Functional Characterization of WaaL, a Ligase Associated with Linking O-Antigen Polysaccharide to the Core of *Pseudomonas aeruginosa* Lipopolysaccharide

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The O antigen of *Pseudomonas aeruginosa* B-band lipopolysaccharide is synthesized by assembling O-antigen-repeat units at the cytoplasmic face of the inner membrane by nonprocessive glycosyltransferases, followed by polymerization on the periplasmic face. The completed chains are covalently attached to lipid A core by the O-antigen ligase, WaaL. In *P. aeruginosa* the process of ligating these O-antigen molecules to lipid A core is not clearly defined, and an O-antigen ligase has not been identified until this study. Using the sequence of *waaL* from *Salmonella enterica* as a template in a BLAST search, a putative *waaL* gene was identified in the *P. aeruginosa* genome. The candidate gene was amplified and cloned, and a chromosomal knockout of PAO1 *waaL* was generated. Lipopolysaccharide (LPS) from this mutant is devoid of B-band O-polysaccharides and semirough (SR-LPS, or core-plus-one O-antigen). The mutant PAO1*waaL* is also deficient in the production of A-band polysaccharide, a homopolymer of D-rhamnose. Complementation of the mutant with pPAJL4 containing *waaL* restored the production of both A-band and B-band O antigens as well as SR-LPS, indicating that the knockout was nonpolar and *waaL* is required for the attachment of O-antigen repeat units to the core. Mutation of *waaL* in PAO1 and PA14, respectively, could be complemented with *waaL* from either strain to restore wild-type LPS production. The *waaL* mutation also drastically affected the swimming and twitching motilities of the bacteria. These results demonstrate that *waaL* in *P. aeruginosa* encodes a functional O-antigen ligase that is important for cell wall integrity and motility of the bacteria.

Pseudomonas aeruginosa is an opportunistic pathogen that typically causes disease only in individuals with impaired host defenses. Such compromised individuals include patients undergoing immunosuppressive therapies (e.g., cancer treatment), receiving treatment for traumatic skin damage (burn wounds), suffering from human immunodeficiency virus infections, and having cystic fibrosis (CF) (20, 33). CF patients in particular are highly susceptible to chronic pulmonary infections with *P. aeruginosa*. The pathogenicity of this organism is attributed to the production of an arsenal of diverse virulence factors, including exotoxin A, phospholipase C, proteases, alginate, and lipopolysaccharide (LPS) (8). LPS also plays an essential structural role in the outer membrane and consists 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* produces two forms of O antigen, known as A band (homopolymer) and B band (heteropolymer). LPS is a complex molecule, the assembly of which requires a number of specific proteins. It has become clear in the last decade that the assembly of homopolymeric and heteropolymeric O antigens are fundamentally different (61). Interestingly, our laboratory has provided substan-

tial evidence that A-band and B-band LPS are assembled via separate pathways in *P. aeruginosa* (12, 50).

Sugar nucleotide precursors for both homopolysaccharides and heteropolysaccharides are synthesized in the cell cytoplasm and used as donor molecules for assembly of the O-polysaccharide units (51). An initial glycosyltransferase serves to transfer the first sugar residue onto a carrier lipid molecule, identified as the C₅₅ polyisoprenoid alcohol derivative undecaprenol phosphate (Und-P) (63). Und-P also serves as a scaffold for peptidoglycan biosynthesis (17). Synthesis of homopolysaccharides requires the activity of an initiating glycosyltransferase that adds only the initial sugar onto Und-P. This sugar apparently acts as a primer and does not form part of the O-repeating unit (61). In contrast, heteropolysaccharides have a requirement of the initiating glycosyltransferase for the formation of each O-repeat unit on Und-P. Thus, the initiating sugar becomes the first sugar of every O unit. WbpL in *P. aeruginosa* (7) is a homologue of WecA, a glycosyltransferase known to initiate the biosynthesis of homopolymeric exopolysaccharides in *Enterobacteriaceae* (2, 48), and it is encoded by a gene in the B-band O-antigen gene cluster in *P. aeruginosa* serotype O5 (37). Interestingly, a *wbpL* chromosomal mutant in serotype O5 is deficient in both A band and B band, thus demonstrating the requirement of WbpL (49). In addition to WbpL, three other glycosyltransferases have been identified for the assembly of the A-band D-rhamnose polymer in *P. aeruginosa* (49). Specifically, these proteins are rhamnosyltransferases, WbpX (PA5449), WbpY (PA5448), and WbpZ (PA5447), located in the A-band O-polysaccharide gene cluster (49),

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which maps between 10.5 and 13.3 min on the PAO1 chromosome (37). Chromosomal mutations in each of *wbpX*, *wbpY*, and *wbpZ* results in a loss of A-band LPS biosynthesis, while B-band LPS is unaffected (49). After assembly of homopolymeric O units, the completed O units must be transported from the cytoplasm to the periplasm. An ATP-binding cassette (ABC) transport system serves to export most homopolymeric O-polysaccharides to the periplasm for ligation to lipid A. Such homopolymer polysaccharide export systems have been identified in *Escherichia coli* O9a (31), *Klebsiella pneumoniae* O1, *Serratia marcescens* O16, *Yersinia enterocolitica* O:3, and *Vibrio cholera* O1 (6, 32, 41, 57, 64).

