Functional Characterization of MigA and WapR: Putative Rhamnosyltransferases Involved in Outer Core Oligosaccharide Biosynthesis of *Pseudomonas aeruginosa*[∇]

Karen K. H. Poon,^{1†‡} Erin L. Westman,^{1†} Evgeny Vinogradov,² Shouguang Jin,³ and Joseph S. Lam^{1*}

Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada¹; Institute for Biological Sciences, National Research Council, Ottawa, Ontario K1A 0A6, Canada²; and Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida 32610³

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Pseudomonas aeruginosa lipopolysaccharide (LPS) contains two glycoforms of core oligosaccharide (OS); one form is capped with O antigen through an α -1,3-linked L-rhamnose (L-Rha), while the other is uncapped and contains an α -1,6-linked L-Rha. Two genes in strain PAO1, *wapR* (PA5000) and *migA* (PA0705), encode putative glycosyltransferases associated with core biosynthesis. We propose that WapR and MigA are the rhamnosyltransferases responsible for the two linkages of L-Rha to the core. Knockout mutants with mutations in both genes were generated. The *wapR* mutant produced LPS lacking O antigen, and addition of *wapR* in *trans* complemented this defect. The *migA* mutant produced LPS with a truncated outer core and showed no reactivity to outer core-specific monoclonal antibody (MAb) 5C101. Complementation of this mutant with *migA* restored reactivity of the LPS to MAb 5C101. Interestingly, LPS from the complemented *migA* strain was not reactive to MAb 18-19 (specific for the core-plus-one O repeat). This was due to overexpression of MigA in the complemented strain that caused an increase in the proportion of the uncapped core OS, thereby decreasing the amount of the core-plus-one O repeat, indicating that MigA has a regulatory role. The structures of LPS from both mutants were elucidated using nuclear magnetic resonance spectroscopy and mass spectrometry. The capped core of the *wapR* mutant was found to be truncated and lacked α -1,3-L-Rha. In contrast, uncapped core OS from the *migA* mutant lacked α -1,6-L-Rha. These results provide evidence that WapR is the α -1,3-rhamnosyltransferase, while MigA is the α -1,6-rhamnosyltransferase.

Pseudomonas aeruginosa is an important gram-negative opportunistic pathogen capable of producing virulence determinants, including secreted toxins and enzymes, as well as cell surface structures, such as adhesins, flagella, and lipopolysaccharide (LPS) (18). LPS is a glycolipid composed of three distinct regions: lipid A, core oligosaccharide, and O antigen. *P. aeruginosa* produces two forms of O antigen, which are the homopolymeric A band and the heteropolymeric B band (15, 21). The gene clusters associated with the biosynthesis of A-band and B-band O antigen in *P. aeruginosa* have been well characterized (for reviews, see references 18 and 22). In contrast, relatively little is known about the biosynthesis of the core oligosaccharide (OS).

The *P. aeruginosa* core OS, like that of other gram-negative bacteria, can be conceptually subdivided into the inner core and the outer core (Fig. 1). The inner core is highly conserved and contains two L-glycero-D-manno-heptose (Hep) residues and two 3-deoxy-D-manno-octulosonic acid (Kdo) residues; the genetics and enzymology of this region are the best understood to date (14). The outer core is composed of four D-glucose

(D-Glc) residues, one L-rhamnose (L-Rha) residue, and one N-(L-alanyl)-D-galactosamine residue, and its biosynthesis is not fully understood. The structures of the core OS of several different serotypes, clinical isolates, and rough mutants of *P. aeruginosa* have been reported previously (2, 3, 12, 17, 24). The results of the previous studies showed that there are minor variations in phosphate and O-acetyl substituents in some of the core sugars. Furthermore, there are two glycoforms of the core OS, depending on the linkage of L-Rha on a D-Glc residue. One of these glycoforms contains L-Rha that is α -1,3 linked to D-Glc and acts as the acceptor molecule for the covalent attachment of the A- or B-band O antigen. This glycoform is known as the “capped core.” The other core glycoform contains L-Rha that is α -1,6 linked to D-Glc and is not substituted with O antigen; therefore, this glycoform is known as the “uncapped core” (24, 25). The glycosyltransferases responsible for attaching the L-Rha residues could play an important role in regulating the relative amount of each glycoform present on the cell surface.

L-Rha is derived from the nucleotide-activated sugar dTDP-L-Rha. Production of dTDP-L-Rha begins with glucose-6-phosphate, a common intermediate for sugar-nucleotide production in *P. aeruginosa*, and is converted by AlgC to glucose-1-phosphate (19). Four additional metabolic steps, catalyzed by proteins encoded by genes of the *mmlBDAC* operon, are then required to form dTDP-L-Rha (for a review, see reference 16). An *mmlC* knockout mutant of *P. aeruginosa* PAO1 was found to produce LPS lacking O antigen and L-Rha, because the precursor compound dTDP-L-Rha was not produced (20). Sugar-nucleotides,

* Corresponding author. Mailing address: Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON N1G 2W1, Canada. Phone: (519) 824-4120, ext. 53823. Fax: (519) 837-1802. E-mail: jlam@uoguelph.ca.

