JOURNAL OF BACTERIOLOGY, Aug. 1997, p. 4713–4724 Vol. 179, No. 15
0021-9193/97/$04.00+0

Identification and Functional Characterization of an ABC Transport System Involved in Polysaccharide Export of A-Band Lipopolysaccharide in *Pseudomonas aeruginosa*

HEATHER L. ROCCHETTA† AND JOSEPH S. LAM*

Department of Microbiology and Canadian Bacterial Diseases Network, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Received 4 March 1997/Accepted 30 May 1997

Pseudomonas aeruginosa **coexpresses two distinct lipopolysaccharide (LPS) molecules known as A band and B band. B band is the serospecific LPS, while A band is the common LPS antigen composed of a D-rhamnose O-polysaccharide region. An operon containing eight genes responsible for A-band polysaccharide biosynthesis and export has recently been identified and characterized (H. L. Rocchetta, L. L. Burrows, J. C. Pacan, and J. S. Lam, unpublished data; H. L. Rocchetta, J. C. Pacan, and J. S. Lam, unpublished data). In this study, we report the characterization of two genes within the cluster, designated *wzm* and *wzt*. The Wzm and Wzt proteins have predicted sizes of 29.5 and 47.2 kDa, respectively, and are homologous to a number of proteins that comprise ABC (ATP-binding cassette) transport systems. Wzm is an integral membrane protein with six potential membrane-spanning domains, while Wzt is an ATP-binding protein containing a highly conserved ATP-binding motif. Chromosomal *wzm* and *wzt* mutants were generated by using a gene replacement strategy in *P. aeruginosa* PAO1 (serotype O5). Western blot analysis and immunoelectron microscopy using A-band- and B-band-specific monoclonal antibodies demonstrated that the *wzm* and *wzt* mutants were able to synthesize A-band polysaccharide, although transport of the polymer to the cell surface was inhibited. The inability of the polymer to cross the inner membrane resulted in the accumulation of cytoplasmic A-band polysaccharide. This A-band polysaccharide is likely linked to a carrier lipid molecule with a phenol-labile linkage. Chromosomal mutations in *wzm* and *wzt* were found to have no effect on B-band LPS synthesis. Rather, immunoelectron microscopy revealed that the presence of A-band LPS may influence the arrangement of B-band LPS on the cell surface. These results demonstrate that A-band and B-band O-antigen assembly processes follow two distinct pathways, with the former requiring an ABC transport system for cell surface expression.**

Pseudomonas aeruginosa is an opportunistic pathogen responsible for many debilitating infections, with one of the most notable being chronic pulmonary infections in cystic fibrosis patients. The pathogenicity of this organism is attributed to the production of such diverse virulence factors as exotoxin A, phospholipase C, proteases, alginate, and lipopolysaccharide (LPS). LPS molecules also play an essential structural role in the outer membrane and consist of three distinct regions: a hydrophobic lipid A, which serves to anchor the LPS in the outer membrane, a core oligosaccharide, and the O antigen (O polysaccharide). *P. aeruginosa* has been shown to coexpress two distinct forms of LPS, known as A band and B band. A-band LPS is an antigenically conserved molecule with an O-polysaccharide region composed of short-chained polymers of D-rhamnose, arranged as trisaccharide repeat units of α1→2, α1→3, and α1→3 linkages (2). In comparison, B-band LPS is serospecific, with variations in the O-antigen structure differentiating *P. aeruginosa* into 20 distinct serotypes (36, 46, 47). During chronic pulmonary infections in cystic fibrosis patients, *P. aeruginosa* isolates become nontypeable due to the loss of B-band O antigen, while A-band LPS and alginate become the primary surface polysaccharides (22, 42). To determine the mechanisms involved in *P. aeruginosa* LPS biosynthesis and regulation, our laboratory has focused on identifying and characterizing genes involved in A-band and B-band synthesis and expression.

LPS biosynthesis has been proposed to follow three different pathways on the basis of O-antigen assembly (30, 66). The first pathway involves growth of the polymer at the reducing terminus, which has been proven for the synthesis of many heteropolysaccharides, such as that of *Shigella dysenteriae* type 1 (35). O-antigen repeat units are individually synthesized on the cytoplasmic face of the inner membrane and are translocated to the periplasmic face by Wzx (RfbX) (note that the nomenclature for LPS biosynthetic genes follows the convention recently suggested by Reeves et al. [52]). Polymerization of the O-antigen units occurs at the periplasmic face through the action of an O-antigen polymerase, Wzy (Rfc), with Wzz (Rol) functioning to regulate O-antigen chain length. Recently, both Wzy and Wzz have been shown to be required for B-band LPS synthesis in *P. aeruginosa* (10, 14).

A second pathway of synthesis involves growth of the polymer at the nonreducing terminus which has been proven for the synthesis of such homopolymeric O antigens as *Escherichia coli* O8 and O9 (27) and *Klebsiella pneumoniae* O1 (12). Initiation of this pathway is dependent upon WecA (Rfe) for transfer of an *N*-acetyl-glucosamine (GlcNAc) residue to undecaprenol phosphate (Und-P) (53). This GlcNAc subsequently serves as the acceptor molecule for the sequential transfer of sugars one residue at a time; however, GlcNAc does not become part of the repeating O-antigen units (66). The WecA protein is also involved in heteropolysaccharide synthesis, serving as both an initial acceptor molecule and a component of the O-antigen repeat unit (35). Recently, Zhang et al. (68)

* Corresponding author. Mailing address: Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1. Phone: (519) 824-4120, ext. 3823. Fax: (519) 837-1802. E-mail: jlam@micro.uoguelph.ca.

† Formerly known as Heather L. Currie.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics[a]	Reference or source
Strains		
P. aeruginosa		
PAO1	Serotype O5; A^+ B^+	21
M1	*wzm*::Gm^r B^+ A^- (cytoplasmic A-band polysaccharide)	This work
M2	*wzm*::Gm^r B^+ A^- (cytoplasmic A-band polysaccharide)	This work
T1	*wzt*::Gm^r B^+ A^- (cytoplasmic A-band polysaccharide)	This work
T2	*wzt*::Gm^r B^+ A^- (cytoplasmic A-band polysaccharide)	This work
E. coli		
DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	GIBCO/BRL
SM10	*thi-1 thr leu tonA lacY supE recA* RP4-2-Tc::Mu Km^r	59
CSR603	*recA1 uvrA6 phr-1 thi-1 thr-1 leuB6 lac-Y1 galK2 ara14 xyl115 mtl1 proA2 argF3 rpsL31 tsx-33 supE44 gyrA98-1* F^-	56
Plasmids		
pFV3	27-kb *Xho*I fragment in cosmid pCP13	44
pFV300	2.3-kb *Kpn*I-*Bam*HI fragment in pUCP26	This work
pFV301	2.5-kb *Bam*HI-*Bam*HI fragment in pUCP26	This work
pFV311	4.0-kb *Pst*I-*Pst*I fragment in pBluescript	This work
pFV312	2.7-kb *Pst*I-*Eco*RI fragment in pUCP26	This work
Bluescript-II KS(+/−)	Ap^r	PDI Biosciences
pUCP26	pUC18-derived broad-host-range vector; Tc^r	64
pEX100T	Gene replacement vector; *oriT*$^+$ *sacB*$^+$ Ap^r	58
pUCGM	Contains the 879-bp Gm^r cassette; Ap^r Gm^r	57
pCP13	RK2 derivative; *cos*$^+$, Mob^+ Tra^- Tc^r Km^r	13

[a] A superscript plus or minus with an A or a B stands for the presence or absence, respectively, of the indicated LPS type.

reported that WecA of *E. coli* may also function to transfer *N*-acetyl-galactosamine (GalNAc) to Und-P. In this second pathway of synthesis, polymerization of the O polysaccharide occurs at the cytoplasmic face of the inner membrane and has been shown to proceed independently of Wzy and Wzz, as exemplified in *K. pneumoniae* O1 (12). Since polymerization occurs at the cytoplasmic face, the completed polymer must be transported from the cytoplasm to the periplasm to allow ligation of the O polysaccharide to core-lipid A by WaaL (RfaL). This translocation of polymer has been shown to occur through an ATP-binding cassette (ABC) transport system.

ABC transporters in general have been found in both prokaryotic and eukaryotic systems and are responsible for the import and export of various proteins, peptides, polysaccharides, and drugs (17). ABC transport systems have recently been identified for the export of LPS O polysaccharides for *Yersinia enterocolitica* O:3, *K. pneumoniae* O1, *E. coli* O9, *Serratia marcescens* O16, *Vibrio cholerae* O1, and *Myxococcus xanthus* (8, 20, 33, 48, 61, 67). These transport systems are comprised of two components: a hydrophilic protein containing a highly conserved ATP-binding domain and a hydrophobic protein with five to six membrane-spanning domains. Two subunits of each component associate, giving rise to four protein domains which form a functional transporter (17). The ATP-binding proteins couple the energy of ATP hydrolysis to transport, while the integral membrane protein components are thought to form a pore-like structure (23, 32). It is the integral membrane proteins, rather than the ATP-binding proteins, that are thought to confer substrate specificity to the transport system (23).

Interestingly, a third pathway of O-antigen synthesis has recently been reported in *Salmonella enterica* serovar Borreze for the homopolymeric O:54 antigen (30). This pathway is a novel Wzy-independent pathway that still requires WecA for initiation; however, export of the polymer does not require a Wzx O-unit transporter or an ABC transport system. Rather, the processive glycosyltransferase RfbB may function to polymerize the *N*-acetyl-mannosamine (ManNAc) homopolymer while coupling polymerization with transport (30). The C-terminal transmembrane region of RfbB has been proposed to form a pore-like structure through which the growing polysaccharide travels (30).

