Evidence that WapB Is a 1,2-Glucosyltransferase of *Pseudomonas aeruginosa* Involved in Lipopolysaccharide Outer Core Biosynthesis^{∇†}

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Pseudomonas aeruginosa is an important opportunistic pathogen infecting debilitated individuals. One of the major virulence factors expressed by *P. aeruginosa* is lipopolysaccharide (LPS), which is composed of lipid A, core oligosaccharide (OS), and O-antigen polysaccharide. The core OS is divided into inner and outer regions. Although the structure of the outer core OS has been elucidated, the functions and mechanisms of the glycosyltransferases involved in core OS biogenesis are currently unknown. Here, we show that a previously uncharacterized gene, *pa1014*, is involved in outer core biosynthesis, and we propose to rename this gene *wapB*. We constructed a chromosomal mutant, *wapB::Gm*, in a PAO1 (O5 serotype) strain background. Characterization of the LPS from the mutant by Western immunoblotting showed a lack of reactivity to PAO1 outer core-specific monoclonal antibody (MAb) 5c-101. The chemical structure of the core OS of the *wapB* mutant was elucidated using nuclear magnetic resonance spectroscopy and mass spectrometry techniques and revealed that the core OS of the *wapB* mutant lacked the terminal β -1,2-linked-D-glucose residue. Complementation of the mutant with *wapB* in *trans* restored the core structure to one that is identical to that of the wild type. Eleven of the 20 *P. aeruginosa* International Antigenic Typing Scheme (IATS) serotypes produce LPSs that lack the terminal D-glucose residue (Glc^{IV}). Interestingly, expressing *wapB* in each of these 11 serotypes modifies each of their outer core OS structures, which became reactive to MAb 5c-101 in Western immunoblotting, suggesting the presence of a terminal D-glucose in these core OS structures. Our results strongly suggested that *wapB* encodes a 1,2-glucosyltransferase.

Pseudomonas aeruginosa is ubiquitous in the environment and generally regarded as a saprophyte, but it is also an important opportunistic human and animal pathogen (34). This bacterium can cause a variety of infections including some unusual ones, such as green nail syndrome associated with the use of community pools (17) and keratitis associated with the use of contact lenses (37), but mainly it infects compromised individuals, such as AIDS patients and those with burn wounds and cystic fibrosis (CF). For CF patients, *P. aeruginosa* is the major cause of morbidity and mortality (11, 32). *P. aeruginosa* is a Gram-negative bacterium and possesses lipopolysaccharide (LPS) as a major constituent of the outer leaflet of the outer membrane. LPS also serves as one of its major virulence factors (7, 30). Due to its proximity in the bacterial outer envelope, LPS plays crucial roles in maintaining structural integrity and interacting with the environment. *P. aeruginosa* LPS is composed of three distinct regions: (i) lipid A, the endotoxic moiety that anchors the LPS molecule in the outer membrane; (ii) the core oligosaccharide (OS); and (iii) the long-chain O polysaccharides (or O antigen) that consist of different repeated sugar units. These features segregate *P.*

aeruginosa strains into 20 International Antigenic Typing Scheme (IATS) serotypes.

The core oligosaccharide (OS) can be divided into inner and outer regions. The inner core is conserved among Gram-negative bacteria and is composed of two 3-deoxy-D-manno-octulosonic acid (Kdo) and two L-glycero-D-manno-heptose (LD-Hep) residues. The outer core region is more species specific, and in the case of *P. aeruginosa*, it contains one L-rhamnose (L-Rha) and one N-(L-alanyl)-D-galactosamine and either three or four D-glucose (D-Glc) residues. A single *P. aeruginosa* cell simultaneously produces two distinct core OS glycoforms. The first glycoform is capped, meaning that it is covalently attached to O antigen, while the second, uncapped core is devoid of O antigen. Besides the presence or absence of an O antigen, the two core glycoforms differ in the outer core region, particularly in the position and linkage of L-Rha, and in the presence/absence of a terminal Glc residue (Glc^{IV}) (Fig. 1). The basic core OS structure is conserved among different *P. aeruginosa* strains; however, variations can be observed in the presence of Glc^{IV} in uncapped outer core and noncarbohydrate substituents (such as phosphorylation of Hep residues or acetylation on certain sugar residues of the core). Immunochemical data produced by our group (9) and the structural elucidation of core OS reported by Bystrova et al. (2) revealed that only 9 out of the 20 IATS serotypes (O2, O5, O7, O8, O10, O16, O18, O19, and O20) reacted with outer core-specific monoclonal antibody (MAb) 5c-101, and the elucidated core OS structures of these serotypes possess terminal Glc^{IV}. Although the chemical structures of the core OS of the wild-type

