Assembly of Cyclic Enterobacterial Common Antigen in *Escherichia coli* K-12

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Received 6 May 2005/Accepted 20 June 2005

We describe here the purification and quantification of a water-soluble cyclic form of enterobacterial common antigen (ECA_{CYC}) from *Escherichia coli* K-12 as well as information regarding its subcellular location and the genetic loci involved in its assembly. Structural characterization of purified ECA_{CYC} molecules obtained from *E. coli* K-12 revealed that they uniformly contained four trisaccharide repeat units, and they were substituted with from zero to four *O*-acetyl groups. Cells from overnight cultures contained approximately 2 µg ECA_{CYC} per milligram (dry weight), and cell fractionation studies revealed that these molecules were localized exclusively in the periplasm. The synthesis and assembly of ECA_{CYC} were found to require the *wzxE* and *wzyE* genes of the *wec* gene cluster. These genes encode proteins involved in the transmembrane translocation of undecaprenylpyrophosphate-linked ECA trisaccharide repeat units and the polymerization of trisaccharide repeat units, respectively. Surprisingly, synthesis of ECA_{CYC} was dependent on the *wzzE* gene, which is required for the modulation of the polysaccharide chain lengths of phosphoglyceride-linked ECA (ECA_{PG}). The presence of ECA_{CYC} in extracts of several other gram-negative enteric organisms was also demonstrated; however, it was not detected in cell extracts of *Pseudomonas aeruginosa*. These data suggest that in addition to ECA_{PG}, ECA_{CYC} may be synthesized in many, if not all, members of the *Enterobacteriaceae*.

Enterobacterial common antigen (ECA) is a cell surface glycolipid that is present in all gram-negative enteric bacteria thus far examined (19, 23, 25, 38). The carbohydrate portion of the polymer contains the amino sugars *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-mannosaminuronic acid (ManNAcA), and 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc). These amino sugars are linked to one another to form linear polysaccharide chains that are comprised of the trisaccharide repeat unit → 3-α-D-Fuc4NAc-(1→4)-β-D-ManNAcA-(1→4)-α-D-GlcNAc-(1→ (22, 24). In addition, the hydroxyl groups at position 6 of the GlcNAc residues are nonstoichiometrically substituted with *O*-acetyl groups (11, 22). Individual ECA polysaccharide chains are covalently linked to diacylglycerophosphate via glycosidic linkage between the potential reducing terminal GlcNAc residue and the phosphate residue of the aglycone (19, 20, 36). The polysaccharide chains of ECA obtained from *Escherichia coli* K-12 comprise a modal population of polymers ranging in chain length from 1 to 14 repeat units, with a modal value of 6 to 7 repeat units (2). The phosphoglyceride portion serves to anchor the polymers in the outer leaflet of the outer membrane (1, 39). Accordingly, phosphoglyceride-linked ECA is referred to as ECA_{PG}, and it has been estimated that it accounts for approximately 0.2% of the cellular dry weight of *E. coli* K-12 (21, 24).

The trisaccharide repeat unit of ECA_{PG} is assembled as the lipid carrier-linked intermediate Fuc4NAc-ManNAcA-GlcNAc-pyrophosphorylundecaprenol (lipid III; Fuc4NAc-ManNAcA-GlcNAc-PP-Und) (3, 4, 37, 38). The genes involved in

the assembly of lipid III, as well many of the other genes involved in the utilization of lipid III for the assembly of ECA_{PG}, are located in the *wec* gene cluster at approximately 85.4 min on the *E. coli* chromosome (2, 7, 24, 32). Our current understanding of the pathway of ECA_{PG} biosynthesis and the function of the genes that comprise the *wec* gene cluster have been recently described (12). Steps of the assembly process that are pertinent to this communication are summarized in Fig. 1.

In addition to containing ECA_{PG} on the cell surface, cell extracts prepared from *Shigella sonnei* phase I (11, 22), *Yersinia pestis* (43), and *Plesiomonas shigelloides* contain water-soluble cyclic polysaccharides comprised of ECA trisaccharide repeat units (6, 42). These polysaccharides have been designated cyclic ECA (ECA_{CYC}). ECA_{CYC} polysaccharides lack the phosphoglyceride aglycone of ECA_{PG}, and they are shorter and more uniform with respect to their degree of polymerization than are the polysaccharides of ECA_{PG}. For example, ECA_{CYC} from *Shigella sonnei* phase I contains four to six trisaccharide repeat units (11). More-recent studies have also demonstrated the occurrence of ECA_{CYC} in cell extracts of *E. coli* B and *E. coli* K-12 (12).

Recent reports have suggested that the ECA_{PG} of *Salmonella enterica* serovar Typhimurium functions as a virulence factor for oral infection in mice by contributing to the resistance of the organism to bile salts (33). In contrast, nothing is known concerning the function of ECA_{CYC}. Furthermore, although the trisaccharide repeat units of ECA_{PG} and ECA_{CYC} are assembled by a common biosynthetic pathway (12), subsequent steps involved in the assembly ECA_{CYC} remain to be established. We describe here the separation, purification, and quantification of ECA_{CYC} molecules obtained from soluble extracts of *E. coli* K-12 using reverse-phase high-pressure liq-

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FIG. 1. Pathway for the assembly of ECA_{PG}. The genes that encode the enzymes that catalyze the individual reactions of the pathway are indicated in italics. Abbreviations are as follows: C₅₅-P, undecaprenylphosphate; C₅₅-PP, undecaprenylpyrophosphate; TDP, dTDP.

uid chromatography (HPLC). Examination of soluble fractions obtained from the cytoplasm and periplasm revealed that ECA_{CYC} is a periplasmic component of *E. coli* K-12. Data are also presented that support the conclusion that the synthesis of ECA_{CYC} requires functional *wzxE*, *wzyE*, and *wzze* genes, which encode proteins involved in the transmembrane translocation of lipid III, the polymerization of ECA trisaccharide repeat units, and the regulation of ECA polysaccharide chain length, respectively. ECA_{CYC} was also detected in extracts obtained from several other members of the *Enterobacteriaceae*, thus suggesting the possibility that ECA_{CYC} is a component of all gram-negative enteric bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Cultures were grown at 37°C with vigorous aeration in either Luria-Bertani (LB) broth (29), proteose peptone beef extract broth (40), or M9 minimal medium (29) containing 0.2% glucose (M9-glucose medium) and other required nutrients. Tetracycline, ampicillin, kanamycin, gentamicin, and chloramphenicol were added to media when appropriate to give final concentrations of 10 µg/ml, 50 µg/ml, 15 µg/ml, and 30 µg/ml, respectively. Transductions were carried out using phage P1 *vir* as described by Silhavy et al. (41).

Construction of plasmids. Plasmid pRL147 containing the wild-type *wecA* allele under the control of the pBAD promoter of plasmid pBAD18 was constructed as previously described (35). Plasmid pRL163 containing the wild-type *wecG* gene under the control of the pBAD promoter was constructed as follows. The wild-type *wecG* allele was obtained by PCR amplification using genomic DNA obtained from strain AB1133 as the template. The polynucleotides 5'-CACCATGAATAACAACACACGGC-3' and 5'-AAAGAGAGGAAATC ATAGGTTGCC-3' were used as forward and reverse primers, respectively. The amplified sequence (758 bp) was cloned into the TA cloning site of the pBAD102/D-TOPO vector (Invitrogen) according to the manufacturer's instructions.

