

# Involvement of the *rml* locus in core oligosaccharide and O polysaccharide assembly in *Pseudomonas aeruginosa*

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**L-Rhamnose (L-Rha) is a component of the lipopolysaccharide (LPS) core, several O antigen polysaccharides, and the cell surface surfactant rhamnolipid of *Pseudomonas aeruginosa*. In this study, four contiguous genes (*rmlBDAC*) responsible for the synthesis of dTDP-L-Rha in *P. aeruginosa* have been cloned and characterized. Non-polar chromosomal *rmlC* mutants were generated in *P. aeruginosa* strains PAO1 (serotype O5) and PAK (serotype O6) and LPS extracted from the mutants was analysed by SDS-PAGE and Western immunoblotting. *rmlC* mutants of both serotype O5 and serotype O6 synthesized a truncated core region which was unable to act as an attachment point for either A-band or B-band O antigen. A *rmd rmlC* PAO1 double mutant (deficient in biosynthesis of both D-Rha and L-Rha) was constructed to facilitate structural analysis of the mutant core region. This strain has an incomplete core oligosaccharide region and does not produce A-band O antigen. These results provide the genetic and structural evidence that L-Rha is the receptor on the *P. aeruginosa* LPS core for the attachment of O polysaccharides. This is the first report of a genetically defined mutation that affects the synthesis of a single sugar in the core oligosaccharide region of *P. aeruginosa* LPS, and provides further insight into the mechanisms of LPS biosynthesis and assembly in this bacterium.**

**Keywords:** L-rhamnose, *Pseudomonas aeruginosa*, lipopolysaccharide, *rml*, *rmd*

## INTRODUCTION

*Pseudomonas aeruginosa* is an important Gram-negative pathogen capable of elaborating a number of virulence determinants including various toxins, alginate and lipopolysaccharide (LPS). The LPS of *P. aeruginosa* is typical of Gram-negative bacteria and consists of lipid A, core oligosaccharide and O antigen repeat units. *P. aeruginosa* can simultaneously produce two distinct forms of LPS designated A-band and B-band LPS (Rivera *et al.*, 1988; Lam *et al.*, 1989). A-band LPS is antigenically conserved and consists of D-rhamnose (D-Rha) residues linked  $\alpha 1 \rightarrow 2$ ,  $\alpha 1 \rightarrow 3$ ,  $\alpha 1 \rightarrow 3$  (Arsenault *et al.*, 1991). B-band LPS is a heteropolymer of di- to pentasaccharide repeat units consisting of a variety of monosaccharides. The composition of the B-band O antigen trisaccharide moiety of the common laboratory strain PAO1 (serotype O5) is di-N-acetylmannosamin-

uronic acid and N-acetyl-6-deoxygalactose (Fuc2NAc; Knirel & Kochetkov, 1994). The gene clusters responsible for A- and B-band LPS have been extensively characterized and encode enzymes involved in the synthesis and transfer of monosaccharide units to nascent polymer chains (reviewed by Rocchetta *et al.*, 1999).

In contrast to the extensive knowledge of O antigen synthesis and assembly in *P. aeruginosa* LPS, relatively little is understood regarding the biosynthesis of the core oligosaccharide region. The *P. aeruginosa* core region, like that of other Gram-negative bacteria, can be subdivided into the inner core and the outer core. The inner core contains L-glycero-D-manno-heptose and 3-deoxy-D-manno-octulosonic acid (Kdo), while the outer core is composed of hexose sugars such as D-glucose (D-Glc) and L-rhamnose (L-Rha). The structures of the core oligosaccharides of *P. aeruginosa* serotypes O5 and O6 have recently been reported by our group (Masoud *et al.*, 1995; Sadovskaya *et al.*, 1998). There are two

**Abbreviations:** Gm, gentamicin; Rha, rhamnose.

distinct core oligosaccharide configurations in *P. aeruginosa*, each of which contains a single L-Rha residue, but attached to D-Glc through different anomeric configurations. In the unsubstituted core (lacking attached O antigen), L-Rha is located in the main oligosaccharide chain and is attached to D-Glc via an  $\alpha$ 1,6 linkage (Sadovskaya *et al.*, 1998). In contrast, the core region substituted with O polysaccharide contains L-Rha linked to a branched D-Glc residue through an  $\alpha$ -1,3 glycosidic bond (Sadovskaya *et al.*, 2000). This latter L-Rha residue apparently serves as the attachment point for B-band O antigen. The unsubstituted outer core of serotype O6 was similar to that of serotype O5 except for the lack of the terminal  $\beta$ -D-Glc residue (Masoud *et al.*, 1995; Sadovskaya *et al.*, 1998). This sugar is the epitope recognized by monoclonal antibody (mAb) 5C-101 (de Kievit & Lam, 1994), and its absence precludes recognition of serotype O6 core LPS by this antibody. The residue(s) involved in the attachment of A- and B-band O antigen remains to be identified in O6, a more clinically prevalent serotype.

In addition to being integral to the outer-core region of the *P. aeruginosa* LPS molecule (Sadovskaya *et al.*, 1998), L-Rha is also a constituent of the O antigen polysaccharides in serotypes O3 and O6 (Knirel & Kochetkov, 1994), and comprises part of the rhamnolipid molecules secreted by *P. aeruginosa* (Jarvis & Johnson, 1949). This methylpentose is synthesized as an activated sugar precursor from deoxythymidine triphosphate (dTTP) and glucose 1-phosphate (Glc-1-P) through the catalysis of four enzymes as described by Kornfeld & Glaser (1961). The primary step in the anabolic pathway involves a reversible bimolecular group transfer reaction which is catalysed by glucose-1-phosphate thymidyltransferase (RmlA; Lindquist *et al.*, 1993). Subsequent reactions in the pathway are catalysed by RmlB (dTDP-D-glucose-4,6-dehydratase), RmlC (dTDP-4-keto-6-deoxy-D-glucose-3,5-epimerase) and RmlD (dTDP-4-keto-L-rhamnose reductase), respectively. With a few exceptions, the genes encoding these enzymes are normally clustered within the bacterial chromosome (Saigí *et al.*, 1999; Hausman *et al.*, 1998; Robertson *et al.*, 1994).

In the present study, we describe the cloning and characterization of the *rml* locus responsible for the synthesis of dTDP-L-Rha in *P. aeruginosa* serotypes O5 and O6. SDS-PAGE and Western immunoblot analyses reveal that *rmlC* mutants exhibit defects in the attachment of A-band and B-band LPS to core-lipid A, and synthesis of a complete outer core. These results provide evidence that the L-Rha residue located in the branch core oligosaccharide chain is the linkage site for both O polysaccharides. Furthermore, data from spectrometry analysis provided definitive evidence of the composition of the outer-core oligosaccharide of the mutant strains.

## METHODS

**Bacterial strains, plasmids and culture conditions.** The bacterial strains and plasmids used are listed in Table 1. Bacterial strains were cultured in Luria Broth (LB; Gibco-

BRL). *Pseudomonas* Isolation Agar (PIA; Difco Laboratories) was used for the selection of transconjugants following mating experiments. For gene replacement experiments, resolving medium containing tryptone (1.2%), yeast extract (0.5%), sucrose (5%) and D-Glc (0.1%) (Ma *et al.*, 1998) was used for enhancing the selection of colonies which had undergone a double cross-over event. Bacto-agar (Difco Laboratories) was added to 1.5% for solid media. When necessary, IPTG (10  $\mu$ l of 200 mg ml<sup>-1</sup> stock; Bioshop Canada) and X-Gal (75  $\mu$ l of 20 mg ml<sup>-1</sup> stock; Gibco-BRL) were added to the medium. Antibiotics (Sigma) were used to supplement selection media at the following concentrations in  $\mu$ g ml<sup>-1</sup> for *Escherichia coli* and *P. aeruginosa*, respectively: ampicillin, 100 and 700; tetracycline, 15 and 50; and gentamicin (Gm), 15 and 300. For long-term storage, bacterial strains were maintained in media containing 15% glycerol (Sigma) at -70 °C.