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FIG. 1. Structures of the two distinct outer core OSs that are simultaneously produced by a single *P. aeruginosa* PAO1 cell. (A) Uncapped core OS is devoid of O antigen and contains an α -1,6-linked L-Rha and 1, 2-linked D-Glc^{IV}. (B) Capped core OS has a substitution of O polysaccharide through an α -1,3-linked L-Rha. GalN, galactosamine; Ala, alanine; Rha, rhamnose; Glc, glucose. Putative glycosyltransferases MigA, WapR, and WapB required for transfer of Rha^A, Rha^B, and Glc^{IV}, respectively, are depicted by arrowheads (adapted from references 18 and 26).

PAO1 strain and the IATS serotypes (2) have been elucidated, knowledge of how outer core residues are transferred to synthesize the core is lacking. We reported earlier that MigA and WapR are two putative rhamnosyltransferases associated with outer core OS biosynthesis, and these two enzymes share 35% identity. Analysis of LPS from *migA*::Gm and *wapR*::Gm mutants suggested that WapR is the α -1,3-rhamnosyltransferase that adds the L-Rha residue to the core, which then becomes the proper acceptor for the attachment of O antigen, whereas MigA is the α -1,6-rhamnosyltransferase required for the assembly of the uncapped core glycoform (26). Other putative outer core glycosyltransferases are WapH and WapG, potentially transferring Glc^{II} and GalN, respectively. However, their functions remain hypothetical since the LPS structure of a *wapH* mutant has not been determined, and a *wapG* mutant could not be constructed even though various strategies were used (23). Apparently, *wapG* is an essential gene, and mutation in this gene is lethal. Based on homology to MigA and WapR, we hypothesized that the product of the uncharacterized *pa1014* gene is also involved in outer core OS biosynthesis. To conform to the widely accepted LPS gene nomenclature and be consistent with other genes associated with core OS biosynthesis as *wap** genes (18, 27), we propose to name *pa1014* as *wapB*. We have constructed a chromosomal *wapB*::Gm mutant in the PAO1 strain (O5 serotype) background, and LPS prepared from this mutant was characterized by Western immunoblotting, nuclear magnetic resonance (NMR) spectroscopy,

and mass spectrometry (MS). The outer core OS of the mutant lacked a terminal 1,2-linked Glc^{IV} residue, suggesting that WapB is a 1,2-glucosyltransferase. We further substantiated this by showing that expression of WapB in all 11 *Pseudomonas aeruginosa* serotypes lacking the terminal Glc^{IV} residue confers a Glc substitution on their outer core OS structures that is recognized by the outer core-specific monoclonal antibody 5c-101. This indicated that WapB can be functional in all *P. aeruginosa* serotypes.

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 grown in lysogeny broth (LB; also commonly known as Luria-Bertani medium) (Invitrogen) at 37°C. Antibiotics were used at the following concentrations: for *Escherichia coli* strains, 100 μ g/ml ampicillin, 15 μ g/ml gentamicin, and 15 μ g/ml tetracycline; and for *P. aeruginosa* strains, 300 μ g/ml carbenicillin, 150 μ g/ml gentamicin, and 90 μ g/ml tetracycline.

General DNA methods. Primers were prepared by Sigma Genosys. PCR was performed according to the KOD hot start procedure (Novagen). Restriction enzymes, Klenow fragment, and T4 DNA ligase were obtained from New England BioLabs. All enzymes were used according to the manufacturers' instructions. Chemically competent *E. coli* DH5 α cells were prepared using the rubidium chloride method outlined by New England BioLabs (24). Constructs were routinely transformed into *P. aeruginosa* strains using the benchtop method (6). Nucleotide sequences of all constructs were verified by the Laboratory Services Division at the University of Guelph (Guelph, Ontario, Canada).