† K.K.H.P. and E.L.W. contributed equally to this work.

‡ Present address: Department of Physiology and Biophysics, University of Calgary, 3330 Hospital Drive N.W., Calgary, AB T2N 4N1, Canada.

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A. Uncapped core oligosaccharide

B. Capped core oligosaccharide (B-band O antigen)

FIG. 1. Structures of the two distinct core OS present in *P. aeruginosa* strain PAO1. (A) Uncapped core OS that is devoid of O antigen and contains an α -1,6-linked L-Rha thought to be added by MigA. (B) Capped core OS that provides the site of O-antigen attachment through an α -1,3-linked L-Rha thought to be added by WapR. Abbreviations: Hep, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-octulosonic acid; FucNAc, N-acetylglucosamine (2-acetamido-2,6-dideoxy-D-galactose); ManNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid; ManNAc3NAmA, 2-acetamido-3-acetamidino-2,3-dideoxy-D-mannuronic acid; GalN, galactosamine; Ala, alanine; Cm, carbamoyl group; P, phosphate. Adapted from references 2, 24, and 25.

such as dTDP-L-Rha, are utilized for core OS biosynthesis by enzymes encoded in the large core OS assembly cluster that spans the region corresponding to PA5012 to PA5001 in the PAO1 genome (27). The first 6 of the 12 open reading frames in the locus are required for biosynthesis of the inner core, but the roles of the last 6 open reading frames of the operon have not been characterized (5, 28).

Minor variations in the outer core OS have been observed in *P. aeruginosa* strains. In particular, the uncapped core OS has a terminal β -D-Glc attached to α -L-Rha in strain PAO1 (serotype O5), but the β -D-Glc is not present in strain 170041 (serotype O6) or in clinical isolate 2192 (3, 12). Previous work using Western immunoblotting demonstrated that monoclonal antibody (MAb) 5C101 reacted to the outer core OS of serotype O5 but not to that of O6, which suggests that this antibody recognizes the terminal β -D-Glc (6). The L-Rha residue may also be O acetylated, as observed in isolate 2192. O acetylation of L-Rha appears to be nonstoichiometric, and the biological significance of the O-acetyl substitution is presently unknown (3, 12).

The other rhamnose enantiomer, D-Rha, is a constituent of the A-band O antigen but is not present in the core OS. The precursor, GDP-D-Rha, is synthesized from glucose-6-phosphate in a six-step pathway that ends with the action of Rmd, a GDP-4-keto-6-deoxy-D-mannose reductase (23). An *rmd* knockout strain, which does not produce GDP-D-Rha, is a useful background with which to pursue analysis of the role of L-Rha in the core OS (20).

Incorporation of L-Rha into the nascent core OS is an important step in LPS biosynthesis. Since the site and type of L-Rha linkage differentiate the core OS into the capped and uncapped glycoforms, this is a possible regulatory step. Identification of the rhamnosyltransferases involved in the specific addition of L-Rha is, therefore, a significant goal for improving our understanding of LPS biosynthesis. Two putative rhamnosyltransferases have been identified and are under investigation. The gene encoding the first rhamnosyltransferase, *wapR* (PA5000), is located 158 bp downstream of the core OS biosynthesis operon, and the enzyme shows similarity to members of glycosyltransferase family 2. The second putative rhamnosyltransferase is encoded by *migA* (PA0705), which was initially identified using an in vivo selection system (29). MigA and WapR share 54% similarity. Enhanced expression of *migA* was observed when *P. aeruginosa* wild-type strain PAO1 or PAK was grown in the presence of sputum from cystic fibrosis patients and was correlated with reduced production of low-molecular-weight LPS (29, 32). Interestingly, overexpression of MigA resulted in a reduction in the amount of capped core OS with one O-antigen repeat unit (called "core-plus-one") but not in the amount of A-band or B-band LPS, which contains capped core OS with many O-antigen repeat units. Also, *P. aeruginosa* isolated from clinical sputum samples invariably exhibited an increase in the expression of *migA* transcripts (32). Although *migA* is known to be regulated by the RhlI/RhlR quorum-sensing regulatory system, the function of MigA has not been described yet (32).