Plasmid pRL164 containing the 5'-*wzyE-wecG*-3' genes on a single DNA fragment of 2.1 kb was obtained by PCR amplification of the *wzyE-wecG* sequence using the genomic DNA of strain AB1133 as the template and 5'-CAC CATGAAGTCTGCTGCAATTCAGTGGCCTG-3' and 5'-TCTAGAGTGACC ACTCCGTCGC-3' as forward and reverse primers, respectively. The amplified sequence was cloned into the TA cloning site of the pBAD102/D-TOPO (Invitrogen) according to the manufacturer's instructions, thus placing both genes under the control of the pBAD promoter.

Plasmid pRL165 containing the wild-type *wzxE* gene under the control of the *lac* promoter was obtained by PCR amplification of the genomic DNA of AB1133 using 5'-CTTTGTGAATTCCTTTTCCTGA-3' and 5'-CCCAGTACGTGGATC CGTACAGTC-3' as forward and reverse primers, respectively. EcoRI and BamHI restriction sites were incorporated into the forward and reverse primers (underlined sequences), respectively. The amplified product (1,267 bp) was digested with EcoRI and BamHI, and the product was ligated into the corresponding sites of the plasmid vector, pBAD24 (15), immediately downstream from the vector-encoded Shine-Dalgarno sequence located in the multicloning site of the vector. The cloned fragment, together with the 5' Shine-Dalgarno sequence, was obtained by PCR amplification with 5'-CTACTGTTTATCGATACCCG-3' and 5'-AGCCAAGCTTGCATGCCTGC-3' as forward and reverse primers, respectively (the ClaI restriction site is underlined). The PCR product was digested with ClaI and BamHI and ligated into the corresponding sites in the multicloning site of the expression plasmid pBluescript KS+ immediately downstream from the *lac* promoter. A fragment containing the 5'-*lac* promoter-Shine-Dalgarno sequence-*wzxE* gene-3' was obtained by PCR amplification with 5'-GTGCTG CAAGGCGTTTAAATTG-3' and 5'-CGATTCTTTAAAGCAGCTGGCA-3' as forward and reverse primers, respectively. A DraI restriction site was incorporated into both primers (underlined). The PCR product was then digested with DraI, and the fragment was ligated into the corresponding site of the vector, pACYC184, to yield plasmid pRL165.

Plasmid pRL166 containing wild-type *wzxC* under the control of the *lac* promoter was obtained by PCR amplification using the genomic DNA of strain AB1133 as the template and 5'-GGTTTCGGTACCAAAGCGG-3' and 5'-CTTATCAGGCCTGCAGGTCC-3' as forward and reverse primers, respectively. KpnI and PstI restriction sites (underlined) were incorporated into the forward and reverse primers, respectively. The PCR product (1.6 kb) was digested with KpnI and PstI, and the resulting fragment was ligated into the corresponding sites located immediately downstream of the *lac* promoter in the multicloning site of the expression vector pBluescript KS+. A fragment including the *lac* promoter of the vector and the downstream wild-type *wzxC* gene was obtained from this construct by digestion with the restriction enzyme PvuII, and the fragment was ligated into the PvuII site of the vector pACYC184, to yield plasmid pRL166.

Plasmid pRL169 containing the wild-type *wzxO₁₆* gene under the control of the *lac* promoter was obtained by PCR amplification using the genomic DNA of strain AB1133 as the template and 5'-CTTATAAGCTTAGGAGGAATTGCA TGAATACG-3' and 5'-CATTCGCACAACTGCAGCA-3' as forward and reverse primers, respectively. HindIII and PstI restriction sites were incorporated into the forward and reverse primers, respectively (underlined sequences). In addition, the forward primer also contained a Shine-Dalgarno sequence (bold sequence) located 6 base pairs upstream of the translational start codon (bold italic sequence). The product (1.3 kb) was digested with the restriction enzymes HindIII and PstI, and the resulting fragment was ligated into the corresponding sites located immediately downstream from the *lac* promoter in the multicloning site of the expression vector pBluescript KS+. A fragment (1.7 kb) including the wild-type *wzxO₁₆* gene and the upstream *lac* promoter of the vector was obtained from this construct by PCR amplification using 5'-GTGCTGCAAGGCGTT TAAATTG-3' and 5'-CGATTCTTTAAAGCAGCTGGCA-3' as forward and reverse primers, respectively. A DraI restriction site was incorporated into both of these primers (underlined). The product was digested with the restriction enzymes PvuII and SmaI, and the resulting 1.6-kb fragment containing the 5'-*lac* promoter-Shine-Dalgarno sequence-*wzxO₁₆* gene-3' was ligated into the DraI site of the vector pACYC184, to yield plasmid pRL169.

Mass spectrometry (MS) studies. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectra were obtained using an Applied Biosystems Voyager-DE STR biospectrometry workstation. Calibrations were conducted in the reflector mode using the peptide adrenocorticotrophic hormone. Samples were analyzed using a laser intensity of 2,200. The matrix was 2,5-dihydroxybenzoic acid at a concentration of 10 mg/ml in 20% acetonitrile. Spectra were acquired in the negative mode.

Isolation of soluble periplasmic and cytoplasmic contents. The soluble periplasmic fraction of cells was released by osmotic shock using a modification of the procedure described by Rech et al. (34). Briefly, cells were grown to