We report in the present work the identification and characterization of an ABC transport system responsible for translocation of A-band polysaccharide in *P. aeruginosa* serotype O5. Nucleotide sequence analysis and amino acid alignments suggest that two genes, *wzm* and *wzt*, code for the integral membrane protein and the ATP-binding component, respectively, to form an ABC transport system. A gene replacement strategy was used to generate *P. aeruginosa* serotype O5 chromosomal mutants in both *wzm* and *wzt* genes. These mutants are essential for assessing the role these two proteins play in the transport of A-band polysaccharide.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were routinely propagated in Luria Broth (Gibco/BRL, Burlington, Ontario, Canada) at 37°C. Pseudomonas Isolation Agar (Difco Laboratories, Detroit, Mich.) was used for selecting transconjugates following mating experiments. For maxicell expression, bacterial strains were grown in Davis minimal media (Difco) supplemented with Casamino Acids (0.5%; ICN Biomedicals, Costa Mesa, Calif.), D-glucose (0.2%), and thiamine-HCl (5 μg ml^{-1}). The following antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used in selection media at the indicated concentrations: ampicillin at 100 μg ml^{-1} for *E. coli*, carbenicillin at 500 μg ml^{-1} for *P. aeruginosa*, gentamicin at 15 and 300 μg ml^{-1} for *E. coli* and *P. aeruginosa*, respectively, and tetracycline at 15 and 45 μg ml^{-1} for *E. coli* and for *P. aeruginosa* (160 μg of tetracycline ml^{-1} in Pseudomonas Isolation Agar), respectively.

DNA procedures. Restriction enzymes were purchased from Gibco/BRL, Boehringer Mannheim (Laval, Quebec, Canada), and Pharmacia (Baie d'Urfé, Quebec, Canada). T4 DNA ligase, Klenow enzyme, and alkaline phosphatase were purchased from Boehringer Mannheim. All enzymes were used according to the suppliers' specifications. Small-scale plasmid DNA preparations were

prepared by the alkaline lysis method of Birnboim and Doly (5), while large-scale preparations were obtained with a plasmid midi kit (Qiagen Inc., Chatsworth, Calif.). Electrocompetent cells of *E. coli* and *P. aeruginosa* were prepared following the methods of Binotto et al. (4) and Farinha and Kropinski (16), respectively. Plasmid DNA was electroporated into *E. coli* and *P. aeruginosa* with a gene pulser (Bio-Rad Laboratories, Richmond, Calif.). Recombinant plasmids were mobilized from *E. coli* SM10 to *P. aeruginosa* according to the method of Simon et al. (59). Genomic DNA was isolated from *P. aeruginosa* by the method of Ausubel et al. (3). Restriction enzyme-digested genomic DNA was separated on 0.7% agarose gel and transferred to a Zeta-Probe membrane (Bio-Rad) by the method of Ford et al. (18). Southern hybridizations were carried out at 42°C for 18 h with a DNA probe previously labelled with dUTP conjugated to digoxigenin (Boehringer Mannheim). Subsequent washes and incubations were carried out according to the manufacturer's recommendations (Boehringer Mannheim). Detection of hybridized DNA was achieved with an antidigoxigenin antibody and the chemiluminescent substrate AMPPD [3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy)-phenyl-1,2-dioxetane] (Boehringer Mannheim), followed by exposure to X-ray film (E. I. Du Pont de Nemours & Co., Wilmington, Del.).

Nucleotide sequencing. The 2.3-kb *Kpn*I-*Bam*HI insert of pFV300 and the 2.5-kb *Bam*HI-*Bam*HI insert of pFV301 were independently cloned into the vector pBluescript-II KS(+/−). Both strands of DNA were sequenced at the MOBIX facility (McMaster University, Hamilton, Ontario, Canada) with an Applied Biosystems (Foster City, Calif.) model 373A DNA sequencing unit. Oligonucleotide primers were synthesized on an Applied Biosystems model 391 DNA synthesizer and purified according to the manufacturer's instructions. All sequencing reactions were performed with the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and were carried out in an Ericomp (San Diego, Calif.) model TCX15 thermal cycler. Excess DyeDeoxy Terminators were removed from the completed sequencing reaction mixtures by passing the mixtures over a 1-ml Sephadex G-50 column (Pharmacia).

Sequence analysis. Nucleotide and amino acid sequence analysis was performed by using the computer software program Gene Runner (Hastings Software Inc., Newark, N.J.). Sequence homologies were determined by using GenBank DNA and protein sequence databases through the National Center for Biotechnology Information BLAST network server (1, 19). Amino acid sequence alignments were carried out by using the PC/GENE package (IntelliGenetics Inc., Mountain View, Calif.), while open reading frame (ORF) analysis and predicted protein characteristics were performed by using the Gene Runner program.

Maxicell analysis of plasmid-encoded proteins. The maxicell protein expression system of Sancar et al. (56) was used to analyze plasmid-encoded proteins. Plasmids were electroporated into *E. coli* CSR603, and maxicells were prepared as previously described by Lightfoot and Lam (45) with the following modifications. Overnight cultures were diluted 1:50 into supplemented Davis minimal media lacking antibiotics. The cultures were grown to mid-logarithmic phase and then irradiated for 30 s at 400 μW cm^{-2} with a germicidal lamp. Expressed proteins were labelled with Trans ^{35}S-Label (ICN Biomedicals).

Mutagenesis of the *wzm* and *wzt* genes of *P. aeruginosa* PAO1 (serotype O5). *P. aeruginosa* PAO1 *wzm* and *wzt* chromosomal knockout mutants were generated by using a gene replacement strategy previously described by de Kievit et al. (14). The 2.3-kb *Kpn*I-*Bam*HI insert of pFV300 and the 2.5-kb *Bam*HI-*Bam*HI insert of pFV301 were blunt ended using Klenow enzyme and independently subcloned into the *Sma*I site of the gene replacement vector pEX100T. An 879-bp Gm^r cassette from pUCGM (57) was cloned into the second *Pst*I site of the insert DNA from pFV300 within the ORF encoding Wzm. This Gm^r cassette was also cloned into the unique *Eco*RV site of the insert DNA from pFV301 within the ORF encoding Wzt. Southern hybridization analysis was performed with the Gm^r cassette to confirm that gene replacement had occurred in the *wzm* and *wzt* mutants.

Preparation of LPS. Two different methods of LPS preparation were used, the proteinase K digest method of Hitchcock and Brown (HB) (26) and the hot-aqueous phenol (HAP) method of Westphal and Jann (65).

SDS-polyacrylamide gel electrophoresis analysis and Western immunoblotting. LPS was analyzed in sodium dodecyl sulfate (SDS)-polyacrylamide gels as described by Hancock et al. (22). The Western immunoblot method of Burnette (9) was used with the following modifications. LPS was transferred to Biotrace nitrocellulose (Gelman, Rexdale, Ontario, Canada) and blocked in 5% skim milk in phosphate-buffered saline (PBS) for 1 h at 37°C. This was followed by an overnight incubation with the monoclonal antibody (MAb) N1F10 (specific for A-band LPS) or MAb MF15-4 (specific for B-band LPS) (42). Detection was achieved by using an alkaline phosphatase-conjugated second antibody and an appropriate substrate as previously described by Lightfoot and Lam (44).

Transmission electron microscopy. (i) Sample preparation. *P. aeruginosa* cells were fixed for 1 h at 4°C in 2% (vol/vol) paraformaldehyde and 0.1% (vol/vol) glutaraldehyde buffered with 0.05 M sodium cacodylate at pH 7.0. Following fixation, the samples were enrobed in 2% (wt/vol) molten Noble agar, washed in buffer, and dehydrated through a stepwise alcohol series. The samples were then embedded in London Resin white resin, and thin sections were obtained by cutting the embedded material with a Servell MT1 Porter-Blum microtome with a diamond knive.

(ii) Immunolabelling of thin sections. Thin sections were labelled on 200-mesh Formvar-carbon-coated nickel grids. Two percent (wt/vol) skim milk in PBS buffer was used for blocking of the sample prior to labelling with the primary antibody (either MAb N1F10 or MAb MF15-4; both immunoglobulin M). The samples were then washed in PBS and immunogold labelled by using a goat anti-mouse immunoglobulin M secondary antibody conjugated to a 15-nm colloidal gold particle (Sigma Chemical Co.) at a 1:20 dilution.

(iii) Staining. Following gold labelling, the sections were washed in PBS and then washed in deionized water. The sections were stained following standard procedures using 2% uranyl acetate, lead citrate, and appropriate washing steps. Control grids were prepared for each sample with the primary antibody excluded. All sections were examined by using a Philips EM300 transmission electron microscope operating at 60 kV under standard conditions with the cold trap in place.

Nucleotide sequence accession numbers. The nucleotide and predicted amino acid sequences of *wzm* and *wzt* have been submitted to GenBank under the accession no. U63722 and U63723, respectively.

RESULTS

Construction of subclones from the cosmid pFV3. The restriction enzyme map of a previously described cosmid clone, pFV3, isolated from a *P. aeruginosa* PAO1 gene library (44), is shown in Fig. 1. The cloning vector pUCP26, which is able to replicate in *P. aeruginosa*, was used to generate three subclones of pFV3: pFV300 (2.3-kb *Kpn*I-*Bam*HI insert), pFV301 (2.5-kb *Bam*HI-*Bam*HI insert), and pFV312 (2.7-kb *Pst*I-*Eco*RI insert). For nucleotide sequencing, pFV300, pFV301, and pFV311 (4.0-kb *Pst*I-*Pst*I) DNA inserts were cloned into the vector pBluescript-II KS(+/−).

Nucleotide sequencing of *wzm* and *wzt*. The 2.3-kb *Kpn*I-*Bam*HI insert from pFV300 and the 2.5-kb *Bam*HI-*Bam*HI insert from pFV301 were subjected to double-stranded nucleotide sequencing. The subclone pFV311 was used to sequence the overlapping junction of pFV300 and pFV301. Nucleotide sequence analysis of this 4.8-kb region revealed the presence of two complete ORFs designated *wzm* and *wzt* (based on homology; see below). The *wzm* ORF is comprised of 797 nucleotides (265 amino acids) and is predicted to encode a 29.5-kDa protein. The *wzt* ORF is comprised of 1,265 nucleotides (421 amino acids) and encodes a protein with a predicted mass of 47.2 kDa. The G+C content of *wzm* is 64.1 mol%, and that of *wzt* is 66.2 mol%, which is consistent with that of the *P. aeruginosa* genome (67.2% [50]). The *wzm* and *wzt* ATG start codons are preceded by potential Shine-Dalgarno sequences of GGG AGG and GGTGGA, respectively. Two partial ORFs were found flanking *wzm* and *wzt* on pFV300 and pFV301, respectively, all of which are arranged contiguously such that the stop (TGA) and start codons (ATG) of each overlap, allowing possible translational coupling. Analysis of the region upstream of *wzm* and *wzt* revealed no potential promoter sequences. Sequence analysis of the region upstream of pFV300 has revealed a putative promoter sequence resembling the *E. coli* σ^{70} consensus preceding the first gene in the A-band cluster (55). This promoter is likely responsible for the transcription of all eight A-band genes, including *wzm* and *wzt*. However, this does not exclude the possibility that a promoter distinct from the σ^{70} consensus motif may be functional upstream of *wzm*. Attempts to clarify this by transcriptional studies are under way.