Generation of *wapB*::Gm mutant and complemented strains. A chromosomal mutant of *wapB* was constructed by a gene replacement strategy using the *aac*(3) gene cassette encoding aminoglycoside acetyltransferase that confers resistance to gentamicin (Gm), as described by Schweizer and Hoang (28). Specifically, the *wapB* gene was amplified from the genome of *P. aeruginosa* PAO1 with the following primers: 14MF, GGGTGGTTCGTCCTCGCTCTG, and 14MR, CAGG GCGAGGCACTGAAGGAG. The amplified gene was inserted into the HincII site of pUC18 to obtain plasmid pUC $wapB$. A Flp recognition target (FRT)-Gm^r-FRT cassette excised from the plasmid pS856 (16) using SacI was blunt ended with Klenow fragment (NEB). This fragment was then inserted into the unique HincII site of the gene *wapB* in plasmid pUC $wapB$. The replacement vector pEX $wapB$ Gm was constructed by insertion of the disrupted allele *wapB*::Gm into the SphI and XmaI sites of the plasmid pEX18AP (16). The pEX $wapB$ Gm vector was then conjugated from *E. coli* SM10 to *P. aeruginosa* PAO1 according to the method described by de Lorenzo and Timmis (10). Single recombination mutants were selected on Difco *Pseudomonas* isolation agar (BD Biosciences) with 300 μ g/ml of carbenicillin. These mutants were then streaked on LB agar with gentamicin and 7% sucrose to select for bacteria that had undergone double recombination. PCR was performed to confirm that allelic exchange between the chromosomal wild-type *wapB* and the *wapB*::Gm had occurred.

Construction of a complementation vector. The *wapB* gene was PCR amplified by the primer pair *wapB*_F/Xba (CTTCTAGACTTCGATTTTCTGTCTCTCG AGC) and *wapB*_R/HindIII (CAGAAGCTTTCATCGACCCTCGACGCCGGT ACC), and PAO1 genomic DNA was used as a template. The PCR product and pUCP26 vector were digested with XbaI and HindIII and ligated overnight at 16°C. The ligation was transformed into *E. coli* cells, and the transformants were selected on tetracycline plates.

Analysis of LPS prepared from the *wapB*::Gm mutant. LPSs from the wild-type and mutant bacteria were prepared by the SDS and proteinase K method described by Hitchcock and Brown (15). The LPS samples were resolved by electrophoresis in Tricine-SDS-PAGE gels as described by Maskell (22). The LPS banding patterns were visualized by the ultrafast silver staining method (13). In the Western immunoblotting procedure, LPS was transferred onto BioTrace NT nitrocellulose membranes (Pall), and the blots were probed with MAb 5c-101 (outer core specific), 5c-7-4 (inner core specific), N1F10 (A band O antigen specific), MF15-4 (B band O antigen specific), and 5c-18-19 (specific for the core OS substituted with one unit of the O antigen [core-plus-one]) (8). The secondary antibody used was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G at a dilution of 1:2,000 (Jackson ImmunoResearch). The blots were developed using standard protocols by incubation with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (1).

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype, phenotype, or properties	Source or reference
<i>E. coli</i> strains		
DH5 α	λ^- ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(r _K ⁻ m _K ⁻) supE44 thi-1 gyrA relA1	Invitrogen
SM10	thi-1 thr leu tonA lacY supE recA RP4-2-Tc::Mu Km ^r	29
<i>P. aeruginosa</i> strains		
PAO1	IATS serotype O5; wild-type type strain	14
wapB::Gm strain	PAO1 derivative; Gm ^r -marked wapB mutant	This study
wapB::Gm/wapB strain	Complemented wapB::Gm strain containing pUCP26wapB	This study
O1	IATS serotype O1, wild-type type strain ATCC 33348	ATCC
O1/wapB	O1 strain transformed with pUCP26wapB; Tc ^r	This study
O3	IATS serotype O3, wild-type type strain ATCC 33350	ATCC
O3/wapB	O3 strain transformed with pUCP26wapB; Tc ^r	This study
O4	IATS serotype O4, wild-type type strain ATCC 33351	ATCC
O4/wapB	O4 strain transformed with pUCP26wapB; Tc ^r	This study
O6	IATS serotype O6, wild-type type strain ATCC 33354	ATCC
O6/wapB	O6 strain transformed with pUCP26wapB; Tc ^r	This study
O9	IATS serotype O9, wild-type type strain ATCC 33356	ATCC
O9/wapB	O9 strain transformed with pUCP26wapB; Tc ^r	This study
O11	IATS serotype O11, wild-type type strain ATCC 33358	ATCC
O11/wapB	O11 strain transformed with pUCP26wapB; Tc ^r	This study
O12	IATS serotype O12, wild-type type strain ATCC 33359	ATCC
O12/wapB	O12 strain transformed with pUCP26wapB; Tc ^r	This study
O13	IATS serotype O13, wild-type type strain ATCC 33360	ATCC
O13/wapB	O13 strain transformed with pUCP26wapB; Tc ^r	This study
O14	IATS serotype O14, wild-type type strain ATCC 33361	ATCC
O14/wapB	O14 strain transformed with pUCP26wapB; Tc ^r	This study
O15	IATS serotype O15, wild-type type strain ATCC 33362	ATCC
O15/wapB	O15 strain transformed with pUCP26wapB; Tc ^r	This study
O17	IATS serotype O17, wild-type type strain ATCC 33364	ATCC
O17/wapB	O17 strain transformed with pUCP26wapB; Tc ^r	This study
Plasmids		
pEX18Ap	Counterselectable, mobilizable suicide vector for gene replacement; contains OriT and sacB; Ap ^r	16
pEXwapB::Gm	Replacement vector; Ap ^r	This study
pPS856	Gm ^r cassette vector for generation of marked or unmarked mutants via gene interruption	16
pUCP26	pUC18-derived <i>E. coli</i> - <i>P. aeruginosa</i> shuttle vector; Tc ^r	33
pUCP26wapB	Complementation plasmid containing wild-type wapB	This study

