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RESEARCH ARTICLE

ElyC and Cyclic Enterobacterial Common Antigen Regulate Synthesis of Phosphoglyceride-Linked Enterobacterial Common Antigen

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ABSTRACT The Gram-negative cell envelope is a complex structure delineating the cell from its environment. Recently, we found that enterobacterial common antigen (ECA) plays a role maintaining the outer membrane (OM) permeability barrier, which excludes toxic molecules including many antibiotics. ECA is a conserved carbohydrate found throughout *Enterobacterales* (e.g., *Salmonella*, *Klebsiella*, and *Yersinia*). There are two OM forms of ECA (phosphoglyceride-linked ECA_{PG} and lipopolysaccharide-linked ECA_{LPS}) and one periplasmic form of ECA (cyclic ECA_{CYC}). ECA_{PG}, found in the outer leaflet of the OM, consists of a linear ECA oligomer attached to phosphoglyceride through a phosphodiester linkage. The process through which ECA_{PG} is produced from polymerized ECA is unknown. Therefore, we set out to identify genes interacting genetically with ECA_{PG} biosynthesis in *Escherichia coli* K-12 using the competition between ECA and peptidoglycan biosynthesis. Through transposon-directed insertion sequencing, we identified an interaction between *elyC* and ECA_{PG} biosynthesis. ElyC is an inner membrane protein previously shown to alter peptidoglycan biosynthesis rates. We found Δ*elyC* was lethal specifically in strains producing ECA_{PG} without other ECA forms, suggesting ECA_{PG} biosynthesis impairment or dysregulation. Further characterization suggested ElyC inhibits ECA_{PG} synthesis in a posttranscriptional manner. Moreover, the full impact of ElyC on ECA levels requires the presence of ECA_{CYC}. Our data demonstrate ECA_{CYC} can regulate ECA_{PG} synthesis in strains wild type for *elyC*. Overall, our data demonstrate ElyC and ECA_{CYC} act in a novel pathway that regulates the production of ECA_{PG}, supporting a model in which ElyC provides feedback regulation of ECA_{PG} production based on the periplasmic levels of ECA_{CYC}.

IMPORTANCE Enterobacterial common antigen (ECA) is a conserved polysaccharide present on the surface of the outer membrane (OM) and in the periplasm of the many pathogenic bacteria belonging to *Enterobacterales*, including *Klebsiella pneumoniae*, *Salmonella enterica*, and *Yersinia pestis*. As the OM is a permeability barrier that excludes many antibiotics, synthesis pathways for OM molecules are promising targets for antimicrobial discovery. Here, we elucidated, in *E. coli* K-12, a new pathway for the regulation of biosynthesis of one cell surface form of ECA, ECA_{PG}. In this pathway, an inner membrane protein, ElyC, and the periplasmic form of ECA, ECA_{CYC}, genetically interact to inhibit the synthesis of ECA_{PG}, potentially through feedback regulation based on ECA_{CYC} levels. This is the first insight into the pathway responsible for synthesis of ECA_{PG} and represents a potential target for weakening the OM permeability barrier. Furthermore, this pathway provides a tool for experimental manipulation of ECA_{PG} levels.

KEYWORDS biosynthesis, enterobacterial common antigen, isoprenoids, metabolic regulation, outer membrane

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The Gram-negative envelope is a complex multilayered structure comprised of the outer membrane (OM), the inner membrane (IM), and the periplasm containing a thin peptidoglycan layer (1, 2). The lipid component of the OM consists of an outer leaflet containing mainly lipopolysaccharide (LPS) and an inner leaflet containing phospholipids. A highly compact hydrophobic layer and highly hydrophilic layer formed by LPS, as well as the presence of transenvelope efflux pumps, render the OM impermeable to both hydrophobic molecules and large hydrophilic molecules (1, 3, 4).