Southern hybridization of the 20 O serotypes with pFV300 and pFV301. Since A-band LPS is a common antigen, it was of interest to determine whether *wzm* and *wzt* were conserved among the 20 *P. aeruginosa* O serotypes. The 2.3-kb *Kpn*I-*Bam*HI insert from pFV300 (*wzm*) and the 2.5-kb *Bam*HI-*Bam*HI insert from pFV301 (*wzt*) were used as DNA probes for Southern hybridization. Chromosomal DNA isolated from the 20 O serotypes of *P. aeruginosa* was digested with *Bam*HI and probed with pFV300 and pFV301. In the case of pFV300, a conserved probe-reactive fragment of 9.4 kb was seen in all serotypes, with four exceptions (data not shown). In serotypes O5 and O20, hybridization to a 20.0-kb fragment was seen,

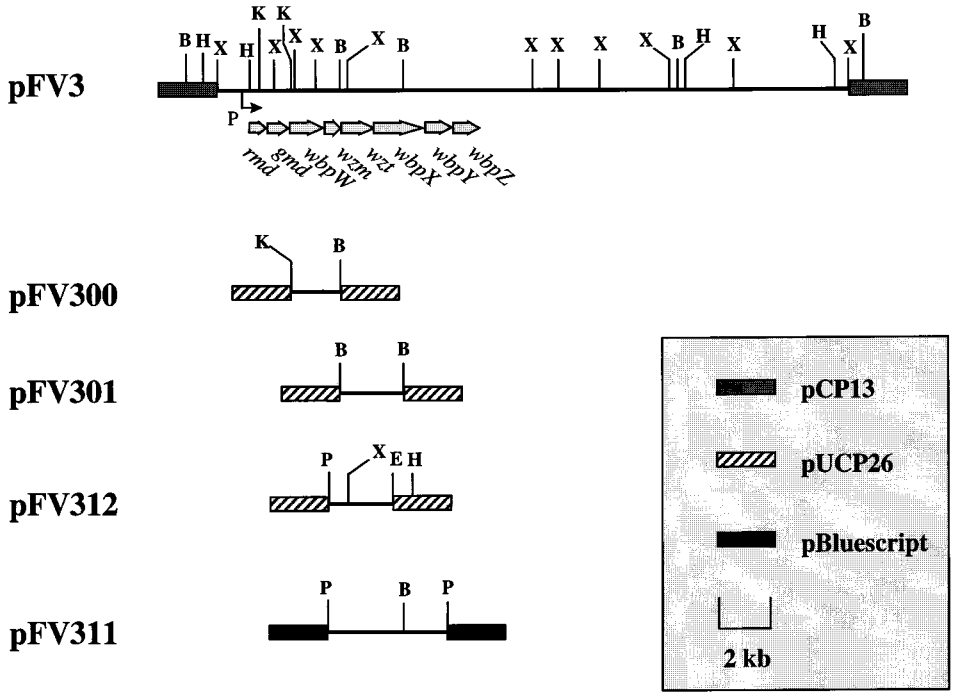

FIG. 1. Restriction maps of pFV3, pFV300, pFV301, pFV311, and pFV312. Eight ORFs are indicated by name on pFV3. The direction of transcription is indicated by the arrows, and the location of the putative promoter region (P) is shown upstream of *rmd*. *rmd* and *gmd* (previously referred to as *gca*) encode proteins which are involved in the synthesis of GDP–D-rhamnose from GDP–D-mannose, while WbpX is involved in GDP–D-mannose biosynthesis (45, 55). *wbpX*, *wbpY*, and *wbpZ* encode glycosyltransferases involved in the assembly of A-band polysaccharide (54). Abbreviations for restriction sites: B, *Bam*HI; H, *Hin*dIII; X, *Xho*I; K, *Kpn*I; P, *Pst*I; E, *Eco*RI.

while serotypes O12 and O13 showed probe-reactive fragments of 3.5 and 7.0 kb, respectively. When pFV301 was used as a probe, a 2.5-kb probe-reactive fragment was seen in all serotypes except O15 and O16, which hybridize to fragments of 4.0 and 11.0 kb, respectively (data not shown).

Amino acid homology of Wzm and Wzt to ABC transport system proteins. Comparison of the sequences of Wzm and Wzt with known proteins revealed significant similarity to a family of ABC transporters involved in polysaccharide export. Wzm is similar to the integral membrane component from a number of LPS O-antigen transporters and capsular polysaccharide transporters (Table 2). Interestingly, Wzm was also similar to an integral membrane protein involved in teichoic acid translocation from the gram-positive organism *Bacillus subtilis* (43). Although these hydrophobic proteins usually display low primary sequence similarity, it was interesting to observe 52.5% amino acid identity between Wzm and ORF261 from *E. coli* O9. The *E. coli* O9 O polysaccharide is a mannose homopolymer with similar glycosidic linkages ($\alpha1\rightarrow2$, $\alpha1\rightarrow3$, $\alpha1\rightarrow3$) to those of the A-band polysaccharide (33). GDP–D-mannose is also the precursor of the A-band O-polysaccharide sugar D-rhamnose (45), with a reduction of CH_2OH occurring at the C-6 position to yield CH_3 in D-rhamnose. These two polysaccharide molecules therefore have common carbohydrate structures and likely require integral membrane components with similar specificities.

In general, these hydrophobic transport proteins have similar secondary structures, with several membrane-spanning regions comprised of hydrophobic α-helices separated by hydrophilic regions (24, 25). Hydrophobicity profiles plotted by the

TABLE 2. Amino acid sequence pairwise comparisons of Wzm and other integral membrane proteins involved in export

Protein	Identity with[a]:						
	ORF261	$KpsM_{K1}$	$KpsM_{K5}$	$RfbA_{KpO1}$	$RfbA_{KpO8}$	$RfbA_{SmO16}$	TagG
Wzm	52.5 (137)	21.7 (56)	21.7 (56)	32.4 (84)	27.5 (71)	31.5 (80)	22.3 (59)
ORF261		10.9 (28)	10.5 (27)	29.3 (76)	28.7 (74)	25.6 (65)	16.9 (44)
$KpsM_{K1}$			97.7 (252)	15.9 (41)	11.2 (29)	10.2 (26)	18.2 (47)
$KpsM_{K5}$				16.3 (42)	15.9 (41)	11.0 (28)	17.8 (46)
$RfbA_{KpO1}$					93.4 (241)	75.6 (192)	14.7 (38)
$RfbA_{KpO8}$						76.0 (193)	15.1 (39)
$RfbA_{SmO16}$							15.7 (40)

[a] Wzm from *P. aeruginosa* serotype O5 is compared to the following integral membrane transport proteins (GenBank accession numbers are given in parentheses): ORF261 from *E. coli* O9 (D43637), KpsM from *E. coli* K1 and K5 (M57382 and U59301), RfbA from *K. pneumoniae* O1 and O8 and *S. marcescens* O16 (L31775, L41518, and L34166), and TagG from *B. subtilis* (U13832). Amino acid sequences were compared by using the P-ALIGN program (49), with an open gap cost of 6 and a unit gap cost of 1. Each amino acid comparison between two proteins is reported as a percent identity, with the value in parentheses signifying the number of identical amino acids.

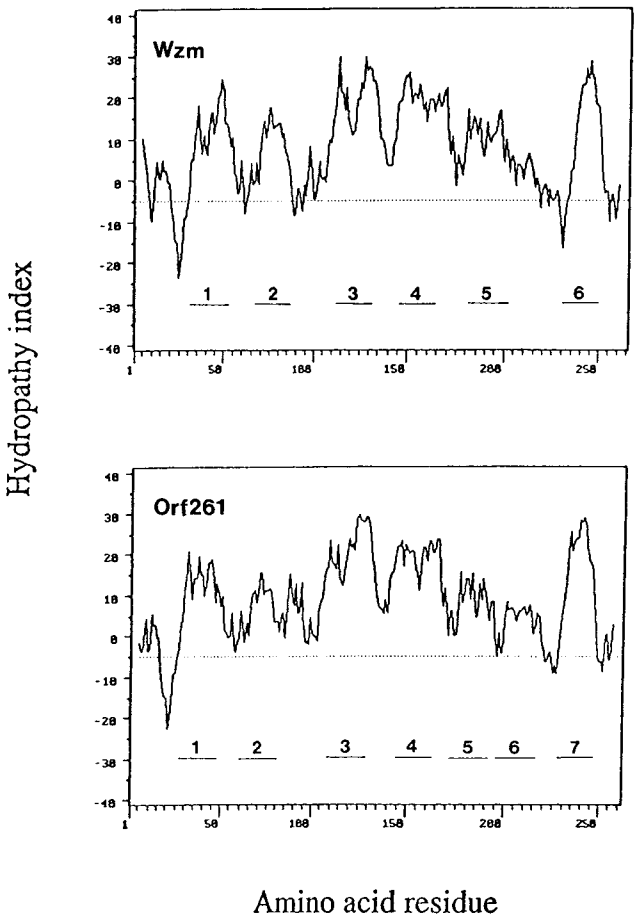

FIG. 2. Hydropathy plots of Wzm and ORF261 of *E. coli* O9, drawn by the method of Kyte and Doolittle (39). The *x* axis corresponds to the amino acid residue, while the *y* axis corresponds to the relative hydropathy index. Wzm and ORF261 contain six and seven potential membrane-spanning domains, respectively, which are indicated above the *x* axis in each panel (15).

method of Kyte and Doolittle (39) demonstrate high similarity between Wzm and ORF261 (Fig. 2). The hydrophobic plot of Wzm predicts six potential membrane-spanning domains, which correspond to regions predicted to form membrane-associated α-helices (15). Wzm was also classified as an integral protein based on the method of Klein et al. (34).