Purification of LPS. Large-scale LPS preparations were obtained by the hot aqueous phenol method as described previously (35).

Preparation of the completely deacylated LPS. LPS samples (80 mg each) in polypropylene vials were dissolved in 4 M KOH (4 ml each), kept overnight at 120°C, and neutralized with 2 M HCl. Precipitated material was removed by centrifugation, and deacylated material was isolated by gel chromatography on a Biogel P10 column (2.5 by 60 cm).

Hydrazine O-deacylation of the LPS. LPS (50 mg) was dissolved in anhydrous hydrazine (1 ml) and kept at 50°C for 1 h, poured into 50 ml of water, dialyzed, and freeze-dried.

NMR experiments. Isolation of the O,N-deacylated core-lipid A backbone of the LPS was performed on a Sephadex G50 column. This column does not allow fine separation between the glycoforms of the core OS, and all glycoforms were collected together (one broad peak). However, NMR spectroscopy is sufficiently sensitive for studying a mixture of the various forms of OS molecules, provided there is not too much signal overlap. This approach has been used in recent studies to distinguish between two different core OS structures (26). NMR experiments were carried out on a Varian INOVA 600 MHz (¹H) spectrometer with a 5-mm gradient probe at 25°C with an acetone internal reference (2.225 ppm for ¹H and 31.45 ppm for ¹³C) using standard pulse sequences: double-quantum filtered correlation spectroscopy (DQCOSY), total correlation spectroscopy (TOCSY; mixing time, 120 ms), rotation-frame nuclear Overhauser effect spectroscopy (ROESY; mixing time, 500 ms), ¹H-¹³C heteronuclear single-quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC; 100-ms long-range transfer delay). Acquisition time (AQ) was kept at 0.8 to 1 s for H-H correlations and at 0.25 s for HSQC; 256 increments were acquired for t1.

CE-MS analysis. Capillary electrophoresis (CE) analysis was performed using a 4000 Q-Trap mass spectrometer (Applied Biosystems/Sciex, Concord, Ontario, Canada) via a CE-MS interface with a 90-cm length of bare fused-silica capillary using 15 mM ammonium acetate in deionized water, pH 7.0. A sheath solution (isopropanol-methanol at 2:1) was delivered at a flow rate of 1.5 μ l/min. The orifice voltage was set at -110 V.

RESULTS

LPS analysis of the wapB::Gm mutant. A BLAST search in the *P. aeruginosa* PAO1 genome revealed that an uncharacterized gene, *pa1014*, which we propose to be renamed *wapB*, encodes a protein that shares a relatively high sequence identity to MigA (58%) and WapR (33%), two putative rhamnosyltransferases required for outer core biosynthesis (see Fig. S1 in the supplemental material). This led us to hypothesize that *wapB* is involved in outer core OS biosynthesis. To characterize the function of *wapB*, a chromosomal knockout based on gene replacement of *wapB* with *wapB*::Gm was constructed in the *P. aeruginosa* PAO1 background. LPS from this mutant was resolved by SDS-PAGE, and silver staining of the gel showed a banding pattern that is very similar to that of the wild-type LPS, i.e., revealing a capped core with visible high-

FIG. 2. SDS-PAGE and immunoblotting analysis of LPS from the PAO1, *wapB*::Gm, and complemented strains. (A) Silver-stained SDS-PAGE. (B and C) Immunoblotting analysis using inner core-specific 7-4 antibody (B) and uncapped core-specific MAb 5c-101 (C). Core of the *wapB*::Gm mutant showed truncation and was not detected by the 5c-101 antibody, whereas the cores of PAO1 and the complemented strain showed identical banding patterns, and both cores were recognized by 5c-101. LPS from the *migA*::Gm mutant was used as a control.

