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Synthesis of a *Klebsiella pneumoniae* O-Antigen Heteropolysaccharide (O12) Requires an ABC 2 Transporter

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A recombinant clone encoding enzymes for *Klebsiella pneumoniae* O12-antigen lipopolysaccharide (LPS) was found when we screened for serum resistance of a cosmid-based genomic library of *K. pneumoniae* KT776 (O12:K80) introduced into *Escherichia coli* DH5α. A total of eight open reading frames (ORFs) (wb_{O12} gene cluster) were necessary to produce *K. pneumoniae* O12-antigen LPS in *E. coli* K-12. A complete analysis of the *K. pneumoniae* wb_{O12} cluster revealed an interesting coincidence with the wb_{O4} cluster of *Serratia marcescens* from ORF5 to ORF8 (or WbbL to WbbA). This prompted us to generate mutants of *K. pneumoniae* strain KT776 (O12) and to study complementation between the two enterobacterial *wb* clusters using mutants of *S. marcescens* N28b (O4) obtained previously. Both *wb* gene clusters are examples of ABC 2 transporter-dependent pathways for O-antigen heteropolysaccharides. The *wzm-wzt* genes and the *wbbA* or *wbbB* genes were not interchangeable between the two gene clusters despite their high level of similarity. However, introduction of three cognate genes (*wzm-wzt-wbbA* or *wzm-wzt-wbbB*) into mutants unable to produce O antigen allowed production of the specific O antigen. The *K. pneumoniae* O12 WbbL protein performs the same function as WbbL from *S. marcescens* O4 in either the *S. marcescens* O4 or *E. coli* K-12 genetic background.

In gram-negative bacteria lipopolysaccharide (LPS) is one of the major structural and immunodominant molecules of the outer membrane (OM). LPS consists of three main regions: lipid A, the core oligosaccharide, and the O antigen. The O antigen is the most external component of LPS, and it consists of a polymer of oligosaccharide repeating units. An interesting feature is the high chemical variability shown by O antigens, which is reflected by the genetic variation in the genes involved in O-antigen biosynthesis, designated the *wb* cluster. The genetics of O-antigen biosynthesis have been intensively studied in the *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 (20, 29).

Escherichia coli K-12 strains, including DH5α, are rough (O^-), unable to produce O-antigen LPS, and serum sensitive. We and others have shown with different gram-negative bacteria (5, 13, 14) that the presence of O-antigen LPS (smooth phenotype) can confer serum resistance. We have used this phenotype to clone the O-antigen LPSs from different bacteria into *E. coli* DH5α.

In a recent study of the prevalence of the O serogroups among clinical *Klebsiella* isolates from different sources and countries, serogroup O12 accounted for 0.3% of the isolates (8). The chemical structure of the *Klebsiella* O12-antigen LPS was reported to be a heteropolymer of rhamnose and *N*-acetylglucosamine (4) and was recently determined to be →4)-β-D-GlcNAc*p*-(1→4)-α-L-Rha*p*-(1→ (26).

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In this study we cloned and sequenced the wb_{O12} gene cluster of *Klebsiella pneumoniae*. As the *K. pneumoniae* wb_{O12} cluster showed a high level of similarity to the *Serratia marcescens* wb_{O4} cluster, complementation analysis was performed with both enterobacterial *wb* clusters by using the previously described O-antigen-deficient mutants of *S. marcescens* N28b (serotype O4) (17).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains, cosmids, and plasmids used are listed on Table 1. Bacteria were grown in Luria-Bertani (LB) broth and LB agar (15). LB medium was supplemented with ampicillin (100 μg/ml or 3 mg/ml), chloramphenicol (25 μg/ml), kanamycin (30 μg/ml), and tetracycline (20 μg/ml) when necessary.

General DNA methods. DNA manipulations were carried out essentially as previously described (18). 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 agar plates containing the appropriate antibiotics.

Construction of a *K. pneumoniae* KT776 (O12:K80) genomic library. *K. pneumoniae* KT776 genomic DNA was isolated and partially digested with *Sau*3A as described by Sambrook et al. (18). 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 (6). Recombinant clones were selected on LB agar plates supplemented with tetracycline (20 μg/ml).

Cell surface isolation and analysis. The OM was isolated as previously described (14). OM proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using the Laemmli procedure (11). Protein gels were fixed and stained with Coomassie blue. LPS was purified by the method of Westphal and Jann (28). For screening purposes LPS was obtained after proteinase K digestion of whole cells by the procedure of Darveau and Hancock (3). SDS-PAGE was performed, and LPS bands were detected by the silver staining method of Tsai and Frasch (25).

Serum killing. The survival of exponential-phase bacteria in nonimmune human serum was measured as previously described (14) or by using a microtiter plate-based assay for screening (27).

