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The Modality of Enterobacterial Common Antigen Polysaccharide Chain Lengths Is Regulated by *o349* of the *wec* Gene Cluster of *Escherichia coli* K-12

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The assembly of the phosphoglyceride-linked form of enterobacterial common antigen (ECA_{PG}) occurs by a mechanism that involves modulation of polysaccharide chain length. However, the genetic determinant of this modulation has not been identified. Site-directed mutagenesis of *o349* of the *Escherichia coli* K-12 *wec* gene cluster revealed that this locus encodes a Wzz protein that specifically modulates the chain length of ECA_{PG} polysaccharides, and we have designated this locus wzz_{ECA}. The Wzz_{ECA}-mediated modulation of ECA_{PG} polysaccharide chains is the first demonstrated example of Wzz regulation involving a polysaccharide that is not linked to the core-lipid A structure of lipopolysaccharide.

Enterobacterial common antigen (ECA) is a unique cell-surface glycolipid that is present in all gram-negative enteric bacteria (14, 17, 21, 30). The carbohydrate portion of ECA consists of a linear heteropolysaccharide chain comprising the trisaccharide repeat unit →3)-α-D-Fuc4NAc-(1→4)-β-D-ManNAcA-(1→4)-α-D-GlcNAc-(1→, where GlcNAc is *N*-acetyl-D-glucosamine, ManNAcA is *N*-acetyl-D-mannosaminuronic acid, and Fuc4NAc is 4-acetamido-4,6-dideoxy-D-galactose (16, 18). The major form of ECA, ECA_{PG}, consists of polysaccharide chains of various lengths that are covalently linked to phosphoglyceride via phosphodiester linkage involving the GlcNAc residue of the first repeat unit (15, 28). The occurrence of two other minor forms of ECA, ECA_{LPS} and ECA_{CYC}, has also been demonstrated. ECA_{LPS} is characterized by the covalent linkage of ECA polysaccharide chains to the core region of lipopolysaccharide (LPS) (12, 14, 31, 34), whereas ECA_{CYC} is a water-soluble cyclic form of ECA containing four to six trisaccharide repeat units (9, 14, 33). Although ECA_{PG} is present in all members of the *Enterobacteriaceae*, the occurrence of ECA_{LPS} and ECA_{CYC} appears to be restricted to certain gram-negative enteric bacteria (12, 14, 31, 34).

The known genetic determinants of ECA are located in the *wec* (formerly *rfe*/*rff*) gene cluster located at min 85 of the *Escherichia coli* chromosome (14, 22, 30). (The genetic nomenclature used here is in accordance with that proposed by Reeves et al. [27] for genes involved in the synthesis of bacterial polysaccharides.) The nucleotide sequence of this gene cluster includes 12 open reading frames (Fig. 1B). Biochemical and genetic studies have identified the function of 7 of these genes (30); however, the functions of *o349*, *wecD*, *o416*, *o359*, and *o450* have not been unequivocally demonstrated. Many of the early steps involved in ECA synthesis have been firmly established (Fig. 1A). The ECA trisaccharide repeat unit is assembled as an undecaprenylpyrophosphate (Und-PP)-linked intermediate (lipid III) by the same general mechanism established for the assembly of the repeat units of Wzy-dependent O-antigens (for a review, see reference 35). Although details concerning subsequent steps of ECA assembly have not yet been determined, the available information suggests that these steps occur by the same general mechanism established for the assembly of Wzy-dependent O antigens. In this regard, previous studies led to the conclusion that *wecF* (*o716*) of the *wec* gene cluster encoded the Fuc4NAc transferase involved in ECA synthesis (5, 8). However, recent examinations of the nucleotide sequence of this region revealed that it actually contains two putative open reading frames, *o359* and *o450* (1a). The results of preliminary investigations support the conclusion that *o359* encodes WecF, and the considerable homology between the predicted *o450* gene product and several Wzy proteins (24) suggests that *o450* of the *wec* gene cluster encodes the Wzy protein involved in ECA polysaccharide chain elongation. Thus, it is likely that lipid III is also synthesized on the cytoplasmic face of the inner membrane and subsequently translocated to the periplasmic face of the inner membrane, where assembly of ECA polysaccharide chains occurs by a Wzy-dependent "block polymerization" mechanism.