molecular-weight O-antigen bands and lower-molecular-weight core-plus-one O-antigen repeat bands (Fig. 2A). As confirmed by Western immunoblotting using monoclonal antibodies (MAb) MF15-4 (B band O-polysaccharide specific), N1F10 (A band O-polysaccharide specific), and 5c-18-19 (core-plus-one O-repeat specific) (see Fig. S2 in the supplemental material), the biosynthesis of these higher-molecular-weight LPS molecules was not affected by the *wapB* mutation. In contrast, the core LPS of the *wapB*::Gm mutant exhibited faster relative mobility in SDS-PAGE than the wild type, suggesting a truncation within the core OS (Fig. 2A and B). Moreover, when LPS molecules were analyzed by immunoblotting using the outer core-specific MAb 5c-101, the core OS of the wild type was recognized by the antibody, whereas LPS of the mutant was not (Fig. 2C). The phenotype was fully restored by complementation with *wapB* *in trans*. The low-molecular-weight core LPS band from the complemented strain showed reactivity with MAb 5c-101 and relative mobility indistinguishable from that of the wild-type core-lipid A LPS band. LPS of the *migA* mutant, which has a deficiency in outer core biosynthesis, was used as a control to allow for the comparison of the mobilities of core LPS bands between the *migA* and *wapB* mutants. The results showed that the mobilities of the low-molecular-weight core LPS bands between these mutant strains were similar in the SDS-PAGE gels. The 16% discontinuous Tricine-SDS-PAGE gel system did not provide sufficient resolution to show the difference between the core OS of

the *migA* mutant and that of the *wapB* mutant. However, the low-molecular-weight bands from both mutants migrated faster than those of the wild-type bacteria. These results support our hypothesis that WapB is required for outer core LPS biosynthesis.

Structural analysis of core OS from a *wapB*::Gm mutant and complemented strains. To further determine the function of WapB, we elucidated the structure of LPS prepared from the *wapB* mutant and the complemented strains by using NMR spectroscopy and mass spectrometry. Completely deacylated LPSs from mutant and complemented strains were prepared from the LPSs of these bacteria by KOH treatment. The products had well-resolved NMR spectra and were analyzed without further purification. A set of two-dimensional spectra (DQCOSY, TOCSY, NOESY, ^1H - ^{13}C gHSQC, ^1H - ^{13}C gradient HMBC [gHMBC], ^1H - ^{31}P HMQC, and ^1H - ^{31}P HMQC-TOCSY) was recorded for each compound and interpreted using the Topspin (Bruker) program. Spectra of the deacylated products from both strains were similar except for the absence of β -Glc (residue N) in the *wapB* mutant. Due to removal of this residue, the H-1 signal of the Rha (K residue) significantly shifted upfield from 5.12 ppm in the complemented strain to 4.82 ppm in the *wapB* mutant (Table 2 and Fig. 3). Positions of ^{13}C signals of Rha (K residue) in the *wapB* mutant strain corresponded to nonsubstituted α -rhamnopyranose. Assignment of the configuration and connectivity (i.e., J/H coupling constants) for each spin system led to the structures presented in Fig. 4. NMR signals for the uncapped core of the OS1 (2519.56 atomic mass units [amu]) and OS3 (2357.5 amu) corresponded well with data published previously, with OS1 being the uncapped core of the complemented strain and OS3 being the uncapped core of the mutant (3, 20). The capped OS2 (2502.6 amu) was identical in the mutant and the complemented strains, and this mass was consistent with that of core OS with a substitution of one unit of the O antigen (also called core-plus-one O antigen LPS glycoform). The initiating sugar of the B band of the O5 serotype linked to L-Rha (Fig. 4, M residue) was β -FucN, because the acetyl group of β -FucNAc was removed by KOH treatment. Fucosamine was detected in a β -configuration as it is always observed with the monosaccharide linking O antigen to the core. When LPS was O deacylated with hydrazine, complete repeating units with two 2,3-diamino-2,3-dideoxymannuronic acids were present in capped glycoforms of both strains, as confirmed by MS. Data from MS analysis of the oligosaccharides are in agreement with the presented structures. The observed masses of the core-plus-one capped glycoform at 3,488.0 amu (Fig. 5A) in the mutant strain and at 3,487.6 amu (Fig. 5B) in the complemented are in accordance with the calculated mass for $\text{Rha}_1\text{Hex}_3\text{Hep}_2\text{HexN}_3\text{HexNNA}_2\text{C12OH}_2\text{P}_5\text{Ala}_1\text{Ac}_4$, which is 3,486.1 amu (the amidine group was removed by the hydrazine treatment). The masses of O-deacylated uncapped glycoforms of both strains were in agreement with the structures determined by NMR. The molecular mass of uncapped glycoform of the mutant strain was 2,826.0 amu (Fig. 5A), which corresponds to chemical composition of $\text{Rha}_1\text{Hex}_3\text{Hep}_2\text{HexN}_3\text{HexNNA}_2\text{C12OH}_2\text{P}_5\text{Ala}_1$, whereas the uncapped core of complemented strain had a molecular mass of 2,986.9 Da (Fig. 5B). Therefore, the shift in molecular masses of the two strains corresponds to addition of one Glc residue in the complemented strain.