The surge of antibiotic resistance in Gram-negative bacteria, especially in *Enterobacterales* (e.g., *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella* sp.) has led to classification of five groups of *Enterobacterales* as urgent or serious threats by the Centers for Disease Control and Prevention (USA) (5–8). However, the study of the Gram-negative envelope, and specifically OM biogenesis, has led to the discovery of several antimicrobials in recent years (reviewed in references 9 and 10). Several antimicrobials have been identified targeting essential pathways in OM biogenesis including LPS biogenesis, protein secretion, OM protein biogenesis, and lipoprotein biogenesis (11–20). The continued success of this approach requires greater understanding of cell envelope biogenesis.

Enterobacterial common antigen (ECA) is a carbohydrate antigen present in the outer leaflet of the OM and in the periplasm and is conserved throughout *Enterobacterales* (reviewed in reference 21). The function of this molecule has remained largely unknown, in part because the biosynthesis pathways for ECA, O-antigen, and peptidoglycan overlap and in part because there are three forms of ECA that cannot currently be genetically separated (see Fig. S1 in the supplemental material). In many *Enterobacterales*, deleting the first gene in ECA biosynthesis, *wecA*, not only prevents ECA biosynthesis but also prevents O-antigen biosynthesis and increases precursor availability for peptidoglycan biosynthesis (22–25). Deletion of downstream genes in ECA biosynthesis, such as *wecE* or *wecF*, leads to accumulation of intermediates in ECA biosynthesis, interfering with peptidoglycan biosynthesis, altering cell shape, increasing envelope permeability, and activating envelope stress response systems (26–30). Three forms of ECA, LPS-linked ECA (ECA_{LPS}), cyclic ECA (ECA_{CYC}), and phosphoglyceride-linked ECA (ECA_{PG}), are made from polymerized ECA chains. As many of the genes responsible for the steps in ECA biosynthesis separating these molecules are unknown (see below), assigning functions to these separate forms remains difficult. Nevertheless, it has become clear that in *Salmonella* sp., ECA plays a role in acid and bile salt resistance (31, 32) and is necessary for pathogenesis in a mouse model (32–35). In addition, we have discovered a role for ECA_{CYC} in maintaining the OM permeability barrier in *E. coli* (36).

The polysaccharide chains of ECA consist of linear repeat units, each unit made of three sugars: GlcNAc (*N*-acetylglucosamine), ManNAcA (*N*-acetyl-D-mannosaminuronic acid), and Fuc4NAc (4-acetamido-4,6-dideoxy-D-galactose) (37, 38). Biosynthesis of ECA is initiated by attachment of GlcNAc-1-phosphate to the isoprenoid carrier, undecaprenyl-phosphate (Und-P), followed by the addition of the two remaining sugars (Fig. S1) (39–42). Und-P is a universal lipid carrier and is required for the biosynthesis of O-antigen, peptidoglycan, and capsular polysaccharides, as well as ECA (43–47). WzxE flips the complete ECA repeat unit linked to Und-P across the IM to the periplasmic face (48), and WzyE polymerizes ECA chains (49). The number of repeat units in the polymerized ECA molecule (chain length) is controlled by WzzE (50). The operon responsible for synthesis of ECA, the *wec* operon, contains the genes responsible for the steps in ECA biogenesis resulting in a polymerized ECA molecule attached to Und-PP located on the outer leaflet of the IM (39, 41, 49).

The steps through which the three forms of ECA are made from this precursor are less well understood (21). ECA_{LPS} is produced when WaaL, the O-antigen ligase, attaches ECA to the core polysaccharide of LPS (43, 51). ECA_{LPS} is presumably transported to the cell surface by the Lpt system responsible for transporting LPS to the cell surface (52). The second form, ECA_{CYC}, a cyclic carbohydrate, remains in the periplasm (29). It is generally made with precise chain length (4 repeat units in *E. coli* K-12), and WzzE is necessary for

its synthesis (29, 53, 54). The final form, ECA_{PG}, is a linear ECA chain linked to diacylglycerol through a phosphodiester bond (55). The mechanism through which ECA_{PG} is formed and transported to the cell surface is completely unknown (21, 56, 57). This lack of knowledge impairs genetic studies of ECA function as mutants cannot be made that synthesize ECA_{LPS} and ECA_{CYC} in the absence of ECA_{PG}.