The chain length of Wzy-dependent O antigens (35, 36), as well as some lipid A-core-linked capsular polysaccharides (10, 11), is regulated by Wzz (Rol, Cld). A number of Wzz homologues have also been identified in a variety of bacteria that are involved in the assembly of bacterial cell surface polysaccharides that are not linked to core-lipid A (36), but none of these homologues has been demonstrated to function in the regulation of polysaccharide chain length. Wzz-mediated regulation results in a modal or multimodal distribution of polysaccharide chain lengths when preparations are analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In contrast, a nonmodal random distribution of chain lengths is observed in *wzz* mutants; however, the mechanism of Wzz-mediated modulation of polysaccharide chain length has not been determined. The degree of polymerization of ECA polysaccharide chains is also modulated, and a single modal distribution of chain lengths has been observed following SDS-PAGE analyses of ECA_{PG} preparations (1, 2, 23). Although the genetic determinant of ECA polysaccharide chain length regulation has not been identified, significant homology between the putative product of *o349* of the *wec* gene cluster and

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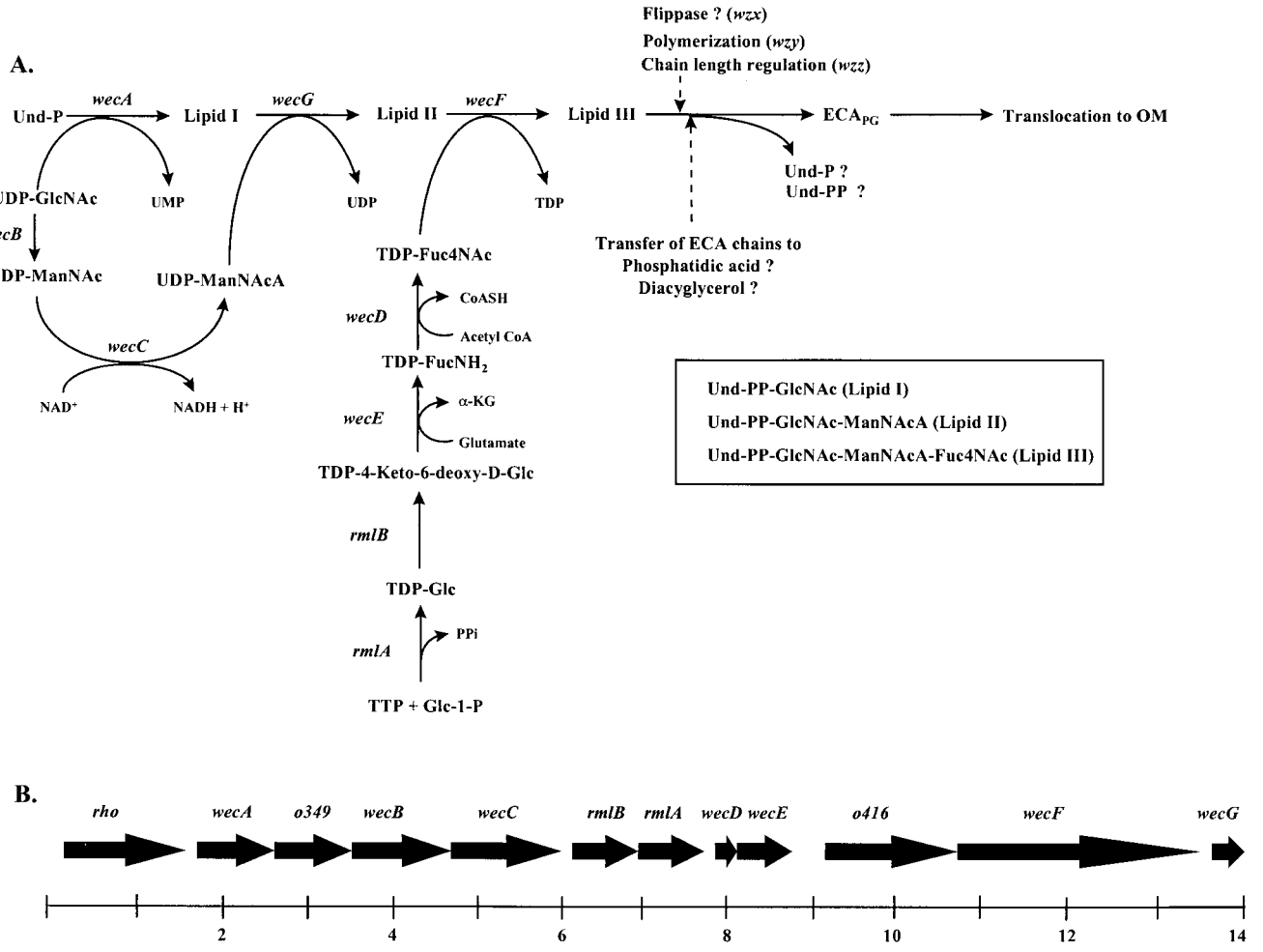