**DNA procedures.** Restriction enzymes were purchased from Gibco-BRL, Boehringer Mannheim, Roche Diagnostics, Pharmacia and New England Biolabs (NEB). Klenow enzyme, T4 DNA ligase and calf intestinal alkaline phosphatase were purchased from Boehringer Mannheim. All enzymes were used according to the manufacturers' directions. Preparative amounts of genomic DNA were extracted using the DNAzol Reagent (Gibco-BRL). Plasmid DNA was isolated using the Boehringer Mannheim High Pure Plasmid Isolation kit according to manufacturer's recommendations. Plasmid DNA was introduced into *E. coli* (Huff *et al.*, 1990) and *P. aeruginosa* (Berry & Kropinski, 1986) by transformation. Purification of DNA from agarose gels was accomplished by the Gene Clean III kit (Bio/Can Scientific).

**PCR amplification.** PCR primers for the *P. aeruginosa* *rml* operon and the *rmlC* gene were designed based on the *P. aeruginosa* PAO1 whole genome database (Pathogenesis) using the computer program GENERUNNER for Windows (Hastings Software, NY). The primer sequences incorporating unique restriction sites for cloning are as follows: P1-RML-BDAC-up (5'-TCGAAGCATGCTACGGACAACC-3'), P2-RML-BDAC-down (5'-TTTTTCTAGAAGCAGGGCG-AGGATGACCA-3'), P3-RML-C-up (5'-GGTGAATTC AACTGGAAAACTCGCCGC-3') and P4-RML-C-down (5'-TTTTTCTAGAAGCAGGGCGAGGATGACCA-3'). The Expand Long Template PCR System (Boehringer Mannheim) was used to amplify the *P. aeruginosa* *rml* locus and the *rmlC* gene. A single master mix was prepared containing approximately 100 ng genomic DNA, 25 pmol each primer, 500  $\mu$ M dNTPs and 1  $\times$  buffer 3 containing 2.25 mM MgCl<sub>2</sub> (Boehringer Mannheim). Cycling reactions were carried out in an automated PowerBlock I thermocycler (ERICOMP). Denaturation of template DNA was accomplished using a hot start at 95 °C for 5 min. This was followed by the addition of 2.5 units of the *Taq*/*Pwo* enzyme mix. The DNA was amplified through 10 cycles of 95 °C (1 min), 60 °C (30 s) and 68 °C (3 min) and 20 cycles of 95 °C (1 min), 60 °C (30 s) and 68 °C (3 min plus an additional 20 s per cycle). After completion of 30 cycles, there was a final elongation time of 7 min at 68 °C. The PCR products were purified using the High Pure PCR Product Purification kit (Boehringer Mannheim) according to manufacturer's specifications.

**Generation of *rmlC* chromosomal mutants in *P. aeruginosa* serotypes O5 and O6.** Chromosomal mutants were generated in *P. aeruginosa* strains PAO1 (serotype O5) and PAK (serotype O6) according to the gene replacement strategy of Schweizer & Hoang (1995). The 3.5 kb *rml*-containing PCR product was digested with *Sph*I and *Xba*I and cloned into the gene replacement vector pEX18Ap (Hoang *et al.*, 1998). A gentamicin-resistance (Gm<sup>R</sup>) cassette from pPS856 (Hoang *et*

**Table 1.** Bacterial strains and plasmids used in this study

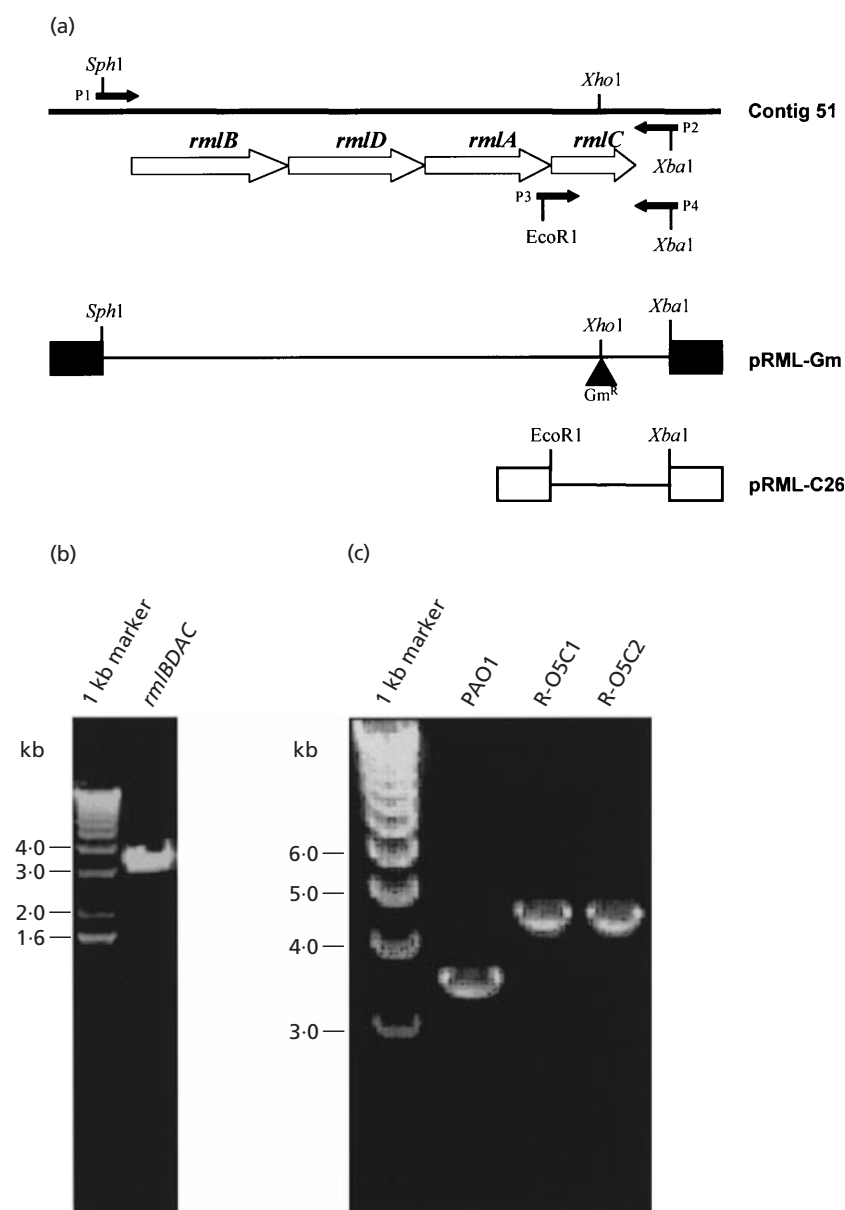
Strain or plasmid	Relevant properties	Reference or source
<b>Strains</b>		
<i>P. aeruginosa</i>		
PAO1	O5 O antigen, A <sup>+</sup> B <sup>+</sup>	Hancock & Carey (1979)
PAK	O6 O antigen, A <sup>+</sup> B <sup>+</sup>	Woods <i>et al.</i> (1997)
R-O5C1 and R-O5C2	PAO1 derivative; <i>rmlC</i> ::Gm <sup>R</sup> , A <sup>-</sup> B <sup>-</sup>	This study
R-O6C1 and R-O6C2	PAK derivative; <i>rmlC</i> ::Gm <sup>R</sup> , A <sup>-</sup> B <sup>-</sup>	This study
RML-ΔC	PAO1 derivative; Gm <sup>R</sup> cassette excised leaving behind <i>FRT</i> sequences interrupting <i>rmlC</i> (unmarked mutant); A <sup>-</sup> B <sup>-</sup>	This study
RML-RMD	PAO1 derivative; Δ <i>rmlC rmd</i> ::Gm <sup>R</sup> ; A <sup>-</sup> B <sup>-</sup>	This study
<i>E. coli</i>		
DH5α	φ80 <i>dlacZ</i> Δ <i>M15</i> Δ( <i>lacZYA-argF</i> ) <i>U169 deoR recA1 hsdR17</i> (r <sub>k</sub> <sup>-</sup> , m <sub>k</sub> <sup>+</sup> ) <i>supE44 λ</i> <sup>-</sup> <i>gyrA96 thi-1 relA1 F</i> <sup>-</sup>	Gibco-BRL
SM10	<i>thi-1 thr leu tonA lacY supE recA</i> RP4-2-Tc::Mu, Km <sup>R</sup>	Simon <i>et al.</i> (1983)
<b>Plasmids</b>		
pUCP26	pUC18-derived broad-host-range vector; Tc <sup>R</sup> (4.9 kb)	West <i>et al.</i> (1994)
pEX18Ap	Gene replacement vector; Ap <sup>R</sup> <i>oriT</i> <sup>+</sup> <i>sacB</i> <sup>+</sup> (5.8 kb)	Hoang <i>et al.</i> (1998)
pPS856	Contains the 830 bp Gm <sup>R</sup> cassette from pUCGM; Ap <sup>R</sup>	Hoang <i>et al.</i> (1998)
pFLP2	Broad-host-range Flp cassette vector; Ap <sup>R</sup> <i>sacB</i> <sup>+</sup> (9.3 kb)	Hoang <i>et al.</i> (1998)
pRML	3.5 kb <i>SphI</i> - <i>XbaI</i> fragment in pEX18Ap; contains the dTDP-L-Rha biosynthetic gene cluster from PAO1	This study
pRML-Gm	Same insert with a Gm <sup>R</sup> cassette inserted at a <i>Sall</i> site within <i>rmlC</i>	This study
pRML-C-26	0.8 kb <i>EcoRI</i> - <i>XbaI</i> fragment in pUCP26; contains <i>rmlC</i>	This study
pFV326-26	1.7 kb <i>PvuI</i> - <i>XhoI</i> fragment from pFV35 in pUCP26; contains <i>rmd</i>	Rocchetta <i>et al.</i> (1998)
pFV327-TGm	1.5 kb <i>EcoRV</i> fragment from pFV326-26 cloned into pEX100T Gm <sup>R</sup> cassette inserted at a <i>KpnI</i> site within <i>rmd</i>	Rocchetta <i>et al.</i> (1998)