The mechanism of heteropolymer assembly differs in many respects from that of homopolymers. In the case of heteropolymers, each O-repeating unit is assembled at the cytoplasmic face of the inner membrane by nonprocessive glycosyltransferases and is translocated to the periplasmic face via the action of the integral protein Wzx (formerly RfbX) (38). Mutation of *wzx* in *P. aeruginosa* abrogated B-band LPS biosynthesis (8). At present, the mechanism of how this translocation, or "flipping," of O units occurs is poorly defined. No ATP-dependent transporter is required for export of individual B-band O units. On the periplasmic face of the cytoplasmic membrane, individual O units are polymerized into chains by the O-antigen polymerase, Wzy (formerly Rfc), to a strain-specific range of lengths determined by the O-antigen chain-length regulator, Wzz (formerly called Rol or Cld). An unusual feature of *P. aeruginosa* serotype O5 is the presence of two separate *wzz* genes on the chromosome; one located adjacent to the B-band LPS biosynthetic genes cluster (7), now designated *wzz*₁, and a second, unlinked version, designated *wzz*₂ (9).

Although studies of LPS biosynthesis in *P. aeruginosa* were initiated several years ago, there are several steps of LPS assembly that still need to be resolved. More specifically, the later stages of assembly, such as attachment of O-polysaccharide to the core oligosaccharide, are poorly understood. In *Enterobacteriaceae*, an enzyme called O-antigen ligase (WaaL) has been shown to be responsible for attachment of a variety of polysaccharides to core lipid A (62). To date, the mechanism of ligation is unknown and there have been no direct demonstrations that purified WaaL has O-antigen ligase activity. WaaL proteins of *E. coli* K-12 and *Salmonella enterica* serovar Typhimurium have low-level primary amino acid sequence similarity. However, both have similar hydropathy plot patterns and appear to be integral membrane proteins with 10 or more potential membrane-spanning domains (35). Mutants in *waaL* of both *E. coli* and *S. enterica* serovar Typhimurium are unable to attach O antigen to the lipid A core. *S. enterica* serovar Typhimurium mutants defective in the *waaL* gene accumulate polymerized O antigen linked to Und-P on the periplasmic surface of the cytoplasmic membrane (44). Transfer of foreign *rfb* genes into the normally rough strain *E. coli* K-12 results in the production of heterologous S-LPS (Smooth-LPS) (40, 15, 43, 47). This indicates that the *E. coli* K-12 WaaL enzyme has relaxed specificity for the O-antigen polymer it attaches to lipid A core. Recent studies have indicated that it is the nature of the acceptor molecule (lipid A core) that is important for the ligation (22, 23). Heinrichs and colleagues

have shown that terminal side groups on the core oligosaccharide (HexIII substitutions) are required for ligation, although different sugar moieties can fulfill this structural requirement. The lack of WaaL homology probably reflects differences in substrate requirement; WaaL would use Und-P-P-O-antigen while known glycosyltransferases use nucleotide diphosphosugars (24).

The WaaL protein is usually encoded within the core oligosaccharide gene cluster, but until this study WaaL has not yet been identified in *P. aeruginosa*. Although the complete chemical structure of the *P. aeruginosa* B-band core oligosaccharide (51) and the entire genome sequence of strain PAO1 (56) is now available, less than half of the genes predicted to be involved in synthesis and assembly of the core oligosaccharide have been identified. In this study, we describe the identification and characterization of a *waaL* gene and provide experimental evidence to show that it encodes a functional O-antigen ligase in *P. aeruginosa* PAO1.

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 (LB; Invitrogen Canada Inc., Burlington, Ontario, Canada) at 37°C. Pseudomonas Isolation Agar (PIA; DIFCO Becton, Dickinson and Company, Sparks, Md.) was used for selecting transconjugants following mating experiments. The following antibiotics were used in selection media at the indicated concentrations: ampicillin at 100 µg ml⁻¹ for *E. coli*, carbenicillin (Cb) at 600 µg ml⁻¹ for *P. aeruginosa*, and gentamicin (Gm) at 15 and 300 µg ml⁻¹ and tetracycline (Tc) at 10 and 100 µg ml⁻¹ for *E. coli* and *P. aeruginosa*, respectively.

