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Characterization of the lipopolysaccharide from a *wbjE* mutant of the serogroup O11 *Pseudomonas aeruginosa* strain, PA103

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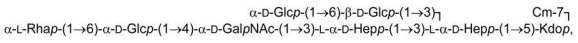
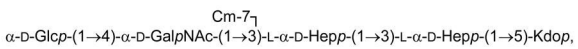
Abstract

The lipopolyaccharide (LPS) of a *wbjE* mutant of *Pseudomonas aeruginosa* PA103, a serogroup O11 strain consists of both high and low molecular weight (HMW and LMW) LPSs. The HMW LPS consisted exclusively of rhamnan A-band LPS however no B-band LPS was detected in the *wbjE* mutant. Interestingly, the LMW LPS from the *wbjE* mutant showed that it contained a variety of oligosaccharides, each with two or three phosphate groups present as mono- or pyrophosphates.

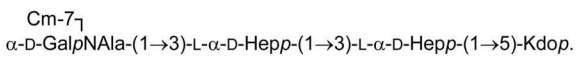
These oligosaccharides consisted of the complete core octasaccharide,



a pentasaccharide,



and a tetrasaccharide,



The GalN residue was present as an *N*-acetylated residue in all of these oligosaccharides except the tetrasaccharide in which it is present as an *N*-alanylated residue. None of these oligosaccharides contained either a D- or L-Fuc*p*NAc residue. These results are discussed with regard to the role of WbjE in the biosynthesis of *P. aeruginosa* PA103 B-band LPS.

Keywords

Pseudomonas aeruginosa; Lipopolysaccharide; Inner core; *wbjE*; Biosynthesis

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen most frequently isolated from chronically infected cystic fibrosis patients and nosocomial infection. It is a major threat to immune-compromised patients. It is also the most common pathogen found in ulcerative keratitis in individuals with extended use of contact lens. This organism is highly adaptable for survival in a wide range of environments.

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As with other Gram-negative bacteria, the lipopolysaccharide (LPS) of *P. aeruginosa* is a major virulence factor and is also reported to be the most immunogenic among the various *P. aeruginosa* cell surface antigens.[1] The LPS from different serogroups of *P. aeruginosa* possess the same general architecture as those from the family *Enterobacteriaceae* being composed of lipid A, a core oligosaccharide, and serologically distinct O-antigenic polysaccharides. The core oligosaccharide is further divided into a relatively conserved inner core and a distal and slightly variable outer core region as was shown by several monoclonal antibody binding assays.[2] *P. aeruginosa* strains produce two antigenically and chemically distinct LPS molecules: namely A-band and B-band LPS. The A-band LPS is a neutral polysaccharide consisting of one α-(1→2), and two α-(1→3) linked D-rhamnopyranosyl units, which is antigenically conserved and known as the common antigen.[3] In contrast, B-band LPSs possess O-antigen polysaccharides that consist of various hetero-oligosaccharide repeating units.[4] The diverse chemical nature of the B-band O-antigen polysaccharides is the basis for the serotype classification of this organism. According to the International Antigen Typing Scheme (IATS) and Lanyi-Bergan classification, all known *P. aeruginosa* strains have been placed into 30 different serotypes.[4]

The synthesis of B-band LPS generally proceeds by the "Wzy-dependent" mechanism.[5,6] This mechanism involves the construction of the repeat unit on the cytoplasmic side of the cell membrane through the transfer of glycosyl residues from activated dinucleotide sugars to undecaprenyl phosphate (Und-P).[6,7] In the case of the serogroup O11 strain PA103, WbpL first catalyzes the transfer of β-D-Fuc*p*NAc to Und-PP,[5] followed by the transfer of α-L-Fuc*p*NAc, and finally β-D-Glc*p* is added by WbjA.[8] The resulting β-D-Glc*p*-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Fuc*p*NAc-1→PP-Und is presumably transferred to the periplasmic side of the cell membrane via Wzx, polymerized by the action of Wzy, and then ligated to the lipid A core oligosaccharide by WaaL.[6,7] We have previously shown that a *wzy* mutant that cannot polymerize the trisaccharide repeat unit is able, likely via WaaL, to ligate the single trisaccharide repeat unit to the core oligosaccharide[5,8] resulting in a low molecular weight (LMW) LPS that is referred to as a "core + 1" LPS. Furthermore, we showed that a *wbjA* mutant is unable to add the β-D-Glc*p* residue to the repeat unit and the result is a LMW LPS where theα-L-Fuc*p*NAc-(1→3)- β-D-Fuc*p*NAc-1→ disaccharide is added to the core; i.e., "core + 2/3".[8] This suggests that the WaaL protein transfers individual and partial repeat units in addition to the B-band polysaccharide to the core. A recent report showed that a *wbjE* mutant added only a single β-D-Fuc*p*NAc to the pilin.[9] Because *P. aeruginosa* glycosylates its pilin by transferring the B-band repeat unit from Und-PP to pilin via the action of PilO,[10–12] this result supports the conclusion that *wbjE* encodes for α-L-Fuc*p*NAc transferase; i.e., the *wbjE* mutant cannot add the α-L-Fuc*p*NAc residue during synthesis of the B-band repeat unit and the result is β-D-Fuc*p*NAc-1→PP-Und.

In this report we characterize the structure of the LPS obtained from the PA103 *wbjE* mutant. Because our previous results showed that a *wzy* mutant and a *wbjA* mutant produced LMW LPS that contained "core + 1" and "core + 2/3" oligosaccharides, respectively, and because the *wbjE* mutant produced a pilin which contained only β-D-Fuc*p*NAc monosaccharide additions, we anticipated that the *wbjE* mutant would produce a LMW LPS that contain a single β-D-Fuc*p*NAc residue added to the core oligosaccharide; i.e., "core + 1/3". Instead, the *wbjE* LPS consisted of LMW LPS that did not contain any detectable Fuc*p*NAc and consisted of a complete core, as well as truncated core oligosaccharides. The implications of these results with regard to LPS synthesis in *P. aeruginosa* PA103 are discussed.

2. Results

2.1. The LPS from *Pseudomonas aeruginosa* wbjE consists of A-band high molecular weight LPS and low molecular weight LPS

The LPS from *P. aeruginosa wbjE* was compared with that from the parent strain, PA103, by DOC-PAGE analysis. The results are shown in lanes 1 and 2 of Fig. 1. The PA103 LPS (lane 1) contains the typical LPS banding pattern for a mixture of A-band and B-band LPSs while the *wbjE* LPS (lane 2) consisted of a high molecular weight (HMW) LPS consistent with A-band LPS, and a low molecular weight (LMW) LPS that migrated at a slightly faster mobility than the LMW LPS band from the PA103 LPS. Notably missing in the *wbjE* LPS were bands that corresponded to the B-band LPS. That the HMW LPS from *wbjE* consisted of A-band LPS was confirmed by the positive reaction with anti-A-band MAb in a Western blot analysis (data not shown).

The *wbjE* HMW and LMW LPS were separated from one another using gel permeation chromatography (GPC) in the presence of deoxycholate (DOC) as described in Experimental section. Lanes 3 and 4 of Fig. 1 show the DOC-PAGE profiles of the separated HMW and LMW LPS, respectively, from the *wbjE* mutant. Composition analysis of the HMW LPS showed that rhamnose (Rha) was the major glycosyl residue, followed by lesser amounts of glucose (Glc), mannose (Man), *N*-acetylgalactosamine (GalNAc), and *N*-acetylglucosamine (GlcNAc), with trace amounts of N-acetylfucosamine (FucNAc) and heptose (Hep). The large amount of Rha found in this HMW LPS is consistent with it being A-band LPS. Composition analysis also showed detectable amounts of 3-hydroxylauric acid, 12:0(3-OH), consistent with the presence of lipid A. The LMW LPS was composed primarily of Glc, GalNAc, *N*-alanylgalactosamine (GalNAla), 3-keto-D-*manno*-oct-2-ulosonic acid (Kdo), with smaller amounts of Rha and Hep. In addition, fatty acids indicative of lipid A were also observed; namely, 10:0(3-OH), 2 and 12:0(3-OH), and 12:0.

