O Acetylation of the Enterobacterial Common Antigen Polysaccharide Is Catalyzed by the Product of the *yiaH* Gene of *Escherichia coli* K-12[∇]

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The carbohydrate component of the enterobacterial common antigen (ECA) of *Escherichia coli* K-12 occurs primarily as a water-soluble cyclic polysaccharide located in the periplasm (ECA_{CYC}) and as a phosphoglyceride-linked linear polysaccharide located on the cell surface (ECA_{PG}). The polysaccharides of both forms are comprised of 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 trisaccharide repeat units with the structure →3-α-D-Fuc4NAc-(1→4)-β-D-ManNAcA-(1→4)-α-D-GlcNAc-(1→. The hydroxyl group in the 6 position of the GlcNAc residues of both ECA_{CYC} and ECA_{PG} are nonstoichiometrically esterified with acetyl groups. Random transposon insertion mutagenesis of *E. coli* K-12 resulted in the generation of a mutant defective in the incorporation of *O*-acetyl groups into both ECA_{CYC} and ECA_{PG}. This defect was found to be due to an insertion of the transposon into the *yiaH* locus, a putative gene of unknown function located at 80.26 min on the *E. coli* chromosomal map. Bioinformatic analyses of the predicted *yiaH* gene product indicate that it is an integral inner membrane protein that is a member of an acyltransferase family of enzymes found in a wide variety of organisms. The results of biochemical and genetic experiments presented here strongly support the conclusion that *yiaH* encodes the *O*-acetyltransferase responsible for the incorporation of *O*-acetyl groups into both ECA_{CYC} and ECA_{PG}. Accordingly, we propose that this gene be designated *wecH*.

The phosphoglyceride-linked form of enterobacterial common antigen (ECA) (ECA_{PG}) is a glycolipid located on the cell surface of all gram-negative enteric bacteria (23, 29, 31, 44, 45). The carbohydrate portion of ECA_{PG} consists of a linear polysaccharide comprised of 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 trisaccharide repeat units with the structure →3-α-D-Fuc4NAc-(1→4)-β-D-ManNAcA-(1→4)-α-D-GlcNAc-(1→ (28, 30). In addition, the 6 position of the GlcNAc residues in the trisaccharide repeat units are nonstoichiometrically replaced with *O*-acetyl groups (14, 28). The polysaccharide chains are covalently linked to diacylglycerophosphate via the glycosidic linkage of the potential terminal reducing GlcNAc residue to the phosphate moiety of the phosphoglyceride (23, 24, 42). The phosphoglyceride aglycone is an integral component of the outer leaflet of the outer membrane, and thus, it serves to anchor the polysaccharide chains to the surface of the cell.

A water-soluble cyclic form of ECA (ECA_{CYC}) has also been demonstrated to be present in many gram-negative enteric bacteria (7, 14, 28, 48), and the available data suggest that ECA_{CYC} may indeed occur in all members of the *Enterobac-*

teriaceae (19). Recent studies have demonstrated that ECA_{CYC} is located exclusively in the periplasm of *Escherichia coli* K-12, and cells from cultures grown overnight were found to contain approximately 2 μg of ECA_{CYC} (dry weight) per milligram (19). Structural characterization of the ECA_{CYC} molecules isolated from *E. coli* K-12 revealed that they uniformly consist of four trisaccharide repeat units, and each molecule of ECA_{CYC} contains from zero to four *O*-acetyl groups (15, 19). Similar to ECA_{PG}, the *O*-acetyl groups of ECA_{CYC} are also linked to the 6 position of GlcNAc residues (14).

It has recently been reported that the ECA_{PG} of *Salmonella enterica* serovar Typhimurium functions as a virulence factor for oral infection in mice by rendering the organism more resistant to bile salts (40). Similarly, ECA_{PG} appears to be required for the resistance of *E. coli* K-12 to bile salts and short-chain fatty acids (unpublished results). However, the role of ECA_{PG} in the resistance of these organisms to these compounds remains to be established. Furthermore, it is not known if ECA_{PG} has a similar function in other gram-negative enteric bacteria. In contrast, there are no reports concerning the function of ECA_{CYC}. In this regard, the periplasmic location and cyclic structure of ECA_{CYC} are similar to those of the osmoregulated periplasmic glucans synthesized by many gram-negative *Proteobacteria* (11). However, unlike the osmoregulated periplasmic glucans, the synthesis of ECA_{CYC} does not appear to be osmoregulated (unpublished results).

Many of the genes and enzymes involved in the synthesis and assembly of both ECA_{PG} and ECA_{CYC} have been identified, and this information has been described in detail in previous reports (4, 5, 15, 19, 43, 44). Briefly, most of the genes known to be involved in the assembly of ECA polysaccharide chains