TABLE 1. Bacterial strains, cosmids, and plasmids used

Strain, cosmid, or plasmid	Relevant characteristics	Source or reference
E. coli strains		
DH5α	F^- *end A hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyr-A96* φ*80lacZ*M15	7
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F′ *proAB lacI*^{q}ZΔM15 Tn*10*)	Stratagene
CLM4	*lacZ trp* (*sbcB-rfb*) *upp rel rpsL recA*	13
K. pneumoniae strains		
KT776	Wild type, serotype O12:K80	I. Ørskov
KT776-1	O^- *wzm-wzr* KT776 deletion mutant obtained with pKO3Wzm-t	This study
KT776-2	O^- *wbbB* KT776 deletion mutant obtained with pKO3WbbB	This study
S. marcescens strains		
N28b	Wild-type O4	17
N28b-2	O^- *wbbL* insertion mutant from N28b, Km^r	17
N28b-3	O^- *wbbA* insertion mutant from N28b, Km^r	17
N28b-4	O^- *wzm-wzt* N28b deletion mutant	17
Plasmids		
pLA2917	Tc^r Km^r	1
pCosKT12	pLA2917 with 20-kb chromosomal KT772 *Sau*3A insert	This study
pST-Blue	Km^r	Novagen
pST1	pST-Blue with complete *wzm-wzt* genes of wb_{KpO12}, Km^r	This study
pST1-m	pST1 plasmid with altered Walker box A in Wzt, Km^r	This study
pST5	pST-Blue with complete *wbbA* gene of wb_{SmO4}, Km^r	This study
pST6	pST-Blue with complete *wzm-wzt* genes of wb_{SmO4}, Km^r	This study
pST7	pST-Blue with complete *wzm-wzt-wbbA* genes of wb_{SmO4}, Km^r	This study
pBR328	Ap^r Cm^r Tc^r	21
pJTO12	pBR328 with complete wb_{KpO12}, Cm^r Tc^r	This study
pBR1	pBR328 with complete *wbbL* gene of wb_{KpO12}, Cm^r Tc^r	This study
pBR2	pBR328 with complete *wbbB* gene of wb_{KpO12}, Cm^r Tc^r	This study
pBR3	pBR328 with *wzm-wzt-wbbB* genes of wb_{KpO12}, Cm^r Tc^r	This study
pKO3	Cm^r, temperature sensitive for replication, *sacB*	12
pKO3Wzm-t	pKO3 with internal fragment of *wzm-wzt* genes	This study
pKO3WbbB	pKO3 with internal fragment of *wbbB* gene	This study

Antisera. Anti-*K. pneumoniae* O12 LPS serum was obtained and assayed as previously described for other LPSs (14, 23). Anti-*S. marcescens* O4 LPS serum was obtained previously (17).

Western immunoblotting. After SDS-PAGE, immunoblotting was carried out by transferring preparations to polyvinylidene fluoride membranes (Millipore Corp., Bedford, Mass.) at 1.3 A for 1 h in the buffer of Towbin et al. (24). The membranes were then incubated sequentially with 1% bovine serum albumin, specific anti-O serum (1:500), alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G, and disodium 5-bromo-4-chloro-indolylphosphate–nitroblue tetrazolium. Incubations were carried out for 1 h, and washing steps with 0.05% Tween 20 in phosphate-buffered saline were included after each incubation step. Colony blotting was performed by using *K. pneumoniae* O12 antiserum as indicated above.

ELISA. Cytosol, whole membrane, inner membrane (IM), and OM fractions were analyzed by enzyme-linked immunosorbent assays (ELISAs). ELISAs were performed by dispensing standardized suspensions of each fraction in coating buffer (pH 9.6) into 96-well microtiter plates. The plates were left to stand overnight at 4°C. The wells were blocked with 1% bovine serum albumin in phosphate-buffered saline for 2 h at 37°C. Anti-O12 polyclonal serum (1:200) was added and incubated for 2 h at 37°C. Detection was performed by using peroxidase-labeled sheep anti-rabbit immunoglobulin G (1:1,000) and 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) as the substrate. Cytosol, whole membrane, IM, and OM were prepared as previously described (17).

DNA sequencing. Double-stranded DNA sequencing was performed by the Sanger dideoxy chain termination method (19) with an ABI Prism dye terminator cycle sequencing kit (Perkin-Elmer). The primers used for DNA sequencing were purchased from Pharmacia LKB Biotechnology. Primers 5′-GACTGGGC GGTTTTATGG-3′ and 5′-CCATCTTGTTCAAT CATGCA-3′, designed from the sequence of cosmid pLA2917 (1), 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) more than 100 bp long were inspected. Deduced amino acid sequences were compared with the deduced amino acid sequences encoded by DNA translated in all six frames from non-redundant GenBank and EMBL databases by using the BLAST network service at the National Center for Biotechnology Information (2). Multiple-sequence alignments were constructed by using the Clustal W program (22). Possible terminator sequences were deduced by using the Terminator program from the Genetics Computer Group package (Genetics Computer Group, Madison, Wis.) in a VAX 4300. Hydropath profiles were calculated by the method of Kyte and Doolittle (10).

***K. pneumoniae* KT776 (*wzm-wzt*) and *wbbB* mutant construction.** The method of Link et al. (12) was used to create chromosomal in-frame deletion mutants. Briefly, plasmid pJTO12 and primers A (5′-CGCGGATCCTGGTGGTTGTCA GTGGGC-3′) and B (5′-CCCATCCACTAAACATGATGAAAGTTTTTGAT GAGG-3′) or primers C (5′-TGTTTAAGTTTAGTGGATGGGCCGTTAAAA ATCATTACAATTG-3′) and D (5′-CGCGGATCCACAATCCCAGCAACTT GG-3′) were used in two sets of asymmetric PCRs to amplify a 785-bp DNA fragment (fragment AB) and a 733-bp DNA fragment (fragment CD) of the *wzm-wzt* region. DNA fragments AB and CD were annealed at the overlapping region and amplified by PCR as a single fragment by using primers A and D. The fusion product was purified, digested with *Bam*HI, ligated to *Bam*HI-digested vector pKO3, electroporated into DH5α, and selected by using Km^r to obtain plasmid pKO3Wzm-t.The same approach was used for the *wbbB* gene by using pJTO12 and primers A1 (5′-CGCGGATCCTTGAGTATGCTGATCGCTGC-3′) and B1 (5′-CCCATCCACTAAACTTAAACACCCTCTGAACGGATATG GAG-3′) or primers C1 (5′-TGTTTAAGTTTAGTGGATGGGGCGGAATTG GGGATTATCTC-3′) and D1 (5′-CGCGGATCCCGGAGCACTAAAAGAAA GCC-3′) amplifying a 734-bp fragment (fragment A1B1) and a 431-bp fragment (fragment C1D1) to obtain plasmid pKO3WbbB. Allelic replacement was performed by the method of Link et al. (12) by using plasmids pKO3Wzm-t and pKO3WbbB.