TABLE 2. NMR data for the outer core of OS1, OS2, and OS3

Unit, sugar, compound(s) ^a	Nucleus	δ (ppm)					
		H/C 1	H/C 2	H/C 3	H/C 4	H/C 5	H/C 6
G, -3,4-GalN, all	H	5.61	3.87	4.48	4.42	3.91	3.80/3.86
	C	98.7	52.0	78.0	77.0	72.8	61.7
J ^b , t- α -Glc, all	H	4.99	3.58	3.77	3.46	3.74	3.74/3.88
	C	99.9	72.7	73.6	70.7	73.6	62.3
J ^b , t- α -Glc, all	H	5.03	3.59	3.72	3.41	3.75	3.74/3.89
	C	99.6	72.7	73.6	71.0	73.6	62.3
L, t- α -Glc, OS2	H	5.06	3.52	3.77	3.53	4.08	3.84/3.86
	C	100.7	73.2	73.6	70.3	72.7	61.6
L, -6- α -Glc, OS1	H	5.02	3.52	3.77	3.62	4.18	3.79/3.91
	C	100.8	73.2	73.6	70.3	71.8	67.7
M, -3- α -Rha, OS2	H	5.19	4.30	3.99	3.65	4.08	1.28
	C	101.8	71.7	81.0	72.6	70.2	17.8
K, t-Rha, OS1	H	4.82	4.03	3.83	3.46	3.75	1.33
	C	102.5	71.2	72.7	73.5	70.2	18.5
H, 6- β -Glc, OS1, OS3	H	4.68	3.28	3.55	3.29	3.72	3.81/3.92
	C	106.1	74.7	77.1	71.8	76.0	69.1
H, -3,6- β -Glc, OS2	H	4.68	3.50	3.67	3.52	3.72	3.81/3.92
	C	106.1	75.0	83.3	69.9	76.0	69.1
X, β -FucN, OS2	H	4.92	3.23	3.91	3.81	3.83	1.30
	C	102.2	54.9	73.0	71.6	72.7	17.0

^a Residue labels and oligosaccharide structures of OS1, OS2, and OS3 correspond to those included in Fig. 4.

^b The residue had two variants with signal overlap.

To summarize the above observations, based on NMR and MS analyses, both strains produced two distinct glycoforms corresponding to the capped and uncapped core OSs (Fig. 4). The outer core OS structures of the capped glycoforms, designated OS2, of the *wapB*::Gm mutant and the complemented strain were identical to the OS structure of PAO1 as shown previously (26). The uncapped cores of the two strains differed in the absence of terminal β -linked D-Glc (Fig. 4, N residue) in the mutant, whereas the structure of the uncapped core OS of the complemented strain was identical to the PAO1 core structures published previously (26). These structural data on LPSs from the mutant and the complemented strain strongly indicate that WapB is a 1,2-glucosyltransferase.

Expression of WapB in serotypes lacking Glc^{IV}. Since not all the *P. aeruginosa* strains contain Glc^{IV} in the core OS, we decided to test if *wapB* in a plasmid construct could be functional and able to confer the transfer of a Glc^{IV} in the strains that lacked this terminal sugar residue in their LPSs. We chose the Glc^{IV}-negative IATS reference strains, O1, O3, O4, O6, O9, O11, O12, O13, O14, O15, and O17, and transformed them with the plasmid pUCP26*wapB*. LPS prepared from each of the transformants reacted positively with MAb 5c-101 in Western immunoblotting (Fig. 6B), whereas the LPS from the wild-type strain of each of these serotypes was not recognized by the MAb (Fig. 6A). Interestingly, the strength of the reactivity of MAb 5c-101 to the LPS core band of all the serotypes expressing WapB was comparable to that observed between the antibody and the complemented *wapB*::Gm/*wapB* strain.