Wzt was found to be similar to the ATP-binding protein component from both LPS O-antigen and capsular polysaccharide ABC transport systems (Table 3). Similarity to AbcA, an ATP-binding protein from *Aeromonas salmonicida* which influences expression of a surface array protein in *E. coli* (11), was also observed. Amino acid sequence analysis of Wzt reveals the presence of an ATP-binding motif, consisting of sites A and B (63) (Fig. 3). Alignment of Wzt with other putative ATP-binding proteins (Fig. 3), demonstrates the conserved nature of this ATP-binding motif. The predicted molecular mass of Wzt is 47.2 kDa, which is considerably greater than that of ATP-binding proteins reported for other systems. Interestingly, the ATP-binding component ORF431 from *E. coli* O9 has a similar predicted size, 47.8 kDa, and shows the highest amino acid similarity to Wzt. AbcA of *A. salmonicida* also has a predicted size, 34 kDa, greater than those of other ATP-binding proteins. The significance of these larger transport proteins is not yet known.

Pairwise comparisons in Tables 2 and 3 reveal that integral membrane proteins and ATP-binding proteins from *K. pneumoniae* O1 and O8 and *S. marcescens* O16 show high primary sequence similarity, likely due to a conserved D-galactan I structure in the O-polysaccharide region (8, 61). KpsM and KpsT from *E. coli* K1 and K5 also demonstrate high similarity, possibly due to common capsular polysaccharide structures. The K1 polymer is composed of *N*-acetylneuraminic acid, which is derived from GlcNAc, while the K5 polymer contains GlcNAc (38, 51). It follows that the similarity observed between *E. coli* O9 and *P. aeruginosa* O5 transport proteins is attributed to the export of common substrates.

Expression of Wzm and Wzt. To determine the molecular mass of the gene products coded for by *wzm* and *wzt*, maxicell analysis was performed. The subclones pFV300 and pFV312 were used for maxicell expression since complete ORFs for *wzm* and *wzt* reside on these two constructs, respectively. pBluescript-II KS(+/−) was also used as the vector control. A unique protein of 47 kDa was detected from pFV312, which is comparable to the predicted size of 47.2 kDa for Wzt (Fig. 4). Attempts to express Wzm using the maxicell system, as well as the T7 RNA polymerase expression system, were unsuccessful (data not shown). This lack of expression may be due to the hydrophobic nature of this protein, since other researchers have been unable to detect integral membrane components

TABLE 3. Amino acid sequence pairwise comparisons of Wzt and other ATP-binding proteins involved in export

Protein	Identity with[a]:									
	ORF431	$KpsT_{K1}$	$KpsT_{K5}$	$RfbB_{KbO1}$	$RfbB_{KbO8}$	$RfbB_{SmO16}$	AbcA	RfbE	CtrD	BexA
Wzt	41.6 (175)	34.4 (75)	32.1 (72)	35.8 (88)	40.2 (99)	35.5 (87)	35.4 (109)	34.3 (82)	36.1 (78)	34.1 (74)
ORF431		34.4 (75)	36.2 (81)	39.8 (98)	41.9 (103)	38.8 (95)	39.0 (120)	32.6 (78)	40.7 (88)	38.7 (84)
$KpsT_{K1}$			72.5 (158)	35.3 (77)	33.5 (73)	34.9 (76)	31.7 (69)	27.5 (60)	46.3 (100)	47.0 (102)
$KpsT_{K5}$				38.8 (87)	36.2 (81)	38.4 (86)	33.0 (74)	30.8 (69)	44.4 (96)	43.8 (95)
$RfbB_{KbO1}$					78.9 (194)	100.0 (245)	42.3 (104)	37.7 (90)	36.1 (78)	36.4 (79)
$RfbB_{KbO8}$						78.8 (193)	46.3 (114)	35.6 (85)	36.1 (78)	37.8 (82)
$RfbB_{SmO16}$							42.0 (103)	36.4 (87)	34.3 (74)	35.9 (78)
AbcA								34.7 (83)	32.4 (70)	30.9 (67)
RfbE									31.5 (68)	33.2 (72)
CtrD										81.0 (175)

[a] Wzt from *P. aeruginosa* serotype O5 is compared to the following ATP-binding transport proteins (GenBank accession numbers are given in parentheses): ORF431 from *E. coli* O9 (D43637), KpsT from *E. coli* K1 and K5 (M57381 and X53819), RfbB from *K. pneumoniae* O1 and O8 and *S. marcescens* O16 (L31775, L41518, and L34166), AbcA from *A. salmonicida* (L11870), RfbE from *Y. enterocolitica* O:3 (Z18920), CtrD from *Neisseria meningitidis* group B (M57677), and BexA from *H. influenzae* type b (X54987). Amino acid sequences were compared by using the P-ALIGN program (49), with an open gap cost of 6 and a unit gap cost of 1. Each amino acid comparison between two proteins is reported as a percent identity, with the value in parentheses signifying the number of identical amino acids.

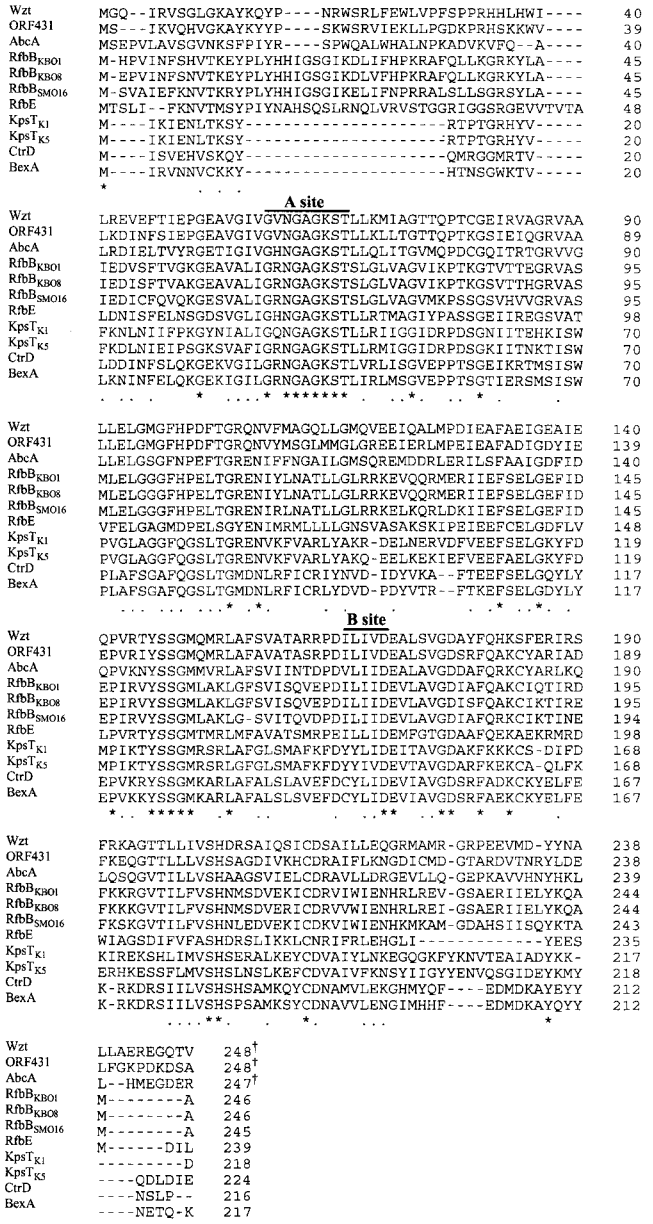

FIG. 3. Amino acid alignment of Wzt with other ATP-binding proteins. Identical amino acids are represented by asterisks, while similar amino acids are represented by periods. Wzt contains the highly conserved ATP-binding motif consisting of sites A and B (17, 63). Alignments were performed by using the CLUSTAL program (PC/GENE package; IntelliGenetics). For amino acid alignments marked with a dagger, only the first 247 or 248 amino acids were used due to the increased sizes of these proteins.

encoded by *bexB* from *Haemophilus influenzae*, *kpsM* of *E. coli* K5, *rfbA*$_{KpO1}$ from *K. pneumoniae* O1, and *rfbA*$_{Sm16}$ from *S. marcescens* O16 (8, 37, 60, 61).

Generation of *P. aeruginosa* chromosomal *wzm* and *wzt* mutants. To investigate the involvement of Wzm and Wzt in A-band polysaccharide transport, chromosomal mutants were generated by using a gene replacement strategy whereby a gentamicin resistance (Gm^r) cassette containing an internal promoter was independently inserted within the *wzm* and *wzt* ORFs, giving rise to a nonpolar mutation in each of these two genes. To ensure that these mutations were nonpolar, the orientation of the Gm^r cassette was determined by using the restriction enzyme *Eco*RV. This enzyme digests the Gm^r cassette 600 bp downstream of the internal promoter. The mutated *wzm* and *wzt* alleles were each mobilized into the parent strain, PAO1, and integrated into the chromosome by homologous recombination. Selection of recombinants which had undergone true gene replacement was achieved by using two vector (pEX100T)-associated markers, for sucrose sensitivity (the *sacB* gene) and carbenicillin resistance (the *bla* gene). Of those Gm^r colonies that were isolated, 15 *wzm* and 38 *wzt* recombinants were found to be carbenicillin sensitive and sucrose resistant. In each case, two *wzm* (M1 and M2) and *wzt* (T1 and T2) putative mutants were randomly chosen for further analysis. To confirm genotypically that allelic replacement had occurred, Southern hybridization analysis was performed by using chromosomal DNA isolated from the parent strain and the four putative *wzm* and *wzt* mutants. The 879-bp Gm^r cassette was used as a probe to screen *Xho*I-digested chromosomal DNA from PAO1, M1, and M2. The probe hybridized to a 2.3-kb fragment in M1 and M2 (Fig. 5A and C) instead of a 1.4-kb *Xho*I fragment due to the insertion of the 879-bp Gm^r cassette. There was no hybridization to chromosomal DNA isolated from the parent strain PAO1. The 879-bp Gm^r cassette was also used as a probe to screen *Bam*HI-digested chromosomal DNA from PAO1, T1, and T2. The probe hybridized to a 3.4-kb fragment in T1 and T2 (Fig. 5B and C) instead of a 2.5-kb *Bam*HI fragment due to the insertion of the 879-bp Gm^r cassette. Thus, for all four mutants, an increase in the size of probe-reactive fragments corresponds to insertion of the 879-bp Gm^r cassette in both *wzm* and *wzt*, demonstrating that M1, M2, T1, and T2 had undergone gene replacement.