*al.*, 1998) was inserted within a unique *Sall* site in the gene of interest (Fig. 3a). pPS856 contains a Gm<sup>R</sup> selectable marker flanked by Flp recombinase target (*FRT*) sites which permits excision of the cassette from its vector, when provided with the Flp recombinase enzyme, leaving behind a 68 bp *FRT*-containing sequence. This allows for the isolation of a second chromosomal mutation in the same strain. The non-replicative knockout construct was introduced into the mobilizer strain *E. coli* SM10, and then conjugally transferred into *P. aeruginosa* PAO1 and PAK according to the method of Simon *et al.* (1983). Transconjugants were selected on Gm-containing media. True recombinants were differentiated from merodiploids/co-integrates by monitoring loss of two vector-associated markers, carbenicillin resistance (*bla*) and sucrose sensitivity (*sacB*). PCR amplification was used to confirm the presence of the Gm<sup>R</sup> cassette within the chromosome of *P. aeruginosa* mutants.

Initial analysis of the chemical structure of the core oligosaccharide of the *rmlC* mutant proved inconclusive due to the strong Rha signal remaining from the D-Rha residues of A-band O antigen. Therefore, to prevent interference it was necessary to disrupt the production of A-band polysaccharide in the *rmlC* mutant by introducing an additional mutation in the A-band biosynthetic gene, *rmd*. To generate consecutive *rmd rmlC* chromosomal mutations in *P. aeruginosa* strain PAO1, the broad-host-range Flp-*FRT* recombination system of Hoang *et al.* (1998) was utilized. The replicative plasmid pFLP2 containing the recombinase structural gene as well as the selectable markers *sacB* (sucrose sensitivity) and *bla* (carbenicillin resistance) was mobilized into PAO1 *rmlC* mutants (containing the *rmlC*::Gm<sup>R</sup>-*FRT* insertion) through

biparental matings with SM10. Transconjugants were selected on carbenicillin-containing media. Deletion of the chromosomally integrated Gm<sup>R</sup> marker by Flp recombinase was monitored by screening recombinants for the loss of Gm resistance. Plasmid pFLP2 was cured from Gm-sensitive isolates by streaking cells on resolving media containing 5 % sucrose (Ma *et al.*, 1998). To monitor loss of the Gm<sup>R</sup> cassette and pFLP2, mutants were replica-plated on carbenicillin- and Gm-containing media. The unmarked PAO1 *rmlC* mutant strains were then used to generate *rmlC rmd* double mutants by introduction of the knockout construct pFV327-TGm (*rmd*::Gm<sup>R</sup>) (Rocchetta *et al.*, 1998) according to the method of Schweizer & Hoang (1995).

**LPS preparation and analysis.** LPS was isolated by either the proteinase K method of Hitchcock & Brown (1983) or the hot-aqueous phenol method of Westphal & Jann (1965). LPS was analysed by standard 12.5 % glycine SDS-PAGE gels or by Tricine SDS-PAGE using commercially available 16.5 % gradient Tricine gels (Novex, Helixx Technologies). LPS was visualized by silver staining according to the methods of Fomsgaard *et al.* (1990) (glycine gels) and Tsai & Frasch (1982) (Tricine gels). The Western immunoblot method of Burnette (1981) was used to transfer LPS separated by SDS-PAGE gels to nitrocellulose (Gelman). After transfer, immunoblots were blocked in 5 % skimmed milk in PBS for 30 min at room temperature. Immunoblots were incubated overnight in one of the following mAbs: N1F10 (specific for A-band LPS; Lam *et al.*, 1989), MF15-4 (specific for O5 B-band LPS; Lam *et al.*, 1987), MF23-2 (specific for O6 B-band LPS; Lam *et al.*, 1987), 5C101 (specific for the O5 outer-core region; de Kievit & Lam, 1994) or 7-4 (specific for the inner-core region; de



**Fig. 1.** Generation and molecular characterization of *P. aeruginosa* serotype O5 *rmlC*::Gm<sup>R</sup> mutants (R-O5C1 and R-O5C2). (a) Schematic representation of the region containing the *rml* locus of *P. aeruginosa* strain PAO1 (nucleotides 5810161–5813561; December 15, 1999 release; *P. aeruginosa* whole genome sequence). Oligonucleotide primers P1 (RML-BDAC-up) and P2 (RML-BDAC-down) were used to PCR-amplify the *rmlBDAC* cluster while primers P3 (RML-C-up) and P4 (RML-C-down) were used to amplify *rmlC*. Constructs pRML-Gm and pRML-C26 were used for the generation of chromosomal mutants and complementation of isogenic mutations, respectively. The site of Gm<sup>R</sup> cassette insertion within *rmlC* is indicated by the black triangle. (b) PCR amplification of the *rmlBDAC* locus using PAO1 chromosomal DNA and primers P1 and P2. (c) Confirmation of allelic replacement using PCR analysis. Oligonucleotide primers (P1 and P2) specific for the *rmlBDAC* cluster were utilized to generate PCR products of 3.5 and 4.6 kb representing the intact *rml* operon of PAO1 and the *rml* cluster of null *rmlC*::Gm<sup>R</sup> mutants (R-O5C1 and R-O5C2), respectively.