Plasmid construction. Plasmids pST5, pST6, and pST7, harboring the *S. marcescens* wb_{O4} genes *wbbA*, *wzm-wzt*, and *wzm-wzt-wbbA*, respectively, were obtained from the pSUB6 plasmid (17) by PCR amplification. Primers Sm1 (5′-A ACAATGGCGAGCGAGAAG-3′) and Sm2 (5′-GGTGAAAGCAAGTCG GAA AG-3′) amplified a 3,900-bp DNA fragment containing *wbbA*; primers Sm3 (5′-CTGCCGTGATCATACAGGG-3′) and Sm4 (5′-CTCTAAAGGGGT AAGCCGG-3′) amplified a 2,212-bp DNA fragment containing *wzm-wzt*; and primers Sm3 and Sm2 amplified a 5,950-bp DNA fragment containing *wzm-wzt-*

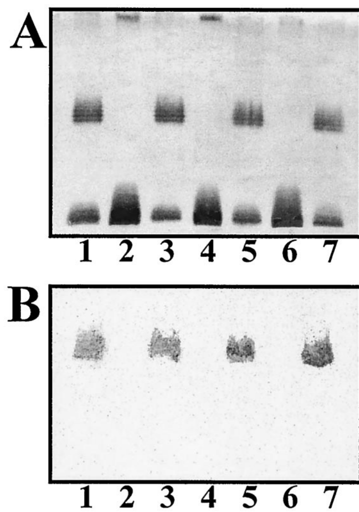

FIG. 1. Silver-stained polyacrylamide gel (A) and Western immunoblot of LPS reacted with *K. pneumoniae* O12-specific antiserum (B). Antiserum was obtained and assayed as described in Materials and Methods. LPS samples were prepared by the method of Darveau and Hancock (3). Lane 1, *K. pneumoniae* KT776; lane 2, *E. coli* DH5α; lane 3, DH5α harboring pCosKT12; lane 4, cured strain from lane 3; lane 5, DH5α harboring pJTO12; lane 6, *E. coli* CLM4 (with *wb* deleted [13]); lane 7, CLM4 harboring pJTO12.

wbbA. The amplified DNA fragments were independently ligated into the vector pST-Blue (Novagen) and introduced into *E. coli* by Km^r selection.

Plasmid pST1 was constructed by using the pJTO12 plasmid (complete *K. pneumoniae* wb_{O12} cluster) and PCR. Primers Kp1 (5′-AACAACTATTGTCC ATGCC-3′) and Kp2 (5′-GGAATCGTCTTCTACCTG-3′) amplified a 3,043-bp DNA fragment containing the *wzm-wzt* genes from *K. pneumoniae* wb_{O12}. The amplified DNA fragment was ligated into the vector pST-Blue to obtain plasmid pST1. Plasmid pST1-m was constructed by using pST1 (*wzm-wzt* from the *K. pneumoniae* wb_{O12} cluster) and PCR. Primers A2bis (5′-TGGTACCATATTCG CTCCG-3′) and B2 (5′-GCAGATAATCTGCAGCAAAGTCGACGCCCCGG C-3′) amplified a 1,220-bp DNA fragment, and primers C2 (5′-ACTTTGCTG CAGATTATCTGCGGCACACTAACC-3′) and D2bis (5′-AATAAGGGGCT TTCACCCC-3′) amplified a 1,149-bp DNA fragment. Using both amplified DNA fragments and primers A2bis and D2bis, we amplified a 2,369-bp DNA fragment that was ligated to vector pST-Blue to obtain plasmid pST1-m. Plasmid pST1-m contains the *wzm* gene and a mutant *wzt* gene from *K. pneumoniae* wb_{O12} with an AAG codon (lysine) substituted for a CGC codon (alanine) (underlined in the primer sequence). DNA sequencing of the pST1-m insert confirmed this change. Because some of the *S. marcescens* $O4^-$ mutants were Km^r and Ap^r is not a suitable marker, we used the following approach to generate plasmids pBR1, pBR2, and pBR3 using pJTO12 for PCR amplification. Primers Kp3 (5′-CGCCAGCTGGTGCT GTCTCTGTCGGAG-3′) and Kp4 (5′-CGCCA GCTGGGCAATACTGCGCACCAC-3′) amplified a 1,075-bp DNA fragment (*wbbL*); primers Kp5 (5′-CGCCAGCTGTCGGGCGATTACCTAATAC-3′) and Kp6 (5′-CGCCAGCTGGCCATTTAGCCTCAAGTAC-3′) amplified a 3,015-bp DNA fragment (*wbbB*); and primers Kp7 (5′-CGCCAGCTGTTGGT ACCATATTCGCTCC-3′) and Kp6 amplified a 5,183-bp DNA fragment (*wzm-wzt-wbbL*). Each of the amplified DNA fragments was independently digested with *Pvu*II (all the primers contained a *Pvu*II restriction site) and ligated to *Pst*I-digested and blunt-ended vector pBR328. This approach allowed us to maintain resistance to chloramphenicol and tetracycline as selective markers.

