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Isolation and Characterization of Two Genes, *waaC* (*rfaC*) and *waaF* (*rfaF*), Involved in *Pseudomonas aeruginosa* Serotype O5 Inner-Core Biosynthesis

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Recent studies have provided evidence to implicate involvement of the core oligosaccharide region of *Pseudomonas aeruginosa* lipopolysaccharide (LPS) in adherence to host tissues. To better understand the role played by LPS in the virulence of this organism, the aim of the present study was to clone and characterize genes involved in core biosynthesis. The inner-core regions of *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium are structurally very similar; both contain two main chain residues of heptose linked to lipid A-Kdo$_2$ (Kdo is 3-deoxy-D-manno-octulosonic acid). By electrotransforming a *P. aeruginosa* PAO1 library into *Salmonella waaC* and *waaF* (formerly known as *rfaC* and *rfaF*, respectively) mutants, we were able to isolate the homologous heptosyltransferase I and II genes of *P. aeruginosa*. Two plasmids, pCOREc1 and pCOREc2, which restored smooth LPS production in the *waaC* mutant, were isolated. Similarly, plasmid pCOREf1 was able to complement the *Salmonella waaF* mutant. Sequence analysis of the DNA insert of pCOREc2 revealed one open reading frame (ORF) which could code for a protein of 39.8 kDa. The amino acid sequence of the deduced protein exhibited 53% identity with the sequence of the WaaC protein of *S. enterica* serovar Typhimurium. pCOREf1 contained one ORF capable of encoding a 38.4-kDa protein. The sequence of the predicted protein was 49% identical to the sequence of the *Salmonella* WaaF protein. Protein expression by the Maxicell system confirmed that a 40-kDa protein was encoded by pCOREc2 and a 38-kDa protein was encoded by pCOREf1. Pulsed-field gel electrophoresis was used to determine the map locations of the cloned *waaC* and *waaF* genes, which were found to lie between 0.9 and 6.6 min on the PAO1 chromosome. Using a gene-replacement strategy, we attempted to generate *P. aeruginosa waaC* and *waaF* null mutants. Despite multiple attempts to isolate true knockout mutants, all transconjugants were identified as merodiploids.

Pseudomonas aeruginosa is an opportunistic pathogen capable of causing fatal infections in immunocompromised and debilitated individuals. The intrinsic resistance of this organism to many common antibiotics necessitates the development of new therapeutic strategies for treatment of infections. *P. aeruginosa* owes much of its success as a pathogen to its ability to elicit a wide variety of virulence determinants, including both secreted and cell-associated factors. Within this latter category is the amphipathic molecule lipopolysaccharide (LPS) (12). Most strains of *P. aeruginosa* coexpress two distinct forms of LPS known as A-band and B-band. A-band is an antigenically conserved molecule made up of D-rhamnose units (3), while B-band is the O-antigen-containing LPS. Chemical and structural differences in B-band LPS form the basis for dividing *P. aeruginosa* into the 20 serotypes recognized in the International Antigenic Typing Scheme (32, 33).

The contribution of wild-type smooth LPS as an important virulence attribute of *P. aeruginosa* has been established in a number of studies using mouse challenge models (12, 48). Smooth LPS is also responsible for the ability of *P. aeruginosa* to resist the bactericidal effect of serum (13, 24, 42) and phagocytic killing (17). Interestingly, results from a number of recent studies which examined the interactions of cultured human airway epithelial cells as well as corneal epithelial cells with rough mutants of *P. aeruginosa* suggested that the core oligosaccharide of *P. aeruginosa* LPS could be a ligand for interaction with host tissues (22, 37, 50). To clearly define the function of core oligosaccharides in host-bacterium interactions, it is important to have a better understanding of the biosynthesis of the core oligosaccharide of *P. aeruginosa* LPS and to obtain a full range of rough mutants comparable to the Ra to Re mutants of *Salmonella* spp.

As with members of the family *Enterobacteriaceae*, *P. aeruginosa* LPS biosynthetic genes tend to be clustered at distinct chromosomal loci. In serotype O5, A-band LPS genes (*psa*) map at 13 min, while genes involved in B-band (*wbp*) synthesis are located at 37 min on the *P. aeruginosa* chromosome (31). To date, the LPS core (*waa*) gene cluster has not been identified. Only one gene has been isolated which appears to be involved in the synthesis of the *P. aeruginosa* core region (20). This gene, called *algC*, is able to restore smooth LPS production in strain AK1012, a core-deficient mutant of PAO1 (serotype O5) (27). The block in LPS synthesis in AK1012 was shown by Jarrell and Kropinski (26) to be at the point of attachment of a Glc residue onto the GalN in the outer core. Interestingly, AlgC is believed to be required for the production of UDP-Glc (11). AlgC is also required for alginate production (11); therefore, it appears to be involved in the synthesis of intermediates of both pathways and is not part of the putative core gene cluster.