DNA procedures. All enzymes were used according to the supplier's specifications. Small-scale plasmid DNA preparations were carried out using a plasmid mini prep kit (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Plasmid DNA was electroporated into *P. aeruginosa* with a Gene Pulser instrument (Bio-Rad). Recombinant plasmids were mobilized from *E. coli* SM10 to *P. aeruginosa* by the method of Simon et al. (55). Genomic DNA was isolated from *P. aeruginosa* PAO1 (21) by the method of Ausubel et al. (4).

PCR amplification was utilized to amplify the *pa4999* of strain PAO1 using primers flanked to the upstream of *pa4999* (P1) and 3' end of the *pa4999* (P2) and standard conditions, such as 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min. However, since the PCR amplification was unsuccessful even using different standard PCR procedures, a method known as plasmid-enhanced PCR strategy (PEP), to perform PCR-mediated mutagenesis (1), was followed to overcome the problem of heterogeneous reactions. The principle behind the PEP method is to amplify smaller segments of the gene and allow subsequent ligation to generate a product consisting of the intact gene. The same procedure was utilized to amplify *waaL* of strain PA14.

DNA sequencing. The 1.4-kb SmaI-PstI insert of *pa4999* and the 1.2-kb SmaI-PstI insert of PA14 *waaL* genes were independently cloned into vector pEX18AP (27). Both strands of DNA were sequenced at the Laboratory Services Division, University of Guelph (Guelph, Ontario, Canada), with an Applied Biosystems model DNA sequencing unit. Oligonucleotide primers were synthesized on an Applied Biosystems model DNA synthesizer by the Laboratory Services Division (University of Guelph) and are available on request.

Sequence analysis. Nucleotide and amino acid sequence analysis was performed using the program Gene Runner (Hasting 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 (3, 19). Comparison of WaaL protein sequences from different bacteria were performed by using Kyte and Doolittle hydropathy plots (36).

Mutagenesis of the *waaL* gene of *P. aeruginosa* PAO1 (serotype O5) and PA14 (serotype O10). *P. aeruginosa* chromosomal knockout mutants PAO1*waaL* and PA14*waaL* were generated by using a gene replacement strategy previously described by Schweizer (52) with minor modifications. Initially, the *pa4999* gene was amplified by PCR (1). The amplified PCR product was cloned into the pEX18Ap vector (53), followed by inserting a gentamicin-resistance (Gm^r) cassette into a unique SalI site within the *pa4999* gene, producing the insertional

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics ^a	Reference or source
Strains		
<i>P. aeruginosa</i>		
PAO1	Serotype O5; A ⁺ B ⁺	21
PAO1waaL	<i>waaL</i> ::Gm ^r A ⁻ B ⁻ derived from strain PAO1	This work
PAO1waaL + pUCP27-PAO1waaL	PAO1waaL::Gm ^r complemented with pUCP27 having PAO1 <i>waaL</i>	This work
PAO1waaL + pUCP27-PA14waaL	PAO1waaL::Gm ^r cross-complemented with PA14 <i>waaL</i> in pUCP27	This work
PA14	Serotype O10; A ⁺ B ⁺	Fred Ausbel (Harvard Medical School)
PA14waaL	<i>waaL</i> ::Gm ^r A ⁻ B ⁻ derived from strain PA14	This work
PA14waaL + pUCP27-PA14waaL	PA14waaL::Gm ^r complemented with pUCP27 having PA14 <i>waaL</i>	This work
PA14waaL + pUCP27-PAO1waaL	PA14waaL::Gm ^r cross-complemented with PAO1 <i>waaL</i> in pUCP27	This work
<i>E. coli</i>		
JM109	<i>recA1 supE44 endA1 hsdR17gyrA96 relA1 thi Δlac-proAB F'[traD36 proAB⁺ lacI^Q lacZΔM15]</i>	
SM10	<i>thi-1 thr leu tonA lacY supE recA</i> RP4-2-Tc::Mu Km ^r	55
Plasmids		
pEX18AP	Ap ^r /Cbr ^r <i>oriT</i> ⁺ <i>sacB</i> ⁺ ; gene replacement vector with multiple cloning site (MCS) from pUC18	27
pPS856	Gm ^r Ap ^r ; <i>aacC1</i> gene (Gm ^r) from pUCP Gm ligated into the EcoRV site of pPS854; Gm ^r cassette is flanked by identical inverted MCS	27
pUCP27	pUC18-derived broad-host-range vector; Tc ^r	59
pPAJL1	PCR product of <i>pa4999</i> upstream DNA (up to SalI site), cloned into pEX18AP using SmaI and SalI restriction sites	This work
pPAJL2	PCR products of <i>pa4999</i> downstream DNA (from SalI to 3' end), cloned into pPA1 using SalI and PstI restriction sites	This work
pPAJL3	PAO1 <i>waaL</i> knockout construct (insertion of Gm ^r cassette into <i>pa4999</i>)	This work
pPAJL4	<i>pa4999</i> cloned into pUCP27 vector using SmaI and PstI restriction sites	This work
pPAJL5	PCR product of PA14 <i>waaL</i> upstream DNA (up to SalI site), cloned into pEX18AP using SmaI and SalI restriction enzymes sites	This work
pPAJL6	PCR products of PA14 <i>waaL</i> downstream DNA (from SalI to 3' end), cloned into pPA1 using SalI and PstI restriction sites	This work
pPAJL7	PA14 <i>waaL</i> knockout construct (insertion of Gm ^r cassette into PA14 <i>waaL</i>)	This work
pPAJL8	PA14 <i>waaL</i> cloned into pUCP27 vector using SmaI and PstI restriction sites	This work

