ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 7, pp. 755-760. © Pleiades Publishing, Ltd., 2011.
Published in Russian in Biokhimiya, 2011, Vol. 76, No. 7, pp. 925-932.

REVIEW

Structural Diversity of the Core Oligosaccharide Domain of *Pseudomonas aeruginosa* Lipopolysaccharide*

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Received January 15, 2011
Revision received February 1, 2011

Abstract—*Pseudomonas aeruginosa* is a Gram-negative bacterium that is ubiquitous in the environment and generally considered to be a saprophyte, but it is also an important opportunistic human pathogen. *Pseudomonas aeruginosa* elaborates a variety of virulence factors, one of which is lipopolysaccharide (LPS). LPS of *P. aeruginosa* is composed of three distinct regions: lipid A, core oligosaccharide (OS), and the long-chain O antigen. The core OS of *P. aeruginosa* is composed of L-*glycero*-D-*manno*-heptose, 3-deoxy-D-*manno*-oct-2-ulosonic acid, D-galactosamine, D-glucose, and L-rhamnose. Non-carbohydrate substituents are also found in the core OS including phosphate, 2-aminoethyl (di)phosphate, acetyl, alanyl and carbamoyl groups. *Pseudomonas aeruginosa* simultaneously synthesizes two core glycoforms, namely, capped and uncapped core. The capped core is covalently attached to an O antigen, whereas the uncapped core is devoid of O antigen. Although the core of *P. aeruginosa* LPS is relatively conserved, strain-to-strain variability of its structure exists. This includes phosphorylation pattern, the level of O-acetylation, and the presence or absence of a fourth glucose residue at the distal end of the uncapped core. A number of studies have been reported on the structures of unique truncated core OS with unusual modifications. This mini-review summarizes the diversity of *P. aeruginosa* complete and truncated core OS structures published over the past fifteen years.

DOI: 10.1134/S0006297911070054

Key words: lipopolysaccharide, core oligosaccharide, *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium, but it is also a pathogen of plants, animals, and an important opportunistic pathogen of humans [1, 2]. This versatile bacterium is a common cause of nosocomial infections and it mainly infects compromised patients, such as burn victims and AIDS or cystic fibrosis patients. To establish an infection, *P. aeruginosa* elaborates a variety of virulence factors, most of which are usually secreted substances including toxins, enzymes, and glycolipids, but one of the major virulence factors is the cell wall-associated lipopolysaccharide (LPS). LPS of *P. aeruginosa* is composed of three distinct regions: lipid A, core oligosaccharide (OS), and the long-chain O polysaccharide (O antigen) that consists of diverse repeat saccharide units. Differences among the sugar constituents and linkages in the O antigens form the basis for classification of *P. aeruginosa* strains into 20 International Antigenic Typing Scheme (IATS) serotypes. In this brief communication, we will summarize the diversity of the core OS structures of *P. aeruginosa* based on studies that have been performed by our group and other laboratories over the past fifteen years.

Abbreviations: GalN, galactosamine; Hep, L-*glycero*-D-*manno*-heptose; IATS, International Antigenic Typing Scheme; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LPS, lipopolysaccharide; MS, mass spectrometry; OS, oligosaccharide; P, phosphate; Rha, rhamnose.

* The authors dedicate this review to Dr. Malcolm Perry in our admiration of the impact that he has made to the field of complex carbohydrate research during his long and fruitful career at the National Research Council of Canada.

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ISOLATION AND STRUCTURAL ANALYSIS OF CORE OS

A prerequisite for studying the core OS structure of the LPS of any Gram-negative bacterium is the ability to prepare a large amount of core OS without contamination of O polysaccharide molecules. There are a number of "standard" methods for preparing LPS from bacteria,

including the hot phenol–water procedure [3], the phenol–chloroform–petroleum ether method [4], and the Darveau and Hancock method [5]. The method developed by the Hancock laboratory [5] utilizes DNase I and RNase A to remove nucleic acids from cell extracts, SDS to solubilize proteins at an elevated temperature, and subsequent pronase digestion to eliminate protein contaminations to enrich for the glycolipid portion.