Kievit & Lam, 1994). Bound first antibody was detected using a goat anti-mouse secondary antibody conjugated to alkaline phosphatase, as described by Lightfoot & Lam (1991).

**Sugar composition and methylation linkage analyses.** Water-insoluble LPS obtained using the hot-aqueous phenol method of Westphal & Jann (1965) was treated with 2% acetic acid at 100 °C for 2 h to cleave the acid-labile ketosidic linkage between lipid A and the core oligosaccharide. Water-insoluble lipid A was removed from the hydrolysate by centrifugation at 6000 g for 30 min. The supernatant containing core oligosaccharide was purified on a column of Bio-Gel P-2 (1 m × 1 cm) with water as the eluent. Fractions containing core oligosaccharide were collected and lyophilized. Sugar composition analysis was performed by the alditol acetate method of Sawardeker *et al.* (1967). Briefly, core oligosaccharide samples were treated with 4 M trifluoroacetic acid for 4 h at 100 °C, reduced in water with NaBD<sub>4</sub>, and acetylated with acetic anhydride. The alditol acetate derivatives were characterized by GLC-MS using a Hewlett Packard chromato-

graph equipped with a 30 m DB-17 capillary column [210 °C (30 min) to 240 °C at 2 °C min<sup>-1</sup>]. MS in the electron impact mode was recorded using a Varian Saturn II mass spectrometer. Methylation linkage analysis was performed by the Ciucanu & Kerek (1984) (NaOH/Me<sub>2</sub>SO-methyl iodide) method. Characterization of the permethylated alditol acetate derivatives was performed using GLC-MS in the electron impact mode using a DB-17 column operated isothermally at 190 °C for 60 min.

## RESULTS

### Cloning of the *rml* locus

A genomic approach was utilized to clone the *rml* genes responsible for the biosynthesis of dTDP-L-Rha in *P. aeruginosa*. The published sequences of the *E. coli* *rml* genes (Liu & Reeves, 1994; Stevenson *et al.*, 1994) were used to identify four homologous, clustered gene

**Table 2.** Amino acid comparisons of RmlB, RmlD, RmlA and RmlC from *P. aeruginosa* with other dTDP-L-Rha biosynthetic enzymes

Protein (organism)	No. of amino acids	Identity*	Accession no./reference
RmlB ( <i>Pseudomonas aeruginosa</i> )	352	100 (352)	This study
RmlB ( <i>Burkholderia pseudomallei</i> )	353	74 (260)	AAD05454
RfbB ( <i>Xanthomonas campestris</i> )	351	68 (238)	P55295
RmlB ( <i>Escherichia coli</i> )	361	62 (219)	AAB88398
RfbB ( <i>Shigella flexneri</i> )	361	62 (219)	P37777
RmlB ( <i>Salmonella enterica</i> )	361	62 (218)	CAA40115
RmlD ( <i>Pseudomonas aeruginosa</i> )	302	100 (302)	This study
RmlD ( <i>Escherichia coli</i> )	299	55 (164)	AAB88399
RfbD ( <i>Shigella flexneri</i> )	300	54 (160)	P37778
RmlD ( <i>Salmonella enterica</i> )	299	51 (153)	CAA40116
RmlD ( <i>Burkholderia pseudomallei</i> )	298	52 (154)	AAD05457
RfbC ( <i>Xanthomonas campestris</i> )	302	47 (142)	C49906
RmlA ( <i>Pseudomonas aeruginosa</i> )	293	100 (293)	This study
RmlA ( <i>Escherichia coli</i> )	293	76 (222)	AAB88400
RfbA ( <i>Shigella flexneri</i> )	290	75 (222)	P37779
RmlA ( <i>Salmonella enterica</i> )	292	75 (218)	CAA40117
RmlA ( <i>Burkholderia pseudomallei</i> )	297	75 (219)	AAD05455
RmlC ( <i>Pseudomonas aeruginosa</i> )	181	100 (181)	This study
RmlC ( <i>Burkholderia pseudomallei</i> )	183	65 (118)	AAD05456
RfbC ( <i>Shigella flexneri</i> )	181	64 (116)	P37780
RmlC ( <i>Salmonella enterica</i> )	183	61 (111)	CAA40118
RfbC ( <i>Synechocystis</i> spp.)	182	61 (111)	BAA18590
RmlC ( <i>Escherichia coli</i> )	185	58 (105)	AAB88401

\* Amino acid comparisons are reported as percentage identities, followed by the number of identical amino acids in parentheses.

sequences (*rmlBDAC*; contig 1, nucleotides 5810161–5813561; December 15, 1999 release) within the *P. aeruginosa* PAO1 genome (www.pseudomonas.com). An additional, unlinked copy of *rmlD* (contig 1, nucleotides 4546491–4547791; December 15, 1999 release) was identified elsewhere on the chromosome of PAO1 (Matewish *et al.*, 1998). To clone the four contiguous *rml* genes, PCR primers were designed based on the nucleotide sequences flanking the *P. aeruginosa* *rml* operon (Fig. 1a). A product of 3.5 kb was amplified from PAO1 chromosomal DNA, which corresponded to the predicted length according to the genome sequence (Fig. 1b). The *rml* locus was subsequently cloned into the broad-host-range vector pEX18Ap (Hoang *et al.*, 1998) using *Sph*I and *Xba*I restriction sites contained within *rml*-specific PCR primers (Fig. 1a).

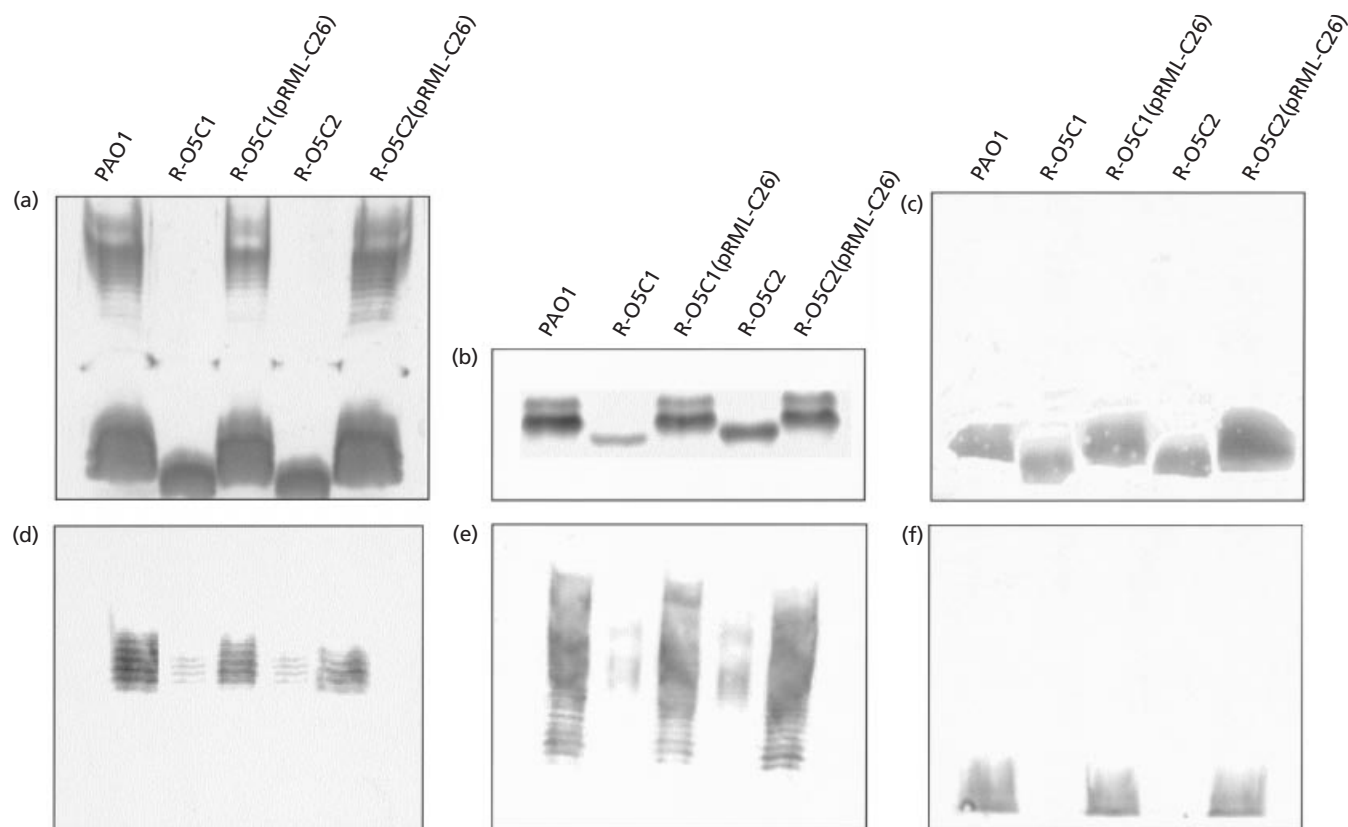
#### Nucleotide and amino acid analysis of the *rmlBDAC* biosynthetic operon