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype, phenotype, or properties	Source or reference
<i>E. coli</i> strains		
JM109	e14 ⁻ (McrA ⁻) <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1</i> Δ (<i>lac-proAB</i>) [F' <i>traD36⁺ proAB⁺ lacI^qZ</i> Δ M15]	Stratagene
DH5 α	F ⁻ ϕ 80d <i>lac</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44</i> λ^- <i>thi-1 gyrA96 relA</i>	Invitrogen
<i>P. aeruginosa</i> strains		
PAO1	IATS serotype O5; <i>P. aeruginosa</i> type strain (ATCC 33351)	9a
KHP-wapR	PAO1 derivative; Gm ^r -marked <i>wapR</i> mutant	This study
KHP-migA	PAO1 derivative; Gm ^r -marked <i>migA</i> mutant	This study
KHP-migA/rmd	PAO1 derivative; Gm ^r -marked <i>migA rmd</i> double mutant	This study
RML- Δ C	PAO1 derivative; unmarked <i>rmlC</i> mutant	20
Plasmids		
pEX18Ap	Counterselectable, mobilizable suicide vector for gene replacement; contains Ori ^T and <i>sacB</i> ; Ap ^r	11
pPS856	Gm ^r -selectable Flp recombinase target (FRT) cassette vector for generation of marked or unmarked mutants via gene interruption	11
pFLP2	bhr FLP recombinase-producing plasmid; contains <i>sacB</i> ; Ap ^r	11
pFV327-TGm	pEX18Ap containing <i>rmd</i> interrupted with a Gm ^r cassette	23
pUCP26	pUC18-derived broad-host-range cloning vector	30a
pKPJL01	<i>wapR</i> in pUCP26 (KpnI/PstI)	This study
pKPJL02	<i>migA</i> in pUCP26 (EcoRI/BamHI)	This study

In this paper, we describe characterization of the putative rhamnosyltransferases WapR and MigA. A combination of genetic and chemical structural evidence indicates that both enzymes are involved in the addition of L-Rha to the outer core OS in *P. aeruginosa* PAO1. However, these rhamnosyltransferases add L-Rha in different positions and linkages to the core OS, giving rise to the two glycoforms of core OS observed in *P. aeruginosa*. WapR transfers L-Rha in an α -1,3 linkage to form a core structure that becomes the receptor for the O antigen, while MigA attaches L-Rha in an α -1,6 linkage at a different site to form the uncapped core OS.

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-Bertani broth medium (Invitrogen Canada Inc., Burlington, Ontario, Canada) at 37°C. *Pseudomonas* isolation agar (Difco Becton, Sparks, MD) was used to select transconjugants from the mating experiments. The following concentrations of antibiotics were used in the selection media: 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, 500 μ g/ml carbenicillin, 300 μ g/ml gentamicin, and 60 μ g/ml tetracycline.

General DNA methods. Primers were prepared by the Laboratory Services Division of the University of Guelph (Guelph, Ontario, Canada) or Sigma Genosys (Mississauga, Ontario, Canada). The sequences of all primers used are available upon request. Restriction enzymes were obtained from Invitrogen (Burlington, Ontario, Canada), and T4 DNA ligase was obtained from New England Biolabs (Pickering, Ontario, Canada). All enzymes were used according to the manufacturers' instructions. Plasmid DNA was introduced into *E. coli* and *P. aeruginosa* by transformation (2). All constructs were verified by nucleotide sequencing at the Laboratory Services Division of the University of Guelph (Guelph, Ontario, Canada).

Generation of knockout mutants and complementation vectors. Nonpolar *wapR* and *migA* knockout mutants were generated by insertional mutagenesis with a gentamicin resistance cassette, followed by allelic replacement as described by Schweizer and Hoang (26). Briefly, *wapR* was amplified from genomic DNA of *P. aeruginosa* PAO1 using primers that incorporate a KpnI site and a PstI site, and the resulting product was ligated into pEX18Ap. The *wapR* gene was disrupted by insertion of the Gm^r cassette from pPS856 into the unique SalI site of *wapR*. Conjugal transfer and phenotypic selection were carried out by

using methods described previously (30), except that *Pseudomonas* isolation agar was supplemented at all stages with 300 μ g/ml gentamicin. Colonies that exhibited a Gm^r Cb^s phenotype were screened by PCR. For the *migA* mutant, the gene was amplified and manipulated as it was for the *wapR* knockout. To generate *rmd migA* double mutations in *P. aeruginosa* strain PAO1, the Flp-FRT recombination system of Hoang et al. was used (11). Briefly, the *migA::Gm^r* knockout strain was transformed with pFLP2 to catalyze excision of the Gm^r cassette, generating an unmarked *migA* knockout strain. The unmarked *migA* PAO1 mutant strain was then used to generate *rmd migA* double mutants by introduction of the pEX18Ap-*rmd::Gm^r* knockout construct (pFV327-TGm [23]) as described above. An *rmlC* knockout strain with the PAO1 background was constructed by our group previously (20). To construct the complementation vectors, the *wapR* and *migA* genes were PCR amplified from *P. aeruginosa* chromosomal DNA and then purified using a HighPure PCR purification kit (Roche, Mississauga, Ontario, Canada). The *wapR* PCR product and pUCP26 were digested with KpnI and PstI and then ligated to generate pKPJL01. The *migA* PCR product and the pUCP26 vector were digested with EcoRI and PstI and then ligated to produce pKPJL02.

Isolation and analysis of crude LPS. Small-scale LPS preparation was performed by using the method of Hitchcock and Brown (10). LPS was separated by using precast 4 to 12% bis-Tris NuPage gels (Invitrogen Canada Inc., Burlington, Ontario, Canada) and the electrophoresis procedures recommended by the manufacturer. LPS was visualized by using the ultrafast silver staining method of Fomsgaard et al. (8). Western immunoblots were probed using the following MAbs: MF15-4, specific for the B band (7, 13); N1F10, specific for the A band (15); 7-4, specific for the inner core (6); 5C101, specific for the outer core (6); and 18-19, specific for core-plus-one (6). The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G at a dilution of 1:2,000 (Jackson ImmunoResearch). Blots were developed using standard protocols for colorimetric detection (Qiagen, Mississauga, Ontario, Canada).