The phenol–water and Darveau and Hancock methods yield a heterogeneous mixture of "smooth" (substituted with O antigen) and "rough" (devoid of O antigen) LPS. Although "rough" LPS containing mainly core OS could be separated from "smooth" LPS by column chromatography, the process is tedious and the yield of core OS is usually low. Therefore, the rule of thumb is to begin such studies by preparing LPS from rough mutants devoid of O polysaccharides. If such a mutant is not available, then various genetic strategies can be used to isolate rough mutants lacking O polysaccharide, or lacking O polysaccharide plus some core sugars resulting in truncated core OS.

To structurally characterize core OS, a combination of two physical techniques are commonly used: (i) mass spectrometry (MS), more specifically matrix assisted laser desorption ionization time-of-flight MS or electrospray ionization MS, and (ii) nuclear magnetic resonance spectroscopy. To increase the solubility and reduce the size of LPS, acyl chains of lipid A can be easily removed by alkaline degradation. This method includes deacylation by mild hydrazinolysis and strong alkaline hydrolysis [6-11]. By virtue of the sites of cleavage, which in this case are the linkages between components of lipid A (acyl chains and the diglucosamine backbone that are N- and O-linked together), the resulting deacylated structure contains the entire core region as well as the diglucosamine. Alternatively, core OS can be delipidated with mild acid hydrolysis (acetic acid or sodium acetate buffer), and the site of cleavage is the acid labile ketosidic linkage between the Kdo residue in the core and lipid A [7, 8, 12, 13]. However, because the bond between two Kdo residues of core OS is acid labile too (for details of core structure, see the next section), the resulting delipidated core structure can be lacking one Kdo residue.

COMMON STRUCTURE OF *P. aeruginosa* COMPLETE CORE OS

The core OS is generally divided into an inner core, which is linked to one of the glucosamine residues of lipid A, and an outer core. The inner core in *P. aeruginosa* is a tetrasaccharide comprised of two residues of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo^{I} and Kdo^{II}) and two residues of L-*glycero*-D-*manno*-heptose (Hep^{I} and Hep^{II}). The inner core of *P. aeruginosa* is highly phosphorylated, and three common phosphorylation sites have been identified, namely at O2 and O4 of Hep^{I} and O6 of Hep^{II}. Besides phosphorylation, Hep^{II} is substituted with a carbamoyl group at O7. The outer core is composed of one D-galactosamine (D-GalN), one L-rhamnose (L-Rha), and three D-glucose residues (Glc^{I}-Glc^{III}). GalN is further substituted on N2 with an alanyl group.

Pseudomonas aeruginosa simultaneously synthesizes two core OS glycoforms, namely capped and uncapped core. The capped core is covalently attached to O antigen, whereas uncapped glycoform is devoid of O antigen. The inner cores of the capped and uncapped forms are conserved and identical, but their outer cores differ in the position and linkage of the L-Rha residue. Capped core is attached to O antigen through $L\text{-}Rha^{B}$ that is 1,3-linked to Glc^{I}, while uncapped core contains $L\text{-}Rha^{A}$ that is 1,6-linked to Glc^{II} (Fig. 1).

VARIABLE SUBSTITUTIONS IN THE COMPLETE CORE OS STRUCTURES

Complete core OS from different strains display a highly conserved structure with relatively low levels of variability. However, three kinds of variable modifications have been described: phosphorylation, acetylation, and the presence of a fourth glucose residue (Glc^{IV}) in the uncapped core.

Bystrova et al. [11] performed a systematic examination of the core OS structures of reference strains of IATS O1-O20 serotypes and showed that most strains, except O4, possess 2-aminoethyl diphosphate at O2 of Hep^{I} in non-stoichiometric amounts in the inner core. In general, each of the three phosphorylation sites of the inner core may be modified with phosphate, diphosphate, or even triphosphate. Moreover, it has been suggested that the actual phosphate and ethanolamine contents on live bacterial cells may be higher than those observed, since these groups could easily be released during mild acid hydrolysis of the LPS prior to structural analysis of core OS [11, 14]. An exception to the presence of three phosphorylation sites in core OS was observed in the LPS of a cystic fibrosis isolate, strain 2192. This strain produces LPS that is devoid of O antigen while core OS structures were apparently intact. In addition to having three common phosphorylation sites, the inner core of strain 2129 possesses a fourth phosphorylation site on O4 of Hep^{II} [7].