Isolation of *P. aeruginosa waa* genes has been partially hampered by a lack of available mutants harboring deficiencies in this region. Some *P. aeruginosa* mutants which express partial core structures have been identified, and these mutants have

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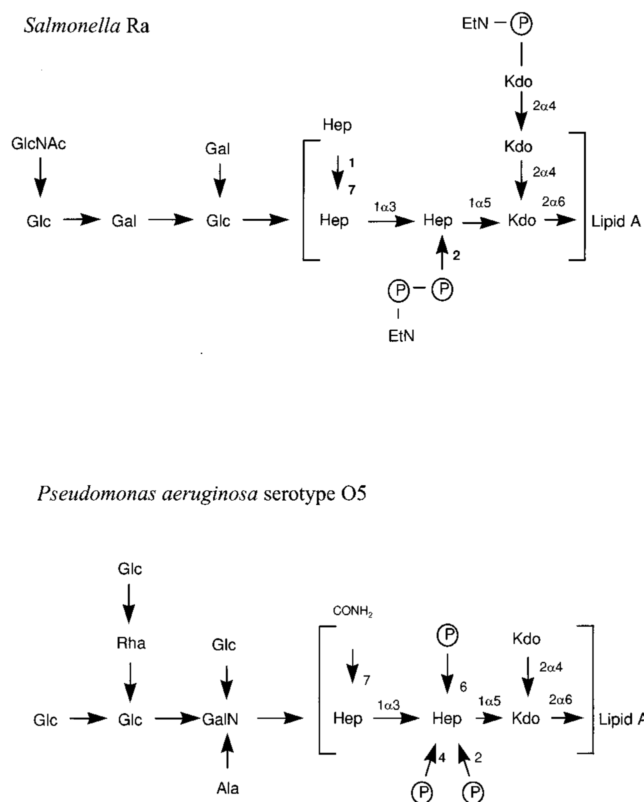

FIG. 1. Proposed structures for the core oligosaccharide of *S. enterica* serovar Typhimurium and *P. aeruginosa* serotype O5. The region which is conserved between the two organisms is indicated in brackets. Linkages between the sugars within this common region are also shown. Glc, D-glucose; Gal, D-galactose; GlcNAc, *N*-acetylglucosamine; Hep, heptose (L-glycero-D-*manno*-heptose); Rha, L-rhamnose; Ala, alanine; GalN, D-galactosamine; P, phosphate group; EtN, ethanolamine; $CONH_2$, *O*-carbamoyl. The *S. enterica* core was adapted from the work of Reeves (38), and the *P. aeruginosa* core was adapted from the work of Altman et al. (1).

been used to elucidate the detailed structure of the serotype O5 and O6 core region (1, 34). Comparison of the O5 and O6 core structures with that of *Salmonella enterica* serovar Typhimurium reveals that while the *P. aeruginosa* outer core differs substantially from that of *Salmonella*, the inner core is quite conserved (Fig. 1). Both organisms have an inner core comprised of two main chain molecules of heptose and at least two molecules of Kdo (3-deoxy-D-*manno*-octulosonic acid). In addition, the linkages between these inner-core sugars are identical.

In *Salmonella* and *Escherichia coli*, genes associated with the transfer of the two inner-core sugars, heptose and Kdo, are located within the *waa* cluster. Based on this finding, we proposed that the isolation of homologous *P. aeruginosa* inner-core genes might lead to the identification of the putative *waa* gene cluster of this organism. To date, no *P. aeruginosa* mutants expressing the deep-rough LPS phenotype, i.e., LPS comprised solely of lipid A-Kdo or lipid A substituted with Kdo and heptose, have been isolated. However, in *Salmonella*, a well-characterized panel of LPS mutants exists, and studies have shown that genes from various bacterial species are capable of complementing *Salmonella* core mutations (9, 29, 51). Structural similarities within the inner-core regions of *P. aeruginosa* and *Salmonella* and the apparent functional exchangeability of inner-core biosynthetic genes prompted us to make use of defined *Salmonella* mutants for isolating *P. aeruginosa* *waa* genes (based on the proposal made by Reeves et al. [39], a new nomenclature was used to replace the *rfa* designations). Through complementation of *Salmonella* strains containing defective *waaC* and *waaF* genes, we were able to clone the homologous heptosyl transferase I and II genes of *P. aeruginosa*. The *P. aeruginosa waaF* and *waaC* genes contain overlapping transcriptional stop and start sequences, suggesting that they form part of an operon. In addition, the chromosomal location of the *waa* genes was determined for the first time. These genes were found to lie between 0.9 and 6.6 min on the 75-min map of strain PAO1.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Miller's Luria broth (Difco Laboratories, Detroit, Mich.) was used for maintenance of bacterial strains. Pseudomonas isolation agar (PIA; Difco) and Davis minimal media (Difco) were used for selection of transconjugants following mating experiments. Antibiotics used in selection media included ampicillin at 100 μg/ml for *E. coli* and carbenicillin at 450 μg/ml for *P. aeruginosa*, tetracycline at 15 μg/ml for *E. coli* and 90 μg/ml for *P. aeruginosa* (250 μg/ml in PIA), and gentamicin at 10 μg/ml for *E. coli* and 300 μg/ml for *P. aeruginosa*. Bacteriophage sensitivity tests were done by the method of Wilkinson et al. (49).