Nucleotide sequence accession number. The nucleotide sequence of the genes described here has been deposited in the GenBank database under accession number AY130997.

RESULTS

Cloning of LPS O12-antigen genes of *K. pneumoniae* (O12: K80). *K. pneumoniae* (O12:K80) strain KT776, like other encapsulated and smooth strains, is serum resistant (14), whereas *E. coli* K-12 strains like DH5α are serum sensitive. A cosmid-based genomic library of *K. pneumoniae* strain KT776 chromosomal DNA was constructed and introduced into *E. coli* DH5α, and recombinant clones were selected on LB agar containing tetracycline. Several serum-resistant clones were isolated by using a microtiter plate based assay (27), and pCosKT12 was representative of these clones. DH5α harboring pCosKT12 was characterized by analysis of the OM protein and LPS profile by SDS-PAGE. No major differences were found in the OM protein pattern, but cosmid pCosKT12 conferred *K. pneumoniae* O12-antigen LPS production to *E. coli* DH5α (Fig. 1). No O12 antigen was detected in strain DH5α with or without the cosmid vector pLA2917. pCosKT12 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 strain lacked the O12-antigen LPS and was serum sensitive.

Subcloning and sequencing of wb_{O12}. By using *Pst*I partial digestion of pCosKT12 and ligation to *Pst*I-digested plasmid vector pBR328, plasmid pJTO12 was obtained and introduced into DH5α. Plasmid pJTO12 contained a 13-kb insert from pCosKT12. As determined by LPS gel analysis and Western blotting with specific anti-O12 antibody, plasmid pJTO12 conferred production of *K. pneumoniae* O12 antigen to DH5α (Fig. 1). When pJTO12 was transferred to *E. coli* CLM4 (a strain with the *wb* cluster deleted [13]), it was still able to confer O12-antigen LPS production (Fig. 1). This result suggested that pJTO12 contains all the genetic information necessary for O12-antigen production. The DNA sequence of the insert was determined. Eight ORFs were deduced, and their characteristics are shown in Table 2. Upstream of each ORF putative ribosome binding sequences were found. No Rho-independent transcription termination sequences were found in the eight ORFs. This feature, in addition to the overlaps between the ORF2 stop codon and the ORF3 start codon and between the ORF6 stop codon and the ORF7 start codon and the short spaces between the ORFs, strongly suggests that these ORFs are part of an operon (wb_{O12}).

Analysis of the ORF deduced amino acid sequences. Analysis of the deduced amino acid sequences showed that the ORF1 to ORF4 sequences are very similar to the sequences of the four enzymes involved in the biosynthesis of dTDP-rhamnose in other enterobacteria (Table 3). Accordingly, we sug-

TABLE 2. *K. pneumoniae* KT776 (O12) *wb* gene cluster

Locus	Base positions	G+C content (%)	Size of protein encoded (kDa)	pI[a]	GRAVY[b]
rmlB (ORF1)	1–1065	57.8	39.4	6.1	−0.287
rmlA (ORF2)	1083–1988	55.6	33.1	5.3	−0.106
rmlD (ORF3)	1985–3875	61.3	31.7	5.8	−0.065
rmlC (ORF4)	2890–3444	54.4	20.2	5.7	−0.217
wbbL (ORF5)	3536–4366	34.5	32.1	8.7	−0.187
wzm (ORF6)	4396–5229	37.5	31.2	9.7	+0.978
wzt (ORF7)	5219–6541	43.8	48.2	6.1	−0.084
wbbB (ORF8)	6545–9346	39.9	106.8	5.8	−0.203

[a] Isoelectric point of the protein calculated by using ProtParam at the Expassy server.

[b] GRAVY, grand average hydropathicity of the protein calculated by using the Kyte-Doolitle method (10).

TABLE 3. Levels of identity and similarity of the amino acid sequences encoded by the *K. pneumoniae* KT776 gene cluster and the most significant other proteins

Protein	No. of amino acids	% Similarity[a]	% Identity[a]	Accession no.
ORF1 (RmlB) of *K. pneumoniae* KT776	354			AY130997
RffG (RmlB) of *Yersinia pestis*	355	81	72	NP407310
RmlB of *E. coli* of O157:H7	355	79	70	NP312748
ORF2 (RmlA) of *K. pneumoniae* KT776	301			AY130997
RmlA of *Yersinia enterocolitica*	289	86	76	P55257
RffH (RmlA) of *Yersinia pestis*	293	86	76	NP407309
ORF3 (RmlD) of *K. pneumoniae* KT776	296			AY130997
RmlD of *Salmonella enterica*	299	58	47	AAG09514
RfbD (RmlD) of *Salmonella enterica* serovar Typhimurium	299	58	47	NP461041
ORF4 (RmlC) of *K. pneumoniae* KT776	184			AY130997
RmlC of *Shigella boydii*	189	86	72	AAL27313
RfbC (RmlC) of *Shigella flexneri*	181	78	68	P37780
ORF5 (WbbL) of *K. pneumoniae* KT776	276			AY130997
WbbL of *S. marcescens* O4	282	69	55	T31088
ORF6 (Wzm) of *K. pneumoniae* KT776	277			AY130997
Wzm of *S. marcescens* O4	277	84	75	T31089
ORF7 (Wzt) of *K. pneumoniae* KT776	440			AY130997
Wzt of *S. marcescens* O4	441	83	71	T31090
ORF8 (WbbB) of *K. pneumoniae* KT776[b]	933			AY130997
WbbA of *S. marcescens* O4	1,191	58	39	T31091