The *rml* locus is composed of four open reading frames (ORFs) designated *rmlB*, *rmlD*, *rmlA* and *rmlC*. These genes are contiguous and transcribed in the same direction and arranged in the same manner as those of *E. coli* (Liu & Reeves, 1994; Stevenson *et al.*, 1994), *Shigella* species (Macpherson *et al.*, 1994; Rajakumar *et al.*,

1994; Klena & Schnaitman, 1993; Sturm *et al.*, 1986) and *Salmonella enterica* (Jiang *et al.*, 1991), suggesting that the *rml* biosynthetic locus evolved along similar lines among these bacteria. The *rmlBDAC* block of genes has a mean G + C content of 66.3 mol %, which is consistent with that of the *P. aeruginosa* genome at 67.2 mol % (Palleroni, 1984). Potential Shine–Dalgarno sequences that precede the translational start codons of all four genes are present and there is a potential  $\sigma^{70}$ -like promoter sequence of 5'-TTACTC-N<sub>15</sub>-TATAAT-3' upstream of *rmlB*. Comparisons of the deduced amino acid sequences of RmlB, RmlD, RmlA and RmlC with those of other proteins in GenBank revealed strong sequence identity among proteins with similar functions (Table 2). Based on these homologies we assigned the putative function of RmlA as glucose-1-phosphate thymidyltransferase, RmlB as dTDP-D-glucose-4,6-dehydratase, RmlC as dTDP-4-keto-6-deoxy-D-glucose-3,5-epimerase and RmlD as dTDP-4-keto-L-rhamnose reductase.

#### Analysis of *P. aeruginosa* serotype O5 (PAO1) chromosomal *rmlC* mutants

Recent structural data reveal a single L-Rha residue located in the distal region of the substituted *P. aeruginosa* O5 core oligosaccharide and illustrate the



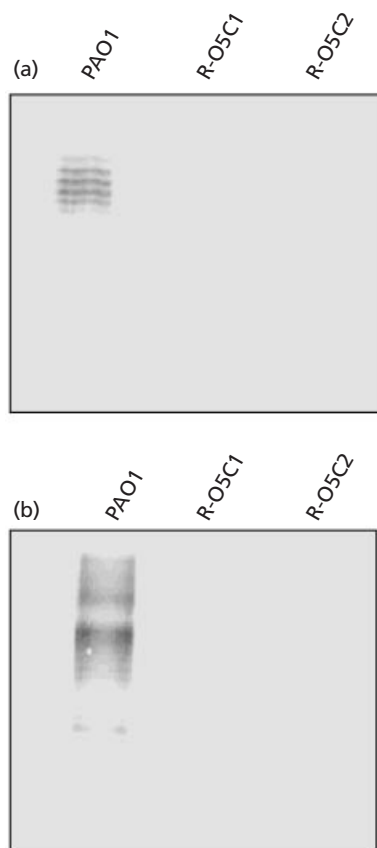
**Fig. 2.** Analysis of LPS from PAO1 (parent strain) and PAO1 *rmlC* mutants (R-O5C1 and R-O5C2). (a) SDS-PAGE glycine gel and silver staining. (b) SDS-PAGE tricine gel and silver staining. (c–f) Western immunoblot results with various mAbs: (c) mAb 7-4 (inner-core-specific); (d) mAb N1F10 (A-band-specific); (e) mAb 15-4 (B-band-specific); and (f) mAb 5c-101 (outer-core-specific). The PAO1 *rmlC* mutants synthesize a truncated core oligosaccharide moiety that is unable to accept either A-band or B-band O polysaccharide, resulting in only minimal, lipid-carrier-linked amounts of these polymers being synthesized. A-band and B-band LPS production was restored to wild-type levels when a construct containing *rmlC* was introduced into the mutants.

involvement of this sugar in the attachment of B-band O antigen (Sadovskaya *et al.*, 1998, 2000). To further investigate the role of L-Rha in LPS biosynthesis and assembly, chromosomal mutants were generated. A  $Gm^R$  cassette was inserted within the last gene of the *rml* biosynthetic operon (*rmlC*), thereby eliminating possible polar effects on the remainder of the biosynthetic alleles (Fig. 1a). The knockout construct was introduced into the PAO1 chromosome by homologous recombination and two putative mutants were chosen for further analysis. To confirm that allelic replacement had taken place, the oligonucleotide PCR primers RML-BDAC-up and RML-BDAC-down were utilized to amplify products of 3.5 and 4.6 kb from parent and mutant genomic DNA, respectively (Fig. 1c). The 4.6 kb product represents the *rml* locus (3.5 kb) plus the  $Gm^R$  cassette (1.1 kb), demonstrating that the two isogenic mutants had undergone gene replacement (Fig. 1c).

LPS from *rmlC* mutants was analysed by SDS-PAGE gels and Western immunoblotting. The *rmlC* mutants exhibited fast migrating core bands in silver-stained SDS-PAGE gels indicative of truncated core oligo-

saccharide moieties (Fig. 2a). The lack of high-molecular-mass bands implied the loss of both A-band and B-band O antigens. Western immunoblots using the outer-core-specific mAb 5C-101 showed no reaction of this antibody with the fast migrating bands, confirming truncation of the outer-core region (Fig. 2f). mAb 5C-101 was shown to recognize the terminal D-Glc moiety present in the outer-core region of PAO1 (de Kievit & Lam, 1994). The inability of the *rmlC*:: $Gm^R$  mutants to synthesize L-Rha probably prevents the addition of the terminal D-Glc residue, resulting in the loss of the epitope recognized by mAb 5C-101. The assembly of the inner core was not affected by the *rmlC* mutation since the same fast migrating bands from both PAO1 (wild-type) and the *rmlC* mutants reacted with mAb 7-4 (inner-core-specific; de Kievit & Lam, 1994; Fig. 2c).