DNA procedures. Plasmid DNA was isolated in small-scale amounts by utilizing the alkaline lysis method of Birnboim and Doly (5), while large-scale preparations were obtained with the Qiagen (Chatsworth, Calif.) midi plasmid kit as described in the manufacturer's instructions. *P. aeruginosa* whole genomic DNA was isolated by the method of Goldberg and Ohman (21). Restriction enzymes were purchased from GIBCO/BRL and Boehringer GmbH (Mannheim, Germany). T4 DNA ligase, T4 DNA polymerase, and alkaline phosphatase were purchased from Boehringer. All enzymes were used as described in the suppliers' recommendations. DNA was transformed into *E. coli* and *S. enterica* serovar Typhimurium by electroporation with a Bio-Rad (Richmond, Calif.) Gene Pulser electroporation unit and by following protocols supplied by the manufacturer. Electrocompetent cells of *E. coli* and *S. enterica* serovar Typhimurium were prepared by the method of Binotto et al. (4). Recombinant plasmids were mobilized from *E. coli* SM10 to *P. aeruginosa* by the method of Simon et al. (46). Genomic DNA was transferred to a Zetaprobe membrane (Bio-Rad) by capillary transfer as described in the manufacturer's instructions. Southern hybridizations were done as described previously (15).

Construction of a *P. aeruginosa* gene library. A genomic library of *P. aeruginosa* PAO1 was constructed by the method of Goldberg and Ohman (21) with the following modifications. Partial *Sau*3AI fragments of predominantly 2 to 10

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Strains		
P. aeruginosa		
PAO1	Serotype O5; A^+ B^+	23
PAK	Serotype O5	W. Paranchych[a]
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	46
S. enterica serovar Typhimurium		
L3770	waa^+	40
SA1377	*waaC630*	8
SL3789	*waaF511*	40
Plasmids		
pBluescript-II vector KS	Ap^r	PDI Biosciences
pEX100T	Gene replacement vector; $oriT^+$ $sacB^+$ Ap^r	45
pUCPGm	Source of Gm^r cassette; Ap^r Gm^r	44

[a] W. Paranchych, University of Alberta, Edmonton, Alberta, Canada.

kb were ligated with *Bam*HI-digested vector pBluescript. The recombinant plasmids were electrotransformed into *E. coli* DH5α. Transformants were allowed to recover in SOC medium (2% Bacto Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) for several hours before being subjected to large-scale plasmid extraction. The plasmid library was then electrotransformed into *waaC* and *waaF* mutants of *S. enterica* serovar Typhimurium.

DNA sequencing. DNA sequence analysis of the O5 *waaF* and *waaC* genes was performed at the MOBIX facility (McMaster University, Hamilton, Ontario, Canada). Sequencing of the 1.5-kb insert of pCOREf1 and the 2.2-kb insert of pCOREc2 was done with a model 373A DNA sequencing unit (Applied Biosystems, Foster City, Calif.). An Applied Biosystems model 391 DNA synthesizer was used to generate oligodeoxynucleotide sequencing primers. The Taq Dye Deoxy terminator cycle sequencing kit (Applied Biosystems) was used for cycle sequencing reactions, which were carried out in an Ericomp (San Diego, Calif.) model TCX15 thermal cycler.

Sequence analysis. The computer software program Gene Runner for Windows (Hastings Software, New York, N.Y.) was used for nucleic acid and amino acid sequence analysis. Homology searches of the nucleotide and amino acid sequences of the *P. aeruginosa waaC* and *waaF* genes were performed with EMBL/GenBank/PDB and SWISS-PROT (release 28.0) databases (2, 19).

Maxicell analysis of plasmid DNA. Analysis of plasmid-encoded proteins was done by the method of Sancar et al. (41). Maxicells were prepared as described previously by Lightfoot and Lam (31), with the following modifications. Plasmids were electroporated into *E. coli* CSR603. Overnight cultures were diluted 1:50 in 10 ml of supplemented Davis medium lacking antibiotics. The cultures were grown to the mid-logarithmic phase, after which time they were irradiated for 30 s at 500 $\mu W/cm^2$ with a germicidal lamp. Expressed proteins were labelled with a Trans^{35}S-labelled methionine (ICN Biomedicals).

PFGE. Procedures for pulsed-field gel electrophoresis (PFGE) were as described by Lightfoot and Lam (31).

Mutagenesis of the *waaC* and *waaF* genes of *P. aeruginosa*. Using a previously described gene replacement strategy (15), we attempted to generate *waaC* and *waaF* null mutants of *P. aeruginosa*. The suicide vector that was used in these experiments, pEX100T, contains a copy of the *Bacillus subtilis sacB* gene which imparts sucrose sensitivity to gram-negative organisms (45). The presence of the vector-associated *sacB* gene in the chromosome of the merodiploids renders them sucrose sensitive. Therefore, streaking cells on sucrose-containing medium allows separation of true recombinants from the more frequently occurring merodiploids.

