WbjA Adds Glucose To Complete the O-Antigen Trisaccharide Repeating Unit of the Lipopolysaccharide of *Pseudomonas aeruginosa* Serogroup O11

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Lipopolysaccharide from a *wbjA* mutant, deficient in a putative glycosyltransferase from *Pseudomonas aeruginosa* serogroup O11, was compared to that from an O-antigen polymerase mutant. Results suggest that WbjA adds the terminal glucose to complete the serogroup O11 O-antigen unit and identifies the biological repeating unit as [-2)- β -D-glucose-(1-3)- α -L-N-acetylglucosamine-(1-3)- β -D-N-acetylglucosamine-(1-].

The lipopolysaccharide (LPS) of *Pseudomonas aeruginosa*, like that of most gram-negative bacteria, is composed of lipid A, an LPS core oligosaccharide, and an O antigen made up of oligosaccharide repeating units extending out from the cell surface. The O antigen is variable, being composed of di- to pentasaccharide heteropolymer repeats, and is immunodominant. On this basis, 20 immunologically distinct *P. aeruginosa* serogroups have been identified (9). The serogroup O11 O antigen is one of the simplest of these and has the chemical repeating unit structure [-3)- α -L-N-acetylglucosamine-(1-3)- β -D-N-acetylglucosamine-(1-2)- β -D-glucose-(1-]. However, this is not necessarily the biological repeating unit, since the initiating sugar in this O-antigen repeating unit was previously unknown (4).

Synthesis of *P. aeruginosa* serogroup O11 (3), O5 (2), and O6 (1) O antigens proceeds by the Wzy (O-antigen polymerase)-dependent mechanism typical of heteropolymeric O antigens. According to a current model (5), individual O-antigen repeating units are synthesized on the cytoplasmic face of the inner membrane through successive addition of sugar residues to the C₅₅-lipid carrier molecule undecaprenol phosphate (Und-PP). The inner membrane protein Wzx acts as a translocase, moving Und-PP-linked repeating units to the periplasmic face of the inner membrane, where they are polymerized into full-length O antigen by Wzy.

Mutants lacking the O-antigen polymerase are unable to combine individual O-antigen repeating units into full-length O antigen. This results in a distinctive LPS profile on silver-stained polyacrylamide gel electrophoresis (PAGE), consisting of an LPS core band and a single larger band representing LPS core containing a single complete O-antigen unit, a phenotype referred to as core + one. The overall distribution of O-antigen chain lengths is controlled by another membrane protein, Wzz. The O-antigen polymer is transferred to the lipid A-LPS core by the O-antigen ligase, WaaL, a protein encoded

outside the cluster containing the genes for O-antigen synthesis.

The O-antigen gene locus of the *P. aeruginosa* serogroup O11 strain PA103 has been characterized (3) and consists of 11 open reading frames (ORFs) (Fig. 1), including the *wzz*, *wzx*, and *wzy* genes required for Wzy-dependent O-antigen synthesis. The remaining genes encode predicted proteins, designated WbjA to WbjF, WbpL, and WbpM, which exhibit homology to polysaccharide biosynthetic proteins that presumably function in concert to synthesize individual O-antigen units (3).

WbpL has been strongly implicated as the initiating glycosyltransferase in *P. aeruginosa* O-antigen synthesis (15). WbjA and WbjE both exhibit significant homology to glycosyltransferases (3). WbjA possesses the conserved regions characteristic of several glycosyltransferase proteins and two conserved aspartate residues within the N-terminal portion of the protein identified as important to β -glycosyltransferase function (18). One of the proteins of this group, ExoM from *Sinorhizobium meliloti*, has been shown to be a β 1-4 glycosyltransferase (10).

In many O-antigen biosynthesis loci, transferase genes are organized in reverse order with respect to the corresponding O-antigen unit, so that the most upstream gene encodes the glycosyltransferase that adds the last sugar to complete the O-antigen unit (14, 19). This suggests that WbjA may transfer

FIG. 1. Organization of the *P. aeruginosa* O-antigen biosynthetic genes of the serogroup O11 strain PA103, showing the site of insertion of the gentamicin resistance determinant (*aacCI*) within the *wbjA* gene. Complementing subclone pCD201, containing an approximately 2-kb *XhoI*-*PstI* fragment from pLPS2 (6) in pUCP18 (20), encompassing *wbjA*, is shown below. Restriction enzyme sites: P, *PstI*; Sn, *SnaBI*; and X, *XhoI*.