Although the level of O-acetylation of core OS might be underestimated due to the chemical treatment of LPS prior to structural analysis (similarly to phosphorylation pattern), some degree of variability between strains have been observed [11, 14]. In all strains that have been characterized so far, O-acetylation on the core OS is non-stoichiometric, which makes identification of all the sites difficult. Core OS of the immunotype 1 strain (equivalent to IATS O6 serotype) was found to contain at least one O-acetylation site [8]. The core of cystic fibrosis isolate 2192, mentioned above, contains at least four acetylation sites

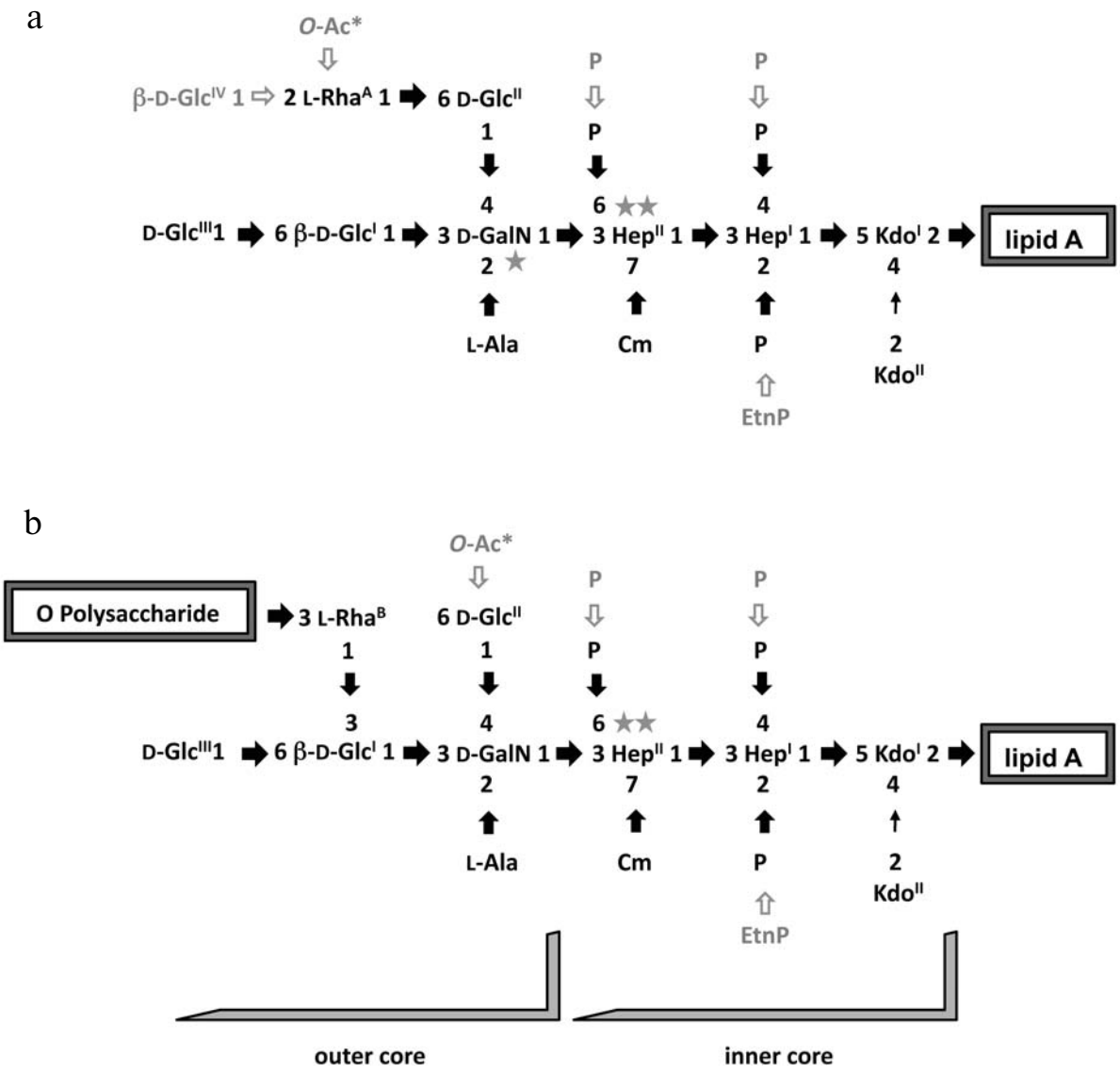

Fig. 1. Structures of the complete uncapped (a) and capped (b) outer core OS of *P. aeruginosa*. Sugars have the α configuration unless otherwise indicated. Common core carbohydrate structure and substitutions are in black. Gray color indicates variable strain-specific modifications. Cm, carbamoyl; Etn, ethanolamine; Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Rha, rhamnose. The asterisks depict the only two sites with non-stoichiometric O-acetylation found in core OS; however, other O-acetyl modification sites also exist. Modifications found uniquely in one single strain: star, L-Ala is replaced by Ac on GalN (OS-I of the *wbjE* mutant); double-star, O4 of Hep^{II} is phosphorylated (the 2192 isolate).

[7]. When O-acetylation of the core OS of reference strains of all IATS O1-O20 serotypes was examined by Bystrova et al. [11], at least one common O-acetylation site was found in a majority of them, but not in serotypes O4, O10, and some strains of serogroup O2, which includes O2, O5, O16, O18, and O20 serotypes (all of them share either identical or similar backbone structures in their O antigens). The highest number of O-acetylation sites, five, was observed in serotypes O9, O11, O15, and O17.

A non-stoichiometric O-acetylation has been found also in the complete capped-core glycoform synthesized by a *migA-rmdA* mutant, which will also be referred to in the next section when we discuss the truncated core OS structures. Noteworthy, the capped core of the *migA-rmd* mutant had an O-acetyl substitution on Glc^{II} [15]. Besides this, the most frequent O-acetyl substitution found in other *P. aeruginosa* strains was on the L-Rha^{A} residue that was 1,6-linked to Glc^{II} in the uncapped core [11, 13].