Characterization of LPS isolated from the *wzm* and *wzt* mutants. To observe the effect of *wzm* and *wzt* mutations on A-band synthesis, LPS samples from the putative mutants M1, M2, T1, and T2 and from the PAO1 control were prepared by both the proteinase K digestion method (HB method [26]) and the HAP method of Westphal and Jann (65). Wild-type *wzm* and *wzt* genes were mobilized into their corresponding mutants by using the constructs pFV300 and pFV312, respectively, to determine if supplying these genes in *trans* restores A-band LPS expression. LPS was also isolated from these recombinants by the above-mentioned methods, and all samples were analyzed by Western immunoblotting using the A-band-specific MAb N1F10 (Fig. 6A and C) and the B-band-specific MAb MF15-4 (Fig. 6B and D). Reactivity on Western immunoblots shows that A-band LPS from the M1, M2, T1, and T2 mutants isolated by the HB method has a molecular weight lower than that of the parent strain PAO1. A visual difference in the LPS banding pattern, roughly equivalent to three O-antigen repeat units, can be discerned between A-band LPS isolated from the mutants and the PAO1 control. LPS HB isolated from the recombinant strains M1 (pFV300), M2 (pFV300), T1 (pFV312), and T2 (pFV312) was found to have reactivity with MAb N1F10 in a pattern resembling that of the parent strain PAO1 (Fig. 6A and C).

To determine if the lower-molecular-weight LPS in the *wzm* and *wzt* mutants was A-band polysaccharide attached to either core-lipid A or to a carrier lipid molecule with an increased mobility, HAP LPS extractions were performed. This approach was taken since Kent and Osborn (31) had previously shown that the polysaccharide-carrier lipid (Und-P) linkage was extremely labile, with cleavage of the pyrophosphate bridge occurring upon treatment with HAP. LPS isolated from *wzm* and *wzt* mutants by the HAP method demonstrates no reactivity on Western immunoblots with MAb N1F10 (Fig. 6A and C). Treatment of the mutants with HAP therefore results in cleavage of the

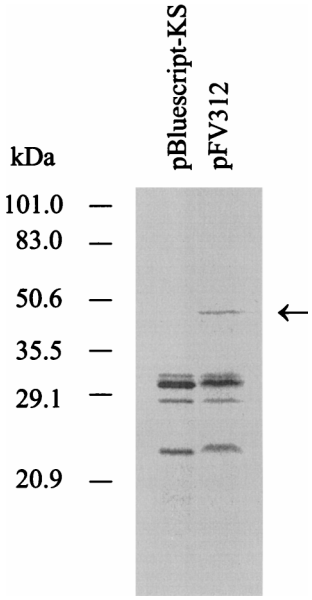

FIG. 4. Autoradiogram showing ^{35}S-labelled proteins expressed by pFV312 and the corresponding plasmid vector pBluescript-II KS(+/−) in the *E. coli* maxicell strain CSR603. The arrow indicates expression of a unique 47-kDa protein from pFV312.

polysaccharide-carrier lipid linkage releasing soluble A-band polysaccharide. LPS HAP extracted from the complemented *wzm* (M1[pFV300], M2[pFV300]) and *wzt* (T1[pFV301], T2[pFV301]) mutants, however, reacts with the A-band-specific MAb N1F10, indicating that the complemented mutants synthesize A-band polysaccharide linked to core-lipid A (Fig. 6A and C). Figure 6B and D demonstrates that LPS isolated from the *wzm* and *wzt* mutants by either method reacts with the B-band-specific MAb MF15-4 in the same manner as that of the parent strain PAO1.

Localization of LPS by immunoelectron microscopy. In order to visualize the location of A-band and B-band LPS molecules on the *wzm* and *wzt* mutants, immunoelectron microscopy was performed with MAbs N1F10 and MF15-4. Since the results for both *wzm* and *wzt* mutants were similar, only the latter are shown in Fig. 7 and 8 and are discussed below. The parent strain PAO1 demonstrated immunogold labelling of the cell surface when either N1F10 or MF15-4 was used as the primary antibody (Fig. 7A and D). B-band LPS could be visualized on the cell surface in both the *wzt* mutant T1 and in the recombinant T1(pFV301) (Fig. 7B and C). The only change observed appeared to be labelling of B-band LPS farther away from the cell surface in T1, compared to PAO1 and T1 (pFV301). While no A-band LPS could be seen on the cell surface of T1, A band was present within the cytoplasm as indicated by immunogold labelling (Fig. 7E). When *wzt* was provided in *trans* to the *wzt* mutant, A-band LPS was expressed on the cell surface of strain T1(pFV301) (Fig. 7F), though at levels lower than that of the parent PAO1. This could be attributed to an imbalance in the stoichiometry between the two components of the transport system, resulting in a less-efficient translocation process. All controls outlined in Materials and Methods were negative for immunogold labelling (data not shown).

P. aeruginosa has been reported recently to release membrane vesicles (MVs) into the medium under normal growth conditions as well as to exhibit enhanced release and an increase in size of MVs upon brief exposure to gentamicin (at four times the MIC, i.e., 8 μg/ml) (28). Specific regions of the outer membrane become loosely attached, forming MVs which detach from the cell and are released into the medium. It has also been suggested that occasionally the cytoplasmic membrane may be breached, resulting in MVs with two lipid bilayers (28). Furthermore, it has been shown that these MVs contain DNA, hydrolytic enzymes, and A-band and B-band LPS (28). Since the *wzm* and *wzt* mutants were constructed with a gentamicin resistance cassette, these strains were cul-

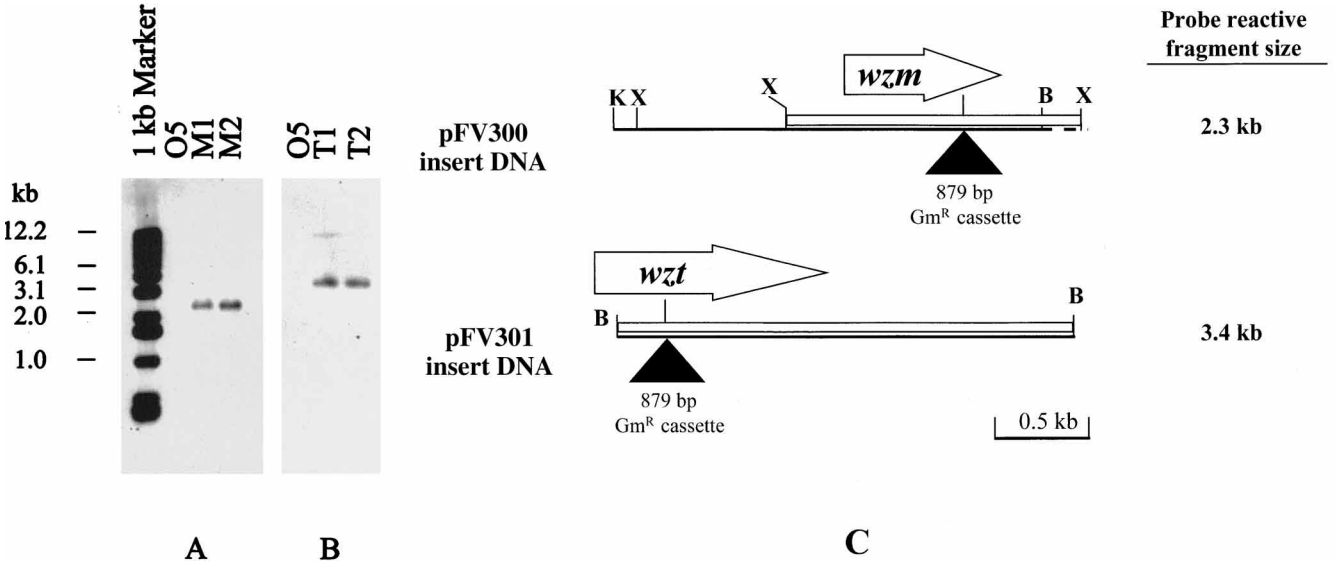

FIG. 5. Southern hybridization of the parent strain PAO1 (O5) and *wzm* and *wzt* mutants with the gentamicin resistance cassette as a DNA probe. (A) Probe reactivity to *Xho*I-digested genomic DNA from PAO1 and the *wzm* mutants (M1 and M2,); (B) probe reactivity to *Bam*HI-digested genomic DNA from serotype O5 and the *wzt* mutants (T1 and T2). Hybridization to the gentamicin cassette was observed for both *wzm* and *wzt* mutants, with probe-reactive fragments of 2.3 and 3.4 kb, respectively. In each case, no hybridization with DNA from the parent strain PAO1 was seen. (C) Restriction maps of pFV300 (*wzm*) and pFV301 (*wzt*) insert DNAs, identifying the locations of the insertion of the 879-bp Gm^r cassette. The broken line represents a segment of *Xho*I probe-reactive DNA which falls outside the *Kpn*I-*Bam*HI pFV300 insert. Abbreviations for restriction sites: B, *Bam*HI; X, *Xho*I; K, *Kpn*I.