Therefore, we set out to identify factors genetically interacting with the biosynthesis of ECA_{PG}. We took advantage of the competition for substrates between the peptidoglycan and ECA biosynthesis pathways to find factors interacting with ECA_{PG} biosynthesis (Fig. S1). Using transposon-directed insertion sequencing (TraDIS), we identified ElyC as a factor interacting with ECA_{PG} biosynthesis. ElyC is an IM protein with two transmembrane domains and a large C-terminal globular DUF218 domain that resides in the periplasm (58, 59). Paradis-Bleau et al. found a Δ*elyC* mutant displays severe growth defects at low temperatures (22°C) and high frequency of cell lysis due to decreased peptidoglycan synthesis (28). They suggested that ElyC regulates the allocation of Und-P between synthesis pathways in *E. coli*.

Here, we have explored the role of ElyC in ECA_{PG} biosynthesis. Our data demonstrate that ElyC posttranscriptionally regulates the synthesis of ECA_{PG}, greatly inhibiting its synthesis during normal growth. Furthermore, we observed that ElyC only had its full effect on ECA_{PG} synthesis in the presence of WzzE, suggesting that WzzE or ECA_{CYC} is involved in this regulatory pathway. In fact, we found that ElyC and ECA_{CYC} act together to regulate ECA_{PG} biosynthesis. Our data demonstrate that the effect of ElyC and ECA_{CYC} on ECA levels is specific to ECA_{PG} and not a result of allocation of Und-P between biosynthesis pathways. Overall, we have deciphered a novel pathway through which ElyC and ECA_{CYC} regulate ECA_{PG} biosynthesis, providing insight into the elusive function of ElyC and demonstrating that ECA_{CYC} plays roles both in maintaining the OM permeability barrier and in regulating ECA biosynthesis.

RESULTS

Identification of candidate genes interacting with ECA_{PG} biosynthesis. There are genes known to be necessary for the biosynthesis of ECA_{CYC} and of ECA_{LPS} specifically: ECA_{CYC} synthesis requires *wzzE*, while ECA_{LPS} synthesis requires *waaL* (29, 36, 43). However, the genes and reactions responsible for producing ECA_{PG}, by transferring the ECA polymer from Und-PP to form phosphoglyceride-linked ECA_{PG}, and for its surface exposure are unknown (55–57). Therefore, we set out to identify factors involved in ECA_{PG} biosynthesis, utilizing interactions between ECA and peptidoglycan biosynthesis.

The ECA and peptidoglycan pathways compete for Und-P and UDP-GlcNAc as depicted in Fig. S1 in the supplemental material (25, 60, 61). Although deletion of *wecA* causes generally mild phenotypes, deletion of later genes in ECA biosynthesis (e.g., *wecB*, *wecG*, *wecF*, or *wzxE*) causes the accumulation of ECA intermediates sequestering Und-P, disrupting peptidoglycan biosynthesis and resulting in increased permeability defects, cell shape defects, and envelope stress response activation (26–30, 62, 63). In fact, deletion of *wzyE*, the ECA polymerase, or all the flippases capable of flipping lipid III^{ECA} across the IM is lethal (29). Thus, we hypothesized that, in a strain making only ECA_{PG}, disruption or dysregulation of the next step in ECA biosynthesis (transfer of polymerized ECA from Und-PP) would also be highly unfavorable due to sequestration of Und-P inhibiting peptidoglycan biosynthesis.

Therefore, we have used TraDIS (transposon-directed insertion sequencing) (64) to compare the favorability of gene disruptions in a mutant which makes ECA_{PG} but not the other forms of ECA (Δ*wzzE* Δ*waaL*) with an isogenic mutant that does not make ECA (Δ*wecA-wzzE* Δ*waaL*) and with wild-type *E. coli* K-12 MG1655. For this approach, we generated high-density transposon libraries in each of these strains and performed Illumina sequencing of the transposon junctions in the initial pooled libraries, as well as after 10 generations of growth in liquid culture. The statistical properties of each data set were similar (Table S1). We then compared the transposon junction reads per

gene between the three strains (Fig. 1A). To confirm we could detect changes in essentiality due to sequestration of Und-P, we analyzed the transposon junction reads in *wzyE*. In strains producing ECA, *wzyE* is essential; however, *wzyE* becomes nonessential when ECA synthesis is disrupted at an earlier step as accumulation of lipid III^{ECA} is prevented (29). In the ECA_{PG}-only strain and wild-type MG1655, we detected very few transposon insertion reads in *wzyE*; however, we observed similar levels of insertions to nearby genes in the strain without ECA (Fig. 1B).