[a] The values were obtained from pairwise comparisions by using the Gap program. The Gap program settings, were as follows: gap weight, 12; and length weight, 12.
[b] The similarity between WbbB (from residue 1 to residue 454) and WbbA (from residue 1 to residue 460) is limited to this region of the two proteins.

gest that these ORFs correspond to the genes *rmlB*, *rmlA*, *rmlD*, and *rmlC*, respectively. The ORF5 product showed homology with the WbbL protein from *S. marcescens* wb_{O4}, which is a rhamnosyl transferase; it also exhibited less homology with other glycosyl transferases (Table 3). ORF5 was designated *wbbL*. The ORF6 and ORF7 products were similar to ABC 2-type transport system integral membrane and ATP-binding proteins, respectively (Table 3). Hydrophobicity analysis and identification of the putative transmembrane domains of the ORF6-encoded protein (amino acid residues 53 to 69, 84 to 100, 133 to 149, 158 to 174, 194 to 210, and 245 to 261), performed by using the method of Klein et al. (9), suggested that this protein is an integral membrane protein. Also, the ORF7-encoded protein contained the sequence GRNGAGKS (amino acid residues 70 to 77), which correspond to Walker box A, a motif present in ATP-binding proteins, as well as the ABC transporter family signature YSSGMYVRLAFAVQA (amino acid residues 158 to 172). Thus, ORF6 and ORF7 were designated *wzm* and *wzt*, respectively. Finally, the ORF8-encoded protein (933 amino acids) exhibited homology only with the WbbA protein (1,191 amino acids) encoded by the *S. marcescens* wb_{O4} cluster. However, homology between the two proteins was present only in the first 460 amino acid residues. Both WbbA and the ORF8 product contained a putative glycosyl transferase motif in the first half of the protein. Both proteins were predicted to be anchored to the membrane (by residues 334 to 350 in the ORF8-encoded protein), and no relevant similarities were found in the C-terminal halves of the proteins. Protein WbbA has been suggested to be bifunctional, and the glycosyl transferase function is also found in the ORF8-encoded protein; thus, we designated ORF8 *wbbB*.

The complete sequence of the *K. pneumoniae* wb_{O12} cluster exhibited an interesting similarity with the sequence of the *S. marcescens* wb_{O4} cluster from ORF5 to ORF8 (or WbbL to WbbA), which prompted us to generate mutants of *K. pneumoniae* strain KT776 (O12) and to study complementation of the two *wb* clusters by using mutants previously created in *S. marcescens* N28b (O4).

Construction of mutant strains KT776-1 (*wzm-wzt*) and KT776-2 (*wbbB*). Mutants KT776-1 (*wzm-wzt*) and KT776-2 (*wbbB*) were obtained by the method of Link et al. (12) to create in-frame deletions. Plasmids pKO3Wzm-t and pKO3WbbB were independently electroporated into *K. pneumoniae* KT776, and colonies were selected for Cm^r at 42°C. Colonies were plated at 30°C in the presence of sucrose (5%). Candidate mutant colonies (chloramphenicol sensitive) were screened by PCR. Mutant KT776-1 (*wzm-wzt*) was characterized by producing an amplified 1,479-bp DNA fragment with primers A and D. Mutant KT776-2 (*wbbB*) was characterized by producing an amplified 1,125-bp DNA fragment with primers A1 and D1. The wild-type strain in similar PCR experiments produced 3,545- and 3,844-bp amplification fragments with primers A and D and primers A1 and D1, respectively. DNA sequencing of the amplified fragments confirmed the deletion of 2,066 bp for KT776-1 and the deletion of 2,718 bp for KT776-2.

Both mutant KT776-1 and mutant KT776-2 were devoid of O12-antigen LPS as determined by using gels and Western

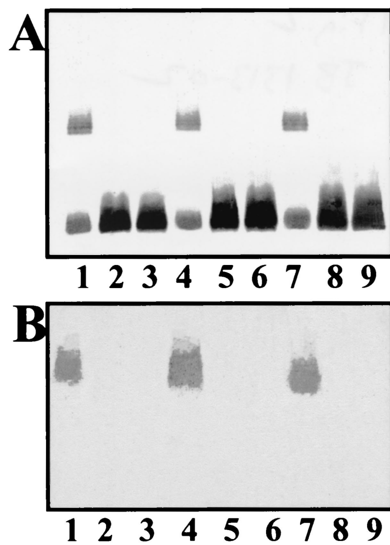

FIG. 2. Silver-stained polyacrylamide gel (A) and Western immunblot of LPS reacted with *K. pneumoniae* O12-specific antiserum (B). Antiserum was obtained and assayed as described in Materials and Methods. LPS samples were prepared by the method of Darveau and Hancock (3) from *K. pneumoniae* strains. Lane 1, KT776 (wild type); lane 2, mutant KT776-1 (*wzm-wzt*); lane 3, mutant KT776-2 (*wbbB*); lane 4, KT776-1 harboring pST1 (*wzm-wzt* of wb_{KpO12}); lane 5, KT776 -1 harboring pBR1 (*wbbL* of wb_{KpO12}); lane 6, KT776-1 harboring pBR2 (*wbbB* of wb_{KpO12}); lane 7, KT776-2 harboring pBR2 (*wbbB* of wb_{KpO12}); lane 8, KT776-2 harboring pBR1 (*wbbL* of wb_{KpO12}); lane 9, KT776-2 harboring pST1 (*wzm-wzt* of wb_{KpO12}).

blots (Fig. 2). No other major differences were observed in the OM molecules and the capsules of these mutants. Analysis of purified LPS from both mutants showed a complete lack of rhamnose, a characteristic sugar of the O12-antigen LPS. ELISAs (Table 4) with *K. pneumoniae* O12-antigen-specific antiserum were performed with different cellular fractions from KT776 (wild type) and mutants KT776-1 and KT776-2. As expected, the OM fraction of wild-type strain KT776 showed a high affinity for the specific antiserum. By contrast, the IM fraction obtained from mutant strain KT776-1 showed a high response to specific antiserum, while no response to the same antiserum was observed with the OM fraction of this mutant strain. Neither the OM fraction nor the IM fraction from mutant strain KT776-2 reacted with the specific antiserum. These results suggest that KT776-2 was unable to form O12-antigen LPS, while KT776-1 was able to form this antigen but was unable to transport it to the OM (i.e., it accumulated in the IM).