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genetic markers or characteristic(s)	Source or reference ^a
Strains		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA</i>	Bethesda Research Laboratories
W3110	F ⁻ λ - IN(<i>rrnD-rrnE</i>) <i>rph-1</i>	CGSC
AB1153	<i>thr-1 leuB6</i> (<i>gpt-proA</i>)66 <i>hisG4 argE3 thi-1 rfbD1 lacY1 ara-14 galK2 xyl-5 mtl-1 mgl-1 rpsL31 kdgK51 supE44</i>	CGSC
EC100	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 endA1 araD139</i> Δ (<i>ara leu</i>)7697 <i>galU galK</i> λ ⁻ <i>rpsL nupG</i>	Epicenter
EC100D	As EC100 but <i>pir</i> ⁺ (dihydrofolate reductase)	Epicenter
KM32	<i>argE3 his-4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 mtl-1 xyl-1 thi-1 rpsL-31 tsx-33 supE44</i> Δ (<i>recC ptr recB recD</i>): <i>P_{lac}-gam-bet-exo cat</i>	38
PR4246	As W3110 but <i>yiaH</i> ::KAN-2 [PR4254 (P1) $\times$ W3110]	This study
PR4254	As KM32 but <i>yiaH</i> ::KAN-2	This study
PR4275	EC100/pJun3	This study
PR4276	As PR4275 but <i>yiaH</i> ::KAN-2	This study
PR4279	EC100D/pRL170	This study
PR4300	PR4246/pRL180	This study
Plasmids		
pGEM-T Easy	PCR cloning vector	Promega
pQE30	Expression vector	QIAGEN
pBR322	Cloning vector	Promega
pJun1	3.73-kb BamHI-HindIII fragment of pRL105 ^b cloned into pBR322	This study
pJun3	Wild-type <i>wec</i> gene cluster on a 12.78-kb EcoRI-BamHI fragment cloned into pBR322	This study
pRL170	Rescue plasmid containing <i>yiaH</i> ::KAN-2 mutant allele	This study
pRL171	Wild-type <i>yiaH</i> gene on a 1.14-kb PCR fragment cloned into pGEM-T Easy	This study
pRL172	<i>yiaH</i> ::KAN-2 on a 3.14-kb PCR fragment cloned into pGEM-T Easy	This study
pRL179	Wild-type <i>yiaH</i> on a 1.10-kb fragment cloned in pGEM-T Easy	This study
pRL180	Wild-type <i>yiaH</i> on a 1.10-kb BamHI-HindIII fragment cloned into pQE30	This study

^a CGSC, *E. coli* Genetic Stock Center and Mary Berlyn, Yale University, New Haven, Conn.

^b See reference 34.

are located in the *wec* gene cluster, which includes 12 genes located at 85.4 min on the *E. coli* chromosome (3, 10, 30, 39). The trisaccharide repeat units of both forms of ECA are assembled by a common pathway on the cytoplasmic face of the cytoplasmic membrane as the undecaprenyl-linked intermediate Fuc4NAc-ManNAcA-GlcNAc-pyrophosphorylundecaprenol (lipid III) (15). Lipid III is then translocated en bloc to the periplasmic face of the membrane by a “flippase” encoded by the *wzxE* gene, and the repeat units are subsequently polymerized by a “block polymerization” mechanism catalyzed by the *wzyE* gene product (6, 19, 41). However, details regarding several other important steps in the assembly of ECA_{PG} and ECA_{CYC} remain to be established. These include the transfer of polysaccharide chains to the phosphoglyceride aglycone to form ECA_{PG}, the subsequent translocation of ECA_{PG} to the outer membrane, the utilization of lipid III for the formation of ECA_{CYC}, and the incorporation of *O*-acetyl groups into both ECA_{PG} and ECA_{CYC}. Although the structural genes for the enzymes that catalyze these reactions have not been identified, it is clear that these genes do not reside within the *wec* gene cluster.

The present study employed random insertion mutagenesis of *E. coli* K-12 in an attempt to identify null mutants defective in the utilization of lipid III for the assembly of ECA_{CYC}. This approach resulted in the isolation of a mutant that was found to be incapable of incorporating *O*-acetyl groups into ECA_{CYC}. Further characterization of the mutant revealed that it was also defective in the *O* acetylation of the polysaccharide chains of ECA_{PG}. The mutant was found to contain an insertion in the

yiaH locus, a putative gene of unknown function. The data presented here support the conclusion that *yiaH* encodes the acyltransferase responsible for the incorporation of *O*-acetyl groups into the ECA polysaccharide chains of both ECA_{CYC} and ECA_{PG}.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Cells were grown at 37°C in Luria-Bertani (LB) broth (36), on LB agar plates (36), in proteose peptone beef extract broth (46), or in M9 minimal medium (36) containing 0.2% glucose (M9-glucose medium) as indicated. SOC medium was prepared as described previously by Miller (36). Tetracycline, ampicillin, kanamycin, and chloramphenicol were added to media when appropriate to give final concentrations of 10 μ g/ml, 50 μ g/ml, 30 μ g/ml, and 30 μ g/ml, respectively. Transductions were carried out using phage P1 *vir* as described previously by Silhavy et al. (47).

Construction of plasmids. The 12.78-kb BamHI-EcoRI nucleotide fragment of plasmid pJun3 containing the entire *wec* gene cluster was assembled from individual smaller nucleotide fragments contained in three different plasmid constructs. The initial step in this assembly process was the restriction enzyme digestion of plasmid pRL105 (34) with enzymes BamHI and HindIII followed by the ligation of the 3.73-kb product into the corresponding sites of plasmid pBR322 to yield plasmid pJun1. The nucleotide fragments containing the remaining genes were subsequently incorporated by the successive ligation of two nucleotide fragments obtained by the digestion of plasmids pCA32 and pCA53 (34) with the restriction enzymes HindIII-ClaI and ClaI-EcoRI, respectively. Plasmid pRL172 containing the *yiaH*::KAN-2 insertion was obtained by PCR amplification using *Taq* polymerase (QIAGEN), the genomic DNA of strain PR4276 as the template, and 5'-CGGAAGGTATAACCGCGCAT-3' and 5'-CATCGGCCCAAAGTAAAGA-3' as forward and reverse primers, respectively. The 3.14-kb product was ligated into the PCR multiple cloning site of the pGEM-T Easy vector (Promega). The 1.10-kb nucleotide insert of plasmid

pRL179 containing the wild-type *yiaH* gene was obtained by PCR amplification of this gene using *Taq* polymerase, the genomic DNA of strain AB1133 as the template, and 5'-GGATCCATGCAGCCCAAAATTAC-3' and 5'-AAGCTTAAAATATTCGTGATGCCGGA-3' as forward and reverse primers, respectively. BamHI and HindIII restriction sites were incorporated into the forward and reverse primers, respectively (underlined sequences). The PCR product was ligated into the PCR multiple cloning site of the pGEM-T Easy vector. Plasmid pRL180 was constructed by digestion of pRL179 with restriction enzymes BamHI and HindIII followed by ligation of the resulting 1.10-kb nucleotide fragment into the corresponding sites located in the multicloning site of the expression vector pQE30 (QIAGEN).