2.2. Structural analysis of *P. aeruginosa* wbjE HMW LPS

The polysaccharide (PS) released by mild acid hydrolysis of the *wbjE* HMW LPS was purified by GPC using Bio-Gel P10 as described in the Experimental section. A major polysaccharide (PS) peak was obtained, HMW PS, which eluted just after the void volume and a very small amount of an oligosaccharide that eluted near the column bed volume. Composition analysis of the PS fraction showed that it contained a 7:2:1 molar ratio of Rha:Man:Glc, and also small amounts of GalNAc, GlcNAc, and FucNAc. Glycosyl linkage analysis of HMW-PS showed the presence of 2- and 3-linked Rha*p* as major residues, which is consistent with the A-band D-rhamnan polysaccharide. Additionally, the HMW PS contained lesser amounts of terminally and 4-linked Man*p*, small amounts of 3- and 4-linked Fuc*p*NAc, and trace amounts of 3- and 4-linked Gal*p*NAc and terminally linked Glc*p*.

The ^{1}H NMR spectrum of the HMW-PS (Fig. 2) is consistent with that reported for the A-band polysaccharide structure;[17] it shows three anomeric signals at δ5.15, 4.97, and 4.90 ppm due to the three Rha*p* residues of the A-band polysaccharide. The Rha*p* 6-deoxy methyl protons were also observed at δ1.26 and 1.22 ppm. In addition, MALDI-TOF MS analysis (data not shown) gives a spectrum showing a series of ions up to *m/z* of about 3000 in which each ion differs from the next by an *m/z* of 146, which is consistent with a single Rha*p* residue difference. In summary, these results are consistent with the HMW LPS from *wbjE* being primarily A-band LPS.

We do not know the source of the minor glycosyl components. The 4-linked Fuc*p*NAc and Man*p* residues are not known components of either A- or B-band LPS, and their presence requires further investigation. There is a 3-linked Fuc*p*NAc residue in the B-band LPS

polysaccharide, however, there is no evidence of the presence of B-band LPS in the *wbjE* mutant; i.e., the LPS does not bind B-band antibody. The Glc*p* and Gal*p*NAc residues could be due to glycosyl residues found in the core oligosaccharide to which the A-band polysaccharide is attached.

2.3. Structural analysis of *P. aeruginosa* wbjE LMW LPS

The *wbjE* LMW LPS was treated with mild acid hydrolysis and the released oligosaccharide (OS) was purified by GPC using Bio-Gel P2. One major component, LMW OS, eluted near the void volume and a minor component eluted at the bed volume. The LMW OS consisted of Rha, Glc, GalNAc, GalNAla, Kdo, and Hep which are known components of the core oligosaccharide portion of this LPS. The minor component consisted of exclusively Kdo and was likely due to the liberation of the branching Kdo residue from the LPS during mild acid hydrolysis.

A ^{1}H NMR spectrum of the LMW OS is shown in Fig. 3. The results indicate that the LMW OS preparation is a heterogeneous mixture of structures as evidenced by the complexity of the resonances in the anomeric region of the spectrum, i.e., between δ 4.5–5.5 ppm, and by overlapping resonances between δ 4.2 and 4.6 ppm that are likely due to heptosyl ring protons at positions of phosphorylation. This spectrum also showed resonances due to both *N*-acetyl and *N*-alanyl components, δ 2.0 and δ 1.52 ppm, respectively, indicating that the LMW OS preparation contained some structures that have a Gal*p*NAc residue and others that have a Gal*p*NAla residue. Resonances were also observed due to the Rha*p* H6 protons, δ 1.2 ppm, as well as the methylene protons of Kdo*p*, δ 1.86 and 2.16 ppm. Notably absent in this spectrum was a resonance at δ 3.2 ppm, which would indicate a phosphoethanolamine (PEA) substituent as was observed for the core oligosaccharide from the *galU* mutant.[18]

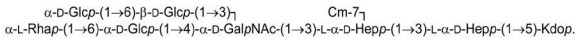
The presence of phosphate in the LMW OS preparation was confirmed by ^{31}P NMR analysis (Fig. 4). The resonances at δ 2.0 to 0.0 ppm are due to monoester phosphate groups and appear to be the major phosphate components of this preparation. The resonances at δ−11.0 ppm are due to pyrophosphate groups. Thus, the ^{31}P and ^{1}H NMR spectra of the LMW OS show that it consists of structures that have phosphate and pyrophosphate groups but is devoid of PEA substituents.

The heterogeneity of the LMW OS was further confirmed by MALDI-TOF MS analysis (Fig. 5 and Table 1). This mixture consists of variously truncated core oligosaccharides. The ion at *m/z* 1136.3 is consistent with a tetrasaccharide that we also identified in the *galU* mutant[18] that consists of Gal*p*NAla, Hep*p*, and Kdo*p*, and has three phosphate groups. One other structure, at *m/z* 1056.3, differs from this in that it has two rather than three phosphate groups. These two ions at *m/z* 1056.3 and 1136.3 are the only two structures that have Gal*p*NAla. All of the remaining structures have Gal*p*NAc, and the most intense of these ions is m/z 1269.3 which is consistent with a pentasaccharide in which a Glc*p* residue has been added to the tetrasaccharide. The ions at *m/z* 1431.4, and 1593.4 are due to structures which have two and three Glc*p* residues, respectively, while that at *m/z* 1739.4 is due to a structure which has added three Glc*p* residues and one Rha*p* residue. All of these ions are consistent with structures that have a carbamoylated Hep*p* residue. These results are consistent with the *wbjE* mutant LMW-LPS having variously truncated core oligosaccharides and one possibly complete core oligosaccharide (i.e., the structure at *m/z* 1739.4). Unlike the core structure for the *P. aeruginosa* serogroup O11 LPS,[8,19] and unlike the truncated core oligosaccharide from the *galU* mutant,[18] the data indicate that the majority of the *wbjE* structures contain Gal*p*NAc rather than Gal*p*NAla.

The LMW-OS was further characterized by treating it with aqueous HF and separating the various oligosaccharides using Bio-Gel P2 GPC. After HF treatment, four oligosaccharide

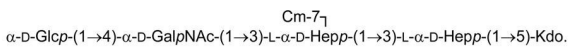
fractions were obtained; HFOS1-HFOS4. Fraction HFOS1 was very minor and was not structurally analyzed. The remaining fractions were analyzed by glycosyl composition, linkage, MS, and NMR analyses.

Glycosyl composition and linkage analysis shows that HFOS2 contains terminally linked Rha*p*, terminally linked Glc*p*, 6-linked Glc*p*, 3-linked Hep*p*, and 3,4-linked Gal*p*NAc. ^{1}H NMR and ^{1}H–^{13}C HSQC analyses, Fig. 6, showed that HFOS2 contained five α-glycosyl residues, one β-glycosyl residue, and a Kdo*p* residue. One of the glycosyl residues had a C2/H2 cross peak at δ 50.5/4.55 ppm, which is consistent with the presence of Gal*p*NAc. Also the cross peak at about δ20/2.10 is due to the methyl protons of an *N*-acetyl, not an *N*-alanyl group. The H6/C6 of Rha*p* was also present, however, the lower intensity of the H6 resonance in comparison to the anomeric resonances indicate that the HFOS2 fraction also contains a structure without Rha. These results are consistent with the published structure of *P. aeruginosa* serogroup 011 core oligosaccharide,[8,19]



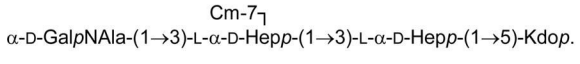
The MALDI-TOF MS analysis of the HFOS2 fraction gave results consistent with this structure showing a major ion of *m/z* 1546, which corresponds to the disodiated, $[MNa + Na]^{+}$, octasaccharide, and a minor ion due to a heptasaccharide that lacks a deoxyhexosyl residue (i.e., Rha*p*).