To identify genes possibly involved in ECA_{PG} biosynthesis, we defined a set of criteria for genes putatively essential only when ECA_{PG} is made without the other forms of ECA (Table S2). These genes had less than 200 reads in the ECA_{PG}-only library under both growth conditions and had at least a 1-standard-deviation decrease in the ECA_{PG}-only strain compared to the other two strains under both growth conditions. In addition, we limited our analysis to genes not known to be essential in wild-type *E. coli* K-12 that make proteins targeted to either the IM or the periplasm (65–67). We identified five genes that fit these criteria: *elyC*, *ynbB*, *ymiB*, *lapA*, and *yoaI* (Table S2). From these hits, we confirmed the data in the literature that *ynbB* was not necessary for the synthesis of ECA_{PG} (68; unpublished data).

In this paper, we focus on *elyC* (Fig. 1C), which encodes an inner membrane protein. Previous work has shown that a Δ*elyC* mutant lyses at room temperature (22°C) due to a peptidoglycan synthesis defect but grows well at 37°C (28). The authors hypothesized this defect is due to competition between peptidoglycan and the ECA biosynthesis pathway, particularly at the step of allocation of Und-P. Data have also suggested that an Δ*elyC* mutant may have a periplasmic protein-folding defect (69) and may experience increased oxidative stress at low temperature (22°C) (70). The experiments described here were performed at 37°C.

***elyC* is essential in a strain producing only ECA_{PG}.** To determine whether *elyC* was essential in a strain producing only ECA_{PG}, we performed genetic linkage-disruption experiments. In these experiments, a Tn*10* marker genetically linked to a deletion in the gene of interest is transduced into strains, selecting for the presence of Tn*10*. Based on the size of DNA packaged by the P1*vir* phage, the gene deletion is cotransduced with a calculable frequency (71). If there is selection against the deletion of the gene (i.e., the gene is essential), the cotransduction frequency observed will decrease. We first measured linkage between *zbj-7230*::Tn*10* and Δ*elyC*::kan. These two markers were approximately 53% linked in wild-type MG1655 and in Δ*waaL* and Δ*wzzE* single mutants (Table 1). However, in a Δ*wzzE* Δ*waaL* double mutant producing only ECA_{PG}, we observed only 1% linkage, demonstrating strong linkage disruption (Table 1). The linkage is restored in a complemented strain. We observed similar linkage disruption when transducing *metE3074*::Tn*10* linked to Δ*wzzE*::kan into a Δ*waaL* Δ*elyC* mutant (Table 1). These data confirm that *elyC* is essential when ECA_{PG} is made in isolation but not in strains making two or more forms of ECA.

Interestingly, we observed only slight linkage disruption when transducing *thd*::Tn*10* Δ*waaL*::kan into a Δ*wzzE* Δ*elyC* strain (Table 1). We confirmed these results by rebuilding the strains from wild-type MG1655 and with two different alleles of Δ*elyC* (Table S3) and through direct transduction of the Δ*waaL*::kan allele. Although the triple deletion mutant, Δ*wzzE* Δ*elyC* Δ*waaL*::kan *thd*::Tn*10*, could be built with Δ*waaL* as the last deletion, the triple mutant colony size was extremely small compared to Δ*wzzE* Δ*elyC thd*::Tn*10* colonies (Fig. S2). These data suggest that, although *elyC* is essential in a strain producing only ECA_{PG}, its function is somehow modified in a Δ*wzzE* strain allowing survival when *waaL* is deleted last (see below).