Structural analysis of large-batch purified LPS. Large-scale LPS preparations were obtained by using two standard methods: the phenol-chloroform-petroleum ether method (9) in the case of the knockout strains and the hot aqueous phenol method (31) for the wild-type strain. The core OS was prepared by hydrolysis of LPS (25 mg) using 2% acetic acid (AcOH) (2 ml, 3 h, 100°C). The water-insoluble lipid A precipitate was removed by centrifugation at 10,000 $\times$ g for 30 min. Soluble products (core OS with or without O antigen) were separated by size exclusion gel chromatography. Polymeric and oligosaccharide fractions were obtained. The core OS fraction was further separated on an anion-exchange column, and fractions were desalted on a Sephadex G-15 column and analyzed by capillary electrophoresis (CE)/electrospray ionization mass spectrometry (ESI-MS). After ESI-MS, samples were separated by anion-exchange chromatography. Fractions were again desalted by gel chromatography on a Sephadex

G-15 column and then analyzed by nuclear magnetic resonance (NMR) spectroscopy and subjected to monosaccharide analysis.

Chromatography. Size exclusion chromatography was carried out using Sephadex G-50 (2.5 by 80 cm) or Sephadex G-15 (1.6 by 80 cm) columns with pyridinium acetate buffer (pH 4.5) (4 ml pyridine and 10 ml AcOH in 1 liter water) as the eluent, and the results were monitored with a refractive index detector. Anion-exchange chromatography of the core OS samples was performed with a Hitrap Q column (Pharmacia) in water for 10 min, and then the samples were eluted using a linear 0 to 1 M NaCl gradient over 60 min with UV detection at 220 nm. Fractions were desalted by gel chromatography with a Sephadex G-15 column.

CE/ESI-MS. A Prince CE system (Prince Technologies, The Netherlands) was coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems/MDS Sciex, Canada). A sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 1.0 μ l/min. Separation was obtained by using an approximately 90-cm-long bare fused silica capillary and 15 mM ammonium acetate in deionized water (pH 9.0). Electrospray ionization voltages of 5 kV and -5 kV were used for the positive ion and negative ion detection modes, respectively.

NMR spectroscopy. NMR spectra were recorded at 25°C in D₂O with a Varian UNITY INOVA 600 instrument, using acetone as a reference for proton (2.225 ppm) and carbon (31.5 ppm) spectra. Varian standard programs for correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY) (mixing time, 200 ms), total correlation spectroscopy (TOCSY) (spinlock time, 120 ms), heteronuclear single quantum coherence (HSQC), and gradient heteronuclear multiple bond coherence (HMBC) (long-range transfer delay, 100 ms) were used.

Monosaccharide analysis. For independent linkage analysis, the core OS was methylated by the Ciucanu-Kerek procedure (6), and then samples (0.5 mg) were hydrolyzed in 0.2 ml of 3 M trifluoroacetic acid at 120°C for 2 h, followed by evaporation to dryness under a stream of air. The residue was dissolved in water (0.5 ml), reduced with NaBH₄ (~5 mg, 1 h), neutralized with AcOH (0.3 ml), and dried, and methanol (1 ml) was added. The mixture was dried twice by addition of methanol, and the residue was acetylated with acetic anhydride (0.5 ml, 100°C, 30 min), dried, and analyzed by gas-liquid chromatography using an HP1 capillary column (30 m by 0.25 mm) with a flame ionization detector (Agilent 6850 chromatograph) and a temperature gradient in which the temperature was increased from 170 to 260°C at a rate of 4°C/min and by gas chromatography-mass spectrometry with a Varian Saturn 2000 ion trap instrument using the same type of column.

RESULTS

Western immunoblot analysis of *wapR* and *migA* PAO1 mutants. LPS from *wapR* and *migA* knockouts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. LPS from wild-type strain PAO1 showed the typical banding pattern on the silver-stained gel and reacted with all of the antibodies tested (Fig. 2). In contrast, for the *wapR* mutant a smaller amount of high-molecular-weight LPS was obtained and the mobility of the low-molecular-weight core bands was greater, as detected by silver staining of the SDS-PAGE gel (Fig. 2). In the Western immunoblots, LPS from the *wapR* mutant showed a weak reaction with MAbs N1F10 (specific for A band) and MF15-4 (specific for B band), no reaction with MAb 18-19 (specific for core-plus-one O-antigen repeat unit), and a very weak reaction with MAb 5C101 (specific for outer core). Complementation with *wapR* (in plasmid pKPJL01) resulted in restoration of the wild-type LPS phenotype, as shown by the reactions with both MAb MF15-4 and MAb 18-19, while the reactivity with MAb 5C101 was not significantly improved compared to that observed for the LPS from the *wapR* knockout mutant (Fig. 2). LPS from the *migA* mutant showed a silver-stained banding profile similar to that of the LPS from the wild type, but it showed no reactivity with MAb 5C101 (Fig. 2). Complementation of the *migA* mutant with *migA* (in pKPJL02) restored recognition of the LPS of this strain by MAb 5C101; however,