Glycosyl composition and linkage analysis of HFOS3 showed the presence of terminally linked Glc*p*, 4-linked Gal*p*NAc, and 3-linked Hep*p*. ^{1}H NMR and ^{1}H–^{13}C HSQC analyses, Fig. 7, indicated that HFOS3 is a pentasaccharide consisting of four α-linked glycosyl residues in addition to the Kdo*p* residue. Resonances due to the β-linked Glc*p* residue and to α-Rha*p* were absent indicating that this oligosaccharide lacked the glucosyl branching disaccharide attached to O3 of the Gal*p*NAc residue as well as the terminal Rha*p* residue. These results are consistent with HFOS3 having the structure,



The MALDI-TOF MS analysis of the HFOS3 fraction gave results consistent with this pentasaccharide, having a major ion at *m/z* 1076, $[MNa + Na]^{+}$.

The ^{1}H and ^{1}H–^{13}C HSQC analyses of HFOS4, Fig. 8, indicate that it is a tetrasaccharide consisting of three α-linked glycosyl residues in addition to the Kdo*p* residue, and that it has a Gal*p*NAla instead of a Gal*p*NAc residue. The data are consistent with the data published structure for the truncated core oligosaccharide of from the *galU* mutant,[18]



The MALDI-TOF MS analysis also showed results consistent with this structure; i.e., a major ion at *m/z* 920 was observed, $[M + Na]^{+}$.

3.0 Discussion

Our results support the following conclusions with regard to the LPS from a *wbjE* mutant of *P. aeruginosa* PA103: [i.] The *wbjE* LPS consists of a HMW A-band LPS and a LMW LPS consisting of complete and variously truncated core oligosaccharides. [ii.] This mutant does not produce B-band LPS. [iii.] The mixture of LMW-LPS core oligosaccharides range from the complete core octasaccharide to a tetrasaccharide that lacks the Glc*p* and Rha*p* residues

and is also found in a *galU* mutant.[18] [iv.] The core oligosaccharides lack PEA substituents and contain phosphate and pyrophosphate groups. [v.] The most truncated tetrasaccharide oligosaccharide contains Gal*p*NAla while all other oligosaccharides contain Gal*p*NAc residues.

We have characterized the structures of the LPS from three *P. aeruginosa* PA103 mutants: *wbjA*,[8] *galU*,[18] and in this report, *wbjE*. These structures are shown in Fig. 9. The *wbjA* gene was proposed to encode for the glucosyl transferase required to incorporate the Glc*p* residue into the B-band trisaccharide repeat unit. Consistent with this proposal, we observed that the LPS from the *wbjA* mutant lacked B-band LPS, produced A-band LPS, and LMW LPS that contained a complete core oligosaccharide and second oligosaccharide that consisted of an α-L-Fuc*p*NAc-(1→3)- β-D-Fuc*p*NAc disaccharide attached to the core oligosaccharide, i.e., core + 2/3.[8] The *galU* mutant is defective in the synthesis of UDP-Glc*p* and, therefore, can not add Glc*p* to the core oligosaccharide. The result is a truncated core that is highly substituted with phosphate and PEA groups and does not contain either B-band or A-band polysaccharide, presumably due to the fact that the truncated core is unable to act as an acceptor for the ligation of these polysaccharides from the bactoprenyl (i.e., undecaprenyl phosphate, Und-P) carrier. In fact, the *galU* mutant appears to produce an A-band polysaccharide that is still attached to the bactoprenyl carrier[18] It has been reported that *wbjE* encodes for the α-L-Fuc*p*NAc transferase during the synthesis of the B-band repeating unit.[9] The ability of the *P. aeruginosa* PA103 *wzy* and *wbjA* mutant to add one repeating unit or 2/3 of a repeat unit, respectively, to the core oligosaccharide[8] would suggest, that the LMW LPS from the *wbjE* mutant should have a a single terminally linked D-FucNAc residue linked to the complete core; i.e., core + 1/3. However, we were unable to detect such a structure from the *wbjE* mutant.

It is known that *P. aeruginosa* glycosylates its pilin with the repeating unit of B-band LPS via the action of PilO using the B-band repeat oligosaccharide-PP-Und as the substrate.[10–12] Horzempa et al.[9] have shown that the *wbjE* mutant of PA103 is able to add a single D-Fuc*p*NAc residue to pilin. Thus, the Horzempa et al. report together with our results indicate that the *wbjE* mutant is able to add this residue to the pilin but is unable to add D-Fuc*p*NAc to the core oligosaccharide. A possible explanation is depicted in Fig. 10 which shows the synthetic scheme for the B-band LPS as well as for the glycosylated pilin for *P. aeruginosa* PA103. In that scheme, the Glc*p*-L-Fuc*p*NAc-D-Fuc*p*NAc-PP-Und, which is the product after the WbjA step, can be used for either B-band LPS synthesis or this trisaccharide can be added to pilin via PilO. B-band LPS synthesis requires Wzx, which flips the glycan-PP-Und across the cytoplasmic membrane followed by polymerization (Wzy) and ligation to the lipid A core oligosaccharide (WaaL). The current evidence would support the hypothesis that that PilO is able to transfer the oligosaccharides from D-Glc*p*NAc-L-Fuc*p*NAc-D-Fuc*p*NAc-PP-Und, L-Fuc*p*NAc-D-Fuc*p*NAc-PP-Und, and D-Fuc*p*NAc-PP-Und, respectively to pilin, while Wzx or WaaL are unable to utilize D-Fuc*p*NAc-PP-Und as a substrate but are able to use L-Fuc*p*NAc-D-Fuc*p*NAc-PP-Und, and D-Glc*p*NAc-L-Fuc*p*NAc-D-Fuc*p*NAc-PP-Und.

A number of additional results in this report require further investigation. We expected that the *wbjE* mutant would produce A-band LPS as we observed. However, our data show that the isolated A-band polysaccharide contains, in addition to the expected D-rhamnan attached to the core, Fuc*p*NAc, Glc*p*NAc, and Man*p* residues. The literature currently support that WbpL is a bifunctional enzyme which can transfer D-Fuc*p*NAc to the bactoprenyl phosphate carrier in the first step toward B-band synthesis and it can also transfer D-Glc*p*NAc to bactoprenyl phosphate as the first step in A-band polysaccharide synthesis.[20,21] Thus, if the rhamnan is synthesized by the addition of Rha*p* residues to Glc*p*NAc, then the presence of small amounts of Glc*p*NAc in the HMW LPS from the *wbjE* mutant is not surprising. However, this does not explain observation of low levels of 3- and 4-linked Fuc*p*NAc as well as terminally and 4-linked Man*p*. We are currently investigating whether or not these residues are, in fact, part of

the rhamnan polysaccharide, or if they are due to the presence of one or more other polysaccharides.

Another surprising result was that the *wbjE* produces a variety of truncated core oligosaccharides. This was not the case for the *wbjA* mutant, which produced two oligosaccharides: the complete core oligosaccharide, and a complete core with the added α-L-Fuc*p*NAc-β-D-Fuc*p*NAc disaccharide.[8] In addition, the various core oligosaccharides in the *wbjE* mutant were highly phosphorylated as found for the truncated core in the *galU* mutant,[18] although none contained a PEA group, and the least truncated oligosaccharides contained a Gal*p*NAc residue rather than a Gal*p*NAla residue. In our prior work with the *wbjA* and *galU* mutants, only Gal*p*NAla was observed in the core. In a previous report by Walsh et al.[22] it was shown that phosphorylation of the core Hep*p* residues are required for viability and drug resistance in *P. aeruginosa.* This may be especially true for mutants that are deficient in either B- or A-band polysaccharide and, therefore, accounts for the high level of phosphorylation in the *galU* and *wbjE* mutant LPSs. The mechanism by which the *wbjE* mutant produces highly phosphorylated, complete and truncated core oligosaccharides that contain GalNAc rather than GalNAla requires further investigation.