Immunoblots reacted with A-band- and B-band-specific mAbs reveal minimal A- and B-band O polysaccharide production in mutant strains R-O5C1 and R-O5C2 (Fig. 2d, e). The polysaccharides detected are probably not linked to core-lipid A (see below). Structural data indicate that the L-Rha residue located in the substituted



**Fig. 3.** Western immunoblots of PAO1 (parent strain) and *rmlC* mutants. LPS was isolated from the strains according to the hot-aqueous phenol method of Westphal & Jann (1965). No reaction could be observed when the LPS is reacted with N1F10 [A-band-specific (a)] and MF15-4 [B-band-specific (b)]. This indicates that the O polysaccharides were linked to undecaprenol in those strains.

outer-core structure serves as the attachment site for long-chain B-band O antigen (Sadovskaya *et al.*, 1998, 2000). However, this schematic does not reveal the attachment site for A-band O antigen. Since the loss of L-Rha affects expression of both A-band and B-band LPS, we propose that L-Rha functions as the linkage site for both polysaccharides. Our repeated observations that more A-band LPS is expressed on the cell surface when B-band O antigen genes are inactivated (reviewed by Rocchetta *et al.*, 1999) probably corresponds to an increase in the number of available attachment sites for A-band polymer in the absence of competition from the B-band polymer. Introduction of *rmlC* on plasmid pRML-C-26 restored the capacity of *rmlC* mutants to synthesize complete core oligosaccharide and to produce A-band and B-band LPS, indicating that the mutant phenotype was specifically due to mutation of the *rmlC* gene (Fig. 2a, b, c, f).

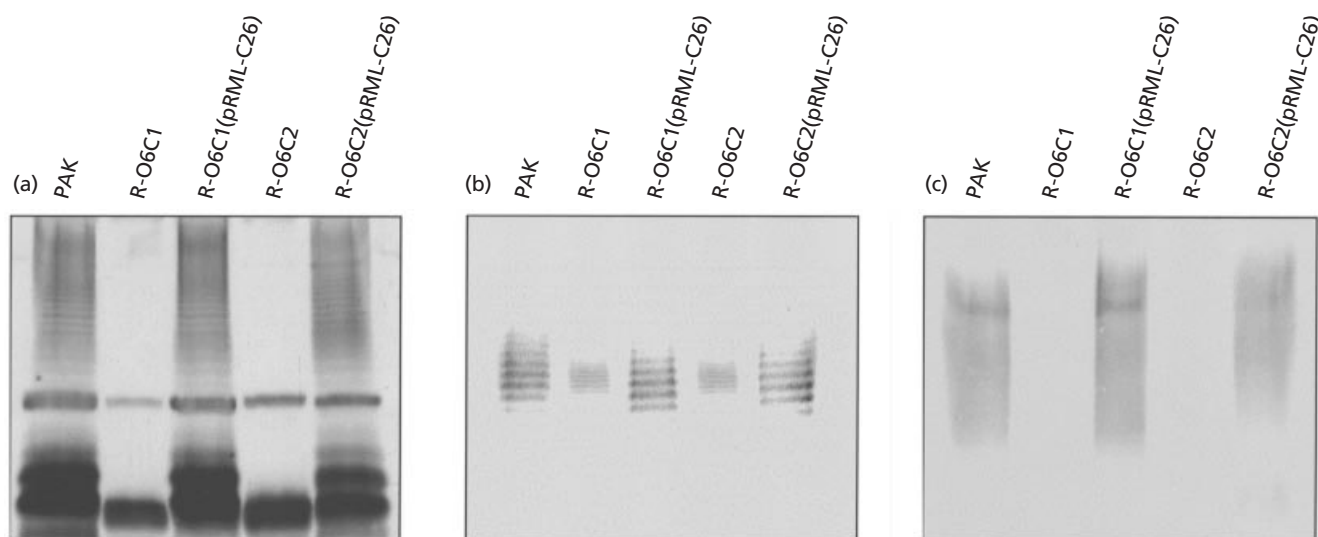
To determine if A-band and B-band polysaccharides observed in the *rmlC* mutants were attached to either core-lipid A or a carrier-lipid molecule, hot-aqueous

phenol extractions were performed. Previous studies by Kent & Osborn (1968) showed that the pyrophosphate linkage between the lipid-carrier molecule (undecaprenol phosphate) and the nascent polysaccharide is extremely labile and is disrupted by hot-aqueous phenol extraction. LPS isolated from *rmlC* mutants using this technique demonstrated no reactivity on Western immunoblots with A-band- and B-band-specific mAbs, implying cleavage of the polysaccharide-carrier lipid linkage and release of the soluble polymers (Fig. 3a, b).

To improve resolution of the low-molecular-mass core oligosaccharide region, Tricine SDS-PAGE and Western immunoblotting were performed. Tricine SDS-PAGE analysis revealed distinct differences in the banding profiles of PAO1 versus *rmlC* mutant LPS preparations (Fig. 2b). The *P. aeruginosa* wild-type strain PAO1 expressed two well-defined bands representing unsubstituted core oligosaccharide and core substituted with a single O antigen repeat unit. Both R-O5C1 and R-O5C2 showed a single band corresponding to the core oligosaccharide region, indicating the loss of three sugar residues analogous to the O5 trisaccharide O repeat unit. In addition, the core LPS of these mutants migrated faster than that of the parent strain PAO1, suggesting a core region modified by the loss of at least one sugar residue (Fig. 2b). Since structural studies indicate that L-Rha precedes the D-Glc residue present in the unsubstituted outer core (Sadovskaya *et al.*, 1998), it is likely that the unsubstituted core region expressed by *rmlC* mutants is deficient in both the terminal D-Glc and L-Rha residues. LPS isolated from the complemented mutants containing the *rmlC* gene *in trans* showed low-molecular-mass bands identical to those of the parental PAO1 LPS on Tricine SDS-PAGE gels, indicating restoration of the core oligosaccharide region and attachment of O antigen (Fig. 2b).

### Generation and analysis of *rmlC* mutants in serotype O6 (PAK)

In previous studies by our group (Masoud *et al.*, 1995; Sadovskaya *et al.*, 1998), L-Rha was shown to be a common constituent in the outer-core region of both IATS serotype O6 and serotype O5 LPS. This sugar is also the terminal residue in the O units of serotype O6 LPS (Knirel & Kochetkov, 1994). Interestingly, recent genetic analysis of the O6 O antigen gene cluster revealed that genes encoding the L-Rha component of the O antigen were not present at that locus (Bélanger *et al.*, 1999). Instead, the synthesis of L-Rha residues of the O antigen was probably encoded by the *rml* locus required for core oligosaccharide synthesis. Taking advantage of the conserved nature of dTDP-L-Rha biosynthetic genes in Gram-negative bacteria, the O5 *rmlC* knockout construct was used to disrupt the homologous gene in strain PAK (serotype O6). LPS isolated from PAK and *rmlC*<sub>O6</sub> mutants was examined on silver-stained SDS-polyacrylamide gels and Western immunoblots. Tricine SDS-PAGE gel analysis indicated that a mutation in *rmlC*<sub>O6</sub> resulted in a truncated core oligosaccharide as



**Fig. 4.** LPS analysis of PAK (parent strain) and PAK *rmlC* mutants (R-O6C1 and R-O6C2). (a) SDS-PAGE and silver staining; (b) Western immunoblotting with mAb N1F10 (A-band-specific); and (c) Western immunoblot with mAb MF23-2 (O6 B-band-specific). The PAK *rmlC* mutants synthesize a truncated core oligosaccharide moiety that is unable to accept A-band O polysaccharide, resulting in production of only lipid-carrier-linked material. O6 B-band polysaccharide is no longer synthesized in *rmlC* mutants. Production of A-band and B-band LPS is restored when a construct containing *rmlC*<sub>O5</sub> is introduced into the mutants.

well as a loss of both A- and B-band LPS (Fig. 4a). As described earlier, truncated core-lipid A molecules are unable to serve as acceptors of O antigen, resulting in the accumulation of O polysaccharides on carrier lipid molecules. Reactivity patterns with the mAbs N1F10 (A-band-specific) and MF23-2 (B-band-specific) on Western immunoblots confirmed the O antigen banding profiles observed on silver-stained gels (Fig. 4b, c). Since L-Rha is a component of O6 B-band O antigen as well as core oligosaccharide, the absence of this residue probably disrupts assembly of both molecules. Complementation of *rmlC*<sub>O6</sub> mutants with *rmlC*<sub>O5</sub> *in trans* restored the production of the core region, A-band LPS and B-band LPS to levels similar to those of the wild-type parent (Fig. 4a).