^a A superscript + or - sign after A or B designates the presence or absence of the particular O-polysaccharide.

construct pPAJL3. This construct was transformed into *E. coli* SM10 and conjugally transferred into *P. aeruginosa* PAO1 (55). Following conjugation, cells were plated onto PIA-Gm150 to select *P. aeruginosa* transconjugants. Subsequently, colonies that were able to grow on PIA-Gm150 plates were streaked on LB (with out salt) containing 10% sucrose. This step was repeated two times to give a selective pressure on meridioids to prevent their growth on sucrose-containing LB medium. Colonies from sucrose selection medium were inoculated onto both PIA-Gm300 and PIA containing 600 µg ml⁻¹ Cb. The colonies, which exhibited a Gm-resistant, Cb-sensitive phenotype, were screened by PCR using primers specific for *pa4999* gene. A similar approach was used to construct PA14 WaaL knockout mutants. For complementation experiments, *waaL* of PAO1 and PA14, respectively, were isolated by digesting pPAJL2 and pPAJL6 vectors with SmaI and PstI restriction enzymes and cloned into pUCP27 vector (59) via the same restriction enzymes sites to yield the complementation constructs (Table 1).

Preparation of LPS. The proteinase K digestion method of Hitchcock and Brown (HB) (25) and the hot-aqueous phenol (HAP) method of Westphal and Jann (60) were used to prepare the LPS.

SDS-PAGE analysis and Western immunoblotting. LPS was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously by de Kievit et al. (12) and visualized by silver staining using the rapid method of Fomsgaard et al. (16). Western immunoblotting was performed as described previously (12). The primary antibodies used in immunoblotting were monoclonal antibody (MAb) N1F10 (specific for A-band LPS),

MAb MF15-4 (specific for B-band LPS), and MAb 18-19 (specific for core-plus-one O-antigen LPS). The secondary antibody was a goat anti mouse F(ab')₂-alkaline phosphatase conjugate (Jackson ImmunoResearch). The blots were developed using a substrate containing 0.33 mg ml⁻¹ nitroblue tetrazolium (NBT; Sigma) and 0.15 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma) in 0.1 M bicarbonate buffer (pH 9.8).

Motility assay. The quantitative assay for twitching motility was adapted from methods described by McMichael (42) and Darzins (11). The strains to be tested were stab inoculated with a needle to the bottom of the 1% LB agar plates poured to an average depth of 3 mm and dried briefly. Plates were incubated at 37°C for 24 h. The twitching zone was fixed and stained with 0.05% Coomassie brilliant blue R250 (in 20% methanol, 10% acetic acid), and excess stain was removed by several washes with an aqueous solution of 40% methanol and 10% acetic acid. After that the zone between the agar and polystyrene was measured. A phenotypic assay for swimming motility was initiated by stab inoculation of bacteria at the center of agar plates containing 0.3% agar to evaluate swimming motility. The plates were then wrapped with Saran wrap to prevent dehydration and were incubated at 30°C for 12 to 18 h. Twitching and swimming assays were performed in triplicates in three independent occasions.

Transmission electron microscopy. To visualize flagella and pili on *P. aeruginosa* cells, a single bacterial colony from an agar plate was suspended in deionized H₂O. An electron microscopy (EM) grid was then submerged in the bacterial suspension for 10 s, blotted onto filter paper to remove any fluid from the grid, and negatively stained with 1% aqueous uranyl acetate. The bacterial cells

on the EM grid was examined by using a Philips EM300 transmission electron microscope operating at 60 kV under standard conditions with the cold trap in place.