Deletion of *elyC* increases ECA_{PG} levels. After confirming *elyC*'s essentiality in a strain making only ECA_{PG}, we asked what the effect of the Δ*elyC* mutation was on surface exposure of ECA, as a Δ*waaL* strain without ECA_{PG} should not have ECA on its surface. We used a dot blot as a qualitative method (72) to detect surface-exposed ECA_{PG} and ECA_{LPS}. ECA_{CYC} is not surface exposed. Surface-exposed ECA was detected in all Δ*elyC* strains including the Δ*waaL* Δ*elyC* strain (Fig. 2A). In fact, the surface-exposed ECA levels appeared higher in the Δ*elyC* and Δ*waaL* Δ*elyC* strains than in the wild-type or Δ*wzzE*

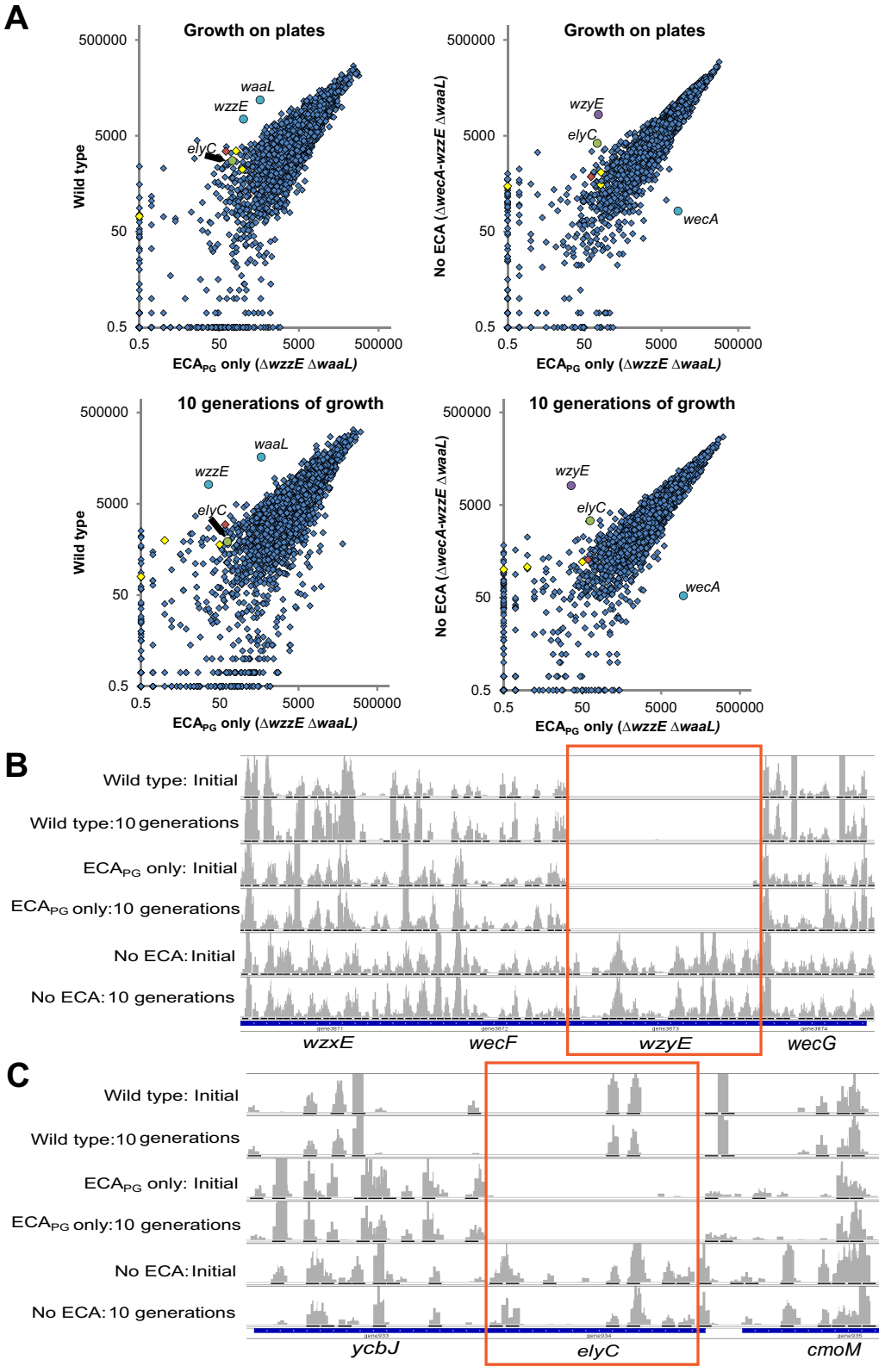

FIG 1 Screening for candidate genes interacting with ECA$_{PG}$ biosynthesis. (A) TraDIS was used to identify genes for which disruption was unfavorable in cells making ECA$_{PG}$ without the other forms of ECA. Scatterplots of transposon junction reads per gene are shown comparing the ECA$_{PG}$ strain (Δ*wzzE* Δ*waaL*) with an isogenic strain without ECA (Δ*wecA* Δ*wzzE* Δ*waaL*) and with wild-type MG1655. Results are shown following initial growth on plates and after 10 additional generations of growth in liquid medium. Putative ECA$_{PG}$ biosynthesis genes are shown in yellow, *elyC* is shown in green, *wzyE* is shown in purple, and *ynbB* is shown in red. Genes deleted in one of the strains are shown in

(Continued on next page)

TABLE 1 *elyC* is essential in strain making only ECA_{PG}

Donor	Recipient	Recipient form(s) of ECA	N[a]	P1*vir* cotransduction frequency[b]
zbj-7230::Tn*10* Δ*elyC*::kan (AM769)	MG1655	ECA_{CYC}, ECA_{LPS}, ECA_{PG}	300	52.7%
	Δ*wzzE* (AM365)	ECA_{LPS}, ECA_{PG}	300	54.3%
	Δ*waaL* (AM366)	ECA_{CYC}, ECA_{PG}	300	53.3%
	Δ*wzzE* Δ*waaL* (AM395)	ECA_{PG}	300	1.0%
