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Formation of a new O-polysaccharide in *Escherichia coli* O86 via disruption of a glycosyltransferase gene involved in O-unit assembly

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Abstract—The majority of hetero-polysaccharide biosynthesis in Gram-negative bacteria utilizes the *wzy*-dependent pathway, in which repeating O-units are first synthesized in the cytosol and then subsequently translocated to the periplasmic face of the inner membrane where polymerization is initiated by the Wzy polymerase. Wzy proteins share little primary sequence homology and are specific for their cognate O-unit structures. Our previous studies on O-polysaccharide biosynthesis in *Escherichia coli* O86 identified the *wbnI* gene, which encodes a galactosyltransferase responsible for the introduction of α-(1→3)-Gal*p* residues as side chains of the polysaccharide. In this work, we functionally inactivated the *wbnI* gene and showed that the mutant strain produced a different polysaccharide without the side chain Gal*p* residue. The yield of the polysaccharide was substantially lower than the one produced by the wild-type strain. This study indicated that the complete O-unit structure is the preferred substrate for the polymerization, thus further confirming the specificity of Wzy. On the other hand, these studies also suggest that the Wzy polymerase might have moderate tolerance of side-chain truncated O-unit substrates.

Keywords: Lipopolysaccharide; Polymerization; Glycosyltransferase; *wzy*-Dependent pathway; O-Repeating unit

1. Introduction

Lipopolysaccharide (LPS) constitutes the major component of the outer membrane in Gram-negative bacteria, and contributes greatly to the structural integrity and pathogenecity of these organisms.[1–3] The structure of LPS has three parts: (i) lipid A, a glucosamine-based phospholipid that serves as a hydrophobic anchor in the lipid bilayer of the membrane; (ii) a core oligosaccharide; and (iii) an O-polysaccharide (O-antigen) that contains multiple copies of an oligosaccharide unit (O-unit).[2] O-polysaccharides have the most structural variations within and among different species.[3] The variations include differences in sugar composition, arrangement within the O-unit, and linkages between O-units.[4,5]

O-Polysaccharide biosynthesis has been studied extensively over the past several decades. Depending on the respective assembly and translocation mechanisms during the polymer elongation, three major pathways have been proposed: *wzy*-dependent,[6–8] ABC-transporter dependent,[9,10] and synthase-dependent.[2] The *wzy*-dependent pathway is involved in the synthesis of the majority of O-polysaccharides, especially hetero-polymeric polysaccharides. The synthesis starts with the sequential addition of sugar residues to undecaprenol phosphate (UndP) on the cytosolic face of the cytoplasmic membrane to form the O-repeating units. This process is catalyzed by specific glycosyltransferases that are predicted to be soluble or peripheral membrane proteins. These O-units, once completed, are translocated by a flippase to the periplasmic face of the membrane[11–13]

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where they become polymerized from the reducing end of the sugar units by a processive polymerase.[6–8]

A characteristic feature of the *wzy*-dependent pathway is the presence of two genes in the biosynthetic gene cluster that encode the putative O-unit flippase (Wzx) and polymerase (Wzy). Both Wzx and Wzy share very little amino acid sequence similarity with their homologs from other bacterial strains. The lack of common motifs in both Wzx and Wzy proteins makes it difficult to decipher the exact mechanism of O-polysaccharide processing on the periplasmic face. Valvano and co-workers[11,12] demonstrated that an incomplete *Escherichia coli* O16 O-unit could be processed and ligated onto the lipid A-core, and that *wzx* from other bacteria could fully or partially complement the *wzx* gene defect in O16. This finding suggested that Wzx could function independent of the chemical structure of O-units. In contrast to the relaxed specificity of Wzx, Wzy proteins possess stricter specificity toward their cognate O-units or structures containing the same linkage between O-units. Early work by Wright et al.[14] on the synthesis of the O-polysaccharide from *S. enterica* serovar *anatum* (group E) showed that the formation of trisaccharide unit (Man*p*-Rha*p*-Gal*p*) was absolutely required for the polymerization. Yuasa et al.[15] also demonstrated that deletion of the side chain abequose from the O-unit in *S. enterica* serova *typhimurium* (group B) completely knocked out the ability of the organism to produce any O-polysaccharides.