N-Acetylation has been found in core OS of a *wbjE* mutant of serotype O11. This mutant produced two truncated LPS cores, but also a complete uncapped core OS with an acetyl group at N2 of the GalN residue in place of

the usual alanyl group as the substituent [16]. The structure of this unusual core OS of *P. aeruginosa* has been included in a recent review by Silipo and Molinaro, who described the diversity of LPS core OS among a broad spectrum of Gram-negative genera [17]. More details of the structure of the two truncated core OS of the *wbjE* mutant will be discussed in the next section.

A variable modification found in uncapped core OS is the addition of Glc^{IV} to O2 of L-Rha^{A}. Nine of the IATS serotypes, O2, O5, O7, O8, O10, O16, O18, O19, and O20, have a terminal residue of Glc^{IV} in the outer core, whereas the other 11 IATS serotypes, O1, O3, O4, O6, O9, O11, O12, O13, O14, O15, and O17, do not have this Glc^{IV} residue [8, 11, 18, 19]. Recently, *wapB*, which encodes a putative glycosyltransferase, has been found to be required for the transfer of Glc^{IV} to form a linkage with L-Rha^{A} in nine of the 20 IATS serotypes (D. Kocincova, Y. Hao, E. Vinogradov, and J. S. Lam, unpublished data).

DIVERSITY IN TRUNCATED CORE OS STRUCTURES

The inner core has been shown to be essential for viability of *P. aeruginosa*, especially since mutation in any one of the genes involved in the biosynthesis of this region of LPS, namely, *waaF*, *waaC*, *waaP*, and *wapP*, had a lethal effect on the bacteria [20, 21]. To date, mutants with inner-core defects have not been isolated in *P. aeruginosa*. Therefore, all core OS structures described in this section only exhibited defects in the outer core. In many cases sugars in core OS have been found to be receptors for various bacteriophages that are LPS specific [22-25], and some of the spontaneous mutants with truncated core were selected under laboratory conditions based on resistance to bacteriophages. As such, bacteriophages have been used to infect bacteria also for the purpose of understanding core OS or lipooligosaccharide biosynthesis.