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genetic markers or characteristic(s)	Source or reference ^a
Strains		
<i>E. coli</i> K-12		
S0864	F ⁻ <i>trp lacZ strA thi upp</i>	31
S0874	Δ(<i>wzx</i> _{O16} <i>wzc</i>), P2 eductant of S0864 with an extended deletion in the region from <i>udk</i> to <i>his</i>	31
W3110	F ⁻ λ ⁻ IN(<i>rrnD-rrnE</i>) <i>rph-1</i>	CGSC
AB1133	<i>thr-1 leuB6 Δ(gpt-proA)66 hisG4 argE3 thi-1 rfbD1 lacY1 ara-14 galK2 xyl-5 mtl-1 mgl-51 rpsL31 kdgK51 supE44</i>	CGSC
21548	Like AB1133, but <i>wecA::Tn10</i>	27
21568	Like AB1133, but <i>wecG::Tn10</i>	27
14.5	λ ⁻ F ⁻ <i>thr-1 leuB6 tonA31 lacY1 tsx-78 supO eda50 his-4 rfbD1 mgl-51 rpsL136 xyl-5 mtl-1 metF159 thi-1 ara-14 wzcE::cm</i>	P. N. Danese
PR4119	Nonpolar insertion of the gentamicin resistance determinant from pUC19 into the <i>AccI</i> site of the <i>wzyE</i> gene of strain 21548	This study
PR4149	Like S0874, but <i>wecA::Tn10</i> [21548(P1) × S0874]	35
PR4150	Like PR4149, but <i>wzcE::cm</i> [14.5(P1) × PR4149]	35
PR4180	PR4150/pRL147 (with wild-type <i>wecA</i> under the control of the pBAD promoter)	35
PR4210	PR4234/pRL147 (with wild-type <i>wecA</i> under the control of the pBAD promoter)	This study
	pRL165 (with wild-type <i>wzcE</i> under the control of the <i>lac</i> promoter)	
PR4214	PR4237/pRL163 (with wild-type <i>wecG</i> under the control of the pBAD promoter)	This study
PR4216	PR4240/pRL163 (with wild-type <i>wecG</i> under the control of the pBAD promoter)	This study
PR4217	PR4240/pRL164 (with wild-type <i>wzyE-wecG</i> under the control of the pBAD promoter)	This study
PR4218	Like PR4237, but <i>wzcE::km</i> (nonpolar insertion of the <i>aphA-3</i> kanamycin resistance determinant from pUC18K [31] into <i>wzcE</i>)	This study
PR4219	PR4218/pRL163 (with wild-type <i>wecG</i> under the control of the pBAD promoter)	This study
PR4220	Like W3110, but <i>wzcE::km</i> (nonpolar insertion of the <i>aphA-3</i> kanamycin resistance determinant from pUC18K [31] into <i>wzcE</i>)	This study
PR4221	PR4220/pCA53 (wild-type <i>wecA-wzcE</i> on a 2.54-kb <i>ClaI</i> fragment cloned in pBR322)	This study
PR4222	Like PR4237, but <i>wzcE::cm</i> [14.5(P1) × PR4237]	This study
PR4223	PR4222/pRL163 (with wild-type <i>wecG</i> under the control of the pBAD promoter)	This study
PR4224	PR4237/pBAD102/D/ <i>lacZ</i>	This study
PR4225	PR4234/pACYC184	This study
PR4226	PR4220/pBR322	This study
PR4227	W3110/pBR322	This study
PR4228	Like PR4227, but <i>wecA::Tn10</i> [21548(P1) × PR4227]	This study
PR4233	PR4234/pRL147 (with wild-type <i>wecA</i> under the control of the pBAD promoter)	This study
	pRL169 (with wild-type <i>wzx</i> _{O16} under the control of the <i>lac</i> promoter)	
PR4234	Like S0874, but <i>wecA::km</i> (nonpolar insertion of the <i>aphA-3</i> kanamycin resistance determinant from pUC18K [31] into <i>wecA</i>) <i>wzcE::cm</i> [14.5(P1)]	This study
PR4236	PR4234/pRL147 (with wild-type <i>wecA</i> under the control of the pBAD promoter)	This study
	pRL166 (with wild-type <i>wzc</i> under the control of the <i>lac</i> promoter)	
PR4237	Like W3110, but <i>wecG::Tn10</i> [21568(P1) × W3110]	This study
PR4240	Like PR4237, but <i>wzyE::gm</i> [PR4119(P1) × PR4237]	This study
<i>Salmonella enterica</i> serovar Typhimurium	Wild type	Laboratory collection
<i>Shigella dysenteriae</i>	Wild type	ATCC
<i>Shigella flexneri</i>	Wild type	ATCC
<i>Enterobacter cloacae</i>	Wild type	ATCC
<i>Klebsiella pneumoniae</i>	Wild type	ATCC
<i>Proteus vulgaris</i>	Wild type	ATCC
<i>Pseudomonas aeruginosa</i>	Wild type	ATCC
Plasmids		
pBR322	Cloning vector	Promega
pBAD18	Expression vector	15
pBAD24	Expression vector	15
pBAD102/D-TOPO ^R	Expression vector	Invitrogen
pBAD102/D/ <i>lacZ</i>	Expression vector	Invitrogen
pACYC184	Cloning vector	10
pRL147	Wild-type <i>wecA</i> under the control of the pBAD promoter in pBAD18	35
pRL163	Wild-type <i>wecG</i> under the control of the pBAD promoter in pBAD102/D-TOPO ^R	This study

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TABLE 1—Continued

Strain or plasmid	Relevant genetic markers or characteristic(s)	Source or reference ^a
pRL164	Wild-type <i>wzyE-wecG</i> under the control of the pBAD promoter in pBAD102/D-TOPO ^R	This study
pRL165	Wild-type <i>wzxE</i> under the control of the <i>lac</i> promoter cloned in pACYC184	This study
pRL166	Wild-type <i>wzxC</i> under the control of the <i>lac</i> promoter cloned in pACYC184	This study
pRL169	Wild-type <i>wzx</i> _{O16} under the control of the <i>lac</i> promoter cloned in pACYC184	This study
pCA53	Wild-type <i>wecA-wzzE</i> on a 2.54-kb <i>Cla</i> I fragment cloned in pBR322	26

^a CGSC, *E. coli* Genetic Stock Center and M. Berlyn, Biology Department, Yale University, New Haven, CT; ATCC, American Type Culture Collection, Manassas, VA.

stationary phase in LB broth at 37°C. The cells were harvested by centrifugation at 4°C and resuspended in osmotic shock buffer (0.5 M sucrose, 0.1 M Tris-HCl, pH 8.2, and 1 mM EDTA) at a ratio of 5 ml buffer to 1 g (wet weight) of cells. The cells were placed on ice for 10 min and then isolated by centrifugation at 4°C. The supernatant solution was discarded, and the cell pellet was resuspended in 5 mM MgSO₄ and incubated on ice for 10 min. The supernatant solution containing the soluble periplasmic contents was isolated by centrifugation at 12,000 × *g* for 10 min at 4°C, and it was either analyzed immediately or stored at −20°C. The particulate fraction containing the osmotically shocked cells was resuspended in 5 mM MgSO₄ in an ice bath, and the soluble cytoplasmic fraction was released by sonication. Cellular debris was removed by centrifugation at 100,000 × *g* for 1 h at 4°C, and the supernatant solution containing the soluble cytoplasmic contents was either analyzed immediately or stored at −20°C. This procedure resulted in the detection of β-galactosidase and β-lactamase activities only in the cytoplasmic and periplasmic fractions, respectively, when these fractions were obtained from strain PR4227 (W3110/pBR322) grown in the presence of 100 μM isopropylthiogalactoside. The concentrations of protein in cell extracts, cytoplasmic fractions, and periplasmic fractions were determined using the Bio-Rad protein assay reagent according to the procedure recommended by the manufacturer.

Identification and isolation of ECA_{CYC} molecules. To identify ECA_{CYC} molecules in cell extracts, cells were grown in 25 ml of M9-glucose medium at 37°C for 12 h and harvested by centrifugation (10,000 × *g*, 4°C). The cells were washed with 5 mM MgSO₄, resuspended in 10 ml of 5 mM MgSO₄, and disrupted by sonication at 0°C. Cellular debris was removed by centrifugation (10,000 × *g*, 4°C) followed by centrifugation of the supernatant solution at 100,000 × *g* for 1 h at 4°C. Absolute ethanol (1.5 volumes) was added to the supernatant solution obtained from the high-speed centrifugation, and the mixture was incubated at 70°C for 5 min. The resulting precipitate was removed by centrifugation (10,000 × *g*, 4°C), and the supernatant solution was reduced to dryness under vacuum at 0 to 4°C. The residue was resuspended in distilled deionized water (ddH₂O; 1 ml), and particulate matter was removed by filtration using a Millex-GV filter unit (Millipore) containing a 4-mm Durapore polyvinylidene difluoride membrane (0.22-μm pore size). Samples of the filtrate (100 μl) were analyzed by reverse-phase high-pressure liquid chromatography using an Agilent 1100 series instrument and a Nucleosil C₁₈ column (4.6 by 250 mm, 5-μm particle size, 300-Å pore size; Thermo Electron Corp.). Chromatographic analyses were carried out at room temperature with a linear gradient of 0 to 30% acetonitrile in an acidic phosphate solution (50 mM NaOH adjusted to pH 2.0 with 85% phosphoric acid and containing sodium azide at a final concentration of 0.0005%) at a flow rate of 0.5 ml/min. Carbohydrate-containing fractions were detected by their absorbance at 206 nm.