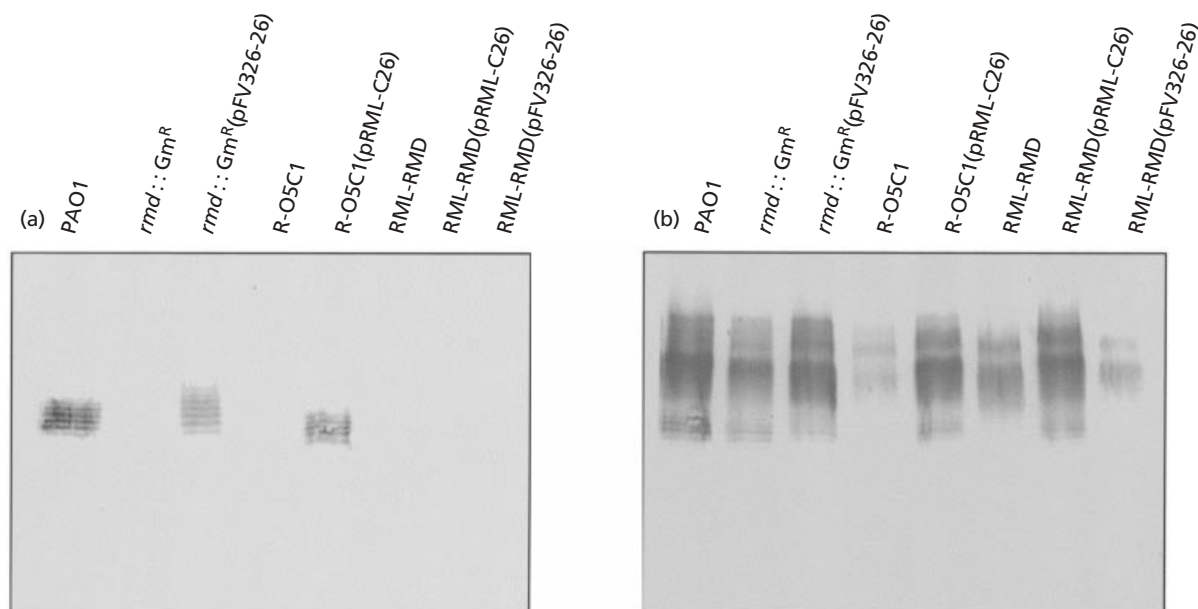
#### Generation of *rmd rmlC* mutants in PAO1

The structure of the core oligosaccharide of the *rmlC* mutant was analysed using compositional and <sup>1</sup>H-NMR methods. Initial composition analysis of partially hydrolysed and fractionated LPS samples indicated the presence of Rha, D-Glc, 2-amino-2-deoxy-D-galactose (D-GalN) and L-glycero-D-manno-heptose residues in core oligosaccharide components of wild-type and mutant strains of *P. aeruginosa* (data not shown). One-dimensional <sup>1</sup>H-NMR spectroscopy revealed a pair of overlapping resonances in the range of 1.30–1.33 p.p.m. indicative of alanine and Rha residues (data not shown). Based on SDS-PAGE and Western immunoblot data, the core region of PAO1- and PAK-derived *rmlC* mutants should be deficient in L-Rha. Since MS- and NMR-based methods lack the ability to distinguish L- and D-enantiomers, the detection of Rha using these techniques

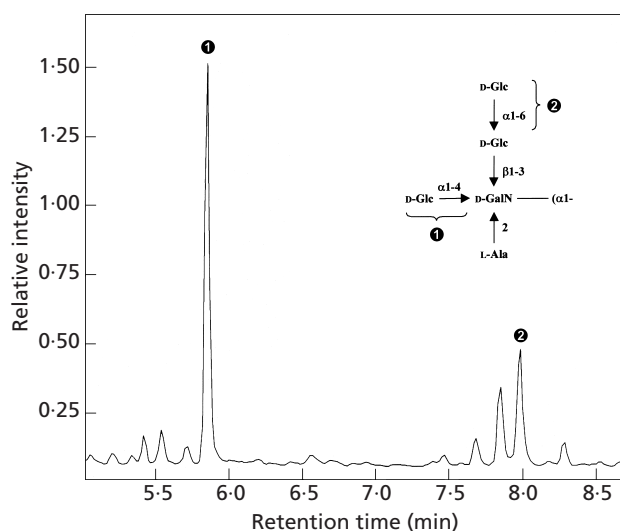
was attributed to contaminating amounts of A-band LPS, a homopolymer of D-Rha (Arsenault *et al.*, 1991).

To prevent D-Rha of A-band polysaccharide from confounding the structural analysis, a double *rmd rmlC* mutant of PAO1 was constructed using the Flp-FRT (Flp recombinase targets) recombination and gene replacement strategy of Hoang *et al.* (1998) as described in Methods. This system enables generation of consecutive chromosomal mutations in the same strain without having to use diverse antibiotic selection markers. The unmarked *rmlC* mutant strain derived following marker excision was designated RML-ΔC. SDS-PAGE analysis revealed that the LPS-banding patterns of RML-ΔC and those of previously isolated PAO1 *rmlC* mutants were identical, indicating that excision of the Gm<sup>R</sup> cassette did not have adverse effects on the LPS profile of the modified PAO1 mutant (RML-ΔC; data not shown). RML-ΔC was then used as the host strain for the generation of A-band-LPS-deficient chromosomal mutants using pFV327-TGm, previously constructed by Rocchetta *et al.* (1998). pFV327-TGm contains a Gm<sup>R</sup> cassette inserted within *rmd*, a gene involved in the synthesis of D-Rha residues comprising A-band polysaccharide (Rocchetta *et al.*, 1998). The knockout construct was conjugally transferred into RML-ΔC to generate the double *rmd rmlC* mutant (RML-RMD) as described in Methods.

LPS extracted from single (RML-ΔC) and double (RML-RMD) mutant strains of *P. aeruginosa* was examined by Western immunoblotting. Reactivity of the LPS of these strains with mAbs N1F10 (A-band-specific) and MF15-4 (B-band-specific) revealed that the *rmd rmlC* mutant does not produce A-band LPS, but does continue to



**Fig. 5.** Western immunoblots of LPS from PAO1 (wild-type) and R-O5C1 (*rmIC* deficient) single and RML-RMD double mutants. The mAbs used are (a) N1F10 (A-band-specific) and (b) MF15-4 (O5 B-band-specific). The RML-RMD double mutant does not have the ability to synthesize A-band LPS, while nominal quantities of B-band O polysaccharide continue to be synthesized.



**Fig. 6.** Methylation linkage analysis of the core oligosaccharides from the double mutant RML-RMD. Peaks 1 and 2 represent the permethylated alditol acetate derivatives of terminal D-Glc residues and 6-substituted D-Glc, respectively. The proposed chemical structure of the outer-core region of the RML-RMD mutant is illustrated.

synthesize nominal amounts of B-band O polysaccharide (Fig. 5a, b). LPS from RML-RMD also failed to react with the outer-core-specific mAb 5C-101 in Western immunoblot experiments, indicating core truncation within the LPS of this mutant (data not shown).

The relative mobility of the core-lipid A band of the double mutant RML-RMD was identical to the truncated core band observed in PAO1 *rmIC* mutants (data not shown). LPS from the double mutant complemented with *rmd*, RML-RMD (pFV326-26), showed a few bands that were weakly reactive with mAb N1F10 (Fig. 5a). However, since the complemented mutant is still deficient in L-Rha biosynthesis, these N1F10-reactive bands are probably undecaprenol-linked A-band polysaccharide. These results indicate that the RML-RMD mutant has the expected phenotype: deficient in both the L and D enantiomers of Rha, expression of an incomplete core region, and lack of A-band LPS synthesis.