An E79 phage-resistant mutant, AK1012, was derived from wild-type strain PAO1 (serotype O5) [23]. The LPS structure of this mutant has been characterized by Sadovskaya et al. [6]. Core OS of AK1012 was significantly truncated with all Glc and Rha residues lacking, and the only sugar residue left in the outer core was GalN (Fig. 2a).

A rough mutant strain H4 was derived from IATS serotype O6 based on its resistance to phage 2 Lindberg [26]. Examination of the LPS from this strain revealed only one core OS structure [12]. Similar to the core OS structure of strain AK1012, all Glc and Rha residues in the core of H4 were absent and the outer core was truncated at GalN (Fig. 2a). However, instead of having an N-linked alanyl substitution, GalN was N-acetylated [12]. This is similar to the modification in the outer core of the *wbjE* mutant that was described above. The core

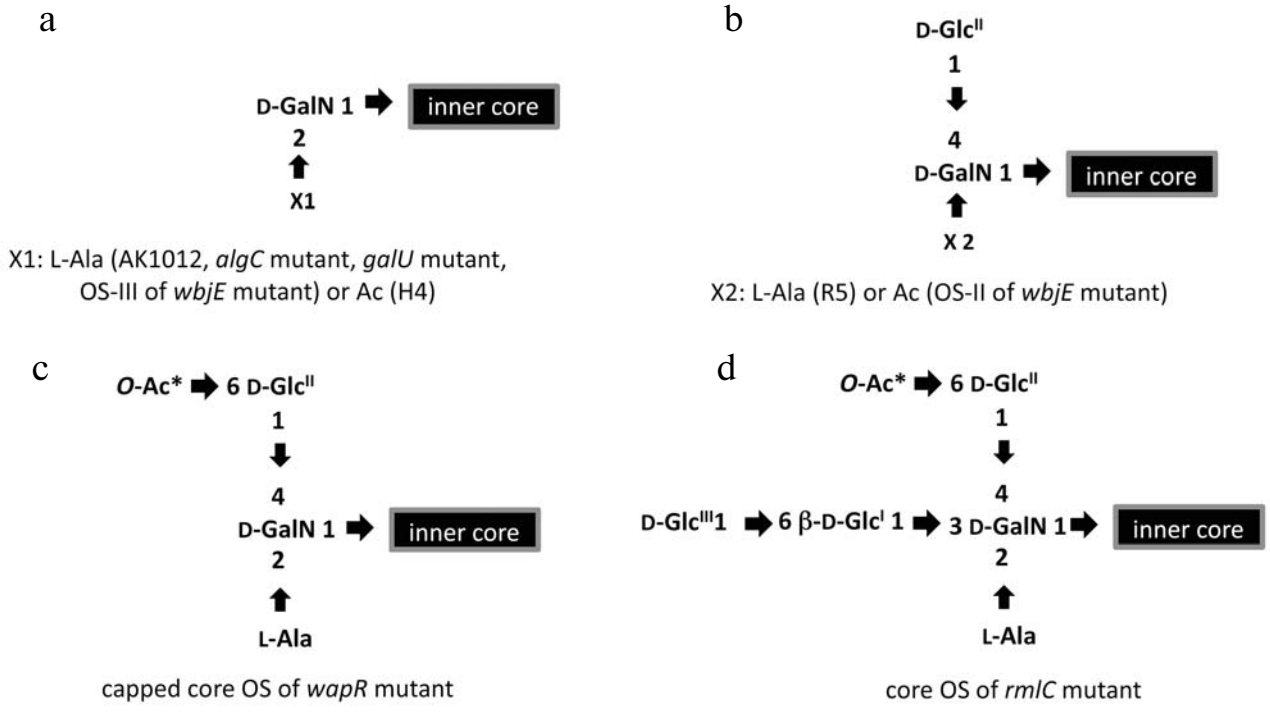

Fig. 2. Diversity of truncated outer core OS structures. a) Oligosaccharide OS-III of the *wbjE* mutant, outer core of the *algC*, *galU*, H4, and AK1012 mutant strains. b) Core OS of the R5 strain and OS-II of the *wbjE* mutant. c) Capped core OS glycoform of the *wapR* mutant. d) Core OS of the *rmlC* mutant. Variable substituents X1 and X2 of the strain-specific core OS structures are as indicated in the figure. Asterisk depicts non-stoichiometric O-acetylation.