The ECA_{CYC} molecules that eluted in peaks 1 to 5 (Fig. 2A) were collected separately for analysis by MALDI-TOF. The material in these fractions was dried under vacuum and subsequently dissolved in 100 μl of ddH₂O. The samples were desalted by passage through a ZipTip_{C18} pipette tip (Millipore Corp.) using a modification of the procedure recommended by the manufacturer. Briefly, the C₁₈ bed in the pipette tips was wetted with 100% acetonitrile and then equilibrated with 0.1% trifluoroacetic acid (TFA) in ddH₂O. The aqueous samples were adsorbed onto the C₁₈ bed and eluted with 5 μl of 50% acetonitrile in ddH₂O.

Purified ECA_{CYC} molecules in peak 1 (Fig. 2A) were isolated on a preparative scale using reverse-phase HPLC. Strain PR4227 was grown with vigorous aeration in 1 liter of LB broth at 37°C for 12 h, and the cells were harvested by centrifugation (10,000 × *g*, 4°C). Subsequent steps were the same as those described for the identification of ECA_{CYC} in cell extracts, with the exception that the volume of the samples injected onto the Nucleosil C₁₈ column was 300 μl, and the column was developed with a linear gradient of 0 to 15% acetonitrile in the previously described acidic (pH 2.0) phosphate solution. The material in peak 1 (Fig. 2A) was collected and then lyophilized. The residue was

FIG. 2. Detection of ECA_{CYC} molecules in whole-cell extracts as determined by reverse-phase HPLC. (A) HPLC analysis of a whole-cell extract obtained from strain PR4227 (wild-type). (B) HPLC analysis of a whole-cell extract obtained from strain PR4228 (*wecA*::Tn10). (C) HPLC analysis of a whole-cell extract obtained from strain PR4227 following treatment of the extract with mild alkali. Details regarding the preparation of whole-cell extracts and the conditions for treatment of the extract with mild alkali are provided in "Materials and Methods." Peaks 1, 2, 3, 4, and 5 contain ECA_{CYC} molecules containing zero, one, two, three, and four *O*-acetyl substituents, respectively. mAU, milliabsorption units.

TABLE 2. MALDI-TOF MS analysis of ECA_{CYC} molecules purified by reverse-phase HPLC

Peak	Retention time (min)	Molecular ion [M-H] ⁻ (Da)		Proposed structure of parent molecule ^a
		Observed ^b	Predicted	
1	79	2,428.9 ± 1	2,429	[ECA _{CYC}] ₄
2	99	2,471.3 ± 1	2,471	[ECA _{CYC}] ₄ + 1 <i>O</i> -acetyl substituent
3	119	2,515.2 ± 1	2,513	[ECA _{CYC}] ₄ + 2 <i>O</i> -acetyl substituents
4	143	2,555.2 ± 1	2,555	[ECA _{CYC}] ₄ + 3 <i>O</i> -acetyl substituents
5	160	2,597.5 ± 1	2,597	[ECA _{CYC}] ₄ + 4 <i>O</i> -acetyl substituents

^a [ECA_{CYC}]₄ refers to a molecule of cyclic ECA containing four trisaccharide repeat units.

^b Values are means ± standard deviations.

dissolved in 500 µl ddH₂O and desalted by reverse-phase HPLC as described above. Chromatography was carried out at room temperature at a flow rate of 1 ml/min using a linear gradient 0 to 100% acetonitrile in ddH₂O. Following sample injection, the column was washed with ddH₂O for 15 min and then developed with a linear gradient of acetonitrile over a period of 30 min. Column fractions were monitored at 206 nm, and a single component was detected. The fractions containing this component were collected and then dried under vacuum. The dried material from three such preparations was dissolved in 500 µl ddH₂O and stored at -20°C.

Treatment of ECA_{CYC} with alkali. Strain PR4227 was grown in 25 ml of M9-glucose medium at 37°C for 12 h and harvested by centrifugation (10,000 × *g*, 4°C). The cells were washed with 5 mM MgSO₄, resuspended in 10 ml of MgSO₄, and then disrupted by sonication at 0°C. Cellular debris was removed by centrifugation (10,000 × *g*, 4°C), followed by centrifugation of the supernatant solution at 100,000 × *g* for 1 h at 4°C. Absolute ethanol (1.5 volumes) was added to the supernatant solution, and the mixture was incubated at 70°C for 5 min. The resulting precipitate was removed by centrifugation (10,000 × *g*, 4°C), and the supernatant solution was reduced to dryness under vacuum at 0 to 4°C. The residue was dissolved in ddH₂O (500 µl), to which was then added 40 µl of 4 N NaOH. The mixture was incubated for 15 min at 37°C and then acidified by the addition of 200 µl of 4 N HCl. The acidified reaction mixture was next filtered using a Millex-GV filter unit (Millipore) containing a 4-mm Durapore polyvinylidene difluoride membrane (0.22-µm pore size). Aliquots of the filtered reaction mixture were then analyzed by reverse-phase HPLC using the methods described above for the identification of ECA_{CYC} in whole-cell extracts.

Quantification of ECA_{CYC}. The amount of ECA_{CYC} in extracts was determined by establishing the relationship between a given chemical quantity of ECA_{CYC} and the corresponding area of the peak generated by its absorbance at 206 nm when analyzed by reverse-phase HPLC. ECA_{CYC} (peak 1) (Fig. 2A) was purified on a preparative scale as described above. The chemical quantity of the ECA_{CYC} in these preparations was determined by quantifying the amount of glucosamine released from aliquots of these preparations following total acid hydrolysis. Samples containing various amounts of ECA_{CYC} in a total volume of 10 µl were mixed with an equal volume of 8 M TFA and incubated for 7 h at 100°C in a sealed tube. The hydrolysate was then dried under vacuum, washed with isopropanol (40 µl), and then dried under vacuum. The free glucosamine in hydrolysates was analyzed by HPLC as the corresponding *p*-aminobenzoic acid ethyl ester (ABEE) monosaccharide derivative using an ABEE labeling kit (Honen Corp., Tokyo, Japan) according to the manufacturer's instructions. Briefly, samples were dissolved in ddH₂O (10 µl), to which was added ABEE labeling reagent (40 µl). The reaction mixtures were incubated for 1 h at 80°C and then cooled to room temperature. Chloroform (200 µl) and ddH₂O (200 µl) were added to reaction mixtures with mixing, the upper aqueous layer was removed, and aliquots were analyzed for the ABEE-glucosamine derivative by reverse-phase HPLC using a Nucleosil C₁₈ column (250 by 4.7 mm, 300-Å pore size; Thermo Electron Corp.) and a linear gradient of 10% to 50% acetonitrile in 0.02% TFA at a flow rate of 1 ml/min at 45°C. The ABEE derivative of glucosamine was detected by fluorescence monitoring (excitation at 305 nm and emission at 360 nm). A standard curve was constructed with known amounts of *N*-acetyl-*D*-glucosamine that were then subjected to total acid hydrolysis and converted to the ABEE derivative using the procedures described above. This assay was linear over the entire range of GlcNAc concentrations examined (0.08 to 1.28 nmol). The amount of ECA_{CYC} in samples was calculated based on the observation that single ECA_{CYC} molecules in *E. coli* K-12 uniformly contain four trisaccharide repeat units (12) and that each repeat unit contains one GlcNAc residue. Accordingly, a standard curve was constructed relating known amounts of ECA_{CYC} to the areas of their corresponding peaks generated by their absor-

bance at 206 nm when analyzed by reverse-phase HPLC. This assay was linear over the entire range of ECA_{CYC} concentrations examined (110 to 710 pmol).

Passive hemagglutination. The presence of ECA in cell extracts was determined by passive hemagglutination assay with monospecific polyclonal rabbit anti-ECA as previously described (37).