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FIG. 2. Analysis of LPS isolated from *P. aeruginosa* PA103 and derivatives. LPS, isolated as previously described (3), was separated on 10% NuPAGE bis-Tris polyacrylamide gels (Novex, Los Angeles, Calif.) and visualized by (A) silver staining, (B) Western immunoblot using serogroup O11-specific polyclonal antiserum (13), and (C) Western immunoblot using serogroup O11-specific monoclonal antibody (Rougier Bio-Tech Ltd., Montreal, Quebec, Canada). Lane 1, PA103; lane 2, PA103 *wzy::aacCI*; lane 3, PA103 *wbjA::aacCI*; and lane 4, PA103 *wbjA::aacCI*(pCD201).

the final sugar to complete synthesis of the serogroup O11 O-antigen unit.

To determine the role of *wbjA* in O-antigen synthesis, a nonpolar insertion in PA103 was constructed. Briefly, an approximately 650-bp *ScaI* fragment containing part of *wbjA* was cloned into *SmaI*-digested pEX100T (22). A gentamicin resistance determinant (*aacCI*), recovered as a *SmaI* fragment from plasmid pUCGM (21), was then ligated into the unique *SnaBI* site within *wbjA* in the same orientation as *wbjA*. This plasmid was introduced into PA103 (11) as previously described (3). Gene replacement was confirmed by PCR amplification of chromosomal DNA from PA103 and PA103 *wbjA::aacCI* using primers flanking the *aacCI* insertion site in *wbjA*. The PCR product from PA103 *wbjA::aacCI* was larger than that generated from wild-type chromosomal DNA by approximately 850 bp, consistent with the size of the *aacCI* gene as estimated by agarose gel electrophoresis.

Compared to LPS from the wild-type strain PA103 (Fig. 2A and 2B, lane 1), PA103 *wbjA::aacCI* is devoid of full-length O antigen (Fig. 2A and 2B, lane 3), establishing a role for WbjA in serogroup O11 O-antigen synthesis. When plasmid pCD201, containing the entire *wbjA* gene (Fig. 1), was transferred to PA103 *wbjA::aacCI*, complementation of the LPS defect was observed (Fig. 2A and 2B, lane 4) confirming that the mutation was nonpolar and that the mutant phenotype resulted solely from the loss of WbjA.

Interestingly, although PA103 *wbjA::aacCI* lacked full-length O antigen, attached to the LPS core appeared to be a single O-antigen repeating unit. The band for PA103 *wbjA::aacCI* (Fig. 2A, lane 3) was observed to migrate slower than the complete core LPS (the lowest band in all the lanes of Fig. 2A) but somewhat faster than the LPS of the PA103 *wzy* (O-antigen polymerase mutant) (Fig. 2A, lane 2), previously found to contain one complete trisaccharide repeating unit attached to the core (3). The migration rate of the *wbjA::aacCI* band thus suggests that it contains an incomplete O-antigen

FIG. 3. MALDI-TOF spectra for fraction II from PA103 *wbjA::aacCI* (top) and from PA103 *wzy::aacCI* (bottom). These spectra were collected in the positive mode.

unit, most likely a disaccharide, attached to the core, and this phenotype was therefore designated core + 2/3. The fact that PA103 *wbjA::aacCI* was not making any detectable higher-molecular-weight forms of the O antigen (Fig. 2B, lane 3) indicates that truncated O-antigen subunits are not recognized as substrates for O-antigen polymerase.

Further supporting the incomplete nature of the core + 2/3 LPS, a serogroup O11-specific monoclonal antibody which preferentially recognizes forms of LPS containing from one to five O repeating units (3) reacted with the core + one band produced by PA103 *wzy::aacCI* (Fig. 2C, lane 2), but failed to react with the core + 2/3 band from PA103 *wbjA::aacCI* (Fig. 2C, lane 3). This suggests that the missing sugar residue of the O-antigen is important for recognition by the monoclonal antibody.