	Δ*wzzE* Δ*waaL* pBAD33-*elyC*[c] (AM1159)	ECA_{PG}	306	62.1%
metE-3074::Tn*10* Δ*wzzE*::kan (AM766)	MG1655	ECA_{CYC}, ECA_{LPS}, ECA_{PG}	300	25.0%
	Δ*elyC* (AM743)	ECA_{CYC}, ECA_{LPS}, ECA_{PG}	300	17.3%
	Δ*waaL* (AM366)	ECA_{CYC}, ECA_{PG}	300	24.3%
	Δ*waaL* Δ*elyC* (AM745)	ECA_{CYC}, ECA_{PG}	300	3.0%
thd::Tn*10* Δ*waaL*::kan (AM735)	MG1655	ECA_{CYC}, ECA_{LPS}, ECA_{PG}	295	79.0%
	Δ*elyC* (AM743)	ECA_{CYC}, ECA_{LPS}, ECA_{PG}	300	72.3%
	Δ*wzzE* (AM365)	ECA_{LPS}, ECA_{PG}	300	76.0%
	Δ*wzzE* Δ*elyC* (AM744)	ECA_{LPS}, ECA_{PG}	232	61.2%

[a]The indicated number of transductants were analyzed. Transductants were harvested from three separate transductions.
[b]P1*vir* was used to transduce the indicated markers into the indicated strain. Cotransduction frequency was determined by selecting the transductants for the presence of Tn*10* and calculating the percentage of colonies containing the gene deletion.
[c]Expression from complementing plasmid was induced with 0.2% arabinose.

Δ*elyC* strains. These observations suggested that there might be an increase in a non-ECA_{LPS}, surface-exposed species of ECA in the Δ*elyC* mutant. As dot blots are not ideal for determining quantitative changes, we performed ECA immunoblot analyses to detect the charged forms of ECA (ECA_{PG} and ECA_{LPS}). ECA_{CYC} is not charged and cannot be observed through immunoblot analysis. We found a very large increase in linear ECA levels in both the Δ*elyC* and Δ*waaL* Δ*elyC* strains (Fig. 2B; compare lanes 5 and 6 with lanes 1 and 4). Similar to the dot blot results, there was much less of an increase in ECA levels in the Δ*wzzE* Δ*elyC* strain (lane 7). These results suggest, when *elyC* is deleted, there is a large increase in a species of ECA which is neither ECA_{LPS} nor ECA_{CYC}.