RESULTS

Identification of *waaL* using hydrophobicity plots. In general, WaaL ligase proteins of bacteria have similar secondary structures, characterized by the presence of several membrane-spanning regions comprised of hydrophobic α -helices separated by hydrophilic regions. Despite a lack of primary sequence homology between WaaL_{*P. aeruginosa*} and WaaL_{*E. coli*}, a comparison of the hydrophobicity profiles plotted by the method of Kyte and Doolittle (36) demonstrated high similarity among WaaL of *E. coli* (accession number AAC69648) (Fig. 1A), *K. pneumoniae* (accession number AAD37765) (Fig. 1B), *P. aeruginosa* strain PA14 (accession number ZP_00141473) (Fig. 1C), and WaaL *P. aeruginosa* strain PAO1 (PA4999) (accession number NP_253686) (Fig. 1D). This strategy of comparison was used earlier by our group to successfully identify the putative Wzy (formerly called Rfc) (12) and Wzm (an ATP transporter protein) among other membrane protein involved in LPS biosynthesis of *P. aeruginosa* (46). Using the same strategy, we were able to demonstrate that the protein encoded by *pa4999* has a strikingly similar secondary structure compared to a group of other WaaL proteins (Fig. 1). This provided the first clue that the protein product of PA4999 could be the O-antigen ligase of *P. aeruginosa* PAO1.

PCR amplification of *waaL* of *P. aeruginosa* strain PAO1 and strain PA14. In the initial attempt to amplify the full length of *pa4999* using primers P1 and P2, broad streaks were observed in the agarose gel analysis. Standard modifications to increase the specificity of the reaction by alterations in annealing temperature, amount of primer, amount of template, magnesium concentration, or the cycle number have provided little improvement. Even specialized approaches, such as hot-start PCR (10), touchdown PCR (14), and overlap extension (26, 54), have not provided a satisfactory solution. This heterogeneity might be attributed by the high G+C (63.01%) content of *pa4999*. To avoid broad streaking bands from the initial PCRs, we used the PEP strategy (1) to amplify smaller fragments and ligating these into a product containing the intact *waaL* gene. This approach has proven to be successful for amplifying *waaL* of both PAO1 and PA14 strains (Fig. 2). To determine the functional role of the cloned *pa4999*, knockout mutants PAO1waaL and PA14waaL were generated using insertional mutation and allelic replacement procedures that are routinely used in our laboratory (as described in Materials and Methods). The presence of the mutant genes was verified by PCR using primers P4 and P3, and the knockout mutated genes in either wild-type background produced a DNA band approximately 1.2 kb larger than the band amplified from the wild-type *waaL* gene. This is consistent with the presence of the Gm^r cassette that has a size of approximately 1 kb (data not shown).

Characterization of LPS isolated from the PAO1 *waaL* and PA14 *waaL* mutants. To examine the effect of *waaL* mutation on LPS synthesis, LPS from the wild-type strains PAO1, PA14, and mutants PAO1waaL and PA14waaL were prepared using the HB method (25). These samples were then analyzed by SDS-PAGE, silver staining, and Western immunoblotting us-

FIG. 1. Comparison of Kyte and Doolittle hydrophathy plots of WaaL proteins from various bacteria. The x axis corresponds to the amino acid residue, while the y axis corresponds to the relative hydrophathy index. Each of the proteins contains 11 potential membrane-spanning domains, which are indicated above the x axis as numbered bars. (A) *E. coli* WaaL accession number AAC69648, (B) *K. pneumoniae* WaaL accession number AAD37765, (C) *P. aeruginosa* strain PA14 WaaL accession number ZP_00141473, (D) *P. aeruginosa* strain PAO1 WaaL (PA4999) accession number NP_253686.

ing A-band-specific MAb (N1F10), B-band-specific MAb MF15-4, and core-plus-one O-antigen-specific MAb 18-19 as immune probes. LPS from PAO1waaL is defective in the production of A-band, B-band, and core-plus-one O-antigen (Fig.

FIG. 2. *pa4999* organization in PAO1 genome and strategy for PCR amplification and cloning of the putative *waaL*, *pa4999*. The gene was amplified as two parts using the PEP strategy. The first PCR product (using primers P1 and P3) was digested with *Sma*I and *Sal*I restriction enzymes and cloned into the pEX18AP plasmid using the same restriction enzymes. The second part of *pa4999* was amplified by using primers P4 and P5 that include the 3' end of *pa5000*. Finally, this PCR product was used as a template to amplify the second half of *pa4999* using P4 and P2 as primers. After that, PCR product was digested with *Sal*I and *Pst*I restriction endonucleases and ligated into the first part of *pa4999* gene construct.