FIG. 1. The ECA biosynthetic pathway and genes of the *wec* gene cluster. (A) Current status of our knowledge concerning the enzymatic reactions involved in the biosynthesis and assembly of ECA. The genetic determinants of the various enzymes are indicated next to the reaction catalyzed by each of the respective enzymes. Abbreviations are as follows: Und-P, undecaprenylmonophosphate; Und-PP, undecaprenylpyrophosphate; Glc, glucose; GlcNAc, *N*-acetyl-D-glucosamine; ManNAc, *N*-acetyl-D-mannosamine; ManNAcA, *N*-acetyl-D-mannosaminuronic acid; Glc-1-P, glucose 1-phosphate; $FucNH_2$, 4-amino-4,6-dideoxy-D-glucose; Fuc4NAc, 4-acetamido-4,6-dideoxy-D-glucose; CoASH, coenzyme A; α-KG, α-ketoglutaric acid; PPi, inorganic pyrophosphate; ECA_{PG}, phosphoglyceride-linked ECA polysaccharide chains. The structures of lipids I, II, and III are defined in the inset. (B) The *wec* gene cluster. Arrowheads designate the direction of transcription. The 14-kb scale below the gene cluster is provided for reference.

various Wzz homologues has been noted (3, 4, 6, 25). The hydropathy profiles of all of these proteins—including that of *o349*—are strikingly similar, and they all possess a large hydrophilic domain that is flanked by apparent transmembrane domains at both the amino and carboxy termini (25). We demonstrate here that *o349* of the *wec* gene cluster of *E. coli* K-12 does indeed encode a Wzz that functions to modulate ECA polysaccharide chain lengths. In addition, the Wzz_{ECA}-mediated modulation of ECA_{PG} polysaccharide chains is the first demonstrated example of Wzz regulation involving a polysaccharide that is not linked to the core-lipid A region of lipopolysaccharide.