Isolation of the *yiaH* insertion mutant. Random transposon mutagenesis of *E. coli* strain PR4275 was carried out using the EZ::TN <R6K γ ori/KAN-2> Tnp transposome kit (Epicenter) according to the manufacturer's instructions. Following the electroporation of the transposome into the cells (50 μ l), the entire mixture was immediately added to 950 μ l of SOC medium and incubated for 1 h at 37°C with vigorous aeration. Randomly generated insertion mutants were then selected by plating the cells onto LB agar plates containing kanamycin, and the plates were incubated overnight at 37°C.

Approximately 3,000 individual kanamycin-resistant colonies were screened for defects in the synthesis of ECA_{CYC} as determined by the absence of ECA_{CYC} in the soluble periplasmic fraction released by osmotic shock. The cells from individual colonies were used to inoculate the wells of 96-well multititer plates (Microtest U-Bottom 35-1177; Falcon), each of which contained 200 μ l of LB agar containing kanamycin. The plates were then covered and incubated overnight at 37°C with gentle shaking. The plates were then subjected to centrifugation (3,000 rpm) for 10 min at 4°C using an Eppendorf 5810R centrifuge, and the supernatant solution in each well was discarded. The cell pellets were resuspended in 100 μ l of osmotic shock buffer (0.5 M sucrose, 0.1 M Tris-HCl [pH 8.2], 1 mM EDTA) and incubated for 10 min at 4°C. The plates were then once again subjected to centrifugation as described above, and the supernatant solutions were discarded. The cell pellets were resuspended in 100 μ l of 5 mM MgSO₄ and incubated at 4°C for 10 min followed by centrifugation of the plates as described above. An aliquot (25 μ l) of the soluble periplasmic fraction from each well was then removed, transferred to the well of a fresh multititer plate, and assayed for the presence of ECA_{CYC} using the passive hemagglutination inhibition (PHI) assay.

Passive hemagglutination inhibition assays. Soluble periplasmic fractions (25 μ l) contained in the wells of multititer plates were mixed with 0.9% saline (15 μ l), monospecific polyclonal rabbit anti-ECA antiserum (10 μ l) (43), and ECA_{PG}-coated sheep erythrocytes (50 μ l). The resulting suspensions were incubated for 1 h at 37°C, and the presence of ECA_{CYC} was detected by its ability to inhibit hemagglutination as determined by visual inspection. ECA_{PG}-coated erythrocytes were prepared using soluble whole-cell extracts obtained from *E. coli* AB1133 as previously described (43), with the exception that sheep erythrocytes were used rather than human erythrocytes. Aliquots of sheep erythrocytes in Alsevers solution (Lampire Biological Laboratories, Pipersville, PA) were washed with 0.9% saline and then resuspended in 0.9% saline to their original volume immediately prior to use.

A modification of the passive hemagglutination inhibition assay described above was used to compare the immunoreactivities of ECA_{CYC} isolated from whole-cell extracts obtained from the *yiaH*::KAN-2 insertion mutant and the wild-type parental strain (PR4275). The total concentration of ECA_{CYC} in samples was adjusted to a final concentration of 5×10^2 pmol/ μ l by the addition of 5 mM MgSO₄. Serial twofold dilutions of the sample in 5 mM MgSO₄ were then prepared to give final concentrations ranging from 5×10^2 pmol/ μ l to 0.049 pmol/ μ l. The appropriate amounts of each of these samples were then added to empty wells in a multititer plate to give a row of wells containing serial twofold decreasing amounts of ECA_{CYC} that ranged from 100 pmol to approximately 0.1 pmol. The volume in each well was then adjusted to a volume of 40 μ l by the addition of 0.9% saline. This was followed by the addition of monospecific polyclonal rabbit anti-ECA antiserum (10 μ l) (43) and ECA_{PG}-coated sheep red blood cells (50 μ l), respectively. The samples were then incubated at 37°C for 1.5 h with gentle shaking, and hemagglutination was determined by visual inspection.

Rescue cloning. Chromosomal DNA was isolated from mutant strain PR4276 by using a MasterPure complete DNA purification kit (Epicenter) according to the manufacturer's instructions. The purified DNA was digested with restriction enzyme EcoRI overnight at 37°C, and the resulting fragments were self-ligated using Fast-Link DNA ligase (Epicenter). The self-ligated products were transformed into electrocompetent cells of *E. coli* EC100D (*pir*⁺) by electroporation, and stable transformants were selected on LB agar plates containing kanamycin. The EZ::TN <R6K γ ori/KAN-2> transposon carries the R6K γ ori origin of rep-

FIG. 1. 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 PR4275 (wild type). (B) HPLC analysis of a whole-cell extract obtained from strain PR4276 (mutant). Details pertaining to the preparation of whole-cell extracts and the methods employed for analyses of these extracts by reverse-phase HPLC have been previously described (19). Peaks 1, 2, 3, 4, and 5 contain ECA_{CYC} molecules that possess zero, one, two, three, and four *O*-acetyl substituents, respectively. mAU, milli-absorption units.

lication; the replication of plasmids containing this origin of replication requires host cell expression of the *pir* gene product π . Accordingly, only those clones that possess closed fragments containing the EZ::TN <R6K γ ori/KAN-2> transposon are able to grow in the presence of kanamycin. Plasmid DNA (pRL170) was isolated from one of the kanamycin-resistant transformants (strain PR4269), and the DNA was sequenced bidirectionally using the forward and reverse primers KAN-2 FP-1 and R6KAN-2 RP-1 (Epicenter), respectively, which are homologous to the ends of the transposon.