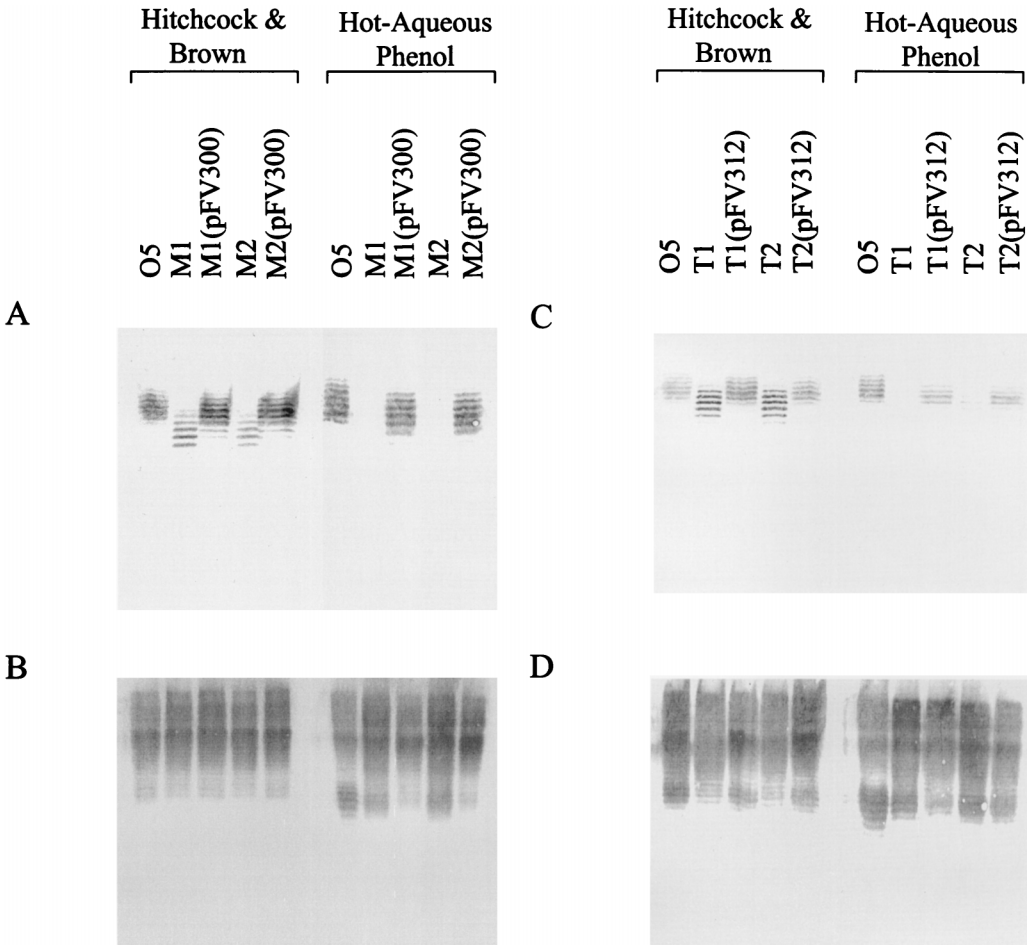

FIG. 6. Western immunoblots of the parent strain PAO1, *wzm* mutants (A and B), and *wzt* mutants (C and D) obtained by using the A-band-specific MAb N1F10 (A and C) and the O5-specific MAb MF15-4 (B and D). Two methods of LPS isolation were used, HB and HAP. Note that HB-isolated A-band LPS from the mutants M1, M2, T1, and T2 has increased rates of migration compared to that of PAO1 and that HAP-isolated A-band LPS from M1, M2, T1, and T2 is not detected (A and C).

tured in a medium containing 300 μg of gentamicin per ml prior to sample preparation for immunoelectron microscopy. As a result, MVs are present and can be used to determine cell surface association of A-band and B-band LPS. The *wzt* mutant T1 and the recombinant strain T1(pFV301) were found to have B-band LPS present on both outer membrane-associated MVs and free MVs found in the surrounding medium (Fig. 8A and B). However, A-band LPS was not found to be present on either the outer membrane-associated MVs or the free MVs in T1 (Fig. 8C). The recombinant strain T1(pFV301) did show A-band LPS on both the outer membrane-associated MVs and free MVs, indicating restoration of the ability to express A-band LPS on the cell surface (Fig. 8D).

DISCUSSION

Consistent with the involvement of Wzy and Wzz proteins in heteropolysaccharide synthesis, our laboratory has recently reported the identification of Wzy and Wzz in *P. aeruginosa* and has demonstrated their involvement in B-band LPS synthesis (10, 14). Chromosomal mutations generated in *wzy* and *wzz* had no effect on A-band LPS synthesis (10, 14). This current study suggests A-band polysaccharide is likely polymerized in the cytoplasm on a carrier lipid molecule and subsequently transported across the inner membrane by an ABC transport system (composed of Wzm and Wzt) for ligation to core-lipid A.

Of the ABC transport systems reported, Wzm and Wzt most closely resemble ORF261 and ORF431, respectively, components of the O-antigen transport system of *E. coli* O9. The integral membrane component Wzm was found to have six potential membrane-spanning domains, while the ATP-binding protein Wzt contains the highly conserved ATP-binding motif. Integral membrane proteins from the transport systems studied thus far reveal multiple membrane-spanning domains which are assumed to fold as α-helices (23). The N and C termini of the integral membrane proteins have been reported to be located on the cytoplasmic face of the inner membrane (23). From the hydropathy plot of Wzm (Fig. 2), it appears that the N and C termini of Wzm are also hydrophilic, indicating a possible cytoplasmic association. ATP-binding proteins are peripheral membrane components which contain the ATP-binding motif consisting of sites A and B which form an ATP-binding pocket (17). Although the mechanism of transport is not fully understood, it is generally thought that the ATP-binding domain may undergo a conformational change during ATP binding and hydrolysis which is transmitted to the integral membrane protein to initiate transport (23).

Analogous ABC transport systems have been reported for

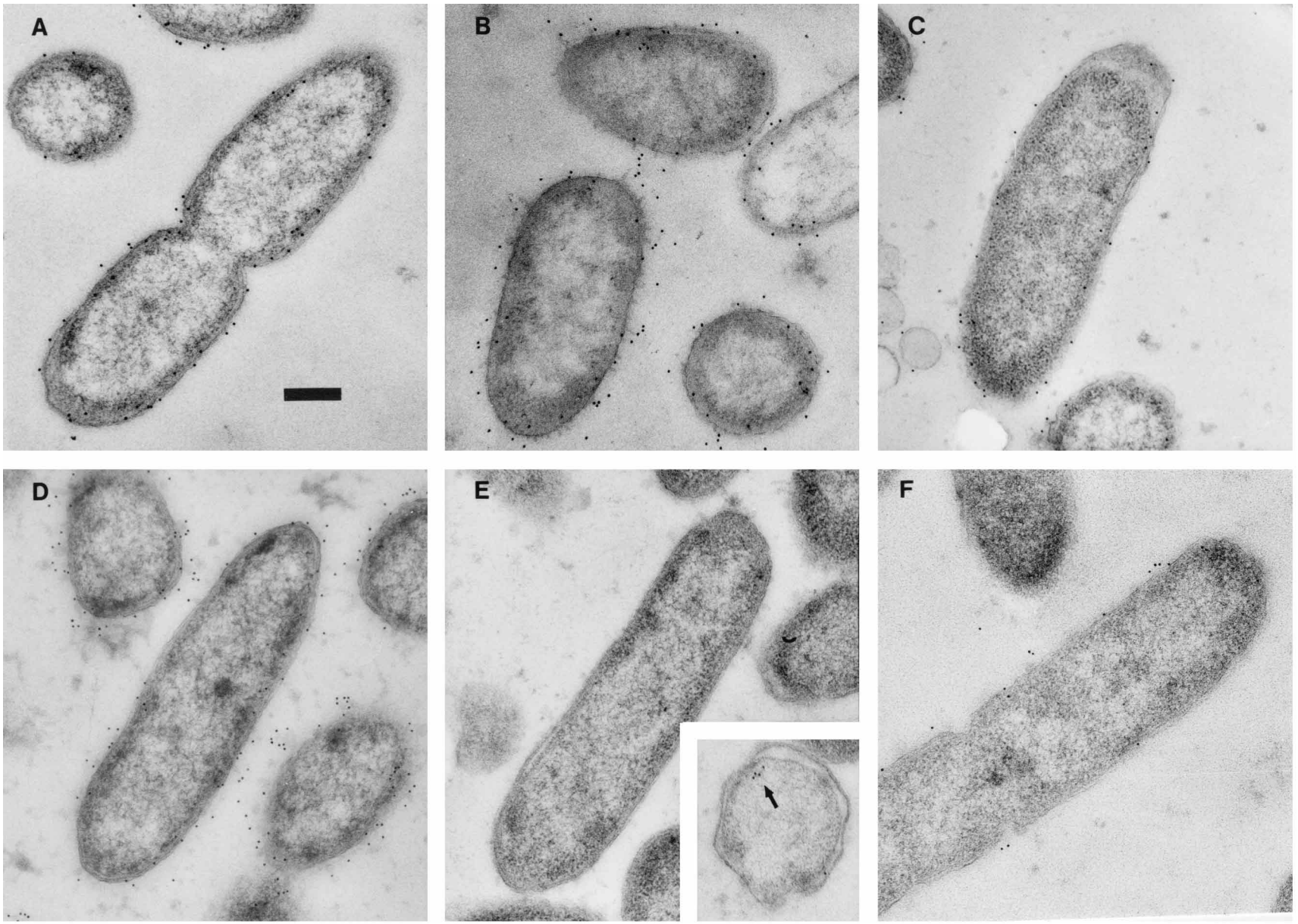

FIG. 7. Immunogold electron microscopic detection of LPS on thin sections of whole cells of PAO1 (A and D), *wzt* mutant T1 (B and E), and T1(pFV301) (C and F) labelled with antibodies specific for B-band LPS (A to C) and A-band LPS (D to F). B-band LPS was visualized on the cell surface of the T1 mutant, whereas A-band was not detected on the surface and instead was seen within the cytoplasm of strain T1 (panel E, insert). (A) Bar = 250 nm.

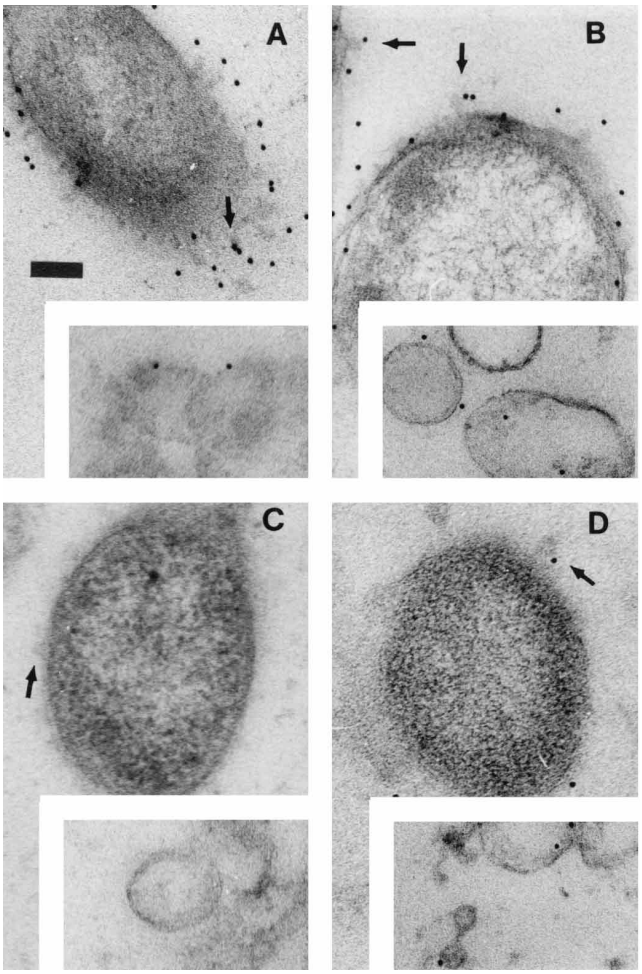

FIG. 8. Immunogold electron microscopy of LPS on thin sections of whole cells showing MVs forming on the cell surface and being released into the surrounding medium. Mutants T1 (A) and T1(pFV301) (B) show labelling of MVs associated with the cell surface (arrows) and free in the medium (inserts) with antibodies to B-band LPS. The T1 mutant shows no labelling of MVs with antibodies to A-band LPS (C), whereas T1(pFV301) shows labelling of both cell-associated (arrows) and free (insert) MVs with antibodies to A-band LPS (D). (A) Bar = 100 nm.