3A to D). When PAO1waaL was complemented with homologous *waaL* gene in *trans*, the production of A-band and B-band LPS was fully restored (Fig. 3A to D). Similar results were obtained regardless of whether the mutant strain PA14waaL was complemented with *waaL* from PA14 or from PAO1 (data not shown). The presence of LPS bands that reacted with MAb MF15-4 (B-band specific) observed in Fig. 3 (panel C) is likely the product of undecaprenol lipid carrier-linked B-band O polymers that have not been ligated to core lipid A. This type of glycolipid band is sensitive to phenol as described in our earlier studies (50). To test this hypothesis, LPS were prepared by the HAP method. This approach was taken since Kent and Osborn (30) 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 PAO1waaL by the HAP method showed no reactivity in Western immunoblotting to MAb N1F10 (A-band specific) (Fig. 3E) and MAb (MF15-4) (B-band specific) (Fig. 3F). These results indicated that treatment of the LPS from mutant strain PAO1waaL with HAP resulted in the cleavage of the linkage between polysaccharide from the carrier lipid releasing soluble A-band and B-band polysaccharides.

Subsurface twitching motility assay. To investigate the potential effects of *waaL* mutation on bacterial motility, we tested the strains using a twitching motility assay by measuring diffuse interstitial zone due to the motile bacterial cells migrating away from the point of inoculation within twitching media. The distance that bacteria of the wild-type strain could move across the twitching motility agar surface, measured as the diameter of the twitching zone, was 10.3333 ± 0.5774 mm. This is approximately fivefold more than that of the twitching distances covered by mutant strains PAO1waaL (diameter of twitching zones are 2.0000 ± 0.0000 mm) and PA14waaL (diameter of twitching zones are 1.8333 ± 0.2887 mm) (Fig. 4).

Swimming motility assay. To investigate the effect of mutation in *waaL* on swimming motility of *P. aeruginosa*, wild-type and mutant bacteria were inoculated onto swimming motility agar medium and the distance that bacterial cells could swim within the motility media was measured (Fig. 5). Wild-type PAO1 and PA14 bacteria used as positive controls showed the distance traveled by swimming motility at 8.3333 ± 0.5774 cm and 9.1667 ± 0.2887 cm, respectively. In contrast, the mutant strains of PAO1waaL and PA14waaL exhibited significantly retarded swimming motility, and the distance traveled by swimming motility were measured at 2.9333 ± 0.2309 cm and 3.3333 ± 0.1155 cm, respectively. Complementation of the

PAO1waaL with plasmid pPAJL4 (pUCP27 containing a *pa4999* insert) restored swimming motility in the recombinants to the similar distance traveled by wild-type PAO1 or PA14 strain (Fig. 5).

Electron microscopic examination of PAO1waaL and PA14waaL mutants. Since there were significant effects in bacterial twitching and swimming motility, the morphology of bacterial cells of PAO1, PA14, and *waaL* mutants were examined by transmission electron microscopy (TEM) and negative staining. Individual cells of PAO1 and PA14 parent strains produce pili and a single polar flagella (Fig. 6A and C). Interestingly, the number of these surface protein appendages produced by the *waaL* mutants was drastically reduced when compared to those produced by the parent strains. Among a group of mutant PAO1waaL bacteria visualized by TEM, only 4 out of 93 cells exhibited pili and only 10 out of 93 cells possessed flagella. Similarly, among PA14waaL cells visualized, 6 out of 104 cells have pili and only 11 out of 104 cells counted possessed flagella. Thus, it is clear that a significantly reduced number of pili and/or flagella could be observed on the cell surfaces of the mutant bacteria compared to their respective wild-type parent strains (Fig. 6B and D).

DISCUSSION

There is overwhelming evidence to suggest that *pa4999* encodes an integral membrane protein with 11 potential membrane-spanning domains. By comparing Kyte and Doolittle hydropathy plots of the sequences of WaaL proteins from various bacteria, we observed that the secondary structures among these proteins are very similar (Fig. 1A to D). Based on these *in silico* analyses, we feel confident that *pa4999* in the *P. aeruginosa* PAO1 genome is the *waaL* gene.