There was one exception: a weak interaction was observed between MAb 5c-101 and the LPS prepared from serotype O6 expressing *wapB* *in trans*. At present, we do not have an explanation for this anomaly. Nevertheless, these data showed that WapB was able to modify the LPS core of all the serotypes lacking the terminal D-Glc^{IV} residue. Interestingly, a search for *wapB* genes in sequenced *P. aeruginosa* genomes of the serotypable strains PA14 and PA7 revealed that the genome of PA14, a Glc^{IV}-positive strain, contains the *wapB* gene in the same location as observed in PAO1, whereas *wapB* is absent from the genome of PA7, a Glc^{IV}-negative strain. This presence and absence of *wapB* in PA14 and PA7 genomes, respectively, is in good agreement with the serotype identities of PA14 (O10; Glc^{IV} positive) and PA7 (O12; Glc^{IV} negative).

DISCUSSION

In this report, we showed that the uncharacterized gene *pa1014* (annotated as *wapB*) of *P. aeruginosa* PAO1 is required for transferring the β -1,2-linked D-Glc^{IV} to the outer core OS. This residue was not present in the LPS core structure of the *wapB*::Gm mutant as determined by NMR. Since the only difference in the core LPSs between the mutant and complemented strains was the absence of Glc^{IV} in the mutant core OS, the evidence suggested that WapB is a putative β -1,2-linkage-specific glucosyltransferase. LPS from the *wapB*::Gm mutant was not recognized by MAb 5c-101, whereas adding *wapB* *in trans* in the mutant restored reactivity to the antibody.

FIG. 3. NMR spectra of the *wapB*::Gm mutant and the complemented strain. The figure shows the overlap of fragments of TOCSY spectra of fully deacylated LPS from the mutant (black) and complemented strain (gray), showing correlations from anomeric protons. Signals of individual spin systems are connected by horizontal lines and labeled at one end by sugar residue labels shown on the structural formula. Note the absence of β -glucose (N residue) in the mutant and a large shift in position of anomeric signal of the Rha (K residue), caused by this difference between the structures of the mutant and complemented strains (arrow). Letters correspond to sugar residues depicted in Fig. 4.

A.

OS1: MW = 2357.5

OS2*: MW = 2502.6

B.

OS3: MW = 2519.56

OS2*: MW = 2502.6 identical to the structure shown above in A.

FIG. 4. LPS structures derived from NMR spectroscopy of the fully deacylated LPS from the *wapB*::Gm mutant (A) and *wapB*::Gm/*wapB* complemented (B) strains. OS1, uncapped core oligosaccharide of the mutant; OS2*, capped (core-plus-one glycoform) oligosaccharides that are present in the mutant and the complemented strain (note that as anticipated, the two elucidated OS2 structures are identical); OS3, uncapped core oligosaccharide of the complemented strain.

Thus, our results have verified that the MAb 5c-101 epitope includes Glc^{IV} of the outer uncapped core, as hypothesized earlier by our group (9). There are still two other Glc residues (Glc^I and Glc^{III}) in the outer core OS that are transferred by unidentified enzymes; therefore, other unknown glucosyltransferases are involved in outer core biosynthesis. This is consistent with what has been reported for the biosynthesis of the *E. coli* and *Salmonella* outer core OSs, where each Glc residue with unique linkage is transferred by a different enzyme (reviewed in reference 36).

The *P. aeruginosa* PAO1 genome contains a gene cluster (PA4996-PA5012) encoding proteins involved in core OS biosynthesis. At present, many of the genes in this cluster have not been fully characterized to determine their roles in core OS biosynthesis (18). A putative glucosyltransferase, PA5001, is localized in this cluster, and it might be required for the addition of Glc^{III} (as proposed by King and coauthors [18]). Our group has recently reported that the encoded product of the *ssg* gene, a homologue of PA5001, in *Pseudomonas alkylphenolia* is a putative glucosyltransferase that is important for both LPS and exopolysaccharide biosynthesis (31). In terms of having the last outer core glucosyltransferase gene located outside the core OS biosynthesis locus, there is precedent to this observation since a gene encoding the putative 1,6-rhamnosyltransferase, MigA, is also localized outside the core cluster. In other *Pseudomonas* species whose genomes have been sequenced, *wapB* homologues could not be detected. However, other