structures seen in AK1012 and H4 strains has by far been the most truncated core OS in all *P. aeruginosa* strains that have been examined to date.

Another mutant derived from IATS O6, the R5 strain, was isolated based on its resistance to bacteriophage E79 [26]. R5 was found to produce one core OS glycoform with the outer region composed of the Glc^{II}-GalN disaccharide [27] (Fig. 2b). Due to the truncation of this core OS (and similarly, the truncation of the outer core of strains AK1012 and H4), the complete outer-core region cannot be synthesized; therefore, capped or uncapped core glycoforms were not recognized in earlier studies on these strains.

Apart from the above list of phage-induced mutants that exhibited truncated core OS, other mutants strains used in more recent studies to shed light on *P. aeruginosa* core OS structures had been constructed using a directed gene inactivation approach. Such studies were conducted to determine if a certain gene was involved in LPS biosynthesis. Kooistra et al. [9] constructed *algC* mutants on PAO1 (serotype O5) and PAC1R (serotype O3) backgrounds. The *algC* gene encodes an enzyme called phosphoglucomutase that is involved in the biosynthesis of UDP-Glc, a nucleotide-activated glucose donor required for the addition of Glc residues to core OS [28]. Hence, as one would anticipate, the core OS of the *algC* mutants was lacking all the Glc residues. In the absence of the appropriate Glc acceptor, Rha cannot form linkages to the core, and therefore the Rha residue is also absent from the core OS in both *algC* mutants (Fig. 2a). However, there was a subtle difference in the core phosphorylation patterns in the PAO1 and PAC1R mutants, i.e. position 2 on Hep^{I} of the *algC* mutant of PAO1 was modified with 2-aminoethyl diphosphate, whereas in the PAC1R mutant, a single phosphate substituent was found.

A *galU* mutant was constructed on strain PA103 (serotype O11) background [29]. Similarly to *algC*, *galU*, encoding Glc-1-P uridyltransferase, is required for the metabolic steps leading to the biosynthesis of UDP-Glc. Accordingly, core OS of the *galU* mutant was also found to be truncated, and the only outer-core residue found was GalN (Fig. 2a). This is similar to the core OS structure of AK1012 and H4 strains as well as the two *algC* mutants described above. In terms of phosphate substitution in the core of the *galU* mutant, three phosphorylation sites were identified on Hep^{I}. The O2 of Hep^{I} was found to be variably substituted with phosphate, diphosphate, 2-aminoethyl diphosphate or possibly triphosphate groups.

In another study [16], a *wbjE* mutant was constructed on PA103 (serotype O11) background. The *wbjE* gene is involved in O antigen biosynthesis; more specifically, the encoded product is responsible for adding a second sugar in O11 O antigen repeat. Surprisingly, the mutant produced three core-OS structures that were, for the sake of clarity, named in this review as OS-I, OS-II, and OS-III. The first, OS-I, was a complete uncapped core that possessed GalNAc instead of GalN-Ala (as mentioned in the previous section). The second, OS-II, has a truncated structure lacking Rha, Glc^{I}, Glc^{III}; therefore the outer core consisted of disaccharide Glc^{II}–GalN that had again an N-acetyl group substituted on GalN in place of an alanyl group (Fig. 2b). The last truncated OS-III structure lacked all the Glc and Rha residues (Fig. 2a) as seen in AK1012, H4, *algC*, and *galU* mutant strains. In this study [16], the reason why inactivation of a gene involved in O antigen biosynthesis resulted in truncation of core OS has not been determined. As the authors have not elucidated the LPS structure from a complemented strain, a potential secondary mutation of genes involved in core OS biosynthesis in the *wbjE* mutant cannot be ruled out.