E. coli O86, belonging to O serogroup of enteropathogenic *E. coli* (EPEC) strains, was previously reported to possess high human blood group B activity.[16] Springer et al. and Kochibe et al. showed that the strong blood group B activity of *E. coli* O86 came from its LPS O-polysaccharide, which contained the blood group B trisaccharide part of its structure.[17] The complete O-polysaccharide repeating unit of *E. coli* O86[18] has five sugar residues, with the polymerization site from β-Gal*p*NAc to α-Fuc*p* via a β-(1→4) linkage (Fig. 1). The terminal trisaccharide motif, α-Gal*p*-(1→3)-[α-Fuc(1→2)]-β-Gal*p*, is the blood group B antigen. The α-(1→3)-linked Gal*p* moiety is the side chain residue from the polymer backbone. Recently, we have sequenced the entire O-antigen gene cluster from *E. coli* O86 and annotated the genes responsible for the biosynthesis of the O-polysaccharide.[19] The *wbnI* gene was shown to encode an α-(1→3)-galactosyltransferase that catalyzes the formation of the α-Gal*p*-(1→3)-Gal*p* linkage.

When we functionally inactivated the *wbnI* gene by replacing it with the chloramphenicol acetyltransferase (CAT) gene, the LPS profile from the mutant strain showed that polysaccharides were still present, although the amount and the extent of polymerization were substantially reduced. Structural determination of O-polysaccharide derived from the mutant strain revealed that the α-(1→3)-linked Gal*p* side chain residue was absent while the O-polysaccharide backbone was intact, suggesting that incomplete O-unit was formed as a result of *wbnI* knockout and that the incomplete O-unit could be polymerized in a less efficient manner. The results of this study indicate that the complete O-unit structure is the preferred substrate for the polymerization, thus further confirming the specificity of Wzy. On the other hand, these results also suggest that the Wzy polymerase might have moderate tolerance toward side-chain truncated O-unit substrates.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli O86:K62:H2 strain was kindly provided by W. F. Vann from the Department of Health and Human Service, Food and Drug Administration. *E. coli* competent cell DH5α [*lacZΔM15hsdR recA*] was from Gibco-BRL Life Technology and *E. coli* competent cell BL21 (DE3) [F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3)] was from Novagen Inc. (Madison, WI). Expression plasmid pET15b was purchased from Novagen (Carlsbad, CA). Plasmids pKD20 and pKK232-8 were kindly provided by L. Wang from Nankai University, China.

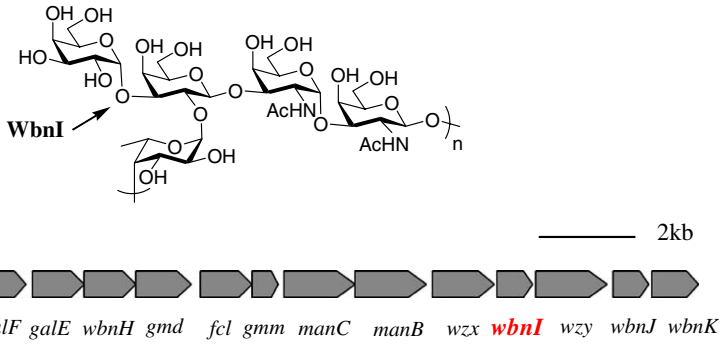

Figure 1. O-antigen structure of *E. coli* O86:K62:H2 and its biosynthetic gene cluster. The glycosyltransferase gene *wbnI* and the glycosidic linkage that it synthesizes are highlighted.

2.2. Functional inactivation of WbnI and complementation

The *wbnI* gene was replaced by a chloramphenicol acetyltransferase (CAT) gene using the RED recombination system of phage lambda.[20,21] The CAT gene was amplified from plasmid pKK232-8 (Invitrogen) using primers binding to the 5′ and 3′ ends of the gene, with each primer carrying the 40 bp that flank *wbnI* gene. The PCR product was then transformed into *E. coli* O86 type strain carrying pKD20, and chloramphenicol-resistant transformants were selected according to the protocol described by Datsenko and Wanner.[21] PCR using primers specific to the CAT gene and *E. coli* O86 mutant genomic DNA flanking the *wbnI* gene was carried out to confirm the replacement. Complementation of *wbnI* mutant was done by introducing the recombinant vector pUC19-*wbnI*.