It is intriguing that *P. aeruginosa* has the unique ability to coproduce a homopolymeric (A-band) and heteropolymeric (B-band) O antigen. This prompted the study of the biosynthetic pathways of these distinct O polymers. Results obtained using genetic, chemical, and biochemical approaches supported the hypothesis that distinct pathways and assembly mechanisms were used for the two O polymers. In this study, our results showed that both PAO1waaL and PA14waaL mutants are incapable of producing LPS containing A-band, B-band, and core-plus-one O-antigen unit. This is in contrast to the function of Wzy (Rfc), which is involved only in the polymerization of B-band but not A-band LPS (12). Although ladder-like banding patterns could be observed in all LPS samples prepared by the HB method when analyzed by Western

FIG. 3. SDS-PAGE and Western immunoblotting analysis of LPS prepared from strain PAO1, mutant PAO1waaL, and complemented transconjugant. LPS from all strains were prepared by the HB method (A to D). Panel A is the silver-stained SDS-PAGE gel, and panels B to D are Western immunoblots with various monoclonal antibodies (MAb). LPS from the mutant PAO1waaL is deficient in the low-molecular-weight core-plus-one O-antigen band. Although panels B, C, and D showed the presence of LPS bands that reacted with MAb N1F10 (A-band specific), MAb MF15-4 (B-band specific), and MAb 18-19 (core-plus-one O-antigen), respectively, we suspect that these bands are undecaprenol lipid carrier-linked glycolipids that have not been ligated to core lipid A. These types of glycolipids are sensitive to phenol treatment, as described in our earlier studies (50). Panels E and F are Western immunoblotting analysis of LPS from strain PAO1, *waaL* mutant, and complemented transconjugant prepared using the hot-aqueous phenol method.

FIG. 4. Determination of twitching motility using the sub-agar-surface translocation assay. The zones of motility cells are as follows: (A) *P. aeruginosa* PAO1, (B) mutant PAO1waaL, (C) PA14, and (D) mutant PA14waaL. Where the cells are nontwitching, only a small "needle-point" at the origin of inoculation can be visualized.

immunoblotting (Fig. 3C and D), the bands in the lanes of samples prepared from mutant bacteria were not attached to core-lipid A because these bands were absent in the samples prepared by the HAP method (Fig. 3E and F). These results showed that the ladder-like bands in the samples prepared from the mutants were cleavable by the hot water-phenol treatment; therefore, these were likely glycolipids attaching to undecaprenol carrier lipids. A similar observation has been made in a previous study by our group in which size variability in the LPS-banding pattern was observed in LPS prepared from *wzm* and *wzt* (ABC transporter components) mutants compared to LPS from parent PAO1 strain (50). The LPS-bands from *wzm* and *wzt* mutants have proved to be composed of glycolipids accumulated in the bacteria, and these bands were absent when the LPS samples were prepared by hot water-phenol treatment. Furthermore, differences in LPS band migration patterns have also been observed in an outer core mutant of *E. coli* K-12 harboring the *rfb*_{EC08} cluster when compared to the LPS banding pattern of *E. coli* wild-type O8 strain (48). Despite the fact that truncated core lipid A molecules are unable to serve as acceptors of O antigen, resulting in accumulation of the *E. coli* O8 O-antigen polysaccharide, separation of O8 polymers was still observed in SDS-polyacrylamide gels, indi-

cating that the O polymers must be attached to charged carrier lipids to afford mobility in SDS-PAGE (48).

Cross-complementation of mutant strain PAO1waaL with *waaL* from strain PA14 restored both A-band and B-band LPS production. The same was observed when mutant PA14waaL was cross-complemented with *waaL* from PAO1. These results are consistent with reports by Heinrichs et al. (22, 24), who showed that WaaL proteins from *Salmonella* and other *Enterobacteriaceae* organisms have a relaxed O-antigen specificity.

Flagella are much more than just organelles for locomotion, they perform multiple roles and contribute to pathogenesis. Flagella biogenesis utilizes a sophisticated export apparatus that is closely related to the type III secretion pathway, and flagella have been shown to be a contributing factor in adhesion, surface colonization, biofilm formation, and invasion (34). Furthermore, flagella-mediated swimming motility is important in the initial approach and attachment to surfaces. O'Toole et al. (45) demonstrated that mutants deficient in the production of pili and flagella had a decreased ability to attach to various substrata. Flagella and type IV pili of *P. aeruginosa* play an important role in the early events of biofilm formation by *P. aeruginosa*. A study by Makin and Beveridge showed that *P. aeruginosa* mutants that are defective in the biosynthesis of

FIG. 5. Examination of swimming motility of *P. aeruginosa* strains. Panels A and B represent the parent strains, *waaL* mutants, and complemented transconjugants of PAO1 and PA14, respectively. Cells were inoculated with a toothpick from an overnight LB agar plate onto a swim plate and photographed after 18 h incubation at 30°C.

B-band LPS exhibited reduced attachment to hydrophilic surfaces but increased attachment to hydrophobic surfaces, while A-band LPS mutation had only mild effects on attachment to various inorganic surfaces (39). In this study, our results showed that wild-type strain PAO1 or PA14 could swim, while this mode of locomotion is abrogated in mutant PAO1waaL. These results indicated that LPS of *P. aeruginosa* plays a role in flagella biogenesis.