FIG. 2. SDS-PAGE and Western immunoblot analysis of LPS isolated from *P. aeruginosa* strain PAO1, as well as *wapR* and *migA* knockout mutants. The inner core is not affected by *wapR* and *migA* knockouts, but the *migA* knockout strain does not react with outer core-specific MAb 5C101. The *wapR* knockout strain and the complemented *migA* strain lack the core-plus-one O-antigen repeat. The *wapR* mutant does not react with A- and B-band-specific MAbs and does not show a characteristic lipid A-linked LPS banding pattern. Providing *wapR* in *trans* restores the *wapR* mutant to the wild-type phenotype.

the complemented strain lacked reactivity to MAb 18-19 (Fig. 2).

Structural analysis of core OS from *wapR*, *migA* *rmd*, and *rmlC* mutants. Core OS were obtained from LPS isolated from *wapR*, *rmlC*, and *migA* *rmd* knockout strains, as well as *P. aeruginosa* wild-type strain PAO1, by hydrolysis with 2% AcOH. LPS from the *migA* *rmd* mutant contained an O chain with the same structure as that of the parent strain (identified by comparison of ¹H NMR spectra); *wapR* and *rmlC* mutants produced no O chain. Soluble products were separated by gel filtration chromatography, and OS fractions were analyzed by ESI-MS and further separated by anion-exchange chromatography. Due to variable phosphorylation, three to five fractions with different charges were isolated. Monosaccharide analysis of isolated core fractions by gas chromatography-mass spectrometry of alditol acetates showed the presence of glucose,

TABLE 2. NMR data for the *P. aeruginosa* LPS core OS^a

Residue, ^b compound	Nucleus	δ (ppm)						
		H/C 1	H/C 2	H/C 3	H/C 4	H/C 5	H/C 6	H/C 7
C, α -Kdo, all	H			1.96/2.26	4.16	4.16	3.90	3.78
	C		96.6	34.3	66.88	73.8	71.9	69.2
E, Hep2P4P, all	H	5.24	4.74	4.22	4.54	3.74	4.12	3.73/3.73
	C	100.1	75.5	76.2	71.6	73.2	69.6	63.8
F, Hep6P7Cm, all	H	5.15	4.42	4.08	4.07	3.99	4.54	3.93/4.53
	C	103.9	70.3	79.0	66.9	71.9	70.8	62.6
G, GalNA1a, all	H	5.35	4.48	4.30	4.35	4.23	3.90/3.90	
	C	100.2	51.1	77.0	77.0	73.4	61.3	
J, α -Glc, all	H	4.98	3.58	3.68	3.46	3.80	3.77/3.90	
	C	99.3	72.6	73.3	70.7	71.5	62.0	
J, α -Glc variant, all	H	5.02	3.58	3.72	3.43	3.74	3.77/3.90	
	C	99.3	72.6	73.3	70.7	71.5	62.0	
L, α -Glc6OAc, <i>migA</i> , <i>rmlC</i> , <i>wapR</i>	H	5.02	3.53	3.85	3.58	4.41	4.36/4.36	
	C	100.3	73.1	73.9	70.9	70.7	64.5	
L, α -Glc, <i>migA</i> , <i>rmlC</i>	H	5.04	3.52	3.85	3.58	4.23	3.84/3.86	
	C	100.3	73.1	73.9	70.9	70.7	61.5	
α -Rha, <i>migA</i>	H	5.16	4.03	3.80	3.50	4.01	1.26	
	C	102.0	71.8	71.5	73.2	70.1	17.8	
H, β -Glc, <i>migA</i>	H	4.58	3.31	3.60	3.47	3.66	3.85/3.85	
	C	105.6	75.0	83.2	69.9	75.5	68.2	
H, β -Glc variant, <i>migA</i>	H	4.57	3.34	3.60	3.52	3.66	3.85/3.91	
	C	105.6	75.0	83.2	69.9	75.5	67.5	
H, β -Glc, <i>rmlC</i> , <i>wapR</i>	H	4.56	3.18	3.47	3.39	3.65	3.82/3.87	
	C	105.8	74.3	77.0	71.7	75.6	68.5	
K, α -Rha, <i>wapR</i>	H	5.12	4.13	3.93	3.50	3.77	1.33	
	C	101.1	81.4	71.3	73.6	70.0	18.3	
N, β -Glc, <i>wapR</i>	H	4.63	3.36	3.50	3.40	3.46	3.72/3.91	
	C	105.5	74.7	76.8	70.8	77.1	62.0	
Ala, all	H		4.09	1.57				
	C	172.4	50.7	17.8				

^a Some monosaccharides gave more than one set of signals due to variations in neighboring sugars (phosphorylation, Kdo derivatives, acetylation, etc.). Significant variants are labeled "variant."