2.3. Isolation of LPS and O-polysaccharide

Dried mutant *E. coli* O86 cells (20 g) were extracted with 50% (w/v) aqueous phenol (200 mL) at 65 °C for 15 min; the extract was cooled to 4 °C, followed by low speed centrifugation (10,000*g*). The top aqueous solution was separated and dialyzed against distilled water until free from phenol. The clear solution was then lyophilized. The products were then dissolved in 0.02 M sodium acetate (pH 7.0, 20 mL) and treated sequentially for 2 h each with DNase, RNase, and proteinase K at 37 °C. After removal of the precipitated materials, the solution was subjected to ultracentrifugation (110,000*g*, 4 °C, 12 h). The precipitated gels were then dissolved in distilled water and lyophilized to 1.1 g pure LPS. The LPS was degraded with aqueous 1% acetic acid at 100 °C for 1.5 h. The lipid precipitate was removed by centrifugation (12,000*g*, 30 min) and the supernatant was then passed through Bio-Gel P2 column. Fractions containing polysaccharide were pooled and lyophilized to yield 30 mg of the products.

2.4. Analysis of LPS by gel electrophoresis and immunoblot

The whole cell extract (3 mL culture) from *E. coli* O86 wild-type, *wbnI*-deficient and complementation strains was treated with proteinase K (40 μg), and subjected to electrophoresis. The gels were visualized with silver staining according to the reported protocol.[22,23] The immunoblot was done by transferring LPS to a nitrocellulose membrane (Invitrogen), followed by probing with a primary anti-B antibody and then a secondary antibody. Commercially available IgM monoclonal anti-B antibody (obtained from clone HEB-29) was purchased from Abcam Ltd (Cambridge, UK). The secondary antibody was goat anti-mouse IgM conjugated to HRP (1:1000) (Sigma). The peroxidase substrate (3,3′,5,5′-tetramethylbenzidine, Sigma) was used to develop the signal.

2.5. Structural analysis of polysaccharide by NMR, MS, and methylation analysis

Mass spectra (ESI) for oligosaccharide determination were run in negative ion mode at the mass spectrometry facility at The Ohio State University. Capillary electrophoresis (CE) was performed using a Prince CE system (Prince Technologies, The Netherlands). The CE system was coupled to an API 3000 mass spectrometer (Applied Biosystems/Sciex, Concord, Canada) via a microspray interface. CE/IS-CID–MS analysis was done at the Institute for Biological Sciences, National Research Council of Canada. Sample separation and mass spectra acquisition of the O-polysaccharide were carried out as described.[24] ^{1}H and ^{13}C NMR spectra were collected using 500-MHz Bruker DRX500 NMR spectrometer. The sample was pretreated with D_2O three times to saturate the hydroxyl groups of the polysaccharide. The product structure was identified through one-dimensional and two-dimensional (COSY, HMQC, and HMBC) $^{1}H/^{13}C$ NMR spectroscopy. Methylation analysis was done at the Complex Carbohydrate Research Center (Georgia).

3. Results and discussion

3.1. Disruption of the *wbnI* in the gene cluster does not result in the complete loss of O-polysaccharide

After demonstrating that *wbnI* encodes a galactosyltransferase responsible for the synthesis of the side chain residue in the O86 O-polysaccharide, we wanted to investigate if this galactose side chain residue is strictly required for further translocation and polymerization. We therefore inactivated the *wbnI* gene from the gene cluster by replacing it with the CAT gene using the RED recombination system of phage lambda as described.[21] The replacement was confirmed by PCR amplification of the region upstream and downstream of the *wbnI* gene in the mutant genomic DNA and the subsequent sequencing of the PCR product. LPSs from mutant and wild-type strains were extracted according to standard procedure and visualized by silver staining.