Wzz_{ECA} is encoded by *o349* of the *wec* gene cluster. The assembly of ECA polysaccharide chains in *E. coli* AB1133 (23) results in a population of polysaccharide chains whose lengths range from approximately 1 to 14 repeat units with an apparent modal value of 5 to 7 (Fig. 2A, lane 1). A chromosomal *o349*::mini-Tn*lacZ* insertion mutant was constructed by allelic exchange in order to evaluate the role of this gene in the regulation of ECA polysaccharide chain length. Briefly, the transposon Tn*lacZ* (Km^r) was used to generate Tn*lacZ* insertions in the coding region of *o349* by the general strategy of Pradel et al. (26). Plasmid pCA53 (Tc^r Amp^r), a pBR322-based construct containing *wecA* and *o349* on a 2.54-kb *Cla*I insertion fragment (Fig. 3) (22), was introduced by transformation into *E. coli* CC170 (19), which carries a chromosomal copy of Tn*lacZ*. Cells in which transposition of Tn*lacZ* to the plasmid had occurred were selected by subsequent growth of the transformants on Luria-Bertani (LB) agar plates containing tetracycline and ampicillin as well as high levels of kanamycin (300 μg/ml). A pool of plasmid DNA prepared from these cells was used to transform strain CC118 (Lac^-) (19), and kanamycin-resistant transformants were selected on LB plates containing kanamycin as well as 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) to differentiate between cells containing potentially in-frame fusions (blue colonies) from those containing out-of-frame insertions (white colonies). Plasmids were isolated from individual transformants, and those containing Tn*lacZ* insertions in *o349* were identified by restriction mapping. One of these plasmids, pJK2885, contained a Tn*lacZ*

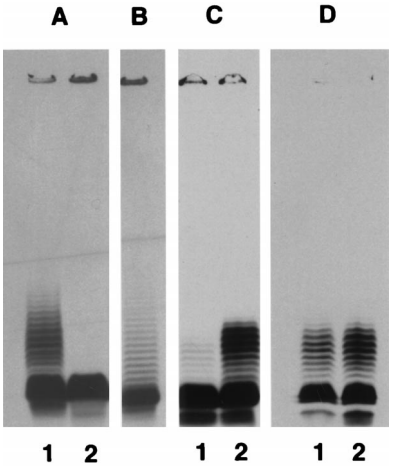

FIG. 2. Effect of mutations in *o349* of the *wec* gene cluster and *waaL* on the modality of ECA polysaccharide chain length. Exponentially growing cultures were incubated with [^{3}H]GlcNAc for 30 min. The incorporation of radiolabeled GlcNAc into ECA was subsequently analyzed by SDS-PAGE and fluorography. (A) Lane 1, strain AB1133 (wild type); lane 2, strain CS2942 (*o349*::mini-Tn*lacZ*). (B) Strain PR4100 (*o349*::mini-Tn*lacZ* + pRL123 [*wecB*$^+$ *wecC*$^+$ *rmlB*$^+$]). (C) Lane 1, strain PR4129 (*o349*::mini-Tn*lacZ* + pRL139 [*o349*$^+$]); lane 2, strain PR4130 (*o349*::mini-Tn*lacZ* + pRL123 [*wecB*$^+$ *wecC*$^+$ *rmlB*$^+$] + pRL139 [*o349*$^+$]). (D) Lane 1, strain AB1133 (wild type); lane 2, strain CS2334 (*waaL*::Tn*phoA*).

insertion in an orientation opposite that of the direction of transcription of the *o349* gene. The insertion in pJK2885 was stabilized by removal of the transposase by digestion with *Xho*I followed by religation to yield plasmid pJK2887 (*o349*::mini-Tn*lacZ*, Km^r).

Introduction of the *o349*::mini-Tn*lacZ* insertion into the *E. coli* K-12 chromosome by allelic exchange was accomplished by transformation of strain VJS803 (*recBC sbcB*) (32) with plasmid pJK2887. Kanamycin-resistant transformants were selected and subsequently screened for sensitivity to ampicillin. The insertion in one of these transformants, strain CS2898 (Km^r Amp^s), was then transduced into strain PR21548 (*wecA*::Tn*10*) (23) by using bacteriophage P1. Kanamycin-resistant transductants were selected, and subsequent screening for sensitivity to tetracycline revealed that all of the kanamycin-resistant transductants were sensitive to tetracycline. The *recA56* allele of JC10240 (*recA56 srl-300*::Tn*10*) (7) was introduced into one of the transductants, CS2900, by conjugation in order to prevent recombination between genes harbored on plasmids and their chromosomal counterparts during subsequent complementation studies. Kanamycin- and tetracycline-resistant transconjugants were selected and screened for sensitivity to UV light. One such UV-sensitive transconjugant, CS2942, was used in all subsequent studies.

