antigen LPS showed approximately a threefold reduction in their ability to adhere to UEC in comparison with the O5$^+$ strains (wild type or CosKT4-complemented mutants). From these experiments, we can conclude that the O5-antigen LPS in *K. pneumoniae* is an important adhesion factor, and we suggested that this is because alterations in the surface charge and hydrophobicity clearly interfere with the bacterial association, a primary step in bacterial adhesion (23). Finally, changes in adhesion should be critical for bacterial colonization. As observed in Table 6, mutants lacking the O5-antigen LPS showed a drastic reduction in their ability to infect the urinary tract in rats, as measured either by the number of animals infected or by the viable bacterial counts found in the kidneys, bladder, or urine, in this experimental infection with respect to the O5$^+$ strains (wild type or Cos-KT4-complemented mutants). Then, the O5-antigen LPS of *K. pneumoniae* is an important colonization factor (at least for urinary tract infections), and this is based at least in part on their role as an adhesion to UEC. It seems clear that adhesion is a critical step for bacterial colonization (infection), as previously described for different bacterium-host interactions (9, 28).

TABLE 5. Adhesion of different *K. pneumoniae* strains to UEC

Strain	% of bacteria adhered[a]
KT769 (O5:K57, wild type)	43.8 ± 3.9
KT769-1 (O5$^-$; *wbdC* defined insertion KT769 mutant)	17.3 ± 3.5
KT769-6 (O5$^-$; *wzm wzt* KT769 deletion mutant)	16.7 ± 3.2
KT769-1C (O5$^+$, KT769-1 complemented with CosKT4)	42.6 ± 3.4
KT769-6C (O5$^+$, KT769-6 complemented with CosKT4)	43.2 ± 4.2

[a] The percentage of bacteria adhering to UEC cells ± standard deviation was calculated as previously described (25). All the assays were done at least in triplicate. Student's *t* test, $P < 0.01$.

TABLE 6. Experimental urinary tract infection of rats by different *K. pneumoniae* strains

Strain	Infectious dose (CFU/rat)	Sample	Infection[a] measured by:	
			% of infected rats[b]	Viable counts[c]
KT769 (O5:K57, wild type)	1.2×10^9	Kidney	100	6.8 ± 0.7
		Bladder	100	6.5 ± 0.5
		Urine	100	8.6 ± 0.5 (6)[a]
KT769-1 (O5$^-$; *wbdC* defined insertion KT769 mutant	0.9×10^9	Kidney	30	3.8 ± 0.3
		Bladder	20	3.6 ± 0.5
		Urine	20	5.8 ± 0.4 (2)
KT769-6 (O5$^-$; *wzm wzt* KT769 deletion mutant	1.1×10^9	Kidney	30	3.9 ± 0.4
		Bladder	30	3.2 ± 0.7
		Urine	20	5.6 ± 0.2 (2)
KT769-1C (O5$^+$, KT769-1 complemented with CosKT4)	1.0×10^9	Kidney	90	6.9 ± 0.6
		Bladder	90	6.3 ± 0.5
		Urine	100	8.4 ± 0.8 (4)
KT769-6C (O5$^+$, KT769-6 complemented with CosKT4)	1.2×10^9	Kidney	100	7.0 ± 0.8
		Bladder	100	6.5 ± 0.3
		Urine	100	8.3 ± 0.6 (6)

[a] A total of 20 kidneys and 10 bladders were studied in each group. Numbers in parentheses represent numbers of urine samples studied.
[b] Percentage of cultures that were positive. The smallest number of organisms detectable by the method was 50 CFU per g (kidney or bladder) or per ml (urine). The values for the O5$^-$ mutants correspond to the percentage of animals infected.
[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.

In summary, all these roles in pathogenicity that we attributed to the *K. pneumoniae* O5-antigen LPS are supported by the behavior of genetically well-characterized isogenic mutants, with no alterations in other surface molecules. It is also clear that some of the roles described here for this O5-antigen LPS have been previously described for other O-antigen LPS from the same bacterium (3, 24, 25) and other members of the *Enterobacteriaceae* (19, 27, 31), but to our knowledge this is the first time this has been done using well-characterized isogenic mutants and cloned *wb* cluster genes for complementation of these isogenic mutants.

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