The wild type *E. coli* O86 strain, as expected, displays the typical LPS structure (Fig. 2A, lane 1), with 12–16 O-repeating units. In contrast, LPS from the *wbnI* knockout strain demonstrates a very different structure (Fig. 2A, lanes 2 and 3), with most of the LPS shifted to low molecular weight. A large portion of the LPS is of the semi-rough phenotype, with one O-unit attached to the lipid-A core. A small amount of LPS contains

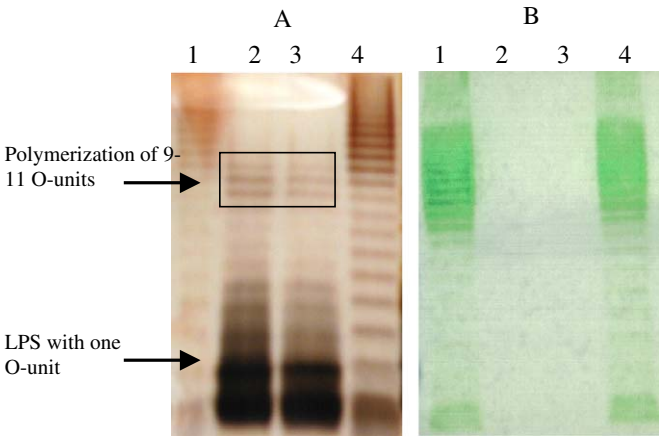

Figure 2. LPS silver staining and immunoblot analysis. (A) Silver-stained SDS-PAGE containing LPS from *E. coli* O86 wild-type strain, *wbnI* deficient and complementation strains. (B) Immunoblot analysis of LPS from *E. coli* O86 wild-type strain, *wbwI* deficient and complementation strains. LPS is transferred to nitrocellulose membrane and probed with primary antibody anti-B (Mouse monoclonal) with dilution of 1:100. The secondary antibody is anti-mouse IgM conjugated with HRP with dilution of 1:2000. Lane 1: *E. coli* O86 wild type; Lanes 2 and 3: *wbnI* deficient strains; Lane 4: *wbnI* complementation with pUC-*wbnI* plasmid.

9–11 O-units, which, from the intensity of the bands, have a much lower concentration compared to the semi-rough LPS. No band was observed in the high molecular weight area, whereas LPS of this size was readily detected in the wild-type strain. Comparison of the LPS bands of the wild-type and mutant strains also revealed that the distance between the corresponding bands is different. In both strains, the band representing the lipid A-core structure migrates to the same level in the gel, indicating that *wbnI* disruption does not affect lipid A-core synthesis. On the other hand, the band with one O-unit attached migrates faster in mutant strain, suggesting that the molecular weight of O-unit in mutant strain is smaller than that in the wild-type strain. The difference between the bands in the two strains becomes more significant with an increase of molecular weight. This suggests that the structure of O-polysaccharide in the two strains is different, and that the *wbnI* gene accounts for the altered structure. Normal LPS was produced when the mutant strain was complemented with recombinant pUC plasmid containing full-length *wbnI* gene.

Because *E. coli* O86 possesses strong blood group B activity, we hypothesize that the *wbnI* gene is essential for immunoreactivity. Therefore, the LPSs from the wild-type and mutant strains were probed with anti-blood group B antibody (Fig. 2B). An immunoblot assay showed that the LPS from wild-type and complement strains had reactivity with anti-B antibody, whereas in the mutant strain, no anti-B activity was detected. This is consistent with the notion that the *wbnI* gene is responsible for the introduction of the α-(1→3)-Gal*p* residue into the blood group B epitope in the O-polysaccharide. From the LPS silver staining and immunoblotting experiments, it is evident that disruption of the *wbnI* gene results in altered expression of the blood group B epitope structure in O86 polysaccharide.

3.2. Structure determination of the new O-polysaccharide

It is evident that the *wbnI* deficient strain still produces O-polysaccharide, although with less efficiency than the wild-type strain. Moreover, the polysaccharide produced from the mutant strain is not the same as the one produced from the wild-type strain, as demonstrated by the band migration distance using the silver staining and immunoblot assays. Therefore, we believe that the O-polysaccharide produced by the mutant strain is structurally different from the one obtained from the wild-type strain. The structural characterization of the new O-polysaccharide was carried out using NMR spectrometry, CE/MS,[24] and methylation analysis.