RESULTS

Characterization of ECA_{CYC} molecules in cell extracts. Erbel et al. (12) reported the occurrence of water-soluble ECA_{CYC} molecules in cell extracts of *E. coli* B and *E. coli* K-12. These cyclic polysaccharides were found to contain four ECA trisaccharide repeat units. Earlier studies established that position 6 of GlcNAc residues in linear ECA_{PG} polymers are nonstoichiometrically substituted with *O*-acetyl groups (11, 22). Analyses of the ECA_{CYC} preparations by mass spectrometry indicated that they also contained *O*-acetyl groups, and five molecular species were identified owing to the nonstoichiometric substitution of molecules with up to four *O*-acetyl residues (12). However, the mass spectrometry data obtained in these studies were obtained from unfractionated ECA_{CYC} pre-preparations. The separation of individual molecular species of ECA_{CYC} has not been reported. Thus, we employed reversed-phase high-performance liquid chromatography and MALDI-TOF MS) to separate and identify the five ECA_{CYC} molecular species in extracts of *E. coli* K-12.

As shown in Fig. 2A, five prominent molecular species were detected when extracts of *E. coli* strain PR4227 (wild type, W3110/pBR322) were analyzed by reverse-phase HPLC. Under the conditions employed, these species eluted at 79 min (peak 1), 99 min (peak 2), 119 min (peak 3), 143 min (peak 4), and 160 min (peak 5) in a ratio of approximately 1.0:2.1:4.6:3.5:1.8, respectively. The molecules in these peaks were tentatively identified as ECA_{CYC} molecules since they were water soluble and they were not detected in extracts of strain PR4228, a derivative of strain PR4227 that is unable to synthesize ECA_{CYC} due to a null mutation in the *wecA* gene (Fig. 2B). The relative amounts of the individual species were very similar to those reported by Erbel et al. (12). MALDI-TOF MS analyses of the molecules in peaks 1, 2, 3, 4, and 5 revealed molecular ions ([M-H]⁻) that were in agreement with those predicted to be derived from ECA_{CYC} parent molecules containing four trisaccharide repeat units substituted with zero, one, two, three, and four *O*-acetyl groups (Table 2). The calculated mass of a linear ECA polysaccharide containing

TABLE 3. Amount of ECA_{CYC} in cell fractions

Fraction	ECA _{CYC} ^a (μg/mg [dry wt] of whole cells)
Cell extract ^b	2.04
Periplasm ^c	1.60
Cytoplasm ^c	0.02

^a Fractions were obtained from strain PR4227 grown in LB broth for 12 h at 37°C. The material contained in the fractions was treated with mild alkali and subsequently analyzed by reverse-phase HPLC. Accordingly, the stated values represent the amount of de-*O*-acetylated ECA_{CYC} in each of the fractions. The quantification of ECA_{CYC} is described in Materials and Methods.

^b Cell extracts were prepared as described in Materials and Methods.

^c Periplasmic and cytoplasmic fractions were obtained as described in Materials and Methods.

four trisaccharide repeat units and lacking *O*-acetyl substituents is 2,448 Da.

Further support for the conclusion that ECA_{CYC} molecules in peaks 2, 3, 4, and 5 are *O* acetylated was provided by treatment of the ECA_{CYC} preparation described above with mild alkali. This treatment resulted in the loss of these peaks and was accompanied by a pronounced increase in the amount of ECA_{CYC} molecules devoid of *O*-acetyl groups in peak 1 (Fig. 2C).

Subcellular localization and the cellular content of ECA_{CYC} molecules. Previous estimates of the amount of ECA_{CYC} in cells of *E. coli* B using indirect methods indicated that cells grown overnight in broth contain approximately 2 μg ECA_{CYC} per milligram of dried cells (12). Assuming that ECA_{CYC} molecules have an average molecular mass of 2,513 Da, this amount corresponds to approximately 795 pmol of ECA_{CYC} per milligram (dry weight) of cells. The ECA_{CYC} content in cell extracts prepared from *E. coli* K-12 cells (strain PR4227) that were grown overnight in broth was determined by direct measurement of the amount of de-*O*-acetylated ECA_{CYC} present in extracts following treatment with mild alkali. These results were in excellent agreement with the data obtained from earlier estimates; cell extracts contained approximately 2 μg of ECA_{CYC} per milligram (dry weight) of cells (Table 3). Examination of subcellular fractions revealed that approximately 80% of the ECA_{CYC} was released from the cells by osmotic shock, and essentially no ECA_{CYC} was found in the cytoplasm. The inability to quantitatively recover ECA_{CYC} in the soluble material released by osmotic shock is most likely due to losses incurred during the preparation of this fraction. In addition, no ECA_{CYC} was detected in cell-free culture filtrates (data not shown). These observations support the conclusion that ECA_{CYC} resides exclusively in the periplasm.

The synthesis of ECA_{CYC} was not restricted to exponentially growing cells. Significant synthesis occurred in cells in the late exponential phase of growth and continued for a prolonged period after the cells had entered stationary phase (Fig. 3). Similar results were obtained with cells grown in M9-glucose minimal medium and proteose peptone beef extract broth (data not shown).

The assembly of ECA_{CYC} requires the *wzyE* gene. Lipid III is a precursor for the synthesis of both ECA_{CYC} and the linear polysaccharide chains of ECA_{PG} (12). The available evidence supports the conclusion that lipid III is synthesized on the inner face of the cytoplasmic membrane and subsequently

translocated en bloc across the membrane to the periplasmic face, where it is utilized for the assembly of the linear polysaccharide chains of ECA_{PG}. Recent experiments have provided support for the conclusion that the transbilayer movement of lipid III across the cytoplasmic membrane is mediated by a translocase or “flippase” encoded by the *wzx* gene (35). Subsequent assembly of ECA polysaccharide chains occurs by the “block polymerization” mechanism described for the assembly of *wzy*-dependent *O*-antigen polysaccharides (17), and this reaction is catalyzed by a polymerase encoded by the *wzyE* gene.

The specific steps involved in the utilization of lipid III for the assembly of ECA_{CYC} molecules remain to be established. Thus, initial experiments were conducted to determine if the synthesis of ECA_{CYC} was dependent on the polymerase *WzyE*. Previous attempts to address this question have been hampered by the loss of cell viability that accompanies the insertional inactivation of *wzyE*; this phenotype is believed to be due to the accumulation of lipid III in the cytoplasmic membrane (unpublished results). However, an experimental approach to this problem was made possible by the construction of strains that possessed null mutations in both *wecG* and *wzyE* but that were unaltered in cell growth when they were grown under specific conditions. Null mutations in *wecG* preclude the synthesis of ECA_{CYC} and ECA_{PG} due to the inability of these mutants to synthesize lipid III (Fig. 1). However, experiments with strain PR4214 (*wecG*::Tn10/pRL163 [with wild-type *wecG* under the control of the P_{BAD} promoter]) revealed that the “leaky” or basal-level expression of wild-type *wecG* from the P_{BAD} promoter in the absence of exogenously supplied arabinose is sufficient to rescue the ability of this strain to synthesize both ECA_{CYC} and ECA_{PG} (Table 4). Normal growth accompanied the basal level expression of wild-type *wecG* from the P_{BAD} promoter in strain PR4216, a derivative of strain PR4214 that also possesses a null mutation in *wzyE*; however,

FIG. 3. Synthesis and accumulation of ECA_{CYC} in strain PR4227 (wild type, W3110/pBR322) as a function of growth stage. ■, absorbance at 600 nm; ○, nanomoles of ECA_{CYC} per 100 ml of culture. Additional details are provided in “Materials and Methods.”