Isolation and quantification of ECA_{CYC}. The isolation of ECA_{CYC} from whole-cell extracts, its quantification by reverse-phase high-pressure liquid chromatography (HPLC), and the isolation of ECA_{CYC} molecules in peak 1 (Fig. 1B) on a preparative scale were carried out as previously described (19).

Isolation and purification of ECA_{PG}. The isolation and purification of ECA_{PG} were accomplished by a modification of the procedure described previously by Lugowski and Romanowska (27). Cells were grown overnight at 37°C in 20 liters of proteose peptone beef extract broth containing 0.4% glucose with vigorous aeration in a BioFlo 5000 fermentor (New Brunswick Scientific) and then harvested by tangential-flow filtration using a Pellicon Cassette filtration system (Millipore). The cells were washed with deionized water, and the concentrated cell suspension was lyophilized. The dried cells were resuspended in 250 ml of 0.05 M phosphate buffer (pH 7.0) containing 0.005 M EDTA and 0.1% lysozyme, and they were then disrupted by sonication. Particulate matter was removed by centrifugation, and absolute ethanol was added to the supernatant to give a final ethanol concentration of 85%. The ethanol-containing supernatant solution was incubated overnight at 20°C, and the resulting precipitate was removed by filtration. The supernatant solution was evaporated to dryness at 30°C under a vacuum, and the dry residue was dissolved in 10 ml of 85% ethanol. Acetone was

then added to the solution to give a final concentration of 85%, and the mixture was incubated for 24 h at 20°C. The resulting precipitate was isolated by centrifugation, dissolved in 1 ml of 85% methanol, and applied to the bed of a DEAE cellulose column (1.5 by 10 cm, acetate form) equilibrated in 85% methanol. The column was washed with 100% methanol (4 ml), and it was then eluted with a step gradient of ammonium acetate ($\text{NH}_4^+\text{OAc}^-$) in 85% methanol. Accordingly, the column was successively eluted with 0.2 M $\text{NH}_4^+\text{OAc}^-$ (1 ml), 0.4 M $\text{NH}_4^+\text{OAc}^-$ (1 ml), 0.6 M $\text{NH}_4^+\text{OAc}^-$ (1 ml), and 1.0 M $\text{NH}_4^+\text{OAc}^-$ (20 ml). Fractions of 1 ml were collected and dried under a stream of nitrogen, and the residues were taken up in 50 μl of deionized water. Fractions containing ECA_{PG} were identified by an immunoblot procedure using mouse anti- ECA_{PG} monoclonal antibody mAb898 (33, 35, 37). The ECA_{PG} -containing fractions were pooled, dried under a vacuum, and then taken up in 100 ml of methanol and applied to the bed of a Sephadex LH-20 column (3 by 22 cm). The column was developed with methanol, and fractions of 2 ml were collected. The fractions were dried under a stream of nitrogen, and the residues were taken up in 50 μl of deionized water. Those fractions containing ECA_{PG} were identified by the above-mentioned immunoblot procedure. The peak fractions containing ECA_{PG} were pooled, and the ECA_{PG} was further purified by preparative thin-layer chromatography on 20- by 20-cm glass plates coated with silica gel N-HR (0.2 mm). The sample was streaked as a continuous band at the origin, and plates were subsequently developed with 70% ethanol. The sample was also applied in the same manner to a separate indicator plate that was handled in the same manner, and the locations of compounds on the indicator plate were detected by exposing the developed plate to iodine vapors. The silica gel from regions of the preparative plate that corresponded to resolved bands on the indicator plate were scraped from the glass and resuspended in 70% ethanol, and the silica gel particles were removed by centrifugation after thorough mixing. The band containing ECA_{PG} was determined by analyzing the resulting supernatant solutions using the immunoblot procedure described above. The ECA_{PG} -containing solution was reduced to dryness under a stream of nitrogen, taken up in deionized water, and lyophilized. The dried residue was repeatedly taken up in water and lyophilized in order to remove any volatile contaminants. The lyophilized ECA_{PG} was stored at -20°C .

Mass spectrometry studies. Matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectra were obtained using an Applied Biosystems Voyager-DE STR biospectrometry workstation. Samples were analyzed using a laser intensity of 2,550 in the reflector mode. The matrix was α -cyano-4-hydroxycinnamic acid at a concentration of 5 mg/ml in 50% acetonitrile–50% 0.1% trifluoroacetic acid in water. The sample preparation consisted of taking 2 μl of the sample and mixing it with 2 μl of the matrix followed by spotting 1.5 μl on a stainless-steel MALDI plate. Spectra were acquired in the negative mode.