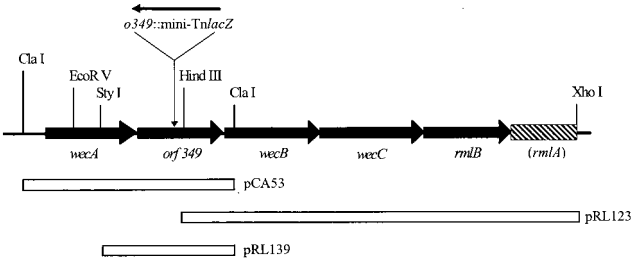

FIG. 3. Physical map of the 5′ half of the *wec* gene cluster. The site and orientation of the mini-Tn*lacZ* insertion in *o349* are indicated. The cross-hatched rectangle for *rmlA* denotes a partial open reading frame. The chromosomal regions contained in plasmids pCA53, pRL123, and pRL139 are indicated by the respective open rectangles.

The precise site of the mini-Tn*lacZ* insertion in the chromosome of strain CS2942 was determined by analysis of the nucleotide sequence of the PCR product obtained by using the *′lacZ* forward primer (5′ GTC ACG ACG TTG TA 3′) and the reverse primer (5′ GCG CTC ACC CAG CAG 3′). The forward primer was complementary to the coding sequence of *′lacZ* at the left end of Tn*lacZ* (19), and the reverse primer was complementary to the flanking nucleotide sequence immediately downstream of the *o349* stop codon (23). Nucleotide sequencing of the PCR product revealed that the mini-Tn*lacZ* was inserted into *o349* between nucleotides 11,400 and 11,401 of the published *wec* gene cluster sequence (GenBank accession no. AE000454).

Wzz_{LPS} function is not required for the synthesis of Wzy-dependent O antigens. In contrast, the null mutation in *o349* resulted in the apparent abrogation of ECA synthesis, as indicated by the lack of incorporation of *N*-acetyl-D-[1-^{3}H]glucosamine into ECA polysaccharide chains in strain CS2942 (*o349*::mini-Tn*lacZ*) (Fig. 2A, lane 2). In vivo incorporation of [^{3}H]GlcNAc (6.6 Ci/mmol) into ECA and analysis of radioactive ECA by SDS-PAGE and fluorography were carried out as previously described (29). The fast-moving broad bands in each lane that migrate immediately ahead of ECA and at the bottom of the gel were not identified; however, they are believed to be radiolabeled lipid A and phospholipid, respectively. Subsequent experiments revealed that the lack of ECA synthesis by strain CS2942 was due to a polar effect of the insertion in *o349* on required downstream genes. The *wecB* and *wecC* genes are located immediately downstream of *o349* (Fig. 1B) (20), and these genes are required for the synthesis of UDP-ManNAcA, the donor of ManNAcA residues for ECA synthesis. In addition, *o349* and the *wecBC* genes appear to be located in the same transcriptional unit (5). Thus, ECA synthesis was restored in strain PR4100, which was constructed by the introduction of plasmid pRL123 containing the *wecBC* genes and the *rmlB* gene into strain CS2942 (Fig. 2B). It should be noted that the $rmlB_{ECA}$ and $rmlA_{ECA}$ genes are located immediately downstream of the *wecC* gene, and these genes are required for synthesis of TDP-Fuc4NAc, the donor of Fuc4NAc residues for ECA synthesis (20). Although the $rmlBA_{ECA}$ genes appear to be located in the same transcriptional unit as *o349* and the *wecBC* genes (5), plasmid pRL123 contained only the $rmlB_{ECA}$ gene (Fig. 3). Thus, it seems likely that synthesis of ECA in strain PR4100 was also dependent on the expression of the chromosomal *rmlA* allele contained in the *wbb* (formerly *rfb*) gene cluster of *E. coli* K-12 (27).