4. Experimental

4.1. Isolation and purification of LPS

LPS from the *P. aeruginosa wbjE* mutant[9] was extracted by using hot phenol water method.[13] Briefly, 5.0 g of dried cells were ground into a powder and suspended in 90 mL of water and pre-heated to 68 °C followed by addition of 90 mL of pre-heated 90% aqueous phenol. The reaction mixture was then stirred at 68 °C for 30 min and immediately cooled to 7 °C in an ice-water bath. The reaction mixture was centrifuged at about 10,000 ×g at 7 °C for 40 min. Centrifugation separated the mixture in three layers; the top aqueous layer (opaque), a middle phenol layer (dark brown), and precipitated cell debris. The middle phenol layer and cell debris were combined, heated to 68 °C and re-extracted two more times with addition of 80 mL of pre-heated water each time and centrifuged as described above. The pooled aqueous layers and the pooled middle layers were each dialyzed (1000 MWCO regenerated cellulose dialysis tubing) against several changes of deionized water for a week until the odor of phenol was removed from the solutions and each layer was concentrated and lyophilized. As a first purification step, the LPS preparations were centrifuged to remove insoluble debris. Each supernatant was treated with DNase, RNase and Proteinase-K to digest contaminating proteins and nucleic acids. After dialysis the LPS was sedimented by ultracentrifugation a 100,000 × g for 4 h. Finally all LPS samples were fractionated by gel permeation chromatography (GPC) using Sephacryl S-200 column. The elution buffer consisted of Tris-HCl buffer at pH 9.2 containing 0.25% of deoxycholic acid (DOC) and the eluent was monitored by an on-line refractive index (RI) detector. RI positive fractions were further analyzed by DOC-PAGE and appropriate fractions were pooled, dialyzed extensively against a solution of 50 mM Tris base with 10% ethanol, then against deionized water, concentrated and lyophilized. The yield of purified HMW and LMW LPSs are 18 mg and 120 mg respectively from 5g of dry cells.

4.2. DOC-PAGE analysis

LPS fractions from the *wbjE* mutant were each suspended in water to a concentration of 10 μg/μL and analyzed using a 18% DOC-polyacrylamide gel electrophoresis (PAGE). The polyacrylamide gels were stained using the silver staining and Alcian blue-silver staining procedures as previously described.[14] Approximately 5 and 10μg of each LPS sample was loaded on two separate gels and electrophoresis was performed at constant current mode of 30 mA. The fixing solutions were ethanol: acetic acid: water (40:5:55) and same solution

containing 0.005% of Alcian blue for silver staining and Alcian blue-silver staining, respectively.

4.3. Separation and purification of the carbohydrate portion of the LPS preparations

LPS from *wbjE* mutant was mild acid hydrolyzed using 2% acetic acid at 100 °C for 3 h. The lipid A was collected by centrifugation at 10,000 × g for 5 min and the supernatant containing the polysaccharide or oligosaccharides was lyophilized. The polysaccharide or oligosaccharide preparations were fractionated by GPC using Sephacryl S100 (HR) column or Bio-gel P2 column, respectively. The eluents from these GPC columns were monitored by an on-line RI detector and appropriate fractions were pooled concentrated and lyophilized.

4.4. De-phosphorylation of the LPS and carbohydrates

The LPS and carbohydrates isolated from the *wbjE* mutant were treated with 48% aqueous hydrofluoric acid (HF) for 48 h at 4°C to remove phosphate substitutions, followed by dialysis against deionized water at 4°C using 500 MWCO dialysis tubing and lyophilized. The precipitate obtained from HF treatment of intact LPS was separated by centrifugation. The supernatant and precipitate were each lyophilized and used for further analysis. The supernatants from HF treated LPS and HF treated carbohydrates were each fractionated by GPC using a Bio-gel P2 column with deionized water as the eluant.

4.5. Composition analysis of LPS

The compositions of the LPS preparations were determined by the preparation and combined gas chromatography-mass spectrometric analysis (GC-MS) of trimethylsilyl (TMS) methyl glycosides.[15] Briefly, 200 μg of LPS samples containing 10 μg of inositol as the internal standard was methanolyzed in methanolic 1 M HCl at 80°C for 18 h. The resulting methyl glycosides were *N*-acetylated using a mixture of pyridine, acetic anhydride, and methanol (4:1:1 v/v) at 100°C for 1 h. The free hydroxyl groups of the methyl glycosides were trimethylsilylated by adding Tri-Sil reagent (Pierce Chemical Company, Rockford, IL) at 80° C for 30 min. The TMS methyl glycosides were extracted into hexane and analyzed by GC-MS using a 30 m DB-1 capillary column (P.J. Colbert Associates, St. Louis, MO). The sugars were identified by comparing the retention time and fragmentation patterns with those of authentic standards.

4.6. Glycosyl linkage analysis by the preparation and GC-MS analysis of partially methylated alditol acetates (PMAAs)

The sugar linkages of the carbohydrate samples were determined by the preparation and GC-MS analysis of PMAAs.[16] Briefy, samples (~0.5 mg) were dissolved in anhydrous dimethylsulfoxide (DMSO) (0.4 mL) and NaOH slurry in DMSO was added followed by stirring vigorously for 2 h. Methylation was accomplished by adding methyl iodide (0.3 mL) in two aliquots at 30 min intervals and the solution was stirred for total of 1.5 h. The partially methylated sample was extracted into chloroform and hydrolyzed by 4 N trifluoroacetic acid (TFA) at 100°C for 6 h. The hydrolyzed sample was reduced using $NaBD_4$ and acetylated using a pyridine: acetic anhydride (1:1 v/v) mixture at 80 °C for 20 min. The resulting PMAAs were analyzed using GC-MS with a DB-1 capillary column for neutral and amino sugar PMAAs, and SP-2330 capillary column (Supelco, Bellefonte, PA) for neutral sugar PMAAs. The linkage positions of each PMAA were determined by comparison of the retention time to those of authentic standards and by its mass spectrometric fragmentation pattern.

4.7. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry

The MALDI-TOF mass spectrometric analysis of the carbohydrate samples was done to determine the intact molecular mass of each oligosaccharide. Each sample was dissolved in deionized water (5μg/μL) and mixed in 1:1 ratio (v/v) with 0.5 M of 2,5-dihydroxy benzoic acid containing 0.1 M of 3-O-methyl salicylic acid, i.e., super-DHB, and spotted on a 100 well stainless MALDI plate. All spectra were acquired in either positive or negative mode using 337 nm N_2 laser. The MALDI-TOF instrument was set at delayed and reflectron mode to give optimum resolution.

4.8. NMR spectroscopy

Each carbohydrate sample (~5 mg) was dissolved in 98.99% D_2O (Cambridge Isotope Laboratories Inc.) and lyophilized to exchange the hydroxyl and amide protons with deuterium. The samples were then dissolved in 0.5 mL of 100% D_2O (Cambridge Isotope Laboratories Inc.) and placed in a 5 mm NMR tube to collect the spectral data. One-dimensional ^{1} NMR spectra were acquired using a Varian Inova 500 MHz instrument at 25°C with a spectral width of 8 kHz. The two-dimensional gradient correlated spectroscopy (gCOSY) spectra were measured using a spectral width of 2.2 kHz and a dataset of ($t_1 \times t_2$) of 256 × 2048 points with 16 scans. The total correlation spectroscopy (TOCSY) spectrum was collected using the same data size as the gCOSY experiment with 32 scans and a mixing time of 80 ms. For the heteronuclear single quantum coherence (HSQC) experiment, the spectral width in the ^{1}H and ^{13}C dimensions were 2.2 and 13.9 kHz, respectively, and total of 96 scans were acquired. ^{31}P NMR spectra were measured at 202.38 MHz using a spectral width of 5 kHz on a BB (broadband)-probe with phosphoric acid (85%) as the external standard (δ_P = 0.0 ppm).