FIG. 5. Mass spectrometry analysis of O-deacylated LPS from the *wapB*::Gm mutant (A) and *wapB*::Gm/*wapB* complemented (B) strains. Electrospray ionization mass spectra are in deconvoluted negative mode. The main peaks corresponding to the capped core OS with one O-antigen repeat (core-plus-one glycoform) and uncapped glycoforms are depicted with stars and rectangles, respectively. The shift in masses of uncapped core OS between the mutant and complemented strains corresponds to one glucose residue that was absent in the mutant. Arrows indicate the following core OS modifications: P, phosphate group; EtN, ethanolamine; PEtN, phosphoethanolamine.

genes sharing a higher level of homology to *migA* than to *wapB* were observed. This observation correlates well with the elucidated core OS structures of *Pseudomonas syringae*, *Pseudomonas stutzeri*, and *Pseudomonas fluorescens*, which do not

contain β -1,2-linked Glc residues (19, 21, 40). Hence, WapB is apparently unique in *P. aeruginosa*; however, it is intriguing that not all the strains in this species produce the terminal Glc^{IV} residue in the outer core. Transforming serotype type

FIG. 6. Immunoblotting analysis of LPS from Glc^{IV}-negative strains expressing WapB and corresponding wild-type Glc^{IV}-negative strains. The reference strains of *P. aeruginosa* serotypes IATS O1, O3, O4, O6, O9, O11, O12, O13, O14, O15, and O17 were transformed with pUCP26wapB, and immunoblots were probed with uncapped core-specific MAb 5c-101. LPSs of wild-type reference strains were not recognized by 5c-101 (A), whereas LPS from transformants were recognized (B). PAO1 and the complemented strain *wapB*::Gm/*wapB* were used as controls.

strains lacking Glc^{IV} in the outer core with plasmid DNA carrying *wapB* has led to a change in their core OS structures, as assayed by the reactivity to MAb 5c-101 in Western immunoblotting. The weakest reactivity between the core LPS and this MAb was observed in serotype O6 expressing *wapB* *in trans*. The outer core structure of the O6 strain has been elucidated, and apart from the absence of Glc^{IV}, it possesses no other apparent structural differences from the outer core OS of PAO1 (4). Hence, we speculate that WapB was not able to effectively transfer Glc to the O6 core acceptor because of the presence of nonsugar modifications, for instance, O-acetylation that may be lost during the sample preparation procedures prior to structural analyses (19). As reported earlier, in some *P. aeruginosa* strains, core OSs are nonstoichiometrically O acetylated, and most O-acetyl groups were located on the terminal L-Rha^A of the uncapped glycoform (2), which may suggest that the core of the O6 strain is highly O acetylated on the L-Rha^A, which would prevent the core from being glucosylated by WapB.

Interestingly, the core OSs have been shown to play an active role in pathogenesis of *P. aeruginosa*, especially in the interaction of the bacterium with epithelial and corneal cells (12, 25, 38, 39). The specific core residues of uncapped or capped core OSs that serve as ligands for interaction with cell receptors have not been identified. Nevertheless, the tissue culture or animal experiments suggested that even though the core OS is a short polymer, it is likely not completely covered by a long-chain O antigen at the cell surface, thus allowing core OS to interact with environment. This observation may be a reason why *P. aeruginosa* simultaneously produces two glycoforms, one of which is not substituted by O polysaccharides, which allows more core OS to be available for interactions with not only cells but also the environment.

WapB is the closest homologue of MigA, the uncapped outer core rhamnosyltransferase. Strikingly, MigA shares more identity with WapB, a putative glucosyltransferase, than with the other outer core rhamnosyltransferase (see Fig. S1 in the supplemental material). All three homologues belong to the same inverting glycosyltransferase (GT-2) family (CAZy database [5]) and may have similar enzymatic mechanisms. In future experiments, it will be necessary to develop an enzymatic assay in order to determine the enzymatic mechanism of WapB as a 1,2-glucosyltransferase.

In conclusion, we have provided genetic and chemical structure evidence to demonstrate that *wapB* is required for the addition of Glc^{IV} to the proximal location in the uncapped outer core OS of *P. aeruginosa* PAO1. We characterized the core OS structure of the *wapB* knockout mutant by Western immunoblotting and NMR and MS analyses. The mutant core OS lacked the terminal β -1,2-linked D-Glc^{IV}, indicating that WapB is a 1,2-glucosyltransferase. Although not all the serotypes of *P. aeruginosa* produce an uncapped core OS with the terminal Glc^{IV}, expressing *wapB* *in trans* in these serotypes can confer reactivity to MAb 5c-101, which strongly suggests addition of Glc^{IV} onto their outer core structures.

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