Twitching motility has been shown to occur in a wide range

of bacteria, and it has been very thoroughly studied in *P. aeruginosa*, in which such locomotion has been referred to as "social gliding motility." Genes affecting twitching motility have been shown to be important in the virulence of *P. aeruginosa* as well as for biofilm formation (28, 29). Studies of *P. aeruginosa* biofilms are crucial to our understanding of chronic infections in the lungs of CF patients (46). In *P. aeruginosa*, twitching motility is associated with the presence of type IV pili. Type IV pilus-mediated twitching motility may also be

FIG. 6. Electron microscopic analysis of flagella and pili from (A) PAO1, (B) mutant PAO1waaL, (C) PA14, and (D) mutant PA14waaL. Open arrows indicate the flagella and closed arrows indicate the pili. Bars equal 653.59 nm for A, 861.11 nm for B, 1503.26 nm for C, and 1638.88 nm for D. Note that in the inset of Panel B, only a few PAO1waaL mutant bacteria within a group of cells were found to possess flagella. No pili could be discerned on the surfaces of any of the mutant bacteria examined.

necessary for the bacterial cells to migrate along various surfaces in the environment or epithelial cell surfaces in host tissues to form the multicellular aggregation characteristic of the wild-type strain. In support of such a role, we present evidence that the wild-type PAO1 strain does move across the surface and form cell aggregation by recruiting cells from the adjacent environment (Fig. 4). It is intriguing to note that mutants PAO1waaL and PA14waaL that are defective in ligase activity exhibited no twitching motility. This suggested a requirement of a functional *waaL* gene for twitching motility to occur. In a recent study by our group, we showed that a *wbpL* mutant, lacking the initial glycosyltransferase required for assembly of the O-antigen repeating unit, was unable to support pilin glycosylation in *P. aeruginosa* strain 1244 (13). The data in that study also suggested that pilin glycosylation does not occur through sequential attachment of O-antigen sugars to pilin, and the glycosylation precursor may be the undecaprenol-bound repeating unit of the LPS. The effect of a *waaL* mutation to swimming and twitching motilities in *P. aeruginosa* is similar to an observation made by Toguchi et al. (58), who showed that the lack of O antigen in *Salmonella enterica* serovar Typhimurium due to a defect in core biosynthesis (*waaC* mutation), core modification (*waaK* mutation), or O-antigen biosynthesis (*waaL* mutation) affected swarming motility in this organism. They suggested that the absence of O antigen affected surface "wettability" that is required for swarm colony expansion. Therefore, apart from the observed defects in fla-

gella and pilin expression on the surface of *P. aeruginosa*, the lack of O antigen and less wettability on the *waaL* mutants provided another explanation for the reduced overall motility in this bacterium. In a study by Genevoux et al. (18), mutation in LPS core biosynthesis genes *rfaG* and *rfaP* in *E. coli* were shown to change the bacterium to exhibit a deep-rough LPS phenotype as anticipated. Interestingly, these mutants also showed a reduced level of production in type 1 fimbriae, motility, and a loss in adherence properties. The latter could be due to an effect on flagella biosynthesis due to the lack of O antigen on the bacterial cell surfaces. Furthermore, a recent report by Bengoechea et al. (5) showed that deficiency in O-antigen biosynthesis in *Yersinia enterocolitica* O:8 affected virulence and caused down-regulation of the expression of a number of well-characterized virulence proteins, namely YadA, Ail, and Inv. On the other hand, upregulation of other virulence genes was also observed, including the expression of *flhDC* operons, which are considered to be master flagella regulatory genes, *yplA* that encode phospholipase A, and type III secretion genes. Based on these findings, these authors suggested that the absence of O antigen in the outer membrane of *Y. enterocolitica* O:8 might cause cellular or membrane stress that could act as a regulatory signal affecting the expression of a number of virulence-associated genes. In the case of *waaL* mutation in *P. aeruginosa*, we detected accumulation of O-polysaccharide intracellularly in the mutant bacteria. This might lead to a disturbance of the outer membrane, thereby causing less stress and ultimately a disruption of flagella biosynthesis.

In conclusion, we have been able to clone *waaL* of *P. aeruginosa* and showed that it encoded a protein possessing ligase activity capable of linking O-antigen polysaccharide to the lipid-A core. We have also made interesting observations by TEM that a *waaL* mutation drastically affected the production of flagella and pili. These results correlate well with the significantly reduced swimming and twitching motilities observed in the mutant bacteria. The identification of WaaL from both *P. aeruginosa* PAO1 and PA14 strains provides a basis for studying the LPS assembly events in *P. aeruginosa*.

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