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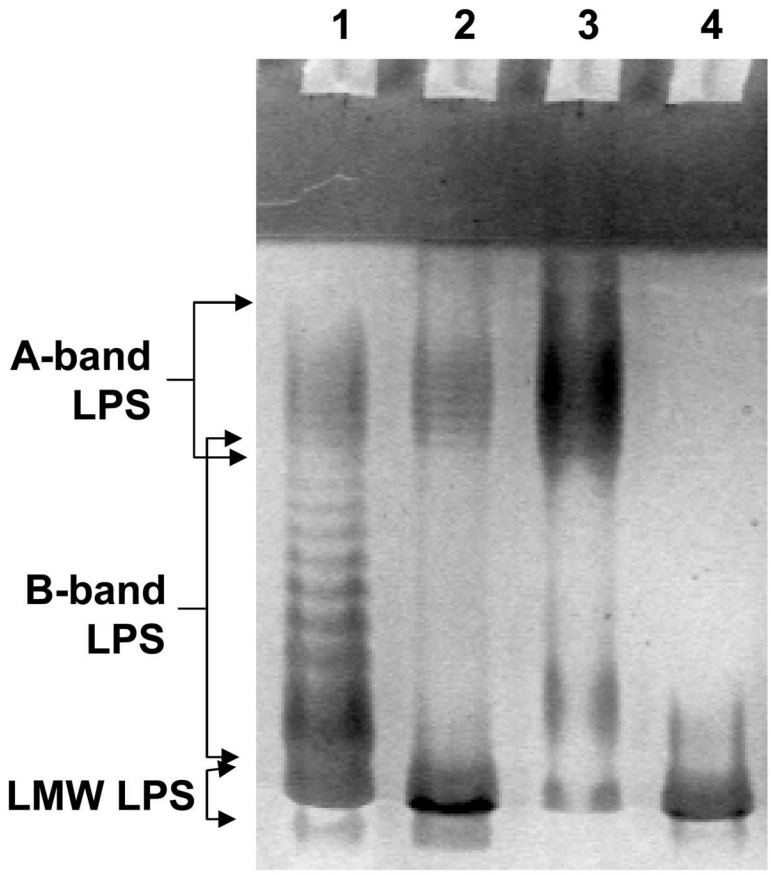

Figure 1.
A DOC-PAGE analysis of the LPS from PA103 (well 1), the *wbjE* mutant (well 2), the HMW *wbjE* LPS (well 3) and LMW *wbjE* LPS (well 4) isolated by S200 DOC GPC of the *wbjE* LPS preparation.

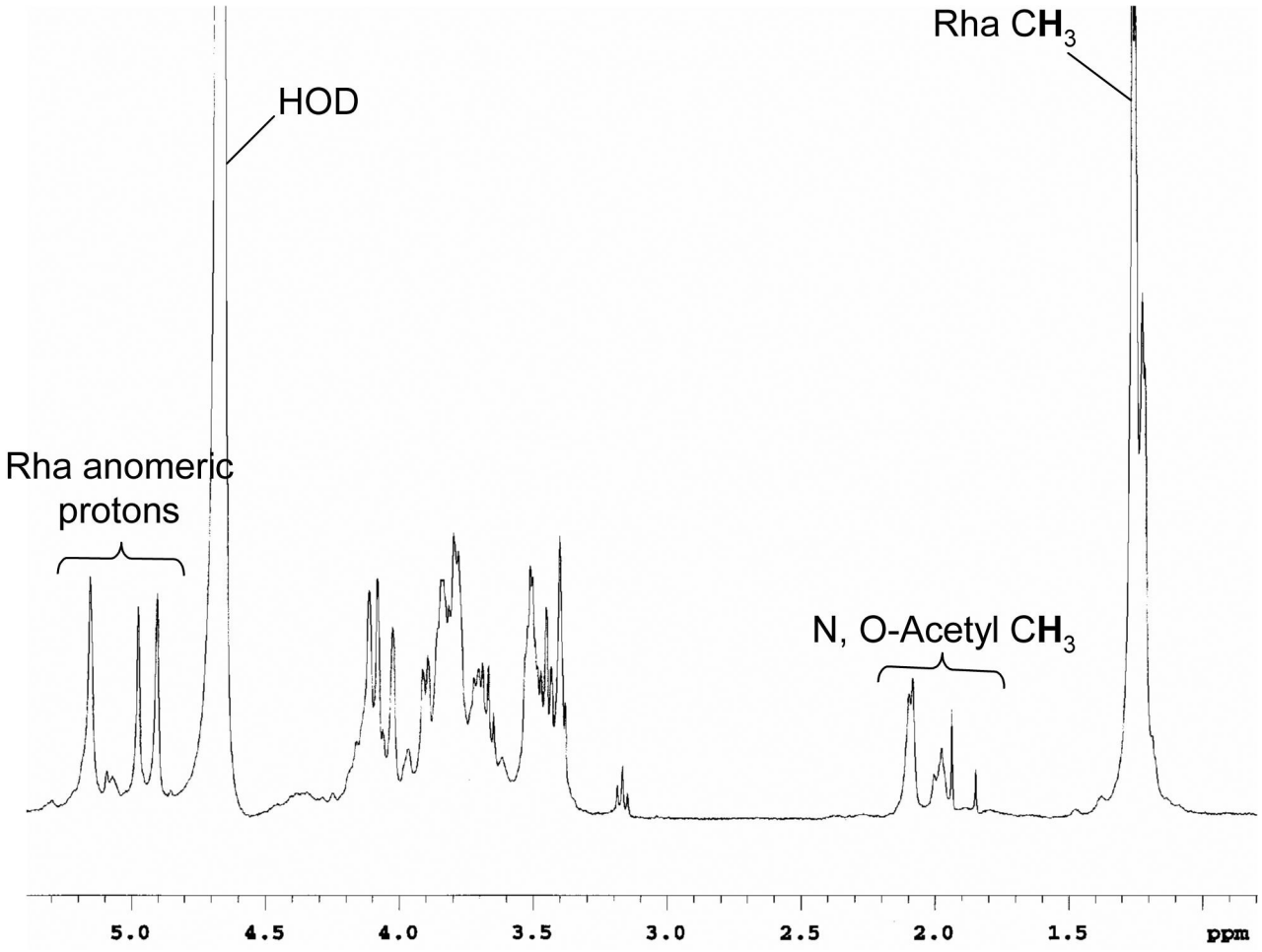

Figure 2.
An ^{1}H NMR spectrum of the HMW PS isolated by mild acid hydrolysis of the HMW LPS and purified by Bio-Gel P10 GPC.