Restoration of ECA synthesis in strain PR4100 resulted in a random nonmodal distribution of ECA polysaccharide chain lengths (Fig. 2B). This distribution pattern is characteristic of the patterns observed for O-antigen synthesis in *wzz* mutants (36). In addition, the phenotype of PR4100 was not due to overexpression of the *wecBC* and *rmlB* genes in pRL123 since introduction of this plasmid into the wild-type parental strain, AB1133, did not result in altered ECA chain length regulation (data not shown). Wild-type modal chain length distribution was rescued in strain PR4130 (wzz_{ECA}::mini-Tn*lacZ*) that contained plasmid pRL123 (*wecBC rmlB*) as well as plasmid pRL139 (*o349*) (Fig. 2C, lane 2). These results clearly support the conclusion that *o349* of the *wec* gene cluster functions as a regulator of ECA polysaccharide chain length; accordingly, we have designated this open reading frame wzz_{ECA}.

A modal distribution of ECA polysaccharide chains was also

observed in strain PR4129, which was constructed by the introduction of only plasmid pRL139 (wzz_{ECA}) into strain CS2942 (Fig. 2C, lane 1). However, the level of [^{3}H]GlcNAc incorporation into ECA chains was significantly reduced in strain PR4129 in comparison to that observed in strain PR4130, which contained plasmid pRL139 (wzz_{ECA}) as well as plasmid pRL123 (*wecBC rmlB*). These observations suggest that wzz_{ECA}::mini-Tn*lacZ* insertion does not completely abolish expression of genes located immediately downstream. Indeed, the apparent absence of radiolabeled chains in strain CS2942 most likely reflects a markedly reduced level of ECA synthesis that was not detected by SDS-PAGE due to the dispersal of the low level of incorporated radioactivity into a randomly distributed population of ECA chains (Fig. 2A, lane 2). However, complementation of the defect in wzz_{ECA} facilitated the detection of the radiolabeled chains by effectively concentrating the radioactivity into a smaller modal population of polymers. It is also important to note that overexpression of wzz_{ECA} in the wild-type parental strain had no effect on either the modality of ECA chains or the amount of incorporation of [^{3}H]GlcNAc into ECA (data not shown). With the exception of the data shown for strains CS2942 (Fig. 2A, lane 2) and PR4129 (Fig. 2C, lane 1), the apparent differences in the amount of radiolabel incorporated into ECA chains primarily reflect variations in the amount of radiolabeled sample analyzed by SDS-PAGE and the length of time the gels were exposed to X-ray film during fluorography.

Wzz_{ECA} regulates the chain length of ECA_{PG} polysaccharides. ECA_{LPS} occurs only in gram-negative enteric bacteria that are able to synthesize a complete R1, R4, or K-12 LPS core structure but that are unable to synthesize O side chains (14, 31, 34). In contrast, all members of the *Enterobacteriaceae* synthesize ECA_{PG}, and ECA_{PG} is the major form of ECA in those organisms capable of synthesizing both ECA_{PG} and ECA_{LPS}. Thus, it has been estimated that less than 5% of *E. coli* K-12 core-lipid A structures are substituted with ECA polysaccharide chains (14). Furthermore, a modal distribution of ECA polysaccharide chains has been observed in extracts obtained from organisms, such as *Salmonella enterica* serovar Typhimurium, that do not synthesize ECA_{LPS} (1).