Compared with the ^{1}H NMR spectrum of the O-polysaccharide from the wild-type O86 strain, the O-polysaccharide isolated from the *wbnI*-deficient strain contained, *inter alia*, four anomeric proton signals at δ 5.23 (2H, $J_{1,2} = 3.8$ Hz), δ 5.08 ($J_{1,2} = 6.5$ Hz), and δ 4.64 ($J_{1,2} = 7.6$ Hz). Due to partial overlap with the HOD peak at 4.69 ppm, these resonances were more clearly visible by collecting the spectrum at 50 °C instead of ambient temperature (Fig. 3). The ^{13}C NMR spectrum of the same material showed signals for the anomeric carbons at 102.2, 98.9, and 92.9 (2 carbons) ppm. Two acetyl groups (C=O: 173.8 and 174.4 ppm; $COCH_3$ (^{1}H/^{13}C): 1.99/22.4 ppm and 1.97/22.1 ppm) and one Fuc*p* residue (1.11 ppm, C6-methyl group) were evident in the NMR spectra. With the aid of 2-D NMR experiments (see Supplementary data), the complete assignment of the spectrum was possible (Table 1). The ^{1}H signals at 5.23 ppm correspond to two protons, the anomeric proton of the Fuc*p* and the Gal*p*NAc

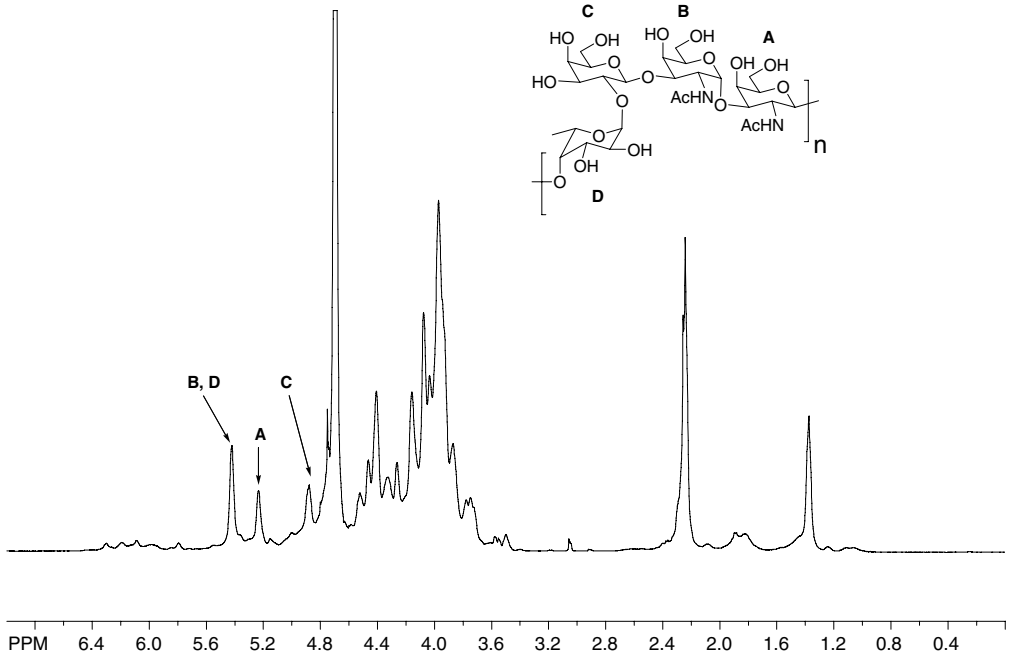

Figure 3. ^{1}H NMR spectra of O-polysaccharide isolated from *wbnI* deficient *E. coli* O86 strain. The spectrum was collected at 50 °C to clarify the anomeric proton signals. The anomeric proton signals were assigned as indicated.