Preparation of LPS. LPS used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) experiments was prepared by the proteinase K digest method of Hitchcock and Brown (25).

SDS-PAGE. The discontinuous SDS-PAGE procedure of Hancock and Carey (23) with 15% running gels was used. LPS separated by SDS-PAGE was visualized by silver staining by the method of Dubray and Bezard (16).

Immunoblotting. The Western blot procedure of Burnette (6) was used with the following modifications. Nitrocellulose blots were blocked with 3% (wt/vol) skim milk and then incubated with polyclonal antisera raised against the wild-type *S. enterica* serovar Typhimurium strain SL3770. The blots were developed at room temperature, with goat anti-rabbit $F(ab')_2$ alkaline phosphatase-conjugated antibody (Jackson Immunoresearch Laboratories, West Grove, Pa.), and a substrate consisting of 30 mg of Nitro Blue Tetrazolium and 15 mg of 5-bromo-4-chloro-3-indolyl phosphate toluidine (Sigma Chemical Co., St. Louis, Mo.) in 100 ml of 0.1 M bicarbonate buffer (pH 9.8).

Immunogen preparation and polyclonal antibody production. For immunizing rabbits, formalin-fixed whole cells of the *S. enterica* serovar Typhimurium wild-type strain SL3770 were used. Immunogen was prepared as described by Lam et al. (28). Two New Zealand White female rabbits were used for production of polyclonal sera. Preimmune serum was collected and pooled to check for preimmune nonspecific antibodies. Immunization and bleeding of the animals were performed as described by Lam et al. (28). All sera were collected and stored at −20°C until used. To determine the optimal dilution of the polyclonal sera, Western blots of LPS from strain SL3770 were incubated with sera which had been serially diluted 10-fold in phosphate-buffered saline. A 1-to-10,000 dilution was used in subsequent Western blot experiments.

Nucleotide sequence accession numbers. The nucleotide sequences of the *P. aeruginosa waaC* and *waaF* genes have been submitted to GenBank under accession numbers U70982 and U70983, respectively.

RESULTS

Isolation of the *waaC* and *waaF* genes of *P. aeruginosa*. A *P. aeruginosa* serotype O5 plasmid library was generated in vector pBluescript and electrotransformed into *S. enterica* serovar Typhimurium SA1377 (*waaC* mutant) and SL3789 (*waaF* mutant). After recovery in SOC medium, *Salmonella* cells were plated on L agar containing novobiocin (100 μg/ml) and ampicillin (100 μg/ml) and incubated at 37°C overnight. Novobiocin was added to the medium because *S. enterica* serovar Typhimurium deep-rough strains are sensitive to this antibiotic. Therefore, cells able to grow on this medium are those that do not have the deep-rough phenotype. Several SA1377 and SL3789 Nb^r Amp^r transformants were isolated. Plasmids were extracted from these transformants and retransformed into the appropriate *Salmonella* mutants to ensure their ability to confer the Nb^r phenotype. Two plasmids which were able to complement the *Salmonella waaC* mutant, SA1377, were identified. Restriction enzyme analysis of the two plasmids revealed that they contained 6.1- and 2.2-kb inserts, and the plasmids were designated pCOREc1 and pCOREc2, respectively. Similarly, a plasmid containing a 1.5-kb insert, designated pCOREf1, was able to restore growth on novobiocin in the *Salmonella waaF* mutant. Transformation of pCOREc1 and pCOREc2 into the *waaF* mutant did not result in restoration of smooth LPS production, indicating that a complete *waaF* gene was not present on either of these plasmids. The restriction maps of pCOREc1, pCOREc2, and pCOREf1 are shown in Fig. 2.

Characterization of LPS expressed by *Salmonella* SA1377 (pCOREc1), SA1377(pCOREc2), and SL3789(pCOREf1) transformants. LPS expressed by the SA1377 and SL3789 transformants, containing the putative *P. aeruginosa waaC* and *waaF* genes, was characterized by phage sensitivity, SDS-PAGE analysis, and Western blot analysis. The phage FFM, which is specific for deep-rough *Salmonella* LPS (49), was added to the freshly inoculated *Salmonella* transformants and the wild-type *S. enterica* strain SL3770. The phage readily lysed the two core mutants, but it had no effect on either the wild-type strain SL3770 or the *Salmonella* transformants containing the *P. aeruginosa waaC* and *waaF* genes (data not shown). Analysis of LPS by SDS-PAGE revealed that transformant strains SL3789 (pCOREf1) and SA1377(pCOREc2) (Fig. 3a), as well as SA1377(pCOREc1) (data not shown), all expressed long-chain LPS. In Western blots, antiserum raised against wild-type *S. enterica* serovar Typhimurium SL3770 reacted with high-molecular-weight LPS from both SL3770 and the transformants (Fig. 3b). These results confirmed the ability of the *P. aeruginosa waa* genes to restore smooth LPS expression in the mutants. A weak reaction of high-molecular-weight LPS bands from the *Salmonella waaC* and *waaF* mutants, strains SA1377 and SL3789, respectively, with the *S. enterica* SL3770-specific antiserum was also observed (Fig. 3b). The presence of long-chain O antigen indicates that these mutants are either leaky or possibly that O hapten, which is not capable of attaching to a heptoseless core on the core-lipid A of these mutants, is present in the samples.