TABLE 4. ECA_{CYC} synthesis in *E. coli* K-12 strains containing null mutations in the *wzyE*, *wzzE*, and *wzxE* genes

Enzyme	Strain	Relevant genotype	Synthesis of ECA _{PG} ^a	Concn of ECA _{CYC} ^b (nmol/mg protein)
Polymerase (WzyE)	W3110	Wild type	+	3.69
	PR4237	Like W3110, but <i>wecG</i> ::Tn10	—	ND
	PR4214	<i>wecG</i> ::Tn10/pRL163 (wild-type <i>wecG</i> under the control of the pBAD promoter)	+	5.14
	PR4216	<i>wecG</i> ::Tn10 <i>wzyE</i> :: <i>gm</i> /pRL163 (wild-type <i>wecG</i> under the control of the pBAD promoter)	—	ND
	PR4217	<i>wecG</i> ::Tn10 <i>wzyE</i> :: <i>gm</i> /pRL164 (wild-type <i>wzyE</i> and <i>wecG</i> under the control of the same pBAD promoter)	+	19.7
	PR4224	<i>wecG</i> ::Tn10/pBAD102/D/ <i>lacZ</i>	—	ND
Chain length regulator (WzzE)	PR4220	Like W3110, but <i>wzzE</i> :: <i>km</i>	+	ND
	PR4219	<i>wecG</i> ::Tn10 <i>wzzE</i> :: <i>km</i> /pRL163 (wild-type <i>wecG</i> under the control of the pBAD promoter)	+	ND
	PR4221	<i>wzzE</i> :: <i>km</i> /pCA53 (wild-type <i>wzzE</i>)	+	2.97
	PR4226	<i>wzzE</i> :: <i>km</i> /pBR322	+	ND
Translocase (WzxE)	S0874	Δ(<i>wzx</i> _{O16} <i>wzxC</i>)	+	2.92
	PR4210	Like S0874, but <i>wecA</i> :: <i>km</i> <i>wzxE</i> :: <i>cm</i> /pRL147 (wild-type <i>wecA</i> under the control of the pBAD promoter), pRL165 (wild-type <i>wzxE</i> under the control of the <i>lac</i> promoter)	+	2.84
	PR4236	Like S0874, but <i>wecA</i> :: <i>km</i> <i>wzxE</i> :: <i>cm</i> /pRL147 (wild-type <i>wecA</i> under the control of the pBAD promoter), pRL166 (wild-type <i>wzxC</i> under the control of the <i>lac</i> promoter)	+	1.51
	PR4233	Like S0874, but <i>wecA</i> :: <i>km</i> <i>wzxE</i> :: <i>cm</i> /pRL147 (wild-type <i>wecA</i> under the control of the pBAD promoter), pRL169 (wild-type <i>wzx</i> _{O16} under the control of the <i>lac</i> promoter)	+	1.92
	PR4225	Like S0874, but <i>wecA</i> :: <i>km</i> <i>wzxE</i> :: <i>cm</i> /pACYC184	—	ND
	PR4223	Like W3110, but <i>wecG</i> ::Tn10 <i>wzxE</i> :: <i>cm</i> /pRL163 (wild-type <i>wecG</i> under the control of the pBAD promoter)	+	3.9

^a Determined by passive hemagglutination assay as described in Materials and Methods.^b Amount in cell extracts. The preparation of cell extracts and the quantification of ECA_{CYC} are described in detail in Materials and Methods. ND, not detected.

synthesis of both ECA_{CYC} and ECA_{PG} were abolished. Presumably, the basal level of *wecG* expression results in the synthesis of sufficient amounts of lipid III to support the synthesis of ECA_{CYC} and ECA_{PG} in strains that possess a wild-type *wzyE* allele. However, this level of *wecG* expression does not appear to result in the accumulation of deleterious amounts of lipid III in mutants lacking a functional *wzyE* gene product. Experiments using strain PR4217 (*wecG*::Tn10 *wzyE*::*gm*/pRL164) revealed that the inability to synthesize ECA_{CYC} and ECA_{PG} was complemented by the basal-level expression of the wild-type *wzyE* and *wecG* alleles from a common P_{BAD} promoter on plasmid pRL164. The amount of ECA_{CYC} synthesized by strain PR4217 was approximately fourfold greater than that observed in strain PR4214, and this most likely reflects a gene dosage-related increase in WzyE activity due to the elevated copy number of the pBAD102/D-TOPO vector. These data support the conclusion that a functional polymerase (WzyE) is required for the assembly of ECA_{CYC}.

The assembly of ECA_{CYC} requires the *wzzE* gene. WzyE-mediated polymerization of the linear chains of ECA_{PG} occurs by a mechanism that involves the modulation of polysaccharide chain length to yield a modal population of polymers, and this modulation is mediated by the *wzzE* gene product (2). Null mutations in *wzzE* do not block the synthesis of ECA_{PG} molecules, but they result in the synthesis of ECA_{PG} molecules

having a random nonmodal distribution of polysaccharide chain lengths (2). In contrast to the synthesis of ECA_{PG}, the synthesis of ECA_{CYC} was abolished in strains PR4219 and PR4220, both of which possess an insertion of a nonpolar cassette carrying the *aphA*-3 kanamycin resistance determinant of *Enterococcus faecalis* (28) in the *wzzE* gene (Table 4). As shown for strain PR4221 (*wzzE*::*km*/pCA53) (Table 3), the defect in ECA_{CYC} synthesis due to the null mutation in *wzzE* was rescued by expression of the wild-type *wzzE* allele in plasmid pCA53. These results indicate that *wzyE*-mediated polymerization of ECA trisaccharide repeat units must be accompanied by *wzzE*-mediated chain length regulation as a prerequisite for the assembly of ECA_{CYC} molecules.

The assembly of ECA_{CYC} requires the *wzxE* gene. The dependence of ECA_{CYC} assembly on functional *wzyE* and *wzzE* gene products supports the conclusion that the assembly of ECA_{CYC} molecules from lipid III occurs on the periplasmic face of the cytoplasmic membrane. Therefore, this assembly process should be dependent on the transbilayer movement of lipid III. Accordingly, attempts were made to examine the role of *wzxE* in the assembly of ECA_{CYC}. Previous such attempts have been severely hampered by the lethal phenotype of *wzxE* null mutants. Thus, the arabinose-induced expression of wild-type *wecA* in *E. coli* PR4180 [Δ(*wzx*_{O16} *wzxC*) *wecA*::Tn10 *wzxE*::*cm*/pRL147, wild-type *wecA* under the control of the P_{BAD} promoter] resulted in a pronounced loss of cell viability,

TABLE 5. Presence of ECA_{CYC} in *Gammaproteobacteria*^a

Organism	ECA _{CYC}
<i>Salmonella enterica</i> serovar Typhimurium	+
<i>Shigella dysenteriae</i>	+
<i>Shigella flexneri</i>	+
<i>Enterobacter cloacae</i>	+
<i>Proteus vulgaris</i>	+
<i>Klebsiella pneumoniae</i>	+
<i>Pseudomonas aeruginosa</i>	—

^a The presence or absence of ECA_{CYC} in cell extracts of organisms was determined by reverse-phase HPLC as described in Materials and Methods.

and this was accompanied by the accumulation of lipid III (35). However, normal cell growth, synthesis of ECA_{PG}, and synthesis of wild-type levels of ECA_{CYC} occurred following expression of both the wild-type *wecA* and *wzx* genes under the control of the *P*_{BAD} and *lac* promoters, respectively, in a similar strain, strain PR4210 [$\Delta(wzx_{O16} wzc)$ *wecA::Tn10 wxE::km/pRL147 pRL165* (Table 4)]. This observation is in agreement with the conclusion that WzxE functions as a translocase for the transbilayer movement of lipid III across the cytoplasmic membrane, where it is subsequently utilized for the synthesis of ECA_{CYC}.