Many *P. aeruginosa* strains produce common antigen or A-band LPS (8). Since most serogroup-specific O-antigen genes, including *wbjA*, are not distributed among the various serogroup strains, it is unlikely that they would participate in synthesis of the conserved common antigen. As expected, and similar to what was previously observed in PA103 *wzy::aacCI* (3), PA103 *wbjA::aacCI* produced normal levels of common antigen (data not shown).

The isolation of core + 2/3 LPS from the PA103 *wbjA::aacCI* mutant is intriguing in that it indicates that the incomplete O-antigen repeating unit can be recognized and translocated to the periplasm by Wzx. Naide et al. first reported the transfer of unpolymerized O-antigen units to the LPS core in *Salmonella* (12). Later, it was proposed that O-antigen units must be completed prior to transport (23). However, the isolation of the LPS core substituted with a truncated O-antigen subunit has been reported in *Escherichia coli* (7) and in *Yersinia enterocolitica* mutated in the O-antigen glycosyltransferase gene *wbcG* (25). Feldman et al. (5) also showed stepwise reconstruction of O repeating unit synthesis by addition of subsets of O-antigen genes in a model *E. coli* strain.

TABLE 1. MS data and proposed compositions of the *P. aeruginosa* OSs in fractions II and III after aqueous HF treatment^a

Experimental ion	Calculated ion	Proposed composition	Bio-Gel P2 fraction
2,246.7 [M + NH ₄] ⁺	2,247.0	Cb ₁ , Ala ₁ , FucNAc ₂ , Glc ₅ , Rha ₁ , GalN ₁ , Hep ₂ , Kdo ₁	II, <i>wzy</i> mutant
2,085.4 [M + NH ₄] ⁺	2,085.0	Cb ₁ , Ala ₁ , FucNAc ₂ , Glc ₄ , Rha ₁ , GalN ₁ , Hep ₂ , Kdo ₁	II, <i>wbjA</i> mutant
1,511.7 [M - H] ⁻	1,511.4	Cb ₁ , Ala ₁ , Glc ₃ , Rha ₁ , GalN ₁ , Hep ₂ , Kdo _{anhydro}	III, <i>wzy</i> and <i>wbjA</i> mutants
1,529.0 [M - H] ⁻	1,529.4	Cb ₁ , Ala ₁ , Glc ₃ , Rha ₁ , GalN ₁ , Hep ₂ , Kdo	
1,365.0 [M - H] ⁻	1,365.3	Cb ₁ , Ala ₁ , Glc ₃ , GalN ₁ , Hep ₂ , Kdo _{anhydro}	III, <i>wzy</i> and <i>wbjA</i> mutants
1,383.0 [M - H] ⁻	1,383.3	Cb ₁ , Ala ₁ , Glc ₃ , Rha ₁ , GalN ₁ , Hep ₂ , Kdo	

^a Cb, carbamoyl; Ala, alanine; FucNAc, *N*-acetylfucosamine; Glc, glucose; Rha, rhamnose; GalN, galactosamine; Hep, heptose; and Kdo, 3-keto-D-manno-2-octulosonic acid. Fraction II was analyzed in the positive mode, while fraction III was analyzed in the negative mode.

This allowed the isolation of an LPS core substituted with as little as a single sugar residue.

The LPS from PA103 *wbjA::aacC1* and PA103 *wzy::aacC1* was isolated from the water layer after hot phenol-water extraction (16). The purified LPS was hydrolyzed in aqueous 1% acetic acid for 2 h at 100°C. The hydrolysate was centrifuged at 10,000 × *g* for 20 min, and the supernatant was collected. The pellet was washed once with water and centrifuged again. The water wash was added to the supernatant, and the total aqueous phase, containing the oligosaccharides (OSs), was lyophilized. The lyophilized OSs were dissolved in water, applied to a Bio-Gel P-2 column (70 by 1.6 cm), and eluted with water containing 1% 1-butanol. Fractions were assayed for carbohydrate by phenol-sulfuric acid assay. Fractions representing the OS peaks were pooled and lyophilized.