Nucleotide sequence determination of *waaC* and *waaF*. The 2.2-kb insert of pCOREc2, containing the *waaC* gene, and the 1.5-kb insert of pCOREf1, containing the *waaF* gene, were subjected to double-strand nucleotide sequencing. Analysis of the DNA sequence encoded by pCOREc2 revealed one open reading frame (ORF) coding for a protein of 355 amino acids with a predicted mass of 39.8 kDa. Sequence analysis of pCOREf1 showed one ORF which could encode a protein of 345 amino acids with a deduced size of 38.4 kDa.

Comparison of the deduced amino acid sequences of the *P. aeruginosa* WaaC and WaaF proteins with those of other reported proteins in the GenBank and SWISS-PROT databases (2, 19) revealed that the WaaC protein of *P. aeruginosa* is 52.7% identical to the WaaC protein of *S. enterica* serovar Typhimurium and 52.4% identical to that of *E. coli* (data not shown). Similarly, the *P. aeruginosa* WaaF protein showed 49.0 and 49.3% identity with the WaaF proteins of *S. enterica* serovar Typhimurium and *E. coli*, respectively (data not shown).

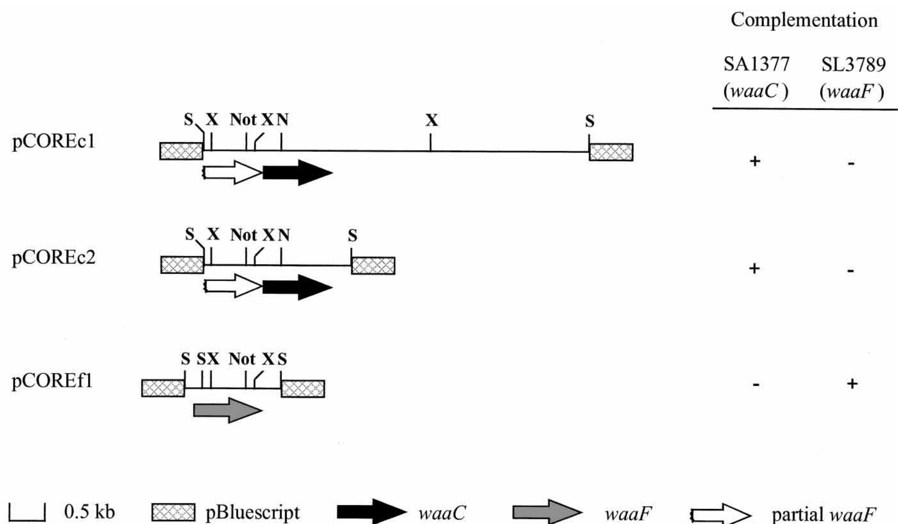

FIG. 2. Restriction maps of the chromosomal inserts of pCOREc1, pCOREc2, and pCOREf1. Arrows indicate the locations of the *waaC* and *waaF* genes. It is important to note that plasmids pCOREc1 and pCOREc2 contain only part of the *waaF* gene. Restriction site abbreviations: S, *Sau*3AI; X, *Xho*I; Not, *Not*I; N, *Nru*I. (Not all of the *Sau*3AI sites are indicated.)

Maxicell in vivo protein expression. Maxicell analysis was performed to confirm that the ORFs contained on the DNA inserts of pCOREc2 and pCOREf1 encoded proteins consistent with the predicted sizes. *E. coli* CSR603, containing pBluescript alone, was used as the vector control. A 31-kDa protein and a 28.5-kDa protein, corresponding to β-lactamase, were found in all of the samples. When pCOREf1 was used in protein expression experiments, a 39-kDa protein (Fig. 4b), corresponding well with the 38.4 kDa deduced from the nucleotide sequence, was observed. In cells expressing pCOREc2, a 40-kDa protein was found, which is consistent with the 39.8 kDa predicted from the sequence data (Fig. 4a). In addition, a 47-kDa protein was observed; however, no ORF corresponding to a protein of this size was identified. Plasmid pCOREc2 contains the entire *waaC* gene plus 176 bp of a downstream gene which is predicted to encode a truncated protein of approximately 7 kDa. Two possibilities exist to account for the presence of this 47-kDa protein. First, the protein may result because the incomplete ORF downstream of *waaC* is being translated into vector sequences. Examination of the downstream region including the pBluescript sequence, however, suggests that this is not the case. Second, a fusion protein could be produced by continued translation of *waaC* into the downstream sequence.