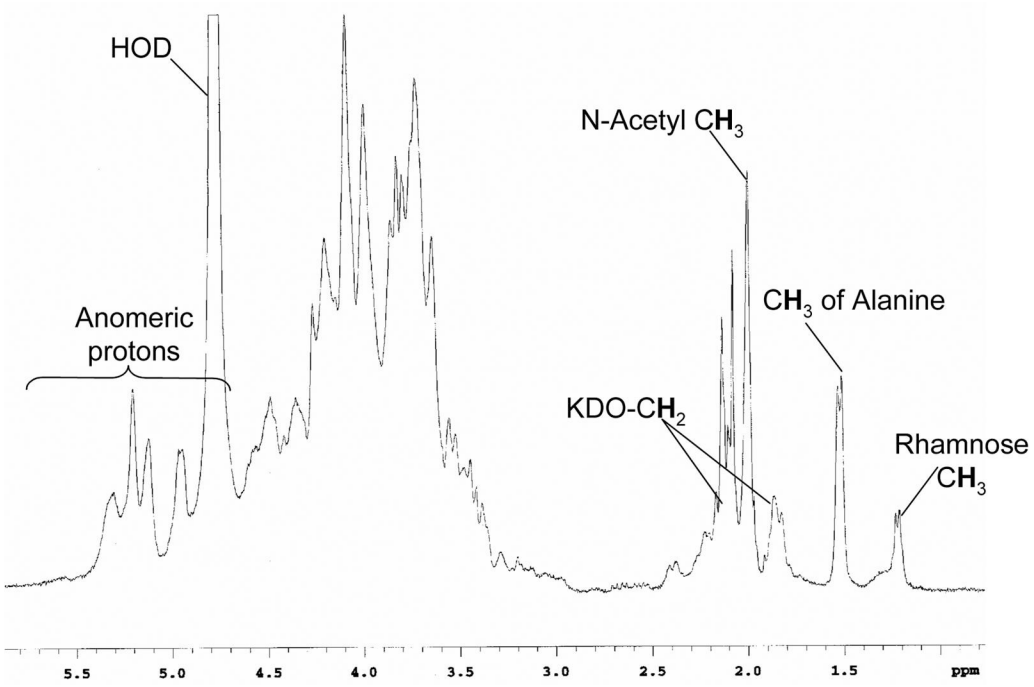

Figure 3.
An ^{1}H NMR spectrum of the LMW OS isolated by mild acid hydrolysis of the LMW LPS and purified by Bio-Gel P2 GPC.

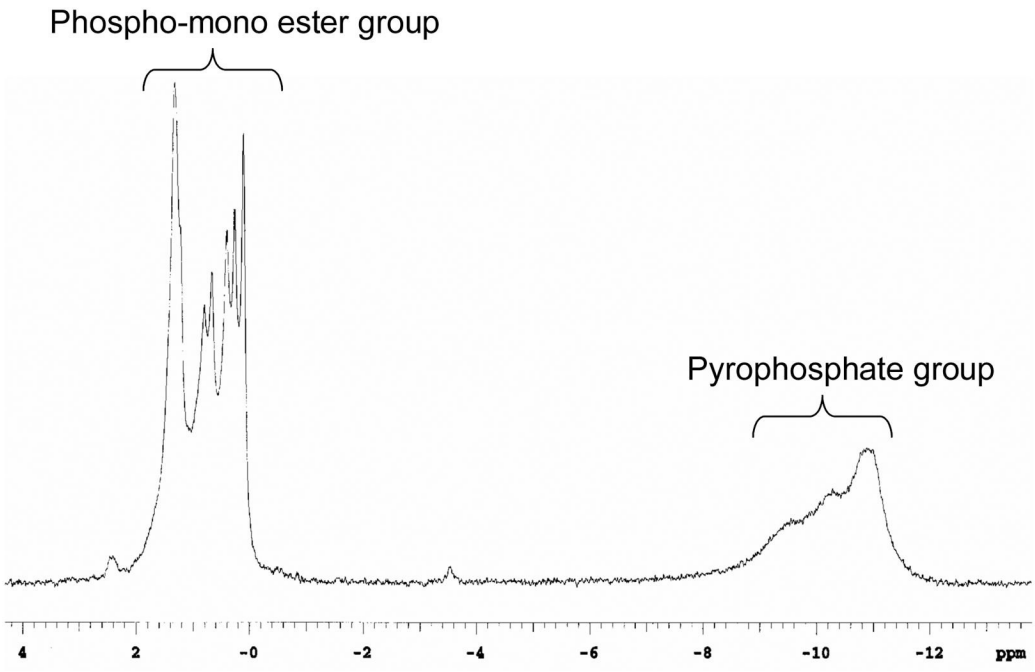

Figure 4.
A ^{31}P-NMR spectrum of the LMW OS isolated by mild acid hydrolysis of the LMW LPS and purified by Bio-Gel P2 GPC.

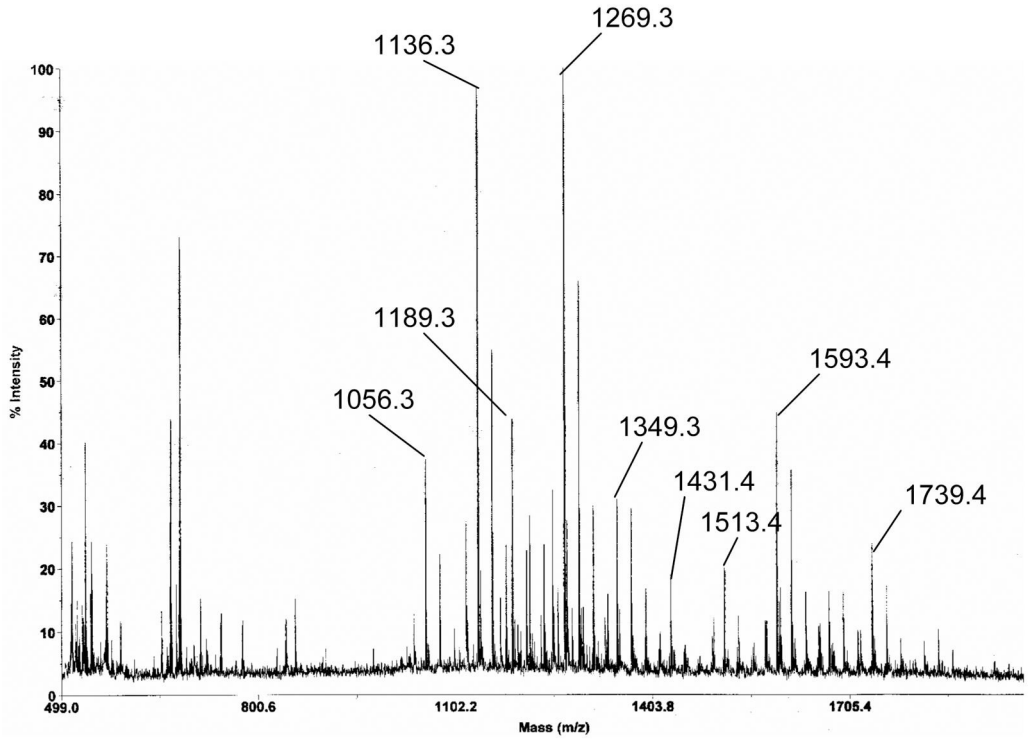

Figure 5.
The MALDI-TOF MS spectrum of the LMW OS isolated by mild acid hydrolysis of the LMW LPS and purified by Bio-Gel P2 GPC. The spectrum was obtained using the negative mode of analysis.