NMR spectroscopy. All nuclear magnetic resonance (NMR) experiments were recorded on Varian Inova 600-MHz NMR spectrometers at 25°C. Samples consisted of 15 mg of lyophilized ECA samples resuspended in 550 μl of 90% H_2O –10% D_2O by volume. Natural-abundance ^{13}C - ^1H heteronuclear single quantum coherence (HSQC) spectra were obtained using standard methods, with 350 to 400 scans in each of 64 t_1 points (^{13}C sweep width = 12 kHz) for total acquisition times of 12 to 16 h each. Alkali-treated ECA_{PG} was prepared by incubating samples with 0.3N NaOH at 35°C for 15 min. The samples were then allowed to cool to room temperature, and the samples were adjusted to pH 7.4 by the addition of HCl. Chemical shift references were based on values used previously by Erbel et al. (15), where DSS (2,2-dimethyl-2-silapentone-5-sulfonate sodium salt) = ^1H = 0.007 ppm and ^{13}C = -1.84 ppm.

RESULTS

Rationale and mutant isolation. The current study was undertaken in an attempt to isolate mutants of *E. coli* K-12 that are unable to synthesize ECA_{CYC} due to a null mutation in the structural gene for the enzyme that catalyzes the cyclization reaction. In order to do so, it was necessary to employ a strategy that decreased the likelihood of isolating mutants defective in the synthesis of ECA_{CYC} due to null mutations in genes residing in the *wec* gene cluster. Accordingly, random transposon mutagenesis was carried out using *E. coli* K-12 strain PR4275, which possesses the entire *wec* gene cluster on the multicopy plasmid pBR322. Transposon mutagenesis employed the $\text{EZ}::\text{TN} <\text{R6K}\gamma\text{ori}/\text{KAN}-2>\text{Tnp}$ transposome

TABLE 2. ECA_{CYC} content of wild-type and mutant strains of *E. coli* K-12 determined by reverse-phase HPLC

Strain	Total amt of ECA_{CYC} ^a (nmol)	Amt of each ECA_{CYC} species ^b (nmol) (no. of <i>O</i> -acetyl groups/molecule)
PR4275 (wild type)	18.59	8.65 (0)
		5.82 (1)
		1.87 (2)
		1.26 (3)
		0.99 (4)
PR4276 (mutant)	17.94	7.94 (0) 0 (1–4)

^a Total amount of ECA_{CYC} in the soluble extracts obtained from cells grown in M9-glucose medium (50 ml) to an A_{600} of 1.0.

^b ECA_{CYC} molecules synthesized by wild-type *E. coli* K-12 contain four trisaccharide repeat units substituted with from zero to four *O*-acetyl groups (15, 19). The numbers in parentheses indicate the number of *O*-acetyl groups/molecule.

(Epicenter) that carries a kanamycin resistance determinant. The resulting kanamycin-resistant insertion mutants were then screened using a PHI assay to identify mutants that are defective in the synthesis of ECA_{CYC} .

A screen of 3,000 kanamycin-resistant mutants using the PHI assay resulted in the identification of one insertion mutant, strain PR4276, that appeared to contain markedly decreased amounts of ECA_{CYC} in the soluble fraction released by osmotic shock (data not shown). Surprisingly, the total amounts of ECA_{CYC} in the soluble fractions obtained from both the mutant and parental strains, as determined by quantitative reverse-phase HPLC, were the same (Table 2). However, quantification of the individual ECA_{CYC} species in the soluble fractions obtained from both the parental and mutant strains revealed that the extracts obtained from the parental strain contained ECA_{CYC} species that were substituted with from zero to four *O*-acetyl groups per molecule (Fig. 1A). In contrast, only a single ECA_{CYC} peak was detected in extracts obtained from the mutant strain, and this material appeared to have the same elution time as ECA_{CYC} molecules that are devoid of *O*-acetyl groups (Fig. 1B). Indeed, MALDI-TOF mass spectrometric analysis of the material that eluted in this peak revealed a molecular ion ($[\text{M} - \text{H}]^-$) of 2,430.6 (± 1) Da, which is in agreement with the molecular ion ($[\text{M} - \text{H}]^-$) of 2,429 (± 1) Da calculated for an ECA_{CYC} molecule comprised of four trisaccharide repeat units and devoid of *O*-acetyl groups.

The apparent decreased total amount of ECA_{CYC} in soluble extracts obtained from the mutant strain as determined by the PHI assay appears to be due to lessened immunoreactivity of ECA_{CYC} molecules devoid of *O*-acetyl groups with the polyclonal rabbit anti- ECA antiserum used in this study. Accordingly, the minimal quantity of ECA_{CYC} obtained from the mutant strain that was required to inhibit the passive hemagglutination assay was approximately 16-fold greater than the amount of ECA_{CYC} obtained from the wild-type parental strain.

Identification of the mutant allele. The location of the transposon insertion in the chromosome of the mutant was determined by “rescue” cloning as described in Materials and Methods. Accordingly, bidirectional sequencing of the regions flanking the transposon in plasmid DNA (pRL170) isolated

from an isolated clone (PR4279) using primers homologous to the ends of the transposon revealed that the transposon was inserted into the 5' region of a putative gene of unknown function designated *yiaH* (GenBank accession number EG12274). The *yiaH* locus is located at 80.26 min on the *E. coli* K-12 chromosomal map (Colibri Web server, Institut Pasteur [http://genolist.pasteur.fr/Colibri/index.html]), and the transposon was inserted into this gene between bp 119 and 120. The location of the transposon insertion was confirmed by PCR amplification of chromosomal DNA obtained from the original mutant isolate, strain PR4276, and wild-type strain PR4275 using the oligonucleotides *yiaH*-1 (5'-CGGAAGGTATAACC GCGCAT-3') and *yiaH*-2 (5'-CCATCGGCCACAGTAA GA-3') as forward and reverse primers, respectively. Primer *yiaH*-1 is identical to the sequence located 410 bp upstream of the translational start codon of the 996-bp *yiaH* locus, and primer *yiaH*-2 is complementary to the sequence located 266 bp upstream of the translational stop codon of *yiaH*. Amplification of the *yiaH* locus in the wild-type and mutant chromosomes using these primers yielded products of approximately 1,140 bp and 3,000 bp, respectively. Taken together, the above-described findings clearly support the conclusion that the transposome (2,001 bp) is inserted into the *yiaH* locus.