Table 1. ^{1}H and ^{13}C NMR assignment for the O-polysaccharide of *wbnI* knockout *E. coli* O86 strain

Sugar residue	Chemical shift (δ, ppm)						
	Nucleus	1	2	3	4	5	6
→3)-β-D-Gal*p*NAc-(1	^{1}H	5.08	4.25	3.96	4.20	3.85	3.79
	^{13}C	92.9	49.7	75.4	69.5	71.9	61.6
→3)-α-D-Gal*p*NAc-(1	^{1}H	5.23	4.14	3.80	4.07	3.67	3.78
	^{13}C	92.9	51.8	75.8	64.6	76.2	61.7
→2)-β-D-Gal*p*-(1	^{1}H	4.64	3.64	3.84	3.93	3.67	3.84
	^{13}C	102.2	77.4	74.4	69.8	74.8	61.8
→4)-α-L-Fuc*p*-(1	^{1}H	5.23	3.81	3.64	3.83	4.28	1.11
	^{13}C	98.9	69.6	70.3	83.3	67.6	15.3

Acetate signals: C═O: 173.8 and 174.4 ppm; COMe (^{1}H/^{13}C): 1.99/22.4 ppm and 1.97/22.1 ppm.

residues. The coupling constant indicates that these two units have α configuration. The ^{1}H signal at 5.08 ppm is assigned to the anomeric proton of the other Gal*p*NAc moiety and the coupling constant (6.5 Hz) indicates the β-stereochemistry. Finally, the ^{1}H signal at 4.64 ppm is assigned to the anomeric proton of the β-Gal*p* residue ($J = 7.6$ Hz). Thus, the ^{1}H and ^{13}C NMR spectra of the polysaccharide indicated a tetrasaccharide repeating unit.

Methylation analysis shows the presence of four major components, namely, 3,4,6-tri-*O*-methyl-galactose, 2,3,4-tri-*O*-methyl-fucose, 2-deoxy-2-*N*-methylacetamido-4,6-di-*O*-methyl-galactose, and 2-deoxy-2-*N*-methylacetamido-3,4,6-tri-*O*-methyl-galactose in a ratio of 0.8:1.0:1.4:0.6. We also observed a trace amount of terminal Fuc*p*. These results, in conjunction with the identified α-(1→3)-galactosyltransferase function of WbnI, suggest that the new polysaccharide structure resulted from the deletion of the terminal α-(1→3)-linked Gal*p* residue in the native O86 polysaccharide.

The structure was further confirmed using CE/MS. A fragment of the O-polysaccharide was obtained based on in-source collision induced dissociation, and the separation of impurities was done by capillary electrophoresis.[24] The CE–IS-CID analysis of the O-polysaccharide is presented in Figure 4. The obtained spectrum was consistent with the proposed new structure. The ion at *m*/*z* 715.3 corresponds to the repeating unit composing Fuc*p*, Gal*p*, and two Gal*p*NAc residues. The ion at *m*/*z* 569.3 arises from the loss of the Fuc*p* residue from the repeating unit; the ion at *m*/*z* 512.3 likely resulted from the fragmentation of the terminal Gal*p*NAc from the repeating unit. The observation of ions at *m*/*z* 366.3 and 407.3 corresponds to different fragmentations of the repeating unit. Therefore, the NMR spectrum and the fragmentation pattern obtained