^b Residues are labeled with letters corresponding to Fig. 3.

galactosamine, Hep, and rhamnose in all strains. To determine the structure of the core OS fractions, all OS were analyzed by NMR.

Core fractions were analyzed by NMR spectroscopy. A set of two-dimensional spectra (COSY, TOCSY, NOESY, ¹H-¹³C HSQC, HMBC, ¹H-³¹P HMQC, and ¹H-³¹P HMQC-TOCSY) were recorded for each compound and interpreted using the Pronto program (Table 2). Monosaccharides were identified using characteristic patterns of TOCSY, NOESY, and COSY cross peaks, as well as chemical shift data. The position of phosphate groups was determined from ¹H-³¹P correlations; proton and carbon signals at the site of phosphorylation were shifted downfield. Additional phosphates visible in mass spectra were present as pyrophosphates; no additional phosphorylated sugars were detected by NMR. Linkage between monosaccharides was determined from NOE and HMBC data (Table 2). All cores showed the following NOE correlations belonging to the common OS fragment: E1:C5, E1:C7, F1:E2, F1:E3, G1:F3, and L1:G4. The following HMBC correlations for this fragment were observed: E1:C5, F1:E3, G1:F3, and L1:G4. Additionally, the following NOE and HMBC correlations were observed: in *wapR*(i), N1:K2, K1:L6, J1:H6, and H1:G3; in *migA rmd*, J1:H6, H1:G3 and M1:H3; and in *rmlC*, J1:H6, H1:G3. Derivatives of all neutral monosaccharides from the outer core, expected from the presented structures,

were detected. Heptose derivatives were not identified due to phosphorylation.

These results allowed us to elucidate the core OS structures of the mutant strains and the wild-type control. LPS from wild-type strain PAO1 contained the α -1,6-linked L-Rha (residue K) (Fig. 3). The *wapR* knockout sample contained two different glycoforms; WapR(i) was the same as the core OS from the PAO1 sample, but WapR(ii) lacked both β -Glc and α -Rha (residues N and K), as well as both α -Glc and β -Glc (residues J and H) (Fig. 3). In the core OS structure of the *migA rmd* knockout the α -1,6-linked L-Rha (residue K) was not present, while the α -1,3-linked L-Rha (residue M) was not detected (Fig. 3). Approximately 50% of terminal α -Glc residue L was 6-O acetylated, and there was a variable number of phosphate groups. The core OS structure of the *rmlC* mutant was totally devoid of L-Rha residues and had a variable number of phosphate groups. Partial acetylation (50 to 70%) of the outer core α -Glc (residue L) was observed in LPS from the *wapR*, *migA rmd*, and *rmlC* knockout strains (Fig. 3).

The structures were further verified by ESI-MS. The spectra of the core OS from the *wapR* knockout contained two sets of signals, corresponding to the structures WapR(i) and WapR(ii), with variable phosphorylation (Fig. 4). The composition of WapR(i) was dHex₁Hex₄Hep₂HexN₁anhKdo₁P₃Cm₁Ala₁ (where anhKdo is anhydro-Kdo and Cm is carbamoyl), and the calculated mass

FIG. 3. LPS structures derived from NMR spectroscopy analysis. The resonances observed were used to identify the sugar residues present and their linkages. *, see Table 2 for selected NMR data (residues are labeled with letters to assist comparison of the different resonances corresponding to the specific structures). MW, molecular weight.

was 1,914.4 Da. The composition of WapR(ii) was Hex₁Hep₂HexN₁anhKdo₁P₃Cm₁Ac₁Ala₁, and the calculated mass was 1,323.9 Da. The composition of the acetylated core from the *migA rmd* knockout mutant was dHex₁Hex₃Hep₂HexN₁anhKdo₁P₄Cm₁Ac₁Ala₁, and the calculated mass was 1,874.3 Da (the quality of the spectra of the OS mixture was poor, and therefore only one of the isolated OS with four phosphate groups is shown [Fig. 4]). Finally, the composition of the base structure of the acetylated core from the *rmlC* knockout mutant was Hex₃Hep₂HexN₁anhKdo₁P₃Cm₁Ac₁Ala₁, and the calculated mass was 1,648.2 Da (Fig. 4).

DISCUSSION

Two putative glycosyltransferase genes, *wapR* (PA5000) and *migA* (PA0705), in the *P. aeruginosa* genome have been identified, and we proposed that the products that they encode are responsible for the addition of L-Rha to D-Glc in the LPS core OS to produce two distinct glycoforms. By generating a knockout mutant with a mutation in each of these two genes, we were able to investigate the role of the encoded proteins in core OS biosynthesis in *P. aeruginosa* using a combination of immunological approaches and biophysical techniques, including

NMR. NMR analysis was used to elucidate the structure of the core OS in each strain. The structure observed wild-type strain PAO1 was in good agreement with previously published data for the uncapped core OS (Fig. 3). As a control, core OS from the *rmlC* mutant, which is defective in the synthesis of dTDP-L-Rha, was also examined. As expected, the *rmlC* mutant core OS structure lacked L-Rha residues. The presence of partial acetylation (50 to 70%) of the outer core sugars, such as residue L, found in LPS from *rmlC*, *wapR*, and *migA rmd* knockout strains and the phosphorylation patterns of all strains were consistent with the results described in a previous report (2).