The *yiaH*::KAN-2 insertion was introduced into competent cells of strain KM32 by linear transformation using NotI-digested DNA isolated from plasmid pRL172 (*yiaH*::KAN-2 on a 3.4-kb fragment cloned into pGEM-T Easy). One of the resulting transformants, strain PR4254, was used as the donor for phage P1-mediated transduction of the insertion mutation into wild-type strain W3110 to yield mutant strain PR4246. Characterization of strain PR4246 confirmed that it possessed the *yiaH*::KAN-2 mutation. Thus, amplification of the *yiaH* gene of strain PR4246 by PCR using primer set *yiaH*-1/*yiaH*-2 yielded a product of 3,000 bp. Analysis of the ECA_{CYC} isolated from strain PR4246 by reverse-phase HPLC also revealed a single peak that eluted at the same time as those previously observed for ECA_{CYC} molecules lacking *O*-acetyl groups (Fig. 2A). In contrast, extracts of the parental wild-type strain W3110 contain ECA_{CYC} molecules that possess from zero to four *O*-acetyl groups (19). Finally, the defect in the *O* acetylation of ECA_{CYC} was rescued in a transformant of strain PR4246, strain PR4300, that contained the plasmid pRL180. Plasmid pRL180 contains the wild-type *yiaH* allele under the control of the phage T5 promoter in the high-level expression vector pQE30. Indeed, high-level expression of the wild-type *yiaH* allele in strain PR4300 resulted exclusively in the synthesis of *O*-acetylated ECA_{CYC} molecules containing three and four *O*-acetyl substituents (Fig. 2B).

Taken together, the above-described data strongly support the conclusion that the *yiaH* locus is the structural gene for the *O*-acetyltransferase that catalyzes the synthesis of *O*-acetyl groups of the ECA trisaccharide repeat unit of ECA_{CYC}. This conclusion was further supported by the results of BLASTP and RPSBLAST searches of the National Center of Biotechnology Information (NCBI) database using the putative *yiaH* gene product as a query. These analyses revealed that the YiaH protein has significant homology (E value = $6e^{-06}$) with the conserved domain characteristic of the pfam01757:Acyl_transf_3 (acyltransferase) family of enzymes found in a variety of organisms (Fig. 3). These analyses also revealed that this

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 PR4246 (*yiaH*::KAN-2). Peak 1 contains ECA_{CYC} molecules devoid of *O*-acetyl substituents. The arrows labeled 2, 3, 4, and 5 indicate the times at which ECA_{CYC} molecules containing one, two, three, and four *O*-acetyl groups, respectively, elute. (B) HPLC analysis of a whole-cell extract obtained from strain PR4300, a derivative of strain PR4246 transformed with plasmid pRL180 that contains the wild-type *yiaH* allele under the control of the phage T5 promoter in the high-level expression vector pQE30. Peaks 4 and 5 contain ECA_{CYC} molecules that possess three and four *O*-acetyl substituents, respectively. The arrows labeled 1, 2, and 3 indicate the times at which ECA_{CYC} molecules containing zero, one, and two *O*-acetyl groups, respectively, elute. mAU, milli-absorption units.

gene product is present in all gram-negative enteric bacteria for which complete genome sequences have been determined.

The *yiaH* gene encodes a putative inner membrane protein of 331 amino acid residues (13). Bioinformatic analysis of the primary structure of YiaH using the hidden Markov model topology predictor TMHMM (22), provided by the support services of the Center for Biological Sequence Analysis, Technical University of Denmark (http://www.cbs.dtu.dk/index.shtml), revealed that the protein contains 10 predicted transmembrane helices (Fig. 3).

Role of the *yiaH* gene product in the *O* acetylation of ECA_{PG}. As stated above, both ECA_{CYC} and the linear polysaccharide chains of ECA_{PG} are nonstoichiometrically replaced with *O*-acetyl groups (14, 28). Thus, experiments were conducted to determine if the *yiaH*-encoded acyltransferase is also responsible for the *O* acetylation of ECA_{PG}. Accordingly, ECA_{PG} preparations isolated from wild-type and *yiaH*::KAN-2 mutant strains were analyzed for the presence and absence, respec-

FIG. 3. Alignment between the predicted *yiaH* gene product of *E. coli* K-12 (GenBank accession number NP_418018) with the conserved domain (CD) of the pfam01757:Acyl_transf_3 (acyltransferase) family of enzymes. Black-shaded amino acid residues are identical in the alignment, and gray-shaded residues show conservative differences. The solid black bars delineate each of the 10 predicted transmembrane (TM) helices in YiaH.

tively, of *O*-acetyl groups. However, the use of reverse-phase HPLC for these analyses was hampered by an inability to identify conditions that allowed the chromatography of intact ECA_{PG} molecules. This was presumably due to the presence of the phosphoglyceride aglycone. Furthermore, all attempts to release the polysaccharide chains from the phosphoglyceride aglycone without a concomitant loss of *O*-acetyl substituents were unsuccessful. Therefore, solution NMR spectroscopy was employed to examine wild-type and mutant ECA_{PG} for the presence and absence, respectively, of *O*-acetyl groups.