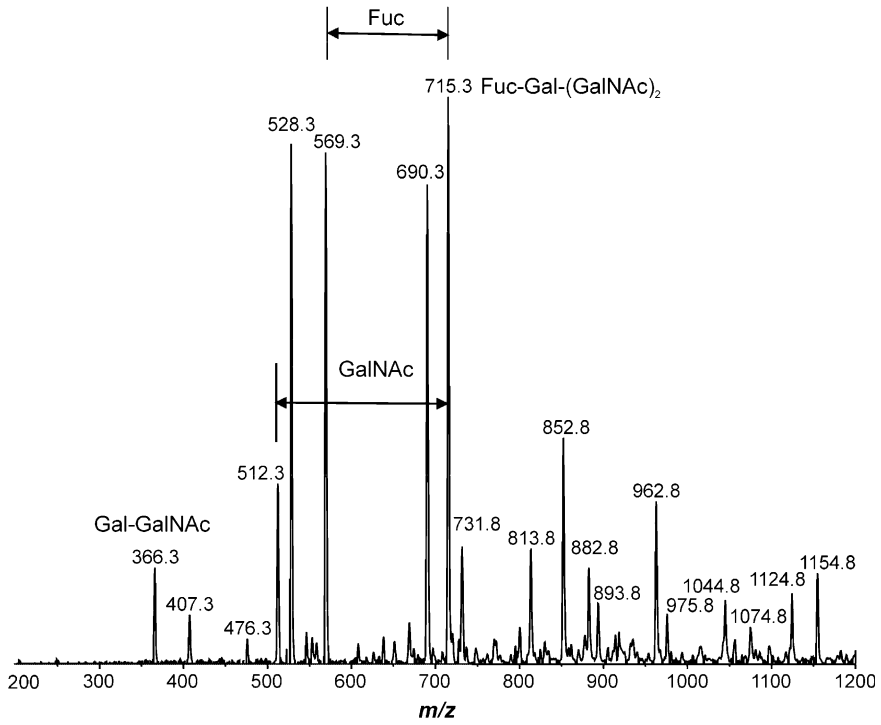

Figure 4. CE/MS analysis of O-polysaccharide from the *wbnI* deficient *E. coli* O86 strain.

by CE/IS-CID–MS indicated that the O-polysaccharide obtained from *wbnI* deficient strain has the repeating unit structure as shown in Figure 5.

From the LPS silver staining and NMR spectroscopy data, it is evident that although the absence of the α-(1→3)-linked Gal*p* side chain in the repeating unit substantially reduces polymerization efficiency, a small portion of shorter LPS molecules, with 9–11 O-chain repeating units, can still be produced. This result reveals some mechanistic aspects in the O-polysaccharide processing pathway.

First, Wzx flippase has relaxed substrate specificity. Disruption of the *wbnI* gene resulted in the formation of truncated O-units lacking the side chain Gal*p* residue. Our results also showed that a large portion of the LPS displayed a semi-rough phenotype with one O-unit attached to the lipid A-core structure, suggesting that the incomplete O-units could be efficiently translocated by Wzx. Furthermore, previous study by Valvano and co-workers[11,12] conclusively demonstrated that Wzx from *E. coli* O16 could function independent of the O-unit structures. Thus our study further confirms the intrinsic relaxed specificity of Wzx.

Second, Wzy has preference for the complete O-unit structure. The strict specificity of Wzy was demonstrated in a previous study of O-polysaccharide synthesis in *Salmonella typhimurium*.[15,25–27] In that study, the complete tetrasaccharide O-unit was required for in vivo polymerization, and mutants lacking the side chain abequose residue were unable to produce any polysaccharide. Although in vitro, structures containing up to two repeating units were observed for the trisaccharide unit lacking abequose; the much higher polymerization rate for the complete tetrasaccharide units indicated that the complete O-unit structure was the preferred substrate for the polymerization system.[25–27] In our study, the specificity of Wzy was further supported by the observation that a large portion of the O-units lacking

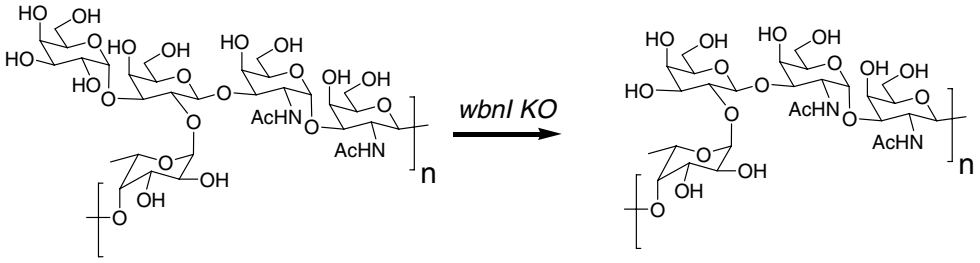

Figure 5. O-Polysaccharide structure resulted from *wbnI* disruption.

the Gal*p* branching structure cannot be polymerized. However, we observed the polymerization of the incomplete O-units up to 11 units in length, suggesting that the Wzy in *E. coli* O86 may have more tolerance toward repeating unit structural modifications than Wzy from *S. typhimurium*. The requirement of the side chain Gal*p* residue for efficient polymerization also indicates that this residue is part of the repeating unit structure and is not introduced following polymerization. This is in contrast with the observations in *Shigella* O-polysaccharide biosynthesis.[28] In *Shigella* O-units, the addition of the side chain glucopyranose residues is serotype-specific and disruption of the glucosylation operon does not affect polymerization. Thus, in *Shigella*, the glucopyranose side chain is more likely a post polymerization modification.

Acknowledgments

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