Three other LPS-deficient strains were constructed by producing gene knockout (a combination of insertional mutation and allelic exchange to create a chromosomal knockout) to target genes associated with LPS biosynthesis. These mutants included *wapR*, *rmlC*, and a double gene knockout of *migA-rmd* [15]. The *wapR* gene encodes a putative rhamnosyltransferase responsible for the transfer of L-Rha^{B} from its precursor dTDP-L-Rha to make it 1,3-linked to Glc^{I} in the capped core. As one would expect, the structure of the capped core glycoform of the *wapR* mutant was truncated. The outer core was composed of GalN and Glc^{II} whereas Glc^{I}, Glc^{III}, and Rha^{B} were lacking (Fig. 2c). However, the absence of the two Glc residues was unpredicted.

The rationale for producing a *migA-rmd* double knockout mutant (based on insertional disruption with an antibiotic resistance cassette and gene deletion, respectively, followed by allelic replacement of the disrupted genes into the chromosome) was to eliminate the function of both MigA (a putative rhamnosyltransferase responsible for the transfer of L-Rha^{A} to make it 1,6-linked to Glc^{II} in the uncapped core) and Rmd (associated with GDP-D-Rha biosynthesis). The removal of background D-Rha signals was important for MS analysis of core OS in this strain. Interestingly, only a complete capped core was found in LPS sample from the *migA-rmd* mutant. The inability to identify truncated uncapped core might be explained by a substrate competition between MigA and WapR enzymes that balances a relative ratio between capped and uncapped core. When MigA was not produced in the *migA* knockout mutant, expression of WapR with the bacteria likely favored the biosynthesis of capped core; as a result, the amount of uncapped core was diminished.

In the *rmlC* mutant, only one core OS glycoform lacking Rha^{A}, Rha^{B}, and Glc^{IV} was identified. The outer core OS therefore consisted of GalN, Glc^{III}, Glc^{II}, and Glc^{I} (Fig. 2d). This is consistent with the function of RmlC as dTDP-4-keto-6-deoxyglucose 3,5-epimerase that is involved in the third enzymatic step of the 4-step pathway for the biosynthesis of dTDP-L-Rha, a donor of L-Rha for the rhamnosyltransferase reaction [30]. All three products, i.e. the complete capped glycoform from

the *migA-rmd* mutant, truncated core from the *wapR* mutant, and truncated core from the *rlmC* mutant, had a non-stoichiometrically O-acetylated Glc^{II} residue.

In contrast to the highly variable O antigen, core OS of *P. aeruginosa* has a conserved backbone carbohydrate structure. The only difference in sugar composition among wild-type strains is Glc^{IV} that is present only in nine of the 20 *P. aeruginosa* IATS serotype strains. Other variable modifications include non-carbohydrate substitution, such as the degree of phosphorylation (including 2-aminoethyl (di)phosphate) and O-acetylation. However, the degree of both modifications might be underestimated because the content of these substituents might be reduced due to LPS preparation steps prior to structural analysis.

Apart from the variability seen in the complete core OS structures, truncation in the core OS is an additional source of core OS diversity. Some of these truncated structures possess an unusual modification, such as replacement of N-linked alanyl group on GalN with an acetyl group. Although a majority of the truncated core OS observed were the results of laboratory experiments and were not seen in clinical or environmental isolates, in the future, these structures may be used for a variety of biochemical or synthetic chemical assays.

This work was supported by an operating grant from the Cystic Fibrosis Canada (CFC). D. K. is a recipient of a fellowship from the CFC, and J. S. L. holds a Canada Research Chair in Cystic Fibrosis and Microbial Glycobiology.

The authors thank the following individuals from the National Research Council of Canada including Malcolm Perry, James Richards, Eleonora Altman, Evgeny Vinogradov, and Jean-Robert Brisson for their friendship over the years and their significant contributions towards elucidating the structures of core OS from the various *P. aeruginosa* mutants generated by the Lam laboratory.

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