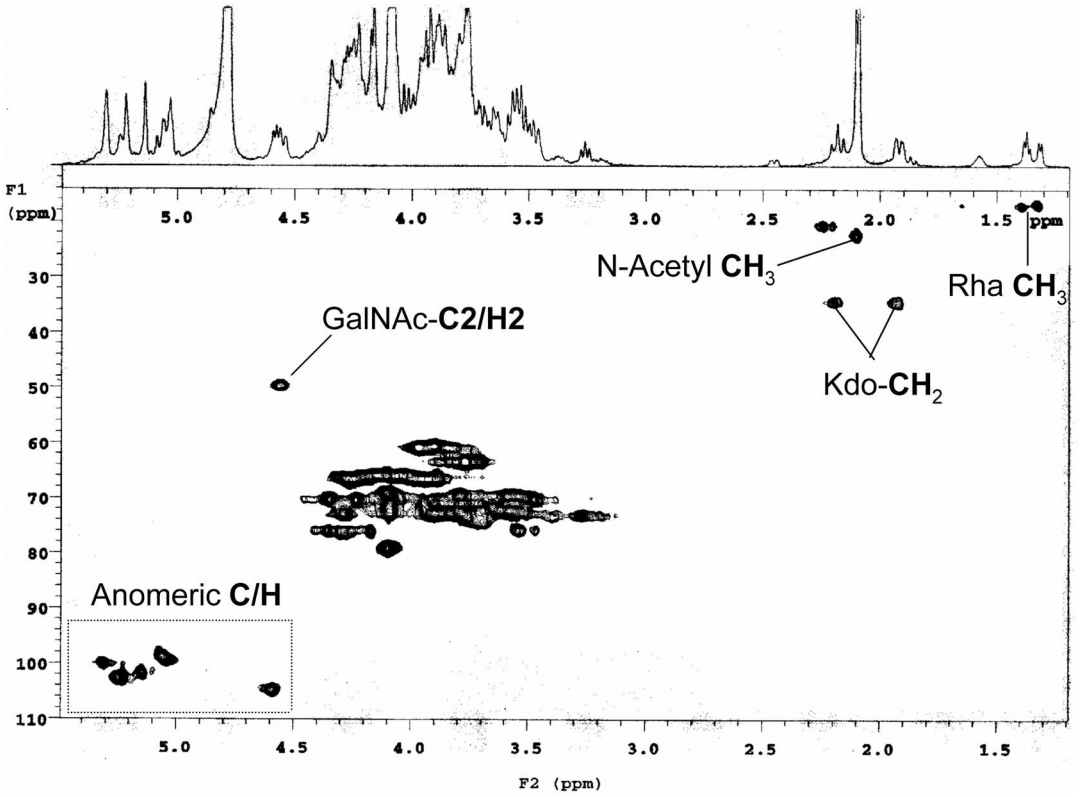

Figure 6.

The $^{1}H–^{13}C$ HSQC spectrum of HF OS2 isolated by Bio-Gel P2 GPC from the LMW OS after treatment with aqueous HF. The boxed region highlights the seven anomeric H1/C1 cross peaks for each of the glycosyl residues present in addition to the Kdo*p* residue. The spectrum also shows the H2/C2 cross peak and CH3 H/C cross peaks for the Gal*p*NAc residue

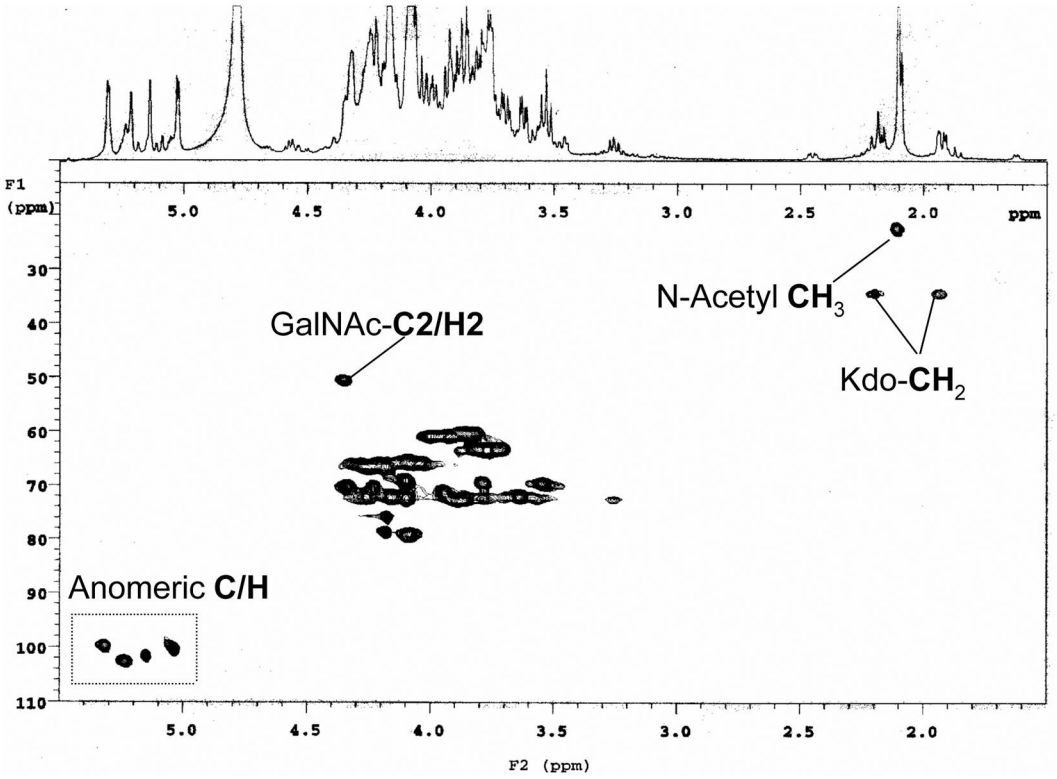

Figure 7.

The $^{1}H–^{13}C$ HSQC spectrum of HF-OS3 isolated by Bio-Gel P2 GPC from the LMW OS after treatment with aqueous HF. The boxed region indicates the four H1/C1 anomeric crosspeaks due to the four glycosyl residues that are present in addition to the Kdo*p* residue. As with the HF OS2, this HF OS3 contains Gal*p*NAc rather than Gal*p*NAla.

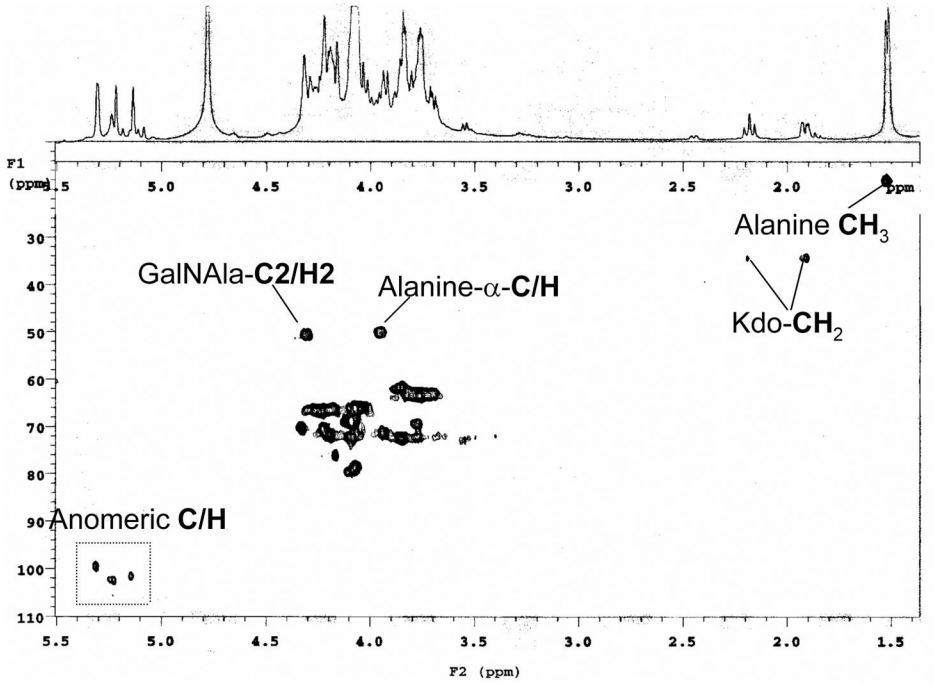

Figure 8.

The ^{1}H–^{13}C HSQC spectrum of HF-OS4 isolated by Bio-Gel P2 GPC from the LMW OS after treatment with aqueous HF. The boxed region indicates the four H1/C1 anomeric crosspeaks due to the three glycosyl residues that are present in addition to the Kdo*p* residue. Unlike HF OS2, this HF OS3 which contain Gal*p*NAc, HF OS4 contains H2/C2 and CH3 H/C cross peaks consistent with Gal*p*NAla.