Taken together, these observations indicate that Wzz_{ECA} is capable of modulating the chain length of ECA_{PG} polysaccharides. These observations also suggest that the data presented in this study primarily reflect the regulation of ECA_{PG}. In order to demonstrate this, the pattern of incorporation of [^{3}H]GlcNAc into ECA polysaccharide chains was examined in strain CS2334 (*waaL*::Tn*phoA*). The transfer of ECA chains from lipid-carrier to core-lipid A receptor requires the same O-translocase (O-ligase) required for translocation of O antigen from lipid-carrier to core-lipid A (14, 30, 31). This enzyme is encoded by the *waaL* gene, and mutations in this gene that preclude synthesis of a functional WaaL are unable to synthesize ECA_{LPS} (31). Radiolabeled GlcNAc was efficiently incorporated into ECA polysaccharide chains in strain CS2334 (Fig. 2D, lane 2), and these chains were distributed in the same modal pattern as were the ECA polysaccharide chains synthesized by wild-type cells (Fig. 2D, lane 1). These data support the conclusion that Wzz_{ECA} does indeed modulate the chain length of ECA_{PG} polysaccharide chains. Furthermore, the data also demonstrate that WaaL is not required for the synthesis of ECA_{PG}. In this regard, it is not known whether the chain length of ECA_{LPS}-linked polysaccharide chains is modulated by Wzz_{ECA} in a manner similar to that observed for ECA_{PG} polysaccharide chains, and experimental approaches to this problem have thus far been hampered by the inability to isolate mutants that are specifically defective in the synthesis of ECA_{PG}. Similarly, water-soluble ECA_{CYC} has been reported to contain four to six trisaccharide repeat units; however, it is not known whether the number of trisaccharide repeat units in this cyclic polymer is regulated in some manner.

The available data support the conclusion that Wzz O-antigen (Wzz_{OAg}) proteins are not O-antigen or species specific; however, modal values appear to be Wzz_{OAg}-dependent (3, 13). In contrast, the activity of Wzz_{ECA} appears to be restricted to the modulation of ECA polysaccharide chain length since the presence of a wild-type wzz_{ECA} gene in mutants that possess a null mutation in Wzz_{OAg} does not complement the defect in O-antigen chain length regulation.

The biochemical mechanism involved in the modulation of polysaccharide chain length is not known; however, two models have been proposed for the assembly of Wzy-dependent O antigens (3, 25). Both of these models suggest that the modulation of O-antigen chain length involves a complex interaction between Wzy_{OAg}, Wzz_{OAg}, WaaL, and lipid-carrier-linked O antigen; however, they differ with regard to the relative importance of interactions between Wzy_{OAg} and either Wzz_{OAg} or WaaL. The results of experiments presented in this study demonstrate that the Wzz_{ECA}-mediated modulation of ECA_{PG} polysaccharide chain length is not dependent on WaaL. The lack of a dependence on WaaL for the Wzz_{ECA}-mediated modulation of ECA_{PG} polysaccharide chain length may indicate that this regulation is primarily dependent on an interaction between Wzz_{ECA} and Wzy_{ECA}. Alternatively, modulation of ECA_{PG} polysaccharide chain length may involve specific interactions between Wzz_{ECA}, Wzy_{ECA}, and an as yet unrecognized protein that is the functional counterpart of WaaL. In this regard, the mechanism involved in the synthesis of the linkage between ECA polysaccharide chains and the phospholipid aglycone of ECA_{PG} has not yet been determined. It seems likely that this reaction involves either the transfer of polysaccharide or polysaccharide-1-phosphate from polysaccharide-pyrophosphorylundecaprenol to phosphatidic acid or diacylglycerol, respectively. In either case, it is possible that the enzyme that catalyzes this reaction functions in agreement with the proposed role of WaaL in the regulation of O-antigen chain length as suggested by Morona et al. (25).

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ADDENDUM IN PROOF

Analysis of the hydropathy profile of Wzz_{ECA}, using the SOAP program of PC/GENE, revealed a large hydrophilic region flanked by two transmembrane regions, TM1 and TM2, located at the amino and carboxy terminus, respectively. This profile is characteristic of Wzz homologs found in other gram-negative bacteria (25). Wzz_{ECA} also contains the proline-rich sequence $R_{306}X_4PX_2PX_4SPRX_2X_7IXGX_3GAG_{339}$ in the carboxy-terminal region, which is in excellent agreement with the consensus sequence $RX_4PX_2PX_4SPKX_2X_7IXGXMXGAG$ found in other Wzz homologs (A. Becker and A. Pühler, J. Bacteriol. **180:**395–399, 1998). Residues predicted to be part of the membrane-spanning helix TM2 are italicized.

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