Examination of natural-abundance two-dimensional ¹³C-¹H HSQC spectra of ECA_{PG} obtained from both wild-type and *yiaH*::KAN-2 mutant strains showed a number of peaks at positions analogous to those previously observed in spectra of ECA_{CYC} (15) (Fig. 4). As shown in the upfield region of the ECA_{PG} spectra, the peaks attributed to the *N*-acetyl groups and the C-5 methyl (C-5 CH₃) groups of Fuc4NAc can be straightforwardly assigned by such a comparison. These data agree with previously published chemical shift assignments for ECA_{CYC} to within 0.03 ppm ¹H and 0.3 ppm ¹³C. Although the peak for the GlcNAc *O*-acetyl group of wild-type ECA_{PG} should be well resolved from the other peaks in this spectrum, we observed a candidate for this peak with chemical shifts slightly upfield of the reported assignments in ECA_{CYC} ($\Delta\delta$ [¹³C], ~ -2.7 ppm). We suggest that this may be due to structural differences between ECA_{PG} and ECA_{CYC}, since unlike ECA_{PG}, the 6-*O*-acetylated GlcNAc of ECA_{CYC} is placed in the center of a highly constrained cyclic structure (16). To bolster our assignment, we treated wild-type ECA_{PG} with mild alkali to verify that this peak exhibited the alkaline sensitivity

expected of an *O*-acetyl group. Indeed, our assignment was confirmed by the absence of detectable peaks in this region of the ¹³C-¹H HSQC spectra of the alkaline-treated glycolipid (Fig. 4B). All of the spectra of ECA_{PG} contain a significant number of unassigned peaks that are most likely due to either copurifying compounds or structural elements not found in ECA_{CYC}, e.g., fatty acyl chains. In addition, the relative amounts of ECA_{PG} and these copurifying compounds in the preparations obtained from the wild-type and *yiaH*::KAN-2 mutant strains appeared to be different. In this regard, it is important that the spectrum obtained from the *yiaH*::KAN-2 mutant (Fig. 4C) was reproduced at a lower contour level than was the case for the spectra shown in Fig. 4A and B; nevertheless, no signal for an *O*-acetyl group was detected even though a very intense peak for the C-5 CH₃ group was observed.

Based on our assignments, we used the peak intensities of the Fuc4NAc C-5 CH₃ and the GlcNAc *O*-acetyl groups of ECA_{PG} to quantify the presence of the *O* acetylation in ECA_{PG} obtained from the wild-type strain and the *yiaH*::KAN-2 mutant. Accordingly, a C-5 CH₃/OAc ratio of ~ 1.08 was observed for wild-type ECA_{PG}, whereas this ratio decreased to 0.07 for ECA_{PG} obtained from the mutant. These data are in agreement with the conclusion that the ECA_{PG} obtained from the mutant is completely devoid of *O*-acetyl groups.

DISCUSSION

Previous studies demonstrated that the hydroxyl group in the 6 position of GlcNAc residues in the polysaccharide moieties of both ECA_{PG} and ECA_{CYC} are nonstoichiometrically

FIG. 4. Natural-abundance ^{13}C - ^1H HSQC spectra recorded on (A) ECA_{PG} obtained from wild-type strain PR4275, (B) mild-alkali-treated ECA_{PG} obtained from wild-type strain PR4275, and (C) ECA_{PG} obtained from *yiaH*::KAN-2 mutant strain PR4276. Treatment of ECA_{PG} with mild alkali was performed as described in Materials and Methods. Boxes indicate ECA-associated peaks. Assignments for the *N*-acetyl groups (NAc) and the Fuc4NAc C-5 methyl group (C-5 CH_3) were obtained from a comparison with ECA_{CYC} (15). Peaks were also observed outside the chemical range shown here, and the GlcNAc *O*-acetyl group (OAc) is assigned by inference as noted in the text. Chemical shift references were based on values used previously by Erbel et al. (15) (DSS ^1H = 0.007 ppm; ^{13}C = -1.84 ppm). It is important that the spectrum obtained from the *yiaH*::KAN-2 mutant (Fig. 4C) was reproduced at a lower contour level than was the case for the spectra in panels A and B; nevertheless, no signal for an *O*-acetyl group was detected even though a very intense peak for the C-5 CH_3 group was observed.

esterified with acetyl groups (14, 19, 28). The data presented here clearly support the conclusion that the *yiaH* gene of *E. coli* K-12 encodes the enzyme responsible for the *O* acetylation of these polysaccharides. This conclusion is supported by the results of both biochemical and genetic experiments that demonstrated that a null mutation in *yiaH* abolished the *O* acetylation of both ECA_{PG} and ECA_{CYC} . Accordingly, we propose that this gene henceforth be designated *wecH*.

It has been reported that WeeH (YiaH) is an inner membrane protein (13), and bioinformatic analysis of the putative primary structure of WeeH predict that it possesses 10 membrane-spanning segments (Fig. 3). Details regarding the reaction catalyzed by WeeH have not yet been determined. Moreover, it is not known at what stage of ECA polysaccharide assembly that WeeH-mediated *O* acetylation occurs. It seems likely that acetyl coenzyme A is the donor of acetyl substituents for the *O* acetylation of the GlcNAc residues of ECA_{PG} and ECA_{CYC} and that this reaction occurs during the assembly of lipid III on the cytoplasmic face of the inner membrane prior to the Wzx-mediated translocation of lipid III across the membrane; however, we have not obtained any experimental data to support this conclusion. Therefore, we cannot formally preclude the possibility that *O* acetylation occurs at some stage in the assembly of ECA_{PG} and ECA_{CYC} following the translocation of lipid III across the inner membrane. In this event, the WeeH-mediated *O* acetylation of lipid III or nascent or completed ECA_{PG} and ECA_{CYC} in the periplasm might possibly occur by a mechanism similar to that postulated for the succinylation of membrane-derived oligosaccharides (MDO) in the periplasm by MdoC as described previously by Bohin (11).