LPS O antigens and the group II class of capsular polysaccharides in several other gram-negative organisms. Various mutational studies using the capsular transport components KpsM and KpsT from both *E. coli* K1 (6, 51, 62), and K5 (7, 38) have shown that inhibition of polymer transport across the inner membrane results in cytoplasmic accumulation of capsular polysaccharide, as seen by electron microscopy. Bronner et al. (8) reported a similar mutational study using the O-antigen transport components of *K. pneumoniae* O1. *E. coli* K-12 harboring the rfb_{KpO1} gene cluster with *rfbA* and *rfbB* deletions was found to accumulate cytoplasmic O antigen, as seen by immunoelectron microscopy (using MAbs and polyclonal sera).

It is intriguing that *P. aeruginosa* has the unique ability to coexpress a homopolymeric and a heteropolymeric O antigen. This permits the study of both systems simultaneously, whereby effects of specific gene mutations can be observed for each of the two independent LPS types. In this study, both *wzm* and *wzt* chromosomal mutations which affected translocation of A-band polysaccharide across the inner membrane were generated in strain PAO1. B-band LPS expression was unaffected in these mutants. The accumulated A-band polymer was analyzed by two different LPS isolation methods and was detected on Western immunoblots. The size variability in migration patterns observed between LPS HB isolated from the *wzm* and *wzt* mutants and the parent strain PAO1 (Fig. 6A and C) is likely attributed to the lack of core residues in A-band polysaccharide isolated from the mutants. Electrically neutral A-band polysaccharide isolated from the *wzm* and *wzt* mutants must be attached to a charged molecule, likely a carrier lipid due to its ability to migrate in SDS-polyacrylamide gels and through its resistance to proteinase K treatment. Considering the steps required in homopolymeric O-antigen synthesis and that the linkage between A-band polysaccharide and the carrier lipid was shown in this study to be phenol labile, it seems likely that the carrier lipid molecule may be Und-P. This variability in migration has previously been observed in an outer core mutant of *E. coli* K-12 harboring the rfb_{EcO8} cluster (53). Truncated core-lipid A molecules are unable to serve as acceptors of O antigen, resulting in accumulation of the O8 polysaccharide, which demonstrates increased electrophoretic mobility in SDS-polyacrylamide gels (53).

Immunoelectron microscopy was performed on the *wzm* and *wzt* mutants to determine the cellular location of A-band and B-band LPS. For both *wzm* and *wzt* mutants, no A-band LPS was observed on the cell surface or associated with MVs (Fig. 7E and 8C). Some reactivity to the A-band-specific MAb could be seen within the cytoplasm (Fig. 7E), indicating that inhibition of transport results in cytoplasmic accumulation of A-band polysaccharide. Low levels of this cytoplasmic A-band polymer were detected by immunoelectron microscopy, possibly due to the limited availability of the carrier lipid molecule Und-P, which is also required for peptidoglycan and B-band LPS biosynthesis. Accumulated A-band polysaccharide–Und-P would therefore result in decreased cycling of Und-P, which may ultimately control the amount of A-band polysaccharide being synthesized.

B-band LPS was found to be maintained on the cell surface in both *wzm* and *wzt* mutants. Immunogold labelling of B-band LPS appeared to occur farther away from the cell surface in the mutant strains than in the parent strain PAO1 and the complemented mutants (Fig. 7A to C). If this anomaly were attributed to rearrangements in the LPS packing due to interruptions in LPS cross-bridging upon gentamicin binding, one would expect to see similar labelling in the complemented mutant, since both were propagated in a medium supplemented with gentamicin. Instead, the increased distance of colloidal gold labels relative to the outer membrane may be due to a lack of cell-surface A band, which could influence the overall architecture of B-band LPS.

To date, little is known about cell surface conformation of LPSs in gram-negative organisms; however, it is recognized as being a dynamic process. The most information has recently come from low-energy modelling and X-ray diffraction studies performed by Labischinski et al. (40) and Kastowsky et al. (29). In the case of *P. aeruginosa*, how the presence or absence of A-band and B-band LPS affects the overall arrangement of cell surface polysaccharides is unknown. Ultrastructural examination of *P. aeruginosa* LPS has been performed by Lam et al. (41) using freeze-substitution and electron microscopy, which together demonstrate the presence of a fibrous layer surrounding cells expressing O antigen. A three-dimensional model on LPS conformation which suggests that the core region may run parallel to the outer membrane, with the O antigen being flexible enough to extend from the cell surface, has been proposed by Kastowsky et al. (29). The longer O antigens may

cross over, providing a stable, feltlike network on the cell surface, giving rise to a protective barrier (29). Since B-band LPS produces longer, negatively charged O side chains, it will likely form this type of network. A-band LPS which produces shorter, neutral O side chains may extend from the cell surface, with a portion of the molecules being drawn within this network formed by B-band LPS. Loss of A-band LPS may influence the spatial arrangement of negative charges as well as overall LPS fluidity. This may result in disruptions in the LPS packing which could influence the formation of the B-band LPS network on the cell surface and could be responsible for the increased distance in colloidal gold labelling observed for the mutants in this study (Fig. 7B).

In conclusion, this study reports the identification of an ABC transport system in *P. aeruginosa* that is required for the export of A-band polysaccharide from the cytoplasm to the periplasm. Mutations in *wzm* and *wzt* result in an A-band polysaccharide located within the cytoplasm which is attached to a carrier lipid molecule, likely Und-P. Although B-band LPS synthesis is not directly affected by these mutations, the overall cell surface arrangement of B band may be affected by the presence or absence of A-band LPS.

ACKNOWLEDGMENTS

This work was supported by grants to J.S.L. from the Canadian Cystic Fibrosis Foundation. H.L.R. is a recipient of a Canadian Cystic Fibrosis Foundation Studentship. The electron microscopy was performed in the NSERC Regional Scanning Transmission Electron Microscopy Facility in the Department of Microbiology, University of Guelph, through the generous assistance of D. Moyles, who was funded by the Canadian Bacterial Disease Network.