Production of uncapped core OS was not affected in the *wapR* mutant, as demonstrated by the interaction of LPS from *wapR* mutants with MAbs specific for the inner and outer core (Fig. 2). This finding was supported by the results of NMR spectroscopy experiments, in which the WapR(i) structure was shown to be identical to the uncapped core structure of wild-type strain PAO1 (Fig. 3). The second core OS structure isolated from the *wapR* mutant, WapR(ii), was severely truncated, and there was partial 6-O acetylation of the terminal α -Glc, residue L. Since only the WapR(i) structure has the

FIG. 4. ESI-MS spectra of LPS fractions. The LPS samples were resolved by anion-exchange chromatography and subjected to mild acid hydrolysis prior to the ESI-MS studies. (A) *wapR* knockout mutant. (B) *rmlC* knockout mutant. (C) *migA rmd* knockout mutant, containing four phosphate groups. Peaks labeled with protein designations

terminal β -D-Glc recognized by MAb 5C101, reduced binding of LPS prepared from the *wapR* mutant by MAb 5C101 was observed (Fig. 2). The truncation of residues J and H was indicated by the Western immunoblotting results showing a lack of reactivity of the LPS of the *wapR* mutant with MAb 18-19 (specific for core-plus-one) (Fig. 2). This suggests that the capped core OS is truncated so that the O antigen cannot be attached. Although it was anticipated that the absence of *wapR* would result in a defect in the capped core OS, it was unclear why D-Glc residues H and J were absent. Nonetheless, the α -1,3-linked L-Rha residue of the capped core OS was missing in the *wapR* mutant, consistent with the prediction that WapR is a rhamnosyltransferase that transfers L-Rha and attaches it to D-Glc in an α -1,3 linkage.

This prediction is substantiated by the observation that LPS from the *wapR* mutant had negligible reactivity with MAb N1F10 (A band specific) and reduced reactivity with MAb MF15-4 (B band specific) (Fig. 2). Reduced reactivity, as opposed to no recognition, by these MABs was expected if O antigen could not be attached to the core OS because it has been shown previously that MABs MF15-4 and N1F10 also recognize undecaprenol phosphate-linked B-band O polymers (precursors for capped core synthesis) that have not been ligated to the core OS. Thus, these results are consistent with the normal production of O-antigen precursors that cannot be attached to the core OS because of a *wapR* knockout. Our group has previously shown that this type of undecaprenol phosphate-linked O-antigens is sensitive to phenol (1, 4, 20), and when LPS from the *wapR* mutant was prepared by the hot aqueous phenol method of Westphal and Jann (31) rather than the fast method of Hitchcock and Brown (10), A-band and B-band O antigens were not detected by either SDS-PAGE or Western immunoblotting (data not shown).

We propose that a pool of nascent core OS molecules lacking any L-Rha is produced and then processed by WapR and MigA to become the capped and uncapped forms of core OS, respectively. Differential expression of MigA and WapR potentially dictates the balance between uncapped core OS, core-plus-one, and long-chain capped core OS on the bacterial cell surface. When MigA is overexpressed, most of the nascent core OS molecules are used as substrates for α -1,6-rhamnosyl transfer, despite competition from WapR for the same substrates. This results in a bacterial cell surface with predominantly uncapped core glycoforms and relatively little LPS with O antigen. When WapR is overexpressed, most of the nascent core OS molecules are used as substrates for α -1,3-rhamnosyl transfer. This favors the production of capped core OS. The level of expression of WapR from the complementation plasmid is likely significantly higher than it is in wild-type cells, and this would explain the reduction in binding of core OS by the outer core-specific MAb 5C101 due to a reduced amount of uncapped core OS.

correspond to the structures shown in Fig. 3. Unlabeled arrows indicate a difference in phosphate groups. Arrows labeled Ac indicate a difference in acetate groups (42 atomic mass units [amu]). Every structure is represented by two variants, one with full Kdo and one with anhydro-Kdo (18-atomic mass unit difference).

In the knockout strains, the phenotype includes overexpression of the active rhamnosyltransferase, due to the difference in the expression levels caused by the knockout. Specifically, in the *migA* knockout strain, all of the nascent core molecules are subject to rhamnosyl transfer by WapR, since no MigA is produced. Thus, all core molecules are modified to be capped core OS glycoforms. Interestingly, a significant increase in O-antigen attachment to these core structures was not observed (Fig. 2). This could be interpreted as due to the fact that the Wzy-dependent B-band O-antigen processing system is not able to keep up with the unusually high level of capped core OS being produced. Since the pool of sugar-nucleotides required to form B-band O-antigen repeat units is unchanged in the knockout strain, the O antigens themselves become limiting, and thus an increase in O-antigen attachment is not expected in *migA* knockout strains. In the *wapR* knockout strain, all nascent core molecules are used as substrates by MigA, which causes all of the molecules to be converted to uncapped core OS glycoforms. Thus, no A-band or B-band O antigens are produced (Fig. 2), because these undecaprenol-linked O polymers cannot be attached to α -1,6-linked L-Rha in the uncapped core. In the complemented *wapR* knockout strain, overexpression of WapR from pKPJL01 caused an increase in core-plus-one O-antigen repeat unit bands, as demonstrated in the silver-stained gel and in the Western blot showing increased binding of the LPS by MAb 18-19 (Fig. 2). The increase in capped core production also explains why the interaction with the uncapped core-specific MAb 5C101 remained poor after complementation. Taken together, these immunochemical data strongly suggest that WapR is the α -1,3-linked L-Rha rhamnosyltransferase required for synthesis of the capped core OS in *P. aeruginosa* PAO1.