For both mutants, Bio-Gel P2 gel filtration chromatography of the carbohydrates released from the LPS by mild acid hydrolysis resulted in four fractions, I to IV. Fraction IV from both samples did not contain sufficient levels of material for analysis. Each OS fraction was then treated with cold aqueous 48% hydrogen fluoride (HF) and kept for 48 h at 4°C. The HF was removed by flushing under a stream of air, followed by the addition of diethyl ether (600 ml) and drying with a stream of air. This step was repeated three times, and the resulting residue was dissolved in deionized water at 4°C and lyophilized. The resulting OSs were analyzed for their glycosyl components and by mass spectrometry (MS).

The glycosyl composition of each sample was determined by the preparation and combined gas-liquid chromatography-MS

analysis of partially methylated alditol acetates and of trimethylsilyl methyl glycosides, as previously described (24). The fraction I samples from both mutants contained only rhamnose (Rha) and glucose (Glc). The various OS fractions from both mutants were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS using a Hewlett Packard LD-TOF system. The samples were dissolved in distilled water at a final concentration of 2 µg/µl, and 1 µl was mixed with the dihydroxy benzoic acid in methanol matrix for analysis.

Analysis of fraction I by MALDI-TOF MS showed that it consisted primarily of a size-heterogeneous polysaccharide up to *m/z* = 2,700. Numerous molecular ions were present between *m/z* 1,000 and 2,700, all of which differed from the others by 146 mass units, the size of a single Rha residue. Thus, fraction I is largely a rhamnan polysaccharide. These data confirm that both mutants make the common antigen (A-band LPS) and, therefore, that neither *WbjA* or *Wzy* is affected in the synthesis of this polysaccharide. The glucose in this fraction may be due to some other type of glucan, since the MALDI-TOF MS spectrum clearly shows that it is not part of the rhamnan (data not shown).

The spectra for both fraction II samples are shown in Fig. 3, and the molecular ions observed for fractions II and III and proposed compositions are given in Table 1. Fractions II and III from both mutants contained Rha, *N*-acetylfucosamine (FucNAc), Glc, heptose (Hep), and galactosamine (GalN), consistent with the components of the LPS core and the O antigen. Fraction III samples from both samples contained

FIG. 4. Proposed structures for the LPS oligosaccharides from PA103 *wbjA::aacC1* and PA103 *wzy::aacC1*. These structures are based on the *P. aeruginosa* serogroup O5 LPS core structure recently reported by Sadovskaya et al. (17).

Rha, Glc, Hep, and GalN, corresponding to the LPS core sugars, and only trace levels of FucNAc, which presumably came from slight contamination with fraction II.

These proposed compositions are consistent with the observed glycosyl components, the masses of the OSs, and reported structures for *P. aeruginosa* LPSs (4, 17). The MS results show that fraction II from PA103 *wzy::aacC1* was larger in mass than fraction II from PA103 *wbjA::aacC1* by 162 mass units. This difference corresponds to the mass of one additional hexose residue, and since the only unmodified or simple hexose observed in the sugar component analysis was glucose (see above), this additional hexose must be a Glc residue.

The exact LPS core structure of *P. aeruginosa* PA103 has not been determined. However, both composition and MS analyses of PA103 *wbjA::aacC1* and PA103 *wzy::aacC1* are consistent with the recently reported *P. aeruginosa* serogroup O5 LPS core structure (17). Based on that structure, the proposed structures for the oligosaccharides present on the LPSs from both PA103 *wbjA::aacC1* and PA103 *wzy::aacC1* are shown in Fig. 4. The structural data for these two mutants supports the conclusion that PA103 *wbjA::aacC1* has only a FucNAc-FucNAc disaccharide attached to the lipid A-LPS core region rather than the complete Glc-FucNAc-FucNAc O-antigen unit.

In conclusion, we have identified WbjA as the enzyme responsible for the transfer of glucose to complete the *P. aeruginosa* serogroup O11 LPS O-antigen subunit, thus defining the biological repeating unit as $[-2)\text{-}\beta\text{-D-glucose-(1-3)-}\alpha\text{-L-N-acetylglucosamine-(1-3)-}\beta\text{-D-N-acetylglucosamine-(1)]$. Accordingly, the order of occurrence of *wbjA*, *wbjE*, and *wbpL* within the O-antigen locus (Fig. 1) is the reverse of assembly of the O-antigen unit, consistent with models for O-antigen assembly in other organisms (14, 19).

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