Thus, we sought to determine ECA_{LPS} levels. Wheat germ agglutinin (WGA) is a lectin protein used to detect glycans (β-GlcNAc or sialic acid multimers) in prokaryotes and eukaryotes (73–78). A beta-linked GlcNAc is present in the glycosidic bond that attaches ECA to LPS to form ECA_{LPS}, but this bond is absent in ECA_{PG}. Therefore, we have found cell surface staining of MG1655 with WGA labels only ECA_{LPS}, providing a specific assay for this ECA species (Fig. S3A). Thus, we assayed WGA staining of *elyC* mutant cells and found deletion of *elyC* caused only a slight increase in the amount of ECA_{LPS}. This increase was similar between the Δ*elyC* and Δ*wzzE* Δ*elyC* strains (Fig. 2C). There are two possible explanations for the smaller increase in ECA_{LPS} levels than linear ECA levels: (i) ElyC plays a role in ECA biosynthesis that is specific to ECA_{PG} or (ii) ECA_{LPS} levels are limited by availability of WaaL. Therefore, we overexpressed *waaL* in the wild-type and Δ*elyC* strains and assayed levels of ECA_{LPS}. Although we observed an increase in ECA_{LPS} levels when *waaL* was overexpressed in a wild-type strain, we did not see an increase in ECA_{LPS} level in the Δ*elyC* strain (Fig. S4A), demonstrating that Δ*elyC* is epistatic to *waaL* expression. These data suggest that the effect of ElyC is specific to ECA_{PG} biosynthesis. Overall, the immunoblot, dot blot, and WGA staining experiments demonstrate that ElyC plays a role in ECA_{PG} biosynthesis that leads to a large increase in the levels of a non-ECA_{LPS} species with deletion of *elyC*.

ElyC posttranscriptionally regulates the production of ECA_{PG} and ECA overall. There are two possible models to explain the increase in ECA observed when *elyC* is

FIG 1 Legend (Continued)
cyan. (B) Histograms of transposon insertion reads in *wzyE* and adjacent genes are shown as a control for detection of changes in essentiality based on Und-P availability. Transposon insertions are observed in *wzyE* only in the strain without ECA. (C) Histograms of transposon insertion reads in *elyC* and adjacent genes. Transposon insertions are observed in the strain without ECA and the wild-type strain but not in the strain making only ECA_{PG}, suggesting essentiality of *elyC* in the ECA_{PG}-only strain.