Chromosomal mapping of cloned *waa* genes. PFGE was used to separate *Spe*I- and *Dpn*I-digested PAO1 chromosomal DNA for mapping of the *P. aeruginosa waa* genes. The inner-core biosynthetic genes were located on the PAO1 chromosome by Southern hybridization with a digoxigenin-labelled probe generated from the 2.2-kb insert of pCOREc2. This DNA insert contains all of the *waaC* gene and most of *waaF*. In Southern blots, the *waa*-specific probe hybridized to a *Spe*I fragment of approximately 450 kb which corresponds to restriction fragment SpB (Fig. 5). SpB spans 0.9 to 6.6 min on the 75-min map (18). In blots of *Dpn*I-digested chromosomal DNA, the probe hybridized to a 269-kb fragment, DpJ, which is actually a doublet composed of two 269-kb fragments (Fig. 5). The two fragments span 75.0 to 3.3 min (DpJ_1) and 3.3 to 6.7 min (DpJ_2) on the map (18). Therefore, genes involved in biosynthesis of the LPS inner-core region lie between 0.9 and 6.6 min.

Southern hybridization of the 20 *P. aeruginosa* serotypes with a *waa*-specific probe. To determine whether the *waaC* and *waaF* genes were present in all 20 serotypes, Southern hybridization analysis was performed. The *waa* probe used to analyze PFGE blots was employed to probe *Bam*HI-, *Eco*RI-, and *Kpn*I-digested chromosomal DNA. The probe hybridized to a common 7.5-kb *Bam*HI fragment in all 20 serotypes except O12, in which case the probe hybridized to a 12.0-kb fragment (data not shown). Similarly, the *waa*-specific probe hybridized to a 4.2-kb *Eco*RI fragment in all serotypes except O12, in which case the probe hybridized to a 5.0-kb band, and serotype

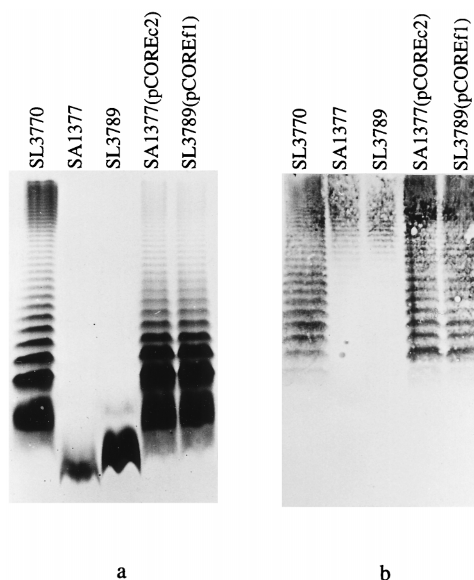

FIG. 3. (a) Silver-stained SDS-PAGE gel of LPS from *S. enterica* serovar Typhimurium SL3770, SA1377 (*waaC* mutant), SL3789 (*waaF* mutant), SA1377 (pCOREc2), and SL3789(pCOREf1) (b) a Western blot corresponding to the gel of panel a reacted with polyclonal antisera raised against the *Salmonella* wild-type strain SL3770. It is important to note that the two transconjugant strains SA1377(pCOREc2) and SL3789(pCOREf1) are expressing smooth LPS like that of the wild type.

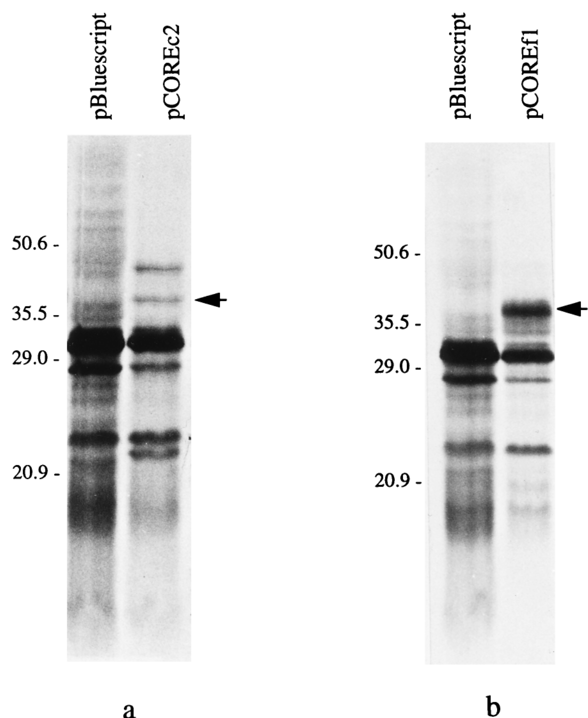

FIG. 4. Autoradiogram showing proteins expressed by pCOREc2 and pCOREf1, and corresponding vector pBluescript, in *E. coli* maxicells. Arrows indicate the 40- and 39-kDa proteins expressed by pCOREc2 and pCOREf1, respectively. Molecular size standards in kilodaltons are indicated to the left of the autoradiograms.