The biological importance of the *O* acetylation of ECA_{PG} and ECA_{CYC} remains to be established. To a large extent, this is due to the fact that the function of ECA_{PG} is not well understood, and essentially nothing is known about the function of ECA_{CYC} . The resistance of *Salmonella enterica* serovar Typhimurium to bile salts is dependent on the ability of the organism to synthesize ECA (40). We have also found this to be the case for *E. coli* K-12; however, it is not known if this is the case for all gram-negative enteric bacteria (unpublished results). The available data support the conclusion that ECA_{CYC} does not appear to play a role in the resistance of *E. coli* to bile salts; rather, resistance to bile salts is dependent on the synthesis of ECA_{PG} . Thus, *E. coli* mutants possessing null mutations in the *wzzE* gene are defective in their ability to regulate the degree of polymerization of the linear polysaccharide chains of ECA_{PG} (3). Although these mutants are still able to synthesize ECA_{PG} , they are unable to synthesize ECA_{CYC} (19); however, the resistance of these mutants to bile salts is unaffected (unpublished results). The specific role of ECA_{PG} in the resistance to bile salts is not known; nevertheless, it does not appear that *O* acetylation of ECA_{PG} is an important structural modification in this regard since mutants of *E. coli* possessing null mutations in *wecH* are unaffected in their resistance to bile salts (unpublished results).

It has been determined that the concentration of ECA_{CYC} in the periplasm of *E. coli* cells growing in a medium of low osmolarity is approximately 2.5 mM (19). In contrast, the concentration of MDO in the periplasm of *E. coli* cells growing in a medium of low osmolarity is approximately 50 mM (20). Moreover, unlike

MDO molecules, the concentration of ECA_{CYC} in the periplasm does not vary as a result of changes in the osmolarity of the environment in which cells are grown. Thus, it does not appear likely that ECA_{CYC} has a function similar to that of MDO or other osmoregulated periplasmic glucans.

A wide variety of bacterial pathogens possess cell surface polysaccharides that are O acetylated (17, 18, 21, 25, 26, 32), and this structural modification appears to be of considerable importance for host-pathogen interactions. In many cases, the O-acetyl groups constitute prominent immunogenic epitopes that are important for the generation of host immune responses against the organism and for the development of protective vaccines (1, 8, 21, 26). In contrast, the virulence of some bacterial pathogens appears to be enhanced by the O acetylation of cell surface polysaccharides (1, 2, 9). ECA_{PG} is a component of all gram-negative enteric bacteria (23, 29, 31, 44), and the available evidence strongly supports the conclusion that this is also the case for ECA_{CYC}. Moreover, the O-acetyl groups of ECA_{CYC} and presumably ECA_{PG} as well, also appear to constitute prominent immunogenic epitopes. Accordingly, our data clearly demonstrate a marked decrease in the immunoreactivity of ECA_{CYC} molecules devoid of O-acetyl groups with the polyclonal rabbit anti-ECA antiserum used in this study. However, the significance of the O-acetyl groups in either ECA_{PG} or ECA_{CYC} as prominent immunogenic epitopes is not understood, since there is no clear evidence that either of these polymers function as virulence factors, and it is not yet known if the absence of O-acetyl groups in these polymers either increases or decreases the susceptibility of gram-negative enteric bacteria to host defense mechanisms. Indeed, speculation as to the significance of this structural modification is made even more difficult by the fact that even though ECA_{PG} and ECA_{CYC} share certain basic structural features, their overall structural and physical properties are quite distinct, and their respective cellular locations are markedly different. Furthermore, it is of interest that the degree to which the ECA_{CYC} molecules of *E. coli* K-12 are O acetylated appears to be highly dependent on whether cells are grown in defined medium or rich broth medium (19). The effect of growth media on the O acetylation of ECA_{PG} has not been examined; however, it does not seem unreasonable to assume that the relationship between medium composition and the degree of O acetylation of ECA_{PG} is similar to that observed for ECA_{CYC}. In this regard, it would be of interest to determine the degree to which both ECA_{PG} and ECA_{CYC} are O acetylated in cells growing within a host environment. In any event, the mechanism by which the O acetylation of these polymers is regulated remains to be established.

The O acetylation of ECA_{PG} and ECA_{CYC} polysaccharide chains most likely increases the hydrophobic character of these chains. Thus, it is possible that a change in the hydrophobicity of ECA_{PG} polysaccharide chains on the cell surface as a result of increased O acetylation may alter the association of the organism with host cells, other bacterial cells, or other components in the environment. Similarly, the O acetylation of ECA_{CYC} might also alter the association of this cyclic polysaccharide with specific periplasmic components. Alternatively, the O acetylation of both of these polymers may render them more resistant to degradative enzymes in a manner similar to that of the increased resistance of a variety of bacteria to

lysozyme and muramidases that accompanies the O acetylation of their respective peptidoglycans (12). In any event, although the functions of both ECA_{PG} and ECA_{CYC} have yet to be definitively established, the restricted occurrence of these polymers in gram-negative enteric bacteria suggests that they have functions that are unique to these organisms. It is anticipated that the determination of these functions will also provide insights into the functional significance of the O acetylation of their respective polysaccharides.

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