It was somewhat surprising to observe that null mutations in *wxE* did not affect the cell viability or the synthesis of ECA_{PG} and ECA_{CYC} in strain PR4223 (*wecG::Tn10 wxE::cm/wild-type wecG* under the control of the *P*_{BAD} promoter) following the expression of *wecG* (Table 4). However, unlike strain PR4180, which was derived from parental strain S0874 [$\Delta(wzx_{O16} wzc)$] (Table 1), strain PR4223 was derived from parental strain W3110, which possesses wild-type *wzx*_{O16} and *wzc* genes; these genes encode the putative translocases involved in the assembly of the O16 O antigen and colanic acid, respectively. These observations suggested the possibility that either Wzx_{O16} or Wzc or perhaps both of these putative translocases are able to mediate the transbilayer movement of lipid III in the absence of functional WzxE. Indeed, as shown by the data obtained with strain PR4236, expression of the wild-type *wzc* gene was able to complement the defects in cell viability, synthesis of ECA_{PG}, and synthesis of ECA_{CYC} due to the null mutation in *wxE* (Table 4). In addition, similar data were obtained with strain PR4233 as a result of the expression of the wild-type *wzx*_{O16} gene in the same genetic background. In both cases, the synthesis of ECA_{CYC} was not fully restored to wild-type levels; however, these data nevertheless suggest that both Wzc and Wzx_{O16} are able to mediate the translocation of lipid III across the cytoplasmic membrane.

Occurrence of ECA_{CYC} in other gram-negative enteric organisms. The lack of a convenient method for the detection of ECA_{CYC} is largely responsible for the fact that ECA_{CYC} has been demonstrated only in the cell extracts of a few gram-negative organisms (6, 11, 22, 42, 43). Accordingly, the possibility that it is present in all members of the *Enterobacteriaceae* has remained open. The recent demonstration of ECA_{CYC} in cell extracts of *E. coli* B and *E. coli* K-12 (12) suggested the possibility of its occurrence among other members of the *Enterobacteriaceae*. A limited survey of other gram-negative enteric bacteria using the assay described here revealed the occurrence of ECA_{CYC} in all of the organisms examined

(Table 5). However, ECA_{CYC} was not detected in the nonenteric gram-negative organism *Pseudomonas aeruginosa*.

DISCUSSION

ECA_{CYC} has been reported to occur in only a few gram-negative enteric organisms owing to the lack of a convenient assay for its detection. The limited survey described here revealed the presence of ECA_{CYC} in all gram-negative enteric bacteria examined. In addition, inspection of complete bacterial genome sequences contained in the National Center for Biotechnology Information database revealed that the *wec* gene cluster is present in all members of the *Enterobacteriaceae*; however, it is not present in other *Gammaproteobacteria*. Thus, there exists the potential for synthesis of ECA_{CYC} in many, if not all, gram-negative enteric bacteria, and it appears likely that it is unique to these organisms.

It is interesting to note that the degree of polymerization of ECA_{CYC} molecules obtained from various gram-negative enteric organisms does not appear to be uniform. Thus, ECA_{CYC} molecules obtained from *Shigella sonnei* phase I have been reported to contain four to six trisaccharide repeat units (11), whereas those of *E. coli* B and *E. coli* K-12 uniformly contain four repeat units (12; this study). However, the basis for this variation is not understood.

It has been previously established that the GlcNAc residues in the linear polysaccharide chains of ECA_{PG} are nonstoichiometrically O acetylated at position 6 (11, 22). Structural analyses of ECA_{CYC} molecules obtained from *E. coli* B and *E. coli* K-12 revealed that they are also O acetylated to various degrees (12). Accordingly, when these organisms were grown in broth cultures, they synthesized ECA_{CYC} molecules that are substituted with from zero to four O-acetyl groups. Although the location of these O-acetyl groups has not been determined, it seems probable that they are also located at position 6 of GlcNAc residues. In this regard, both the degree to which the ECA_{CYC} molecules of *E. coli* K-12 are O acetylated and the total amount of ECA_{CYC} synthesized by cells appear to be highly dependent on the composition of the culture medium in which the cells are grown. The synthesis of molecules containing one to four O-acetyl groups occurs to a much greater degree when cells are grown in M9-glucose minimal medium than when cells are grown in rich broth (unpublished results). In contrast, the synthesis of all ECA_{CYC} species is significantly reduced when cells are grown in MOPS (morpholinepropane-sulfonic acid)-glucose minimal medium (13). The basis for the relationship between ECA_{CYC} synthesis and the composition of the growth medium remains to be established.

Previous studies established that lipid III is used for the synthesis of both ECA_{PG} and ECA_{CYC} (12). The assembly of ECA_{PG} involves translocation of lipid III across the cytoplasmic membrane by the flippase WzxE and its subsequent utilization as a substrate for WzyE-mediated "block polymerization" of trisaccharide repeat units (Fig. 1). In addition, the polymerization process is accompanied by the modulation of polysaccharide chain lengths by WzzE to yield a modal population of linear polymers comprised of approximately 1 to 14 repeat units with an apparent modal value of 5 to 7 (2). The linear polysaccharide chains are then transferred from the lipid carrier to an as-yet-unidentified diacylglycerol-containing ac-

FIG. 4. Proposed model for the assembly of ECA_{CYC} in *Escherichia coli* K-12. It is proposed that WzxE mediates the translocation of lipid III to the periplasmic face of the cytoplasmic membrane. The transmembrane translocation of lipid III is followed by the WzyE catalyzed "block polymerization" of ECA trisaccharide repeat units and cyclization to yield ECA_{CYC} molecules containing four repeat units. The assembly of ECA_{CYC} from lipid III also appears to be dependent on a functional polysaccharide chain length regulator (WzzE); however, the role of WzzE in the assembly process has not been established. The cyclization reaction presumably involves synthesis of an intramolecular glycosidic linkage between the potential reducing terminal GlcNAc residue of Fuc4NAc-ManNAcA-GlcNAc-(Fuc4NAc-ManNAcA-GlcNAc)₃-Fuc4NAc-ManNAcA-GlcNAc-P-P-Und and the hydroxyl group in position 3 of the nonreducing terminal Fuc4NAc residue, thus resulting in the release of undecaprenylpyrophosphate. Neither the putative enzyme that catalyzes this reaction nor its structural gene has been identified. It is possible that the cyclization reaction is dependent on the formation of a complex between the putative "cyclase," WzyE and WzzE. Indeed, this complex may also include WzxE. Alternatively, it is possible that WzyE or WzzE is bifunctional and is able to catalyze the cyclization reaction as well as polymerization or chain length regulation, respectively. In either case, the assembly of ECA_{CYC} from lipid III may be dependent on the formation of a membrane-bound complex that includes some or all of these proteins.

ceptor to yield ECA_{PG}; however, the mechanism of this reaction remains to be established. Data obtained in the current study demonstrate that ECA_{CYC} is also assembled by a mechanism that requires WzxE- and WzyE-mediated transmembrane translocation and polymerization, respectively. Figure 4 depicts a proposed model for the assembly of ECA_{CYC} that is based on the currently available data. Accordingly, following the WzxE-mediated translocation of lipid III across the cytoplasmic membrane, subsequent reactions involve the WzyE-mediated polymerization of trisaccharide repeat units and cyclization. Nothing is known concerning the mechanism of the cyclization reaction or the enzyme that catalyzes this reaction. It is possible that cyclization involves the formation of an *O*-glycosidic linkage as a result of the transfer of the potential reducing terminal GlcNAc residue from an undecaprenylpyrophosphate-linked linear polysaccharide chain to the nonreducing terminal Fuc4NAc residue of the same chain. As previously stated, the ECA_{CYC} molecules synthesized by *E. coli* B and *E. coli* K-12 uniformly contain four trisaccharide repeat units (12). Thus, in the case of the mechanism proposed above, close coordination between the cyclization and polymerization reactions would be necessary in order to maintain stringent control of the degree of polymerization. Although this model predicts that lipid III is a precursor of ECA_{CYC}, a direct precursor-product relationship has not yet been demonstrated.