O4, in which case the probe hybridized to an additional 9.5-kb band (data not shown). In Southern blots of *Kpn*I-digested chromosomal DNA, the probe hybridized to various-sized fragments from the 20 serotypes. Therefore, the two *waa* genes appear to be present in all 20 *P. aeruginosa* serotypes, although the sizes of the restriction enzyme fragments are not strictly conserved.

Generation of *P. aeruginosa* chromosomal *waaC* and *waaF* mutants. Using a gene replacement strategy, we attempted to generate *waaC* and *waaF* mutants of *P. aeruginosa*. Our first approach involved cloning the 2.2-kb insert of pCOREc2 into gene replacement vector pEX100T (45). An 875-bp Gm^r cassette was cloned into a unique *Nru*I site within the *waaC* coding region, and the resulting plasmid was designated pCOREk1. pCOREk1 was mated independently into two strains of *P. aeruginosa*, namely, PAO1 and PAK. During selection of transconjugants, various growth conditions were used to overcome possible deleterious effects associated with the deep-rough mutations. Conditions included growing cells at 30°C as well as 37°C, plating cells on minimal medium containing gentamicin in addition to PIA-gentamicin to select for *P. aeruginosa* harboring the Gm^r cassette, and, finally, plating cells on medium supplemented with 20% sucrose to increase the osmotic strength of the medium for stabilization of outer membranes. Despite the fact that we were able to isolate numerous merodiploids, no true *waaC* recombinants were identified. The next approach involved cloning the larger 6.1-kb insert of pCOREc1 into pEX100T. A larger piece of DNA was used to increase the likelihood of a double-crossover event. This time, the Gm^r cassette was cloned in both orientations into a *Not*I site within the *waaF* coding region. The Gm^r cassette contains a promoter but no transcriptional terminator (44). If genes downstream of *waaF* are transcribed from an upstream promoter, cloning the cassette promoter in the direction opposite to that of transcription (plasmid pCOREk2) should affect expression of downstream genes as well as *waaF*. Conversely, if the cassette is cloned in the other orientation (plasmid pCOREk3), transcription of downstream genes should occur. Plasmids pCOREk2 and pCOREk3 were mated into *P. aeruginosa*, and transconjugants were grown under the conditions described above. Again, no true recombinants were obtained. Insertion of the cassette within the chromosome of the merodiploids was verified by Southern blot analysis and a probe specific for the Gm^r cassette (data not shown). In all cases, the insertion occurred downstream of *waaC*.

DISCUSSION

Salmonella deep-rough mutants have an increased sensitivity to hydrophobic agents such as antibiotics, detergents, and bile salts. This change in membrane permeability enabled us to utilize these mutants for isolating *P. aeruginosa* inner core biosynthetic genes. The ability of the two *P. aeruginosa* genes to complement defined *Salmonella* mutants, as well as the observed homology between the deduced *P. aeruginosa* proteins and the WaaC and WaaF proteins of other species, prompted us to designate these genes *waaC* and *waaF*.

Because the *P. aeruginosa waaC* and *waaF* genes readily complement corresponding *S. enterica* serovar Typhimurium mutants, sufficient similarity must exist between the proteins of these two organisms to allow them to be functionally exchangeable. Inspection of the protein alignments reveals that there is a region near the beginning of the WaaC sequence, corresponding to the N terminus of the protein, of markedly high similarity. Fifty-four of the first 64 amino acids (84%) in the *P. aeruginosa* WaaC protein are identical to those found in *E. coli* and *S. enterica* serovar Typhimurium. Other regions throughout the WaaC protein are highly homologous; however, none is as significant as the N terminus. In contrast,

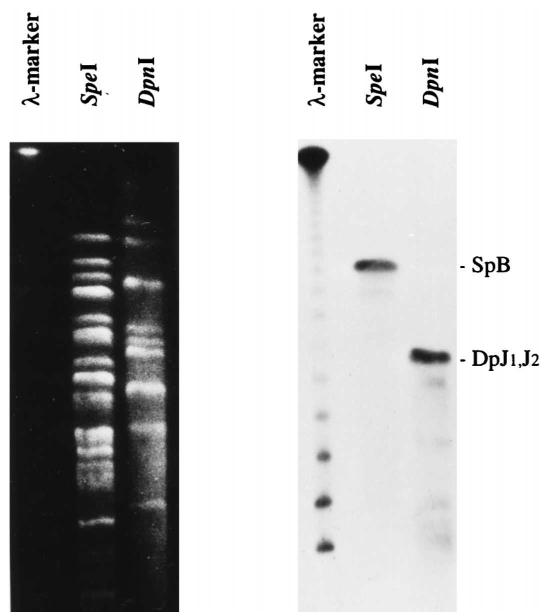

FIG. 5. (a) PFGE analysis of PAO1 chromosomal DNA digested with *Spe*I and *Dpn*I; (b) autoradiogram of the restriction enzyme fragments that hybridize with the *waa*-specific probe. Molecular weight markers include concatamerized lambda DNA (molecular weights are not indicated).

regions of homology between the *P. aeruginosa* WaaF protein and those of *S. enterica* serovar Typhimurium and *E. coli* are more evenly distributed throughout the sequence. These conserved regions likely represent functionally important domains in the two heptosyltransferase proteins. Interestingly, the WaaC protein of *Neisseria gonorrhoeae* has even less identity (36%) with that of *S. enterica* serovar Typhimurium, and yet the gene specifying this protein is able to complement a *Salmonella waaC* mutant (51).