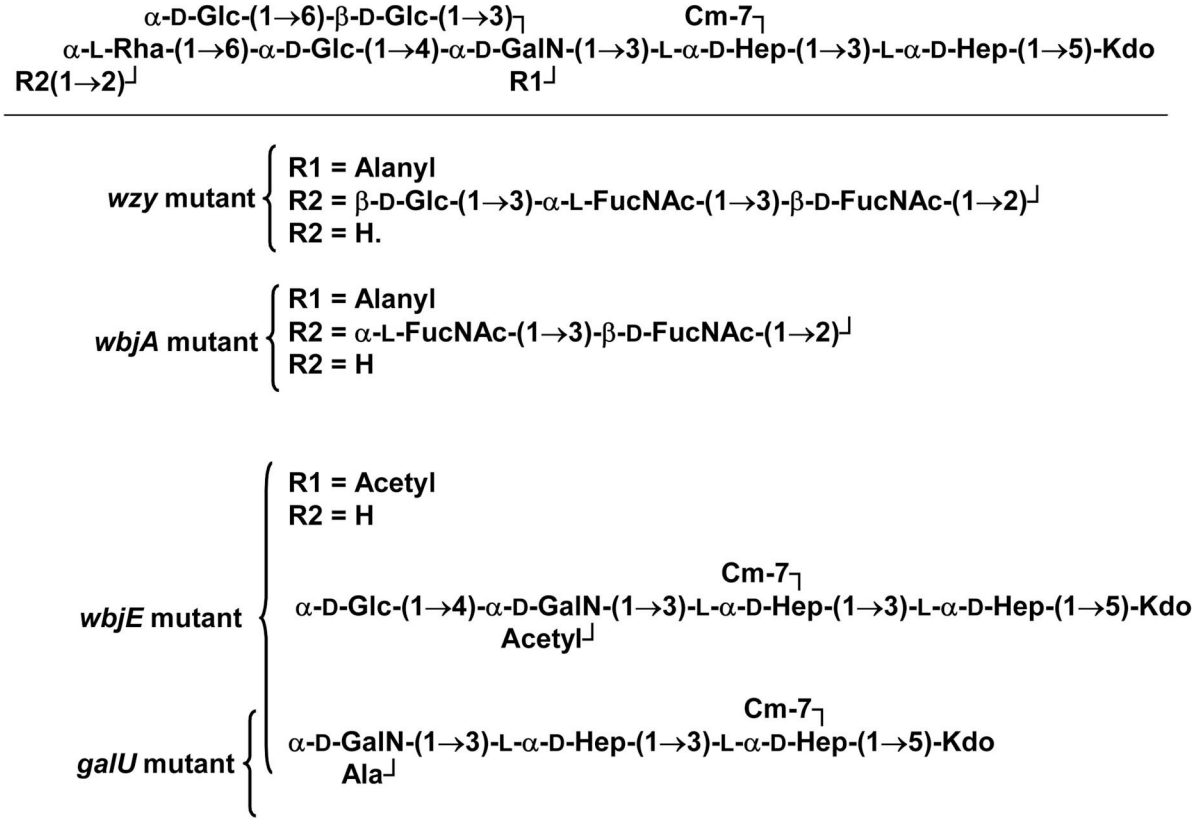

Figure 9.

A summary of the oligosaccharide structures isolated from the LPSs of the *wzy*, *wbjA*, *wbjE*, and *galU* mutants of PA103. Because the discussion of these structures largely concerns the glycosyl residues, the locations of the variable phosphoryl substituents are not shown, but occur on the Hep*p* residues.

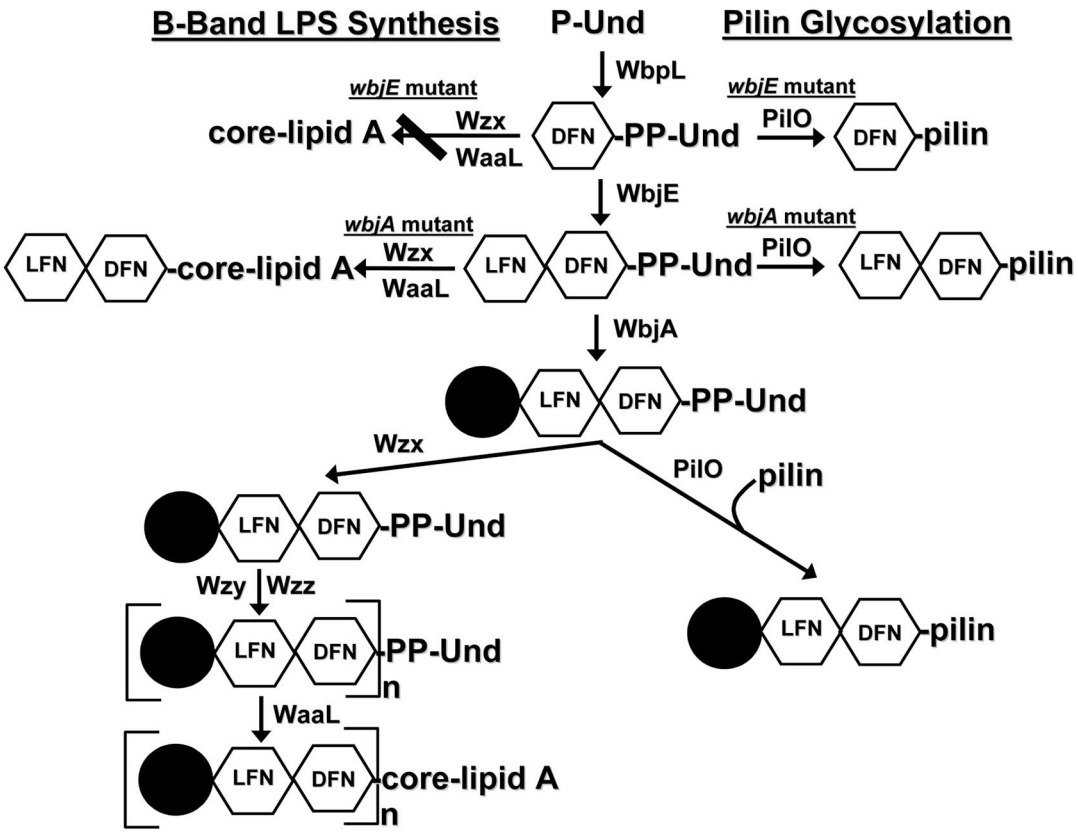

Figure 10.

The biosynthetic scheme for B-band LPS synthesis and pilin glycosylation in PA103. This scheme proposes that PilO is able to transfer the glycosyl residues from D-Fuc*p*NAc-PP-Und, L-Fuc*p*NAc-D-Fuc*p*NAc-PP-Und, and Glc-L-FucNAc-D-FucNAc-PP-Und to the pilin, but that Wzx is unable to "flip" D-Fuc*p*NAc-PP-Und across the cytoplasmic membrane or WaaL us unable to transfer the D-Fuc*p*NAc to the core-Lipid A. Thus, in the *wbjE* mutant the D-Fuc*p*NAc can be added to PilO as has been reported [9], but is not added to the core oligosaccharide of the LPS as we report here.

● = Glc; DFN = D-Fuc*p*NAc; LFN = L-Fuc*p*NAc.

Table 1

MALDI-TOF mass of OS from *wbjE* LMW LPS

Observed Mass $[M-H]^-$	Proposed structure	Calculated Mass, $[M-H]^-$
1136.3	Gal*p*NAla.Hep*p*(Cm).Hep*p*(P_3).Kdo*p*	1136.7
1056.3	Gal*p*NAla.Hep*p*(Cm).Hep*p*(P_2).Kdo*p*	1055.7
1269.3	Glc*p*.Gal*p*NAc.Hep*p*(Cm).Hep*p*(P_3).Kdo*p*	1268.8
1189.3	Glc*p*.Gal*p*NAc.Hep*p*(Cm).Hep*p*(P_2).Kdo*p*	1188.8
1431.4	Glc*p*.Glc*p*.Gal*p*NAc.Hep*p* (Cm).Hep*p* (P_3).Kdo*p*	1431.0
1593.4	Glc*p*.Glc*p*.Glc*p*.Gal*p*NAc.Hep*p*(Cm).Hep*p*(P_3).Kdo*p*	1593.1
1739.4	Rha*p*.Glc*p*.Glc*p*.Glc*p*.Gal*p*NAc.Hep*p*(Cm).Hep*p*(P_3).Kdo*p*	1739.2