In the *migA* knockout strain, the formation of capped core OS was not affected by the deficiency in *migA*, as shown by reactivity to MAbs N1F10 and MF15-4 (specific for A-band and B-band O antigens, respectively) (Fig. 2). Data from the NMR analysis supported this finding, as the core OS structure was shown to be similar to the capped core structure without the O antigen (Fig. 1). A definitive role for MigA in core OS biosynthesis was revealed by the observation that LPS of the *migA* knockout strain did not react with MAb 5C101. In an NMR analysis of an LPS sample prepared from the *migA rmd* double mutant, uncapped core OS could not be detected. In accordance with the MigA/WapR competition model, we interpreted this to mean that no α -1,6-linked L-Rha was present in the core OS of the *migA* mutant, since only the capped core OS glycoform with α -1,3-linked L-Rha is produced due to the activity of WapR. This was demonstrated by the increased recognition by MAb 18-19 in the *migA* knockout strain (Fig. 2). LPS from this mutant contained both A-band and B-band O antigens (Fig. 2). The α -1,3-linked L-Rha is known to be the receptor for the B-band O antigen, and this analysis provides further evidence that the same receptor is used for A-band O-antigen attachment since the presence of α -1,3-linked L-Rha is directly correlated with the presence of both O antigens.

Complementation of the *migA* knockout mutant with *migA* in *trans* restored reactivity of the LPS with MAb 5C101, but the LPS no longer reacted with MAb 18-19 (Fig. 2). Since expression of MigA from the pUCP26 complementation vector is constitutive, whereas the chromosomal *migA* gene is regulated

by the RhlI/RhlR quorum-sensing regulatory system (32), the complemented cells contain a larger amount of expressed MigA than wild-type cells. Overexpression of MigA results in a reduction in the core-plus-one level in the cell (32), possibly because a larger amount of MigA outcompetes the activity of WapR to add L-Rha to nascent core OS, which is then committed to forming the uncapped core glycoform with an α -1,6-linked L-Rha. This explains the loss of reactivity with core-plus-one-specific MAb 18-19, as the limited supply of capped core OS is processed to produce the high-molecular-weight LPS with O antigen observed (Fig. 2). Thus, *migA* upregulation by the quorum-sensing system may be the reason that *P. aeruginosa* clinical isolates from the lungs of cystic fibrosis patients express little or no O antigen (32). These results provide further evidence that MigA is a rhamnosyltransferase responsible for the α -1,6 linkage of L-Rha to the LPS core in *P. aeruginosa*.

Biochemical characterization of MigA and WapR by our group is under way. Before LPS rhamnosyltransferase assays can be carried out, many challenges must be addressed. The substrate for both enzymes, dTDP-L-rhamnose, is not commercially available and therefore must be synthesized. The biosynthesis of dTDP-L-rhamnose from glucose-1-phosphate requires four enzymatic reactions, as previously explained (for a review, see reference 16). Thus, RmlA, RmlB, RmlC, and RmlD must first be purified, and parameters for the enzymatic synthesis must be determined. Once the proposed product, dTDP-L-Rha, has been synthesized, it must be purified by high-performance liquid chromatography, and then its chemical structure must be verified by mass spectrometry and NMR analyses. Only then will dTDP-L-Rha be available for use as one of the substrates. The receptor core OS structure required for in vitro rhamnosyltransferase activity assays is the truncated core OS from an *rmlC* mutant, lacking any L-Rha residues. This receptor molecule also must be purified.

This study reports the identification and characterization of two glycosyltransferases involved in attaching L-Rha from dTDP-L-Rha to the lipid A core OS in *P. aeruginosa*. To our knowledge, this is the first report describing the importance of *migA* and *wapR* for producing specific glycoforms of the outer core OS in *P. aeruginosa*. Evidence was obtained through construction of knockout mutants and from results of immunochemical studies of the LPS prepared from these mutants. The chemical structures of the core OS from *wapR* and *migA rmd* knockout strains were elucidated by mass spectrometry and NMR analyses, and collectively, the results showed that *wapR* mutants produce core OS lacking α -1,3-linked L-Rha and *migA rmd* mutants produce core OS lacking α -1,6-linked L-Rha. In conclusion, our results showed that WapR is an α -1,3-rhamnosyltransferase responsible for the synthesis of the capped core OS, while MigA is an α -1,6-rhamnosyltransferase required for assembly of the uncapped core glycoform.

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