In *S. enterica* serovar Typhimurium, the *waaF* and *waaC* genes are contiguous and cotranscribed from an upstream promoter (47). *gmhD* (formerly *rfaD*) lies upstream of *waaF*, and *waaL* (formerly *rfaL*) is located downstream of *waaC*. These four genes together comprise one of the three *Salmonella waa* operons. A similar contiguous arrangement of the *waaF* and *waaC* genes was observed in *P. aeruginosa. waaF* lies upstream of *waaC*, and the two genes have overlapping termination and initiation codons. In *P. aeruginosa*, there appears to be a gene directly upstream of *waaF*, the stop codon of which overlaps the *waaF* start sequence. We are currently investigating whether this gene is homologous to *gmhD* or is otherwise involved in LPS biosynthesis. Only 176 bp have been sequenced downstream of the *P. aeruginosa waaC* gene; however, this region has amino acid homology with the *waaG* (formerly *rfaG*) gene product of *E. coli* (74% over 174 of the 176 bp) (10, 36). *waaG* encodes a glucosyltransferase which adds the first hexose, a glucose residue, to the inner core. In *Salmonella* and *E. coli* K-12, *waaG* is located at the distal end of another *waa* operon (43). Although the inner core of *P. aeruginosa* is quite similar to that of *S. enterica* and *E. coli*, the outer-core region differs substantially. The first hexose sugar found in the outer core of both *S. enterica* serovar Typhimurium and *E. coli* is Glc, whereas in *P. aeruginosa*, it is a GalN residue (Fig. 1). Another unique feature of the *P. aeruginosa* outer core is the presence of the amino acid L-alanine. In light of these and other structural differences, it is not surprising that the genetic arrangement of the *waa* locus may differ in *P. aeruginosa*, particularly with respect to genes involved in the synthesis of the outer-core region.

Using a gene replacement strategy previously employed to generate knockout mutants of *wzy* (15), *wbpL* (14), and numerous other genes involved in *P. aeruginosa* LPS biosynthesis (7), we were unable to obtain either *waaC* or *waaF* null mutants. To date, no mutants of *P. aeruginosa* which express the deep-rough LPS phenotype have been isolated. Although numerous strategies, including chemical mutagenesis, transposon mutagenesis, and resistance to LPS-specific phages, have been employed in our laboratory (13, 30) and in others, the deepest core deficiency that has been observed occurs in strain AK1282 (27). This mutant was shown to express LPS lacking the first proximal hexose residue and therefore contains only heptose and Kdo (26). Heptose residues are critical sites for phosphate substitution (Fig. 1). Moreover, the LPS core region of *P. aeruginosa* has been reported to have an unusually high phosphate content (35). Since cross-linking of phosphate groups by divalent cations is important for maintaining the integrity of the bacterial outer membrane, it is possible that the absence of heptose, and subsequently phosphate groups, may destabilize the membrane to the extent that the *P. aeruginosa* cells are no longer viable. Although it is possible that the Gm^r cassette insertion may be exerting polar effects on a downstream gene(s) which is critical for cell viability, this alternative explanation is ruled out since we have previously demonstrated the isolation of nonpolar chromosomal mutants of other LPS biosynthetic genes when the Gm^r cassette was inserted in the same orientation as the direction of transcription of the gene cluster (14, 15).

In summary, similarities in the LPS inner-core structures of *P. aeruginosa* and *S. enterica* serovar Typhimurium enabled us to make use of deep-rough *Salmonella* mutants to isolate the *waaC* and *waaF* genes of *P. aeruginosa*. As well as being functionally homologous, the two genes are arranged in a fashion similar to that which is observed in *S. enterica* serovar Typhimurium and *E. coli*. Since bacterial strains expressing truncated LPS are generally more susceptible to phagocytosis and the bactericidal effect of serum, uncovering mechanisms involved in LPS core biosynthesis may lead to the discovery of new antimicrobial agents. The finding that inner-core genes can be functionally interchanged among a variety of gram-negative species is exciting because it suggests that antimicrobial agents which function through blocking LPS inner-core biosynthesis may be broadly cross-reactive against a number of gram-negative bacteria. The isolation of *waaC* and *waaF* in *P. aeruginosa* also facilitated the mapping of *waa* genes to the 0.9- to 6.6-min region on the *P. aeruginosa* PAO1 chromosome. This *waa* map location further adds to our previous observations that the clusters of genes for the synthesis of the two forms of O polymers are mapped at discreet locations, 10.5 to 13.3 min for A-band and 37 min for B-band (31).

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