Complementation studies with *K. pneumoniae* O12$^-$ mutants. *K. pneumoniae* KT776-1 and KT776-2 were fully complemented for O12-antigen production by plasmid pJTO12 (wb_{Kp}) but not by the pBR328 vector, as determined by using LPS gels and Western blots with anti-O12-specific serum. Furthermore, mutant KT776-1 (*wzm-wzt*) was complemented by pST1 (*wzm-wzt* from *K. pneumoniae* wb_{O12}) but not by plasmid pST-Blue, pBR1 ($wbbL_{Kp}$), or pBR2 ($wbbB_{Kp}$). Mutant KT776-2 (*wbbB*) was complemented by pBR2 (*wbbB* from *K. pneumoniae* wb_{O12}) but not by the pBR328 vector, pBR1 ($wbbL_{Kp}$), or pST1 ($wzm\text{-}wzt_{Kp}$) (Fig. 2). Furthermore, the OM fractions of mutants KT776-1 and KT776-2 transformed with pST1 ($wzm\text{-}wzt_{Kp}$) and pBR2 ($wbbB_{Kp}$), respectively, again showed affinity for the O12-specific antiserum (Table 4). The results obtained so far suggest that an ABC 2-type dependent transporter is involved in the export of the *K. pneumoniae* O12-antigen LPS. In order to further examine this possibility, a Wzt mutant was obtained on plasmid pST1-m. The mutation resulted in substitution of the conserved lysine (amino acid residue 76) from the Walker box A of Wzt for an alanine. Mutant KT776-1 harboring pST1-m was unable to export the O12-antigen LPS, as determined by using LPS gels (data not shown) and the export assay with ELISA experiments (Table 4). Due to the similarities between the WbbA and WbbB proteins encoded by the *S. marcescens* wb_{O4} and *K. pneumoniae* wb_{O12} gene clusters, respectively, we decided to study the complementation of these mutants with plasmids harboring *S. marcescens* wb_{O4} genes. Mutants KT776-1 and KT776-2 were not complemented by plasmids pST5 ($wbbA_{Sm}$) and pST6 ($wzm\text{-}wzt_{Sm}$), as judged by using LPS gels or Western blots with specific anti-O12 serum. However, both mutants were able to produce O-antigen LPS when either pSUB6 (wb_{Sm}) or pST7 ($wzm\text{-}wzt\text{-}wbbA_{Sm}$) plasmids were introduced by transformation (Fig. 3A). The O-antigen LPS produced was not able to react with specific *K. pneumoniae* anti-O12 serum (data not shown) but reacted with specific *S. marcescens* anti-O4 serum (Fig. 3B). No O-antigen LPS production was observed when these mutants were transformed with the plasmid vectors.

Complementation studies with *S. marcescens* O4$^-$ mutants. Mutant N28b-2 (*wbbL*) was complemented by plasmid pBR1 harboring the homologous *wbbL* gene from *K. pneumoniae* wb_{O12} (Fig. 4). Furthermore, as happens with the *S. marcescens* *wbbL* gene, plasmid pBR1 is able to produce O-antigen LPS in *E. coli* DH5α. This O-antigen LPS was unable to react with

TABLE 4. *K. pneumoniae* O12-antigen export assays[a]

Strain	A_{405} of fraction extract			
	Crude extract	Whole membrane	IM	OM
KT776 (wild type)	0.1 (0.01)	0.5 (0.04)	0.1 (0.03)	0.7 (0.13)
KT776-1 (*wzm-wzt*)	0.3 (0.05)	0.6 (0.08)	0.8 (0.12)	<0.1
KT776-2 (*wbbB*)	<0.1	<0.1	<0.1	<0.1
KT776-1(pST1) (harboring $wzm\text{-}wzt_{Kp}$)	0.1 (0.03)	0.4 (0.06)	0.1 (0.02)	0.8 (0.07)
KT776-1(pST1-m) (pST1 with altered Wzt)	0.2 (0.06)	0.5 (0.04)	0.7 (0.10)	<0.1
KT776-2(pBR2) (harboring $wbbB_{Kp}$)	0.1 (0.03)	0.6 (0.04)	0.1 (0.05)	0.6 (0.15)

[a] The (A_{405}) values are means (standard deviations) calculated from three independent determinations (ELISAs with different cellular fractions and *K. pneumoniae* O12-specific antiserum).