We thank our colleagues, particularly C. Whitfield, L. L. Burrows, T. J. Beveridge, and J. L. Kadurugamuwa, for many valuable discussions.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Arsenault, T. L., D. W. Huges, D. B. MacLean, W. A. Szarek, A. M. B. Kropinski, and J. S. Lam.** 1991. Structural studies on the polysaccharide portion of 'A-band' lipopolysaccharide from a mutant (AK14O1) of *Pseudomonas aeruginosa* strain PAO1. Can. J. Chem. **69:**1273–1280.
3. **Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl (ed.).** 1992. Short protocols in molecular biology, p. 61. John Wiley and Sons, New York, N.Y.
4. **Binotto, J., R. MacLachlan, and K. E. Sanderson.** 1991. Electrotransformation in *Salmonella typhimurium* LT2. Can. J. Microbiol. **37:**474–477.
5. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmids. Nucleic Acids Res. **7:**1513–1523.
6. **Boulnois, G. J., I. S. Roberts, R. Hodge, K. R. Hardy, K. B. Jann, and K. N. Timmis.** 1987. Analysis of the K1 capsule biosynthesis genes of *Escherichia coli*: definition of three functional regions for capsule production. Mol. Gen. Genet. **208:**242–246.
7. **Bronner, D., V. Sieberth, C. Pazzani, A. Smith, G. Boulnois, I. Roberts, B. Jann, and K. Jann.** 1993. Synthesis of the K5 (group II) capsular polysaccharide in transport-deficient recombinant *Escherichia coli*. FEMS Microbiol. Lett. **113:**279–284.
8. **Bronner, D., B. R. Clarke, and C. Whitfield.** 1994. Identification of an ATP-binding cassette transport system required for translocation of lipopolysaccharide O-antigen side-chains across the cytoplasmic membrane of *Klebsiella pneumoniae* serotype O1. Mol. Microbiol. **14:**505–519.
9. **Burnette, W. N.** 1981. 'Western blotting': electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
10. **Burrows, L. L., D. Chow, and J. S. Lam.** 1997. *Pseudomonas aeruginosa* B-band O-antigen chain length is modulated by Wzz (Rol). J. Bacteriol. **179:**1482–1489.
11. **Chu, S., and T. J. Trust.** 1993. An *Aeromonas salmonicida* gene which influences A-protein expression in *Escherichia coli* encodes a protein containing an ATP-binding cassette and maps beside the surface array protein gene. J. Bacteriol. **175:**3105–3114.
12. **Clarke, B. R., and C. Whitfield.** 1992. Molecular cloning of the *rfb* region of *Klebsiella pneumoniae* serotype O1:K20: the *rfb* gene cluster is responsible for synthesis of the D-galactan I O polysaccharide. J. Bacteriol. **174:**4614–4621.
13. **Darzins, A., and A. M. Chakrabarty.** 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. J. Bacteriol. **159:**9–18.
14. **de Kievit, T. R., T. Dasgupta, H. Schweizer, and J. S. Lam.** 1995. Molecular cloning and characterization of the *rfc* gene of *Pseudomonas aeruginosa* (serotype O5). Mol. Microbiol. **16:**565–574.
15. **Eisenberg, D., R. M. Weiss, and T. C. Terwilliger.** 1982. Definition of helical hydrophobic moment computed in HELWHEEL and HELIXMEM. Nature **299:**371–374.
16. **Farinha, M. A., and A. M. Kropinski.** 1990. High efficiency electroporation of *Pseudomonas aeruginosa* using frozen cell suspensions. FEMS Microbiol. Lett. **70:**221–226.
17. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol. Rev. **57:**995–1017.
18. **Ford, N., C. Nolan, and M. Ferguson.** 1989. Transfer of DNA to nitrocellulose filters, p. 9.38–9.40. *In* J. Sambrook, E. F. Fritsch, and T. Maniatis (ed.), Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Gish, W., and D. J. States.** 1993. Identification of protein coding regions by database similarity search. Nat. Genet. **3:**266–272.
20. **Guo, D., M. Gabriela Bowden, R. Pershad, and H. B. Kaplan.** 1996. The *Myxococcus xanthus rfbABC* operon encodes an ATP-binding cassette transporter homolog required for O-antigen biosynthesis and multicellular development. J. Bacteriol. **178:**1631–1639.
21. **Hancock, R. E. W., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140:**902–910.
22. **Hancock, R. E. W., L. M. Mutharia, L. Chan, R. P. Darveau, D. P. Speert, and G. B. Pier.** 1983. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. Infect. Immun. **42:**170–177.
23. **Higgins, C. F.** 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8:**67–113.
24. **Higgins, C. F., M. P. Gallagher, S. C. Hyde, M. L. Mimmack, and S. R. Pearce.** 1990. Periplasmic binding protein-dependent transport systems: the membrane-associated components. Philos. Trans. R. Soc. Lond. Ser. B **326:**353–365.
25. **Hiles, I. D., M. P. Gallagher, D. J. Jamieson, and C. F. Higgins.** 1987. Molecular characterization of the oligopeptide permease of *Salmonella typhimurium*. J. Mol. Biol. **195:**125–142.
26. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide types in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
27. **Jann, K., and B. Jann.** 1984. Structure and biosynthesis of O-antigens, p. 138–186. *In* E. T. Rietschel (ed.), Handbook of endotoxin. Elvsevier Science, Amsterdam, The Netherlands.
28. **Kadurugamuwa, J. L., and T. J. Beveridge.** 1995. Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme section. J. Bacteriol. **177:**3998–4008.
29. **Kastowsky, M., T. Gutberlet, and H. Bradaczek.** 1992. Molecular modelling of the three-dimensional structure and conformational flexibility of bacterial lipopolysaccharide. J. Bacteriol. **174:**4798–4806.
30. **Keenleyside, W. J., and C. Whitfield.** 1996. A novel pathway for O-polysaccharide biosynthesis in *Salmonella enterica* serovar Borreze. J. Biol. Chem. **271:**28581–28592.
31. **Kent, J. L., and M. J. Osborn.** 1968. Properties of the O-specific hapten formed in vivo by mutant strains of *Salmonella typhimurium*. Biochemistry **7:**4396–4408.
32. **Kerppola, R. E., V. K. Shyamala, P. Klebba, and G. F.-L. Ames.** 1991. The membrane-bound proteins of periplasmic permeases form a complex. J. Biol. Chem. **266:**9857–9865.
33. **Kido, N., V. I. Torgov, T. Sugiyama, K. Uchiya, H. Sugihara, T. Komatsu, N. Kato, and K. Jann.** 1995. Expression of the O9 polysaccharide of *Escherichia coli*: sequencing of the *E. coli* O9 *rfb* gene cluster, characterization of mannosyl transferases, and evidence for an ATP-binding cassette transport system. J. Bacteriol. **177:**2178–2187.
34. **Klein, P., M. Kanehisa, and C. DeLisi.** 1985. The detection and classification of membrane-spanning proteins. Biochim. Biophys. Acta **815:**468–476.
35. **Klena, J. D., and C. A. Schnaitman.** 1993. Function of the *rfb* gene cluster and the *rfe* gene in the synthesis of O antigen by *Shigella dysenteriae* 1. Mol. Microbiol. **9:**393–402.
36. **Knirel, Y. A.** 1990. Polysaccharide antigens of *Pseudomonas aeruginosa*. Crit. Rev. Microbiol. **17:**273–304.
37. **Kroll, J. S., B. Loynds, L. N. Brophy, and E. R. Moxon.** 1990. The *bex* locus in encapsulated *Haemophilus influenzae*: a chromosomal region involved in capsule polysaccharide export. Mol. Microbiol. **4:**1853–1862.
38. **Kröncke, K.-D., G. Boulnois, I. Roberts, D. Bitter-Suermann, J. R. Golecki, B. Jann, and K. Jann.** 1990. Expression of the *Escherichia coli* K5 capsular antigen: immunoelectron microscopic and biochemical studies with recom-

binant *E. coli*. J. Bacteriol. **172:**1085–1091.
39. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
40. **Labischinski, H., G. Barnickel, H. Bradaczek, D. Naumann, E. T. Rietschel, and P. Giesbrecht.** 1985. High state of order of isolated bacterial lipopolysaccharide and its possible contribution to the permeation barrier property of the outer membrane. J. Bacteriol. **162:**9–20.
41. **Lam, J. S., L. L. Graham, J. Lightfoot, T. Dasgupta, and T. J. Beveridge.** 1992. Ultrastructural examination of the lipopolysaccharides of *Pseudomonas aeruginosa* strains and their isogenic rough mutants by freeze-substitution. J. Bacteriol. **174:**7159–7167.
42. **Lam, M. Y., E. J. McGroarty, A. M. Kropinski, L. A. MacDonald, S. S. Pedersen, N. Høiby, and J. S. Lam.** 1989. Occurrence of a common lipopolysaccharide antigen in standard and clinical strains of *Pseudomonas aeruginosa*. J. Clin. Microbiol. **27:**962–967.
43. **Lazarevic, V., and D. Karamata.** 1995. The *tagGH* operon of *Bacillus subtilis* 168 encodes a two-component ABC transporter involved in the metabolism of two wall teichoic acids. Mol. Microbiol. **16:**345–355.
44. **Lightfoot, J., and J. S. Lam.** 1991. Molecular cloning of genes involved with expression of A-band lipopolysaccharide, an antigenically conserved form, in *Pseudomonas aeruginosa*. J. Bacteriol. **173:**5624–5630.
45. **Lightfoot, J., and J. S. Lam.** 1993. Chromosomal mapping, expression and synthesis of lipopolysaccharide in *Pseudomonas aeruginosa*: a role for guanosine diphospho (GDP)-D-mannose. Mol. Microbiol. **8:**771–782.
46. **Liu, P. V., H. Matsumoto, H. Kusama, and T. Bergan.** 1983. Survey of heat-stable major somatic antigens of *Pseudomonas aeruginosa*. Int. J. Syst. Bacteriol. **33:**256–264.
47. **Liu, P. V., and S. P. Wang.** 1990. Three new major somatic antigens of *Pseudomonas aeruginosa*. J. Clin. Microbiol. **28:**922–925.
48. **Manning, P. A., U. H. Stroeher, L. E. Karageorgos, and R. Morona.** 1995. Putative O-antigen transport genes within the *rfb* region of *Vibrio cholerae* O1 are homologous to those for capsule transport. Gene **158:**1–7.
49. **Myers, E. W., and W. Miller.** 1988. Optimal alignments in linear space. Comp. Appl. Biosci. **4:**11–17.
50. **Palleroni, N. J.** 1984. Genus I. *Pseudomonas* Migula 1894, 237^{AL} (nom. cons. opin. 5, jud. comm. 1952, 237), p. 141–199. *In* N. R. Krieg, and J. C. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore, Md.
51. **Pavelka, M. S., Jr., L. F. Wright, and R. P. Silver.** 1991. Identification of two genes, *kpsM* and *kpsT*, in region 3 of the polysialic acid gene cluster of *Escherichia coli* K1. J. Bacteriol. **173:**4603–4610.
52. **Reeves, P. R., M. Hobbs, M. A. Valvano, M. Skurnik, C. Whitfield, D. Copin, N. Kido, J. Klena, D. Maskell, C. R. H. Raetz, and P. D. Rick.** 1996. Bacterial polysaccharide synthesis and gene nomenclature. Trends Microbiol. **4:**495–503.
53. **Rick, P. D., G. L. Hubbard, and K. Barr.** 1994. Role of the *rfe* gene in the synthesis of the O8 antigen in *Escherichia coli* K-12. J. Bacteriol. **176:**2877–2884.
54. **Rocchetta, H. L., L. L. Burrows, J. C. Pacan, and J. S. Lam.** Unpublished data.
55. **Rocchetta, H. L., J. C. Pacan, and J. S. Lam.** Unpublished data.
56. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-encoded proteins. J. Bacteriol. **137:**692–693.
57. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. BioTechniques **15:**831–833.
58. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:** 15–22.
59. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad-host-range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.
60. **Smith, A. N., G. J. Boulnois, and I. S. Roberts.** 1990. Molecular analysis of the *Escherichia coli* K5 *kps* locus: identification and characterization of an inner-membrane capsular polysaccharide transport system. Mol. Microbiol. **4:**1863–1869.
61. **Szabo, M., D. Bronner, and C. Whitfield.** 1995. Relationships between *rfb* gene clusters required for biosynthesis of identical D-galactose-containing O antigens in *Klebsiella pneumoniae* serotype O1 and *Serratia marcescens* serotype O16. J. Bacteriol. **177:**1544–1553.
62. **Vimr, E. R., W. Aaronson, and R. P. Silver.** 1989. Genetic analysis of chromosomal mutations in the polysialic acid gene cluster of *Escherichia coli* K1. J. Bacteriol. **171:**1106–1117.
63. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases, and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.
64. **West, S. E. H., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and the sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **128:**81–86.
65. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5:**83–91.
66. **Whitfield, C.** 1995. Biosynthesis of lipopolysaccharide O antigens. Trends Microbiol. **3:**178–185.
67. **Zhang, L., A. Al-Hendy, P. Toivanen, and M. Skurnik.** 1993. Genetic organization and sequence of the *rfb* gene cluster of *Yersinia enterocolitica* serotype O:3: similarities to the dTDP-L-rhamnose biosynthesis pathway of *Salmonella* and to the bacterial polysaccharide transport systems. Mol. Microbiol. **9:**309–321.
68. **Zhang, L., J. Radziejewska-Lebrecht, D. Krajewska-Pietrasik, P. Toivanen, and M. Skurnik.** 1997. Molecular and chemical characterization of the lipopolysaccharide O-antigen and its role in the virulence of *Yersinia enterocolitica* serotype O:8. Mol. Microbiol. **23:**63–76.