Quite surprisingly, our studies revealed that synthesis of ECA_{CYC} is dependent on WzzE, the modulator of ECA polysaccharide chain length. Although two models have been proposed for how Wzz proteins function to regulate the chain length of lipopolysaccharide O antigens (5, 30), essentially

nothing is known concerning the mechanism of this process. Similarly, the mechanism involved in the WzzE-mediated modulation of the chain lengths of ECA_{PG} polysaccharides remains to be established. Null mutations in *wzzE* result in a random nonmodal distribution of ECA_{PG} polysaccharide chains in which the amount of polymer of a given chain length appears to be inversely related to chain length (2). Thus, although ECA_{PG} molecules containing polysaccharides comprised of four repeat units are relatively more abundant than ECA_{PG} molecules with polysaccharides having greater degrees of polymerization, the abundance of ECA_{PG} molecules, regardless of their polysaccharide chain length, appears to be dramatically reduced in *wzzE* null mutants. Accordingly, it is possible that the cyclization reaction involved in ECA_{CYC} synthesis is essentially abolished in *wzzE* null mutants as a result of a decreased availability of undecaprenylpyrophosphate-linked polysaccharide substrate. Alternatively, the activity of the enzyme responsible for the cyclization reaction may be dependent on the formation of a complex between WzyE and WzzE. Indeed, this complex may also include WzxE. As previously stated, neither the enzyme that catalyzes the cyclization reaction nor its structural gene has been identified. The available data do not exclude the possibility that either WzyE or WzzE is bifunctional and is able to catalyze the cyclization reaction as well as polymerization or modulation of polysaccharide chain length, respectively.

Previous studies determined that the flippases involved in the assembly of Wzy-dependent O antigens appear to have relaxed specificities for the structure of the oligosaccharide moiety of their substrates (14). This conclusion is supported by

the data presented in this study that demonstrate that the Wzx and Wzx_{O16} translocators involved in the assembly of colanic acid and the O16 O antigen of *E. coli*, respectively, also appear able to translocate lipid III across the cytoplasmic membrane *in vivo*. However, the extent to which these translocators function *in vivo* for lipid III transbilayer movement is not clear. In contrast, the available data suggest that the substrate specificity of Wzx is rather stringent. Thus, Wzx appears to function only *in vivo* for the translocation of lipid III.

The dependence of ECA_{CYC} synthesis on Wzx, Wzy, and Wzz clearly support the conclusion that this polymer is assembled in the periplasm. The data presented here also support the conclusion that ECA_{CYC} resides in the periplasm; it was not detected in either in the cytoplasm or in culture filtrates. It is not known at what stage in the assembly of ECA_{CYC} that the O acetylation of GlcNAc residues occurs. It seems likely that acetyl-coenzyme A is the donor of acetyl substituents, and it is possible that these groups are transferred to lipid III molecules prior to their Wzx-mediated translocation across the cytoplasmic membrane. Alternatively, O acetylation of nascent or completed ECA_{CYC} molecules on the periplasmic side of the membrane might occur in a manner similar to that proposed for the transfer of succinyl residues by MdoC (8). In either case, the enzyme responsible for catalyzing O acetylation remains to be identified.

It has recently been reported that the resistance of *Salmonella enterica* serovar Typhimurium to bile salts is dependent on the ability of cells to synthesize ECA (33). However, it was not determined if this resistance was related to the occurrence of ECA_{PG} or ECA_{CYC} or to the presence of both forms of ECA. Data obtained in the present study revealed that the synthesis of ECA_{CYC} is abolished in null mutants that lack the chain length regulator Wzz. In contrast, the synthesis of ECA_{PG} is not abolished in these mutants, but its assembly in these mutants occurs without chain length modulation (2). These mutants possess wild-type levels of resistance to bile salts (unpublished results). Therefore, it seems likely that the resistance of gram-negative enteric bacteria to bile salts is unrelated to the occurrence of ECA_{CYC} in the periplasm, but it appears to be due to the presence of ECA_{PG} in the outer membrane.

The function of ECA_{CYC} in gram-negative enteric bacteria is not known. In some respects ECA_{CYC} appears to be similar to the osmoregulated periplasmic glucans (OPGs) synthesized by many gram-negative *Proteobacteria* (8). Although there are considerable differences in the structures of the OPGs synthesized by various species of *Proteobacteria*, all OPGs have the following characteristics: (i) glucose is the only carbohydrate component, (ii) all are oligosaccharides containing from 5 to 24 glucose residues that are primarily linked through β -glycosidic linkages, and (iii) they are localized exclusively in the periplasm. In addition, the OPGs of many *Proteobacteria* are cyclic glucans; however, several species synthesize linear glucans. Perhaps the best known of the linear OPGs are the membrane-derived oligosaccharides that were first discovered in *E. coli* (18). A general characteristic of the OPGs synthesized by the vast majority of *Proteobacteria*, regardless of their structure, is that their concentrations within the periplasm increase in response to decreases in the osmolarity of the environment (8). In this regard, the cyclic 1,2- β -glucan synthe-

sized by *Brucella abortus* appears to be an exception (16). The function of OPGs has not been clearly defined, and mutants unable to synthesize OPGs display widely varied phenotypes (8). Nevertheless, the periplasmic location and cyclic structures of ECA_{CYC} and many OPGs make it tempting to speculate that they have similar functions. However, this appears unlikely since the composition and other structural features of ECA_{CYC} and cyclic OPGs differ markedly, and the concentration of ECA_{CYC} within the periplasm is considerably less than is the case for MDO and other OPGs. Assuming that *E. coli* cells growing in a medium of low osmolarity have a periplasmic volume of 0.31 μ l per milligram (dry weight) (9), the concentration of ECA_{CYC} in the periplasm is about 2.5 mM. In contrast, the periplasmic concentration of membrane-derived oligosaccharides in cells grown in medium of low osmolarity is about 50 mM (18). Furthermore, the concentration of ECA_{CYC} within the periplasm does not appear to be affected by the osmolarity of the environment (unpublished results). Finally, the available data suggest that ECA_{PG} and ECA_{CYC} are synthesized only in gram-negative enteric bacteria. Therefore, unlike OPGs, which are found in a wide variety of gram-negative bacteria, it appears that ECA_{CYC} serves an as-yet-unknown function that is unique to members of the *Enterobacteriaceae*.

ACKNOWLEDGMENTS

This research was supported by NIGMS grant GM52882.

We acknowledge the generous gift of *Escherichia coli* strain 14.5 (*wzx::cm*) from Paul N. Danese. We also thank Colleen Rogers, Kathy Barr, James Hsu, and Kenneth Gable for their helpful suggestions and support. Finally, we acknowledge Michael Flora for his excellent assistance in obtaining the MALDI-TOF data for this study.

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