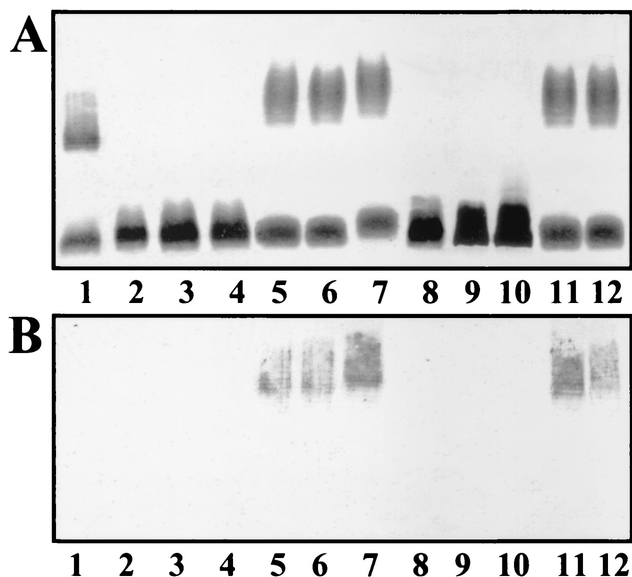

FIG. 3. Silver-stained polyacrylamide gel (A) and Western immunoblot of LPS reacted with *S. marcescens* O4-specific antiserum (B). LPS samples were prepared by the method of Darveau and Hancock (3). Lane 1, *K. pneumoniae* KT776 (wild type); lane 2, *K. pneumoniae* mutant KT776-1 (*wzm-wzt*); lane 3, KT776-1 harboring the pST-Blue vector; lane 4, KT776-1 harboring pST6 (*wzm-wzt* of wb_{SmO4}); lane 5, KT776-1 harboring pST7 (*wzm-wzt-wbbA* of wb_{SmO4}); lane 6, KT776-1 harboring pSUB6 (complete *S. marcescens* wb_{O4} cluster); lane 7, *S. marcescens* N28b (serotype O4, wild type); lane 8, *K. pneumoniae* mutant KT776-2 (*wbbB*); lane 9, KT776-2 harboring the pST-Blue vector; lane 10, KT776-1 harboring pST5 (*wbbA* of wb_{SmO4}); lane 11, KT776-1 harboring pST7 (*wzm-wzt-wbbA* of wb_{SmO4}); lane 12, KT776-2 harboring pSUB6 (complete *S. marcescens* wb_{O4} cluster).

K. pneumoniae anti-O12 serum or *S. marcescens* anti-O4 serum, but it reacted with specific *E. coli* anti-O16 serum (data not shown). This feature is the same as that described for the *S. marcescens wbbL* gene introduced into *E. coli* (16).

Introduction of plasmid pJTO12 (*K. pneumoniae* wb_{O12}) into the *S. marcescens* N28b-3 and N28b-4 mutants allowed the mutants to produce O-antigen LPS, as judged by using LPS gels. This O-antigen LPS was unable to react with *S. marcescens* anti-O4 serum, but it reacted with *K. pneumoniae* anti-O12 serum. Identical results were obtained when both *S. marcescens* mutants were transformed with plasmid pBR3 (*wzm-wzt-wbbB* from *K. pneumoniae* wb_{O12}) but not when they were transformed with plasmid vector pBR328 (Fig. 4). Mutant N28b-3 was unable to produce O-antigen LPS (as determined by using gels) when it was transformed with plasmid pBR2 ($wbbB_{Kp}$) (Fig. 4). Also, mutant N28b-4 was unable to produce O-antigen LPS when it was transformed with plasmid pST1 ($wzm\text{-}wzt_{Kp}$). However, mutant N28b-4 was fully complemented when it was transformed with the pST6 plasmid ($wzm\text{-}wzt_{Sm}$) but not when the pST-Blue vector was used (Fig. 4).

DISCUSSION

One recombinant clone encoding *K. pneumoniae* O12-antigen LPS was found when we screened for serum resistance of a cosmid-based genomic library of *K. pneumoniae* KT776 (O12:K80) introduced into *E. coli* DH5α. A total of eight ORFs (wb_{O12} gene cluster) were necessary to produce *K. pneumoniae* O12-antigen LPS in *E. coli* K-12, as judged by the fact that pJTO12 (wb_{O12}) in *E. coli* CLM4 (a strain with a deletion in the *wb* cluster [13]) was able to confer O12-antigen LPS production. This result suggested that pJTO12 contains all the genetic information necessary for O12-antigen production.

Sugar nucleotides are the activated precursors for cell surface polysaccharides. To synthesize O antigens, monomers are assembled on a lipid carrier (undecaprenol phosphate) by enzymes encoded in the *wb* gene cluster before their incorporation into the LPS molecule. Two known pathways for O-antigen export have been established, the Wzx dependent pathway for heteropolysaccharide structures and the ABC 2 transporter-dependent pathway for homopolysaccharides (29). However, the similarities found between the ABC 2-type pathway and the group II capsular heteropolysaccharide export pathway suggest that not only homopolysaccharide O antigens could be exported by an ABC 2 pathway. The first example of a heteropolysaccharide exported through an ABC 2 export system that was described was the *S. marcescens* O4-antigen LPS (17). In this study we showed that the *K. pneumoniae* wb_{O12} cluster is also able to export a heteropolysaccharide