#### Structural analysis of the *rmd rmIC* double mutant

To further characterize the LPS defects in RML-RMD, sugar linkage analysis was performed. GLC-MS of permethylated alditol acetate derivatives identified terminal Glc residues (two units) as well as a 6-substituted Glc unit. A peak corresponding to 4-substituted Glc was also detected (Fig. 6). By sugar linkage analysis of LPS from the RML-RMD mutant, we detected the presence of Glc-1 (substituted in carbon 1 of the Glc residue), 6-Glc-1 and 4-Glc-1 in approximate molar ratios of 2:2:1:0:0.7. According to Sadovskaya *et al.* (1998), the core oligosaccharide moiety of *P. aeruginosa* strain PAO1 lacks Glc residues substituted at the fourth carbon. Therefore, the observation of these sugars in core oligosaccharides of the RML-RMD mutant may be due to compensatory sugar substitutions resulting from

*rmd rmlC* null mutations. However, this hypothesis remains to be experimentally validated. Rha could not be detected from LPS isolated from RML-RMD. This reflects a truncated PAO1 core oligosaccharide lacking main chain Glc and Rha residues. Methylation linkage analysis failed to detect certain core oligosaccharide components such as galactosamine (GalN) and heptose (Hep) due to the presence of certain substituents (e.g. phosphates). These results illustrate the requirement of L-Rha for outer-core assembly and show unequivocally the presence of a Glc-1-6-Glc- antenna in the absence of core-associated L-Rha residues (Fig. 6).

Sugar composition analysis of RML-RMD LPS detected the presence of Glc, L-glycero-D-manno-heptose and GalN respectively in the following molar ratios: 3:1.2:0.8. These data are consistent with those obtained using sugar linkage analysis and confirm the absence of L-Rha within core oligosaccharide moieties of RML-RMD.

## DISCUSSION

Despite the rare occurrence of L-Rha in nature, it is a frequently observed constituent of the LPS of Gram-negative bacteria. L-Rha is synthesized as an activated nucleotide derivative (i.e. dTDP-L-Rha) by four enzymes designated RmlA, RmlB, RmlC and RmlD (Kornfeld & Glaser, 1961). Using *E. coli rml* genes as probes, we were able to localize *rml* genes in the *P. aeruginosa* (PAO1) whole genome sequence. The four *P. aeruginosa rml* genes are clustered and homologous to those of other bacteria. The identification of an additional, unlinked copy of *rmlD* has not previously been reported for other bacteria, and it will be interesting to determine whether it is functional. The *rmlC* gene inactivated in this study appeared to be the only copy in the chromosome, and the effects of its mutation on LPS structure confirm that there are no additional functional homologues present.

Since dTDP-L-Rha is common in bacteria, the enzymes involved in its synthesis are potential targets for the design of novel therapeutic inhibitors. To date, Rml proteins have been examined in numerous bacterial species (Table 2). Importantly, three of the four Rml proteins (RmlB, RmlC and RmlD) of *S. enterica* sv. Typhimurium have been crystallized (Allard *et al.*, 2000; Giraud *et al.*, 1999a, b). Our group has recently crystallized RmlA of *P. aeruginosa* (Blankenfeldt *et al.*, 2000) in the presence of its substrate, Glc-1-P. The knowledge derived from these protein crystals will contribute towards a better understanding of the structure and function of these enzymes.

To assess the role of L-Rha in LPS biosynthesis and assembly, chromosomal mutants were generated in strain PAO1 (serotype O5) and PAK (serotype O6). SDS-PAGE gels reveal that PAO1 *rmlC* mutants (R-O5C1 and R-O5C2) lack the ability to synthesize complete core molecules, resulting in the apparent loss of A-band and B-band O antigen attachment (Fig. 2a). However, the use of mAbs N1F10 (A-band-specific) and

MF15-4 (B-band-specific) in Western immunoblots showed nominal amounts of A-band and B-band polysaccharide production by the mutants (Fig. 2d, e). These polysaccharides were shown to be phenol-labile (Fig. 2a, b), suggesting that they are linked to undecaprenol phosphate and not to core-lipid A. Based on the current understanding of A-band and B-band LPS biosynthesis, O polysaccharides are synthesized on undecaprenol phosphate and transported from the cytoplasm to the periplasm by two distinct mechanisms to allow for ligation to core-lipid A by the O antigen ligase, WaaL (Rocchetta *et al.*, 1999). As mentioned, PAO1 *rmlC* mutants synthesize a truncated core oligosaccharide moiety devoid of L-Rha. Since truncated core-lipid A molecules are unable to serve as acceptors for O antigen, we propose that carrier-lipid-linked A- and B-band O antigens accumulate in the periplasm. Limitations imposed by undecaprenol phosphate availability within the cell may explain why only small amounts of polymer are synthesized in core mutants. In a previous study by our group (Rocchetta & Lam, 1997), we observed that mutations in genes encoding the ABC transporter for A-band O polysaccharide translocation across the bacterial inner membrane also resulted in accumulation of A-band polysaccharide linked to lipid carrier molecules. Structural data indicate that L-Rha serves as the attachment point for B-band O antigen (Sadovskaya *et al.*, 1998, 2000). However, the schematic does not reveal the acceptor for A-band O antigen. Based on the data in this study, we propose that L-Rha (located on the branched carbohydrate chain) functions as the linkage site for both O polysaccharides. This is a difficult point to clarify structurally, since the processive mechanism of A-band synthesis does not allow for the isolation of semi-rough mutants containing only one O unit (Rocchetta *et al.*, 1999). However, we have noted repeatedly that in the absence of B-band O antigen synthesis, more core-linked A-band LPS is expressed. This observation is consistent with more sites being available for attachment of the A-band polymer when B-band is not present.

Recent structural data reveal that L-Rha is a constituent of the outer-core region of *P. aeruginosa* serotype O6 LPS (Masoud *et al.*, 1995; Sadovskaya *et al.*, 1998). This methylpentose occupies terminal positions within the outer-core region and is hypothesized to be involved in linking B-band O antigen to the rest of the LPS molecule (Sadovskaya *et al.*, 2000). SDS-PAGE and Western immunoblot analysis demonstrate that PAK *rmlC* mutants synthesize an incomplete core oligosaccharide region that is unable to accept A-band O antigen. These results are consistent with those obtained for PAO1 *rmlC* mutants and suggest that L-Rha is the attachment site for A-band polymer in this serotype. The attachment of B-band O antigen cannot be evaluated in O6 *rmlC* mutants, since no complete B-band O units are synthesized.

In conclusion, this study reports the identification and molecular characterization of the dTDP-L-Rha biosynthetic operon in *P. aeruginosa*. To our knowledge,

this is the first report describing the structural analysis of genetically defined mutations affecting the LPS core oligosaccharide region of *P. aeruginosa*. Chromosomal mutations within the *rmlC* allele of PAO1 (serotype O5) and PAK (serotype O6) resulted in core truncation that prevented the attachment of A-band and B-band O antigen. Chemical and structural data confirmed the LPS defects in serotype O5 *rmlC* mutants and illustrated the importance of L-Rha residues in LPS synthesis and assembly. Recent studies by Pier *et al.* (1996a, b) demonstrate that the outer-core region of *P. aeruginosa* LPS mediates the uptake of *P. aeruginosa* by host epithelial cells. Use of genetically defined *P. aeruginosa* mutants with known defects in the core LPS structure will help elucidate specific host–bacterium interactions and broaden our knowledge with respect to the mechanisms involved in the pathogenesis of this versatile organism.

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