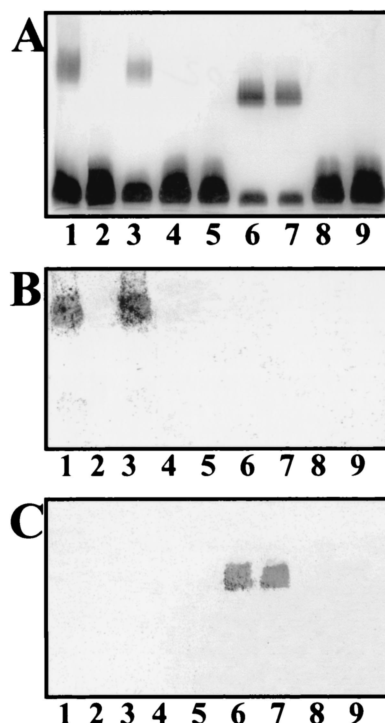

FIG. 4. Silver-stained polyacrylamide gel (A) and Western immunoblots of LPS reacted with *S. marcescens* O4-specific antiserum (B) and *K. pneumoniae* O12-specific antiserum (C). LPS samples were prepared by the method of Darveau and Hancock (3) from *S. marcescens* strains. Lane 1, *S. marcescens* N28b (serotype O4, wild type); lane 2, *S. marcescens* mutant N28b-2 (*wbbL*); lane 3, N28b-2 harboring pBR1 (*wbbL* of wb_{KpO12}); lane 4, *S. marcescens* mutant N28b-4 (*wzm-wzt*); lane 5, *S. marcescens* mutant N28b-4 harboring pST1 (*wzm-wzt* of wb_{KpO12}); lane 6, *S. marcescens* mutant N28b-4 harboring pBR3 (*wzm-wzt-wbbB* of wb_{KpO12}); lane 7, *S. marcescens* mutant N28b-3 harboring pBR3 (*wzm-wzt-wbbB* of wb_{KpO12}); lane 8, *S. marcescens* mutant N28b-3 harboring pBR2 (*wbbB* of wb_{KpO12}); lane 9, *S. marcescens* mutant N28b-3 (*wbbA*).

(with two monosaccharides) by using an ABC 2 transport system. This conclusion was supported by the results of the KT776-1 complementation experiments. Plasmid pST1 (*wzm-wzt*$_{KpO12}$) was fully able to complement the KT776-1 mutant. Plasmid pST1-m (coding for a wild-type Wzm and a Walker box A Wzt mutant) was unable to restore export of O12-antigen LPS. These results allowed us to generalize that the ABC 2 transporter-dependent pathway can export not only O-antigen homopolysaccharides but also heteropolysaccharides (containing only two monosaccharides).

The high level of similarity between the *S. marcescens* *wb*$_{O4}$ and *K. pneumoniae* *wb*$_{O12}$ gene clusters and the similarity between the O4 and O12 chemical structures [in *S. marcescens* O4, →4)-β-D-Glc*p*-(1→4)-α-L-Rhap-(1→; and in *K. pneumoniae* O12, →4)-β-D-GlcNAc*p*-(1→4)-α-L-Rhap-(1→] gave us the opportunity to study the specificity of both systems by performing cross complementation experiments with genetically defined mutants.

The gene encoding the WbbL putative rhamnosyl transferase is interchangeable between the two *wb* gene clusters. Furthermore, when the *K. pneumoniae* *wb*$_{O12}$ *wbbL* gene was introduced into *E. coli* K-12 strain DH5α, this strain was able to produce O16-antigen LPS, as previously described for *S. marcescens* *wb*$_{O4}$ *wbbL* in *E. coli* K-12 (16). These results strongly suggest that the WbbL proteins can transfer L-Rha residues to the O4 position of D-Glc or D-GlcNAc, thus indicating that this enzyme is not highly specific.

Despite the high level of similarity between the ABC 2 export proteins for the two O-antigen gene clusters, neither the integral membrane protein (Wzm) nor the ATP-binding protein (Wzt) is interchangeable between the organisms. This fact indicates the high specificity for O-antigen export by the membrane components of the ABC 2 transporter-dependent pathway. The last proteins encoded by the two gene clusters (WbbA for *S. marcescens* *wb*$_{O4}$ and WbbB for *K. pneumoniae* *wb*$_{O12}$) exhibited similarity only in their first halves, and these two proteins were not interchangeable. This is not surprising, since WbbA should transfer a Glc residue by a β-1,4 linkage to α-L-Rha, whereas WbbB should transfer a GlcNAc residue by a β-1,4 linkage to α-L-Rha. It can be speculated that the N-terminal similarity between the WbbA and WbbB proteins could be related to the glycosyl transferase activity of both proteins. The overall results suggest that the genetic determinants responsible for the differences in the structures of these rather simple heteropolysaccharide O antigens are the last genes of the clusters (*wbbA* and *wbbB*). Despite the similarities in chemical O-antigen structure, the two heteropolysaccharides appear to use their own, noninterchangeable ABC 2 transport systems. The O antigen could be changed in both genetic backgrounds (*Serratia* and *Klebsiella*) when the three genes (*wzm*, *wzt*, and *wbbA* or *wbbB*) were introduced into a mutant unable to produce O antigen. Thus, both the ABC 2 export system and the putative D-GlcNAc transferase are specific for the *K. pneumoniae* O12 antigen.

To summarize, *K. pneumoniae* *wb*$_{O12}$ contains four genes (*rmlA*, *rmlB*, *rmlC*, and *rmlD*) involved in the biosynthesis of dTDP-L-Rha, two genes involved in the sequential transfer of L-Rha (*wbbL*) and D-GlcNAc (*wbbB*), and two genes (*wzm* and *wzt*) coding for a specific O12 ABC 2 export system. These proposed enzymatic activities could explain the biosynthesis and export of *K. pneumoniae* O12-antigen LPS. It can be hypothesized that *K. pneumoniae* O12-antigen LPS biosynthesis begins with the transfer of *N*-acetylglucosamine to the acyl carrier protein, followed by sequential transfer of L-Rha and D-GlcNAc to obtain the complete O12-antigen. This O antigen is exported through the specific ABC 2 transport system and finally is ligated to the core LPS by the O-antigen ligase (WaaL) located in the *K. pneumoniae* *waa* cluster.

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