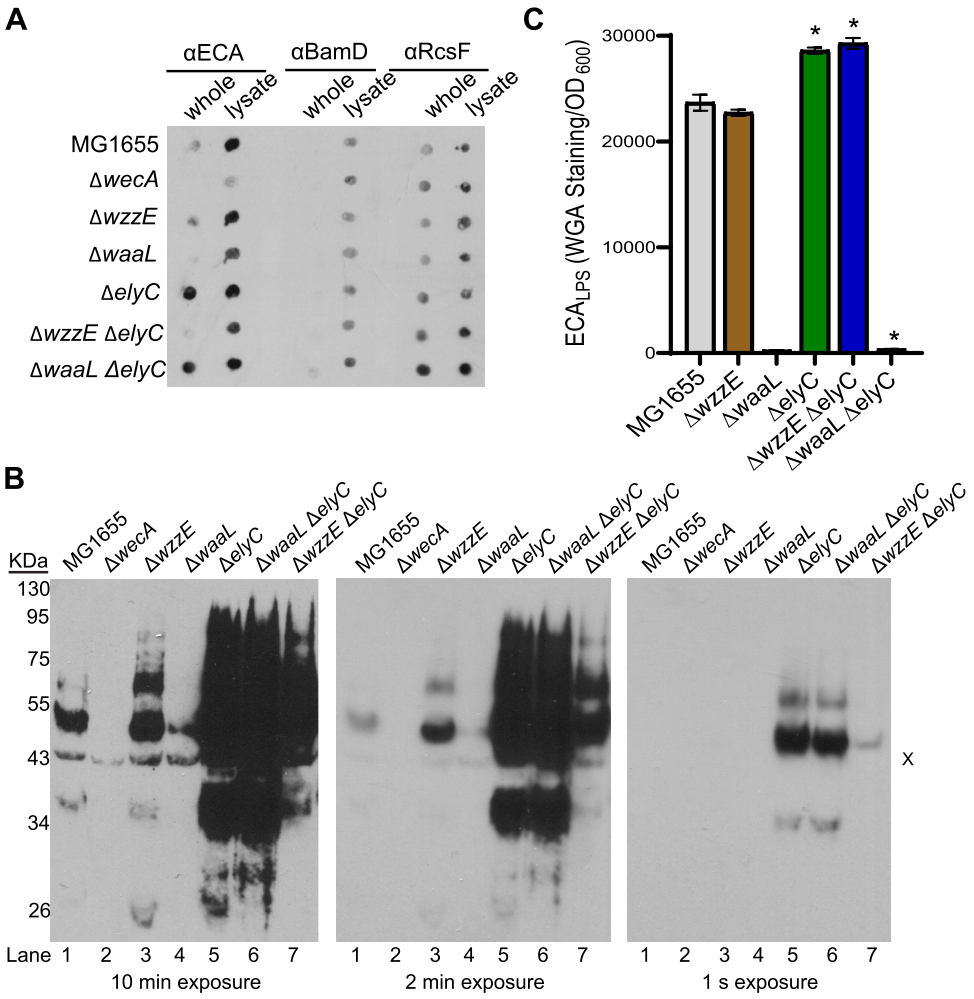

FIG 2 Deletion of *elyC* increases levels of ECA_{PG}. (A) The surface exposure of ECA_{PG} and ECA_{LPS} was detected through dot blot assay. Whole cells or a whole-cell lysate was probed for ECA, BamD, or RcsF. BamD acted as a negative control for surface exposure, while RcsF acted as a positive control for surface exposure. Δ*wecA* served as a negative control for the presence of ECA. Surface-exposed ECA was detected in all Δ*elyC* strains including the Δ*waaL* Δ*elyC* strain, suggesting ECA_{PG} is present on the cell surface in these strains. (B) Immunoblotting was performed to examine ECA levels and chain length. A very large increase in ECA levels was observed in Δ*elyC* and Δ*waaL* Δ*elyC* strains, but less of an increase was observed in the Δ*wzzE* Δ*elyC* strain. The nonspecific "X" band serves as a loading control. Δ*wecA* serves as a negative control for the presence of ECA. (C) ECA_{LPS} quantification was performed in indicated strains by WGA staining. Data are shown as fluorescence relative to OD_{600}. The Δ*waaL* and Δ*waaL* Δ*elyC* strains serve as negative controls for the presence of ECA_{LPS}. There was a small but significant increase in ECA_{LPS} levels in the Δ*elyC* and Δ*wzzE* Δ*elyC* strains compared to their parent strains. Data are shown as the mean from three biological replicates ± standard error of the mean (SEM). *, $P < 0.05$ by the nonparametric Mann-Whitney test compared to $elyC^+$ parent strain.

deleted. First, ElyC may decrease ECA_{PG} levels by inhibiting the production of ECA_{PG}. Second, there might be more than one step necessary to produce ECA_{PG} and ElyC is responsible for a later step leading to the accumulation of a biosynthetic intermediate that is not distinguishable from ECA_{PG} on an immunoblot. To differentiate between these models, we determined the effect of overexpressing *elyC* on ECA levels. In the first model, overexpression of ElyC should decrease ECA_{PG} levels, while, in the second model, overexpression of ElyC should either not effect or increase production of ECA_{PG}, depending on the rate-limiting step in synthesis.

We assayed ECA levels by immunoblotting in strains overexpressing *elyC* in a wild-type or Δ*wzzE* background. We utilized the pCA24N-*elyC* plasmid from the ASKA collection, which expresses *elyC* under a leaky, isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter (79). We observed that increasing *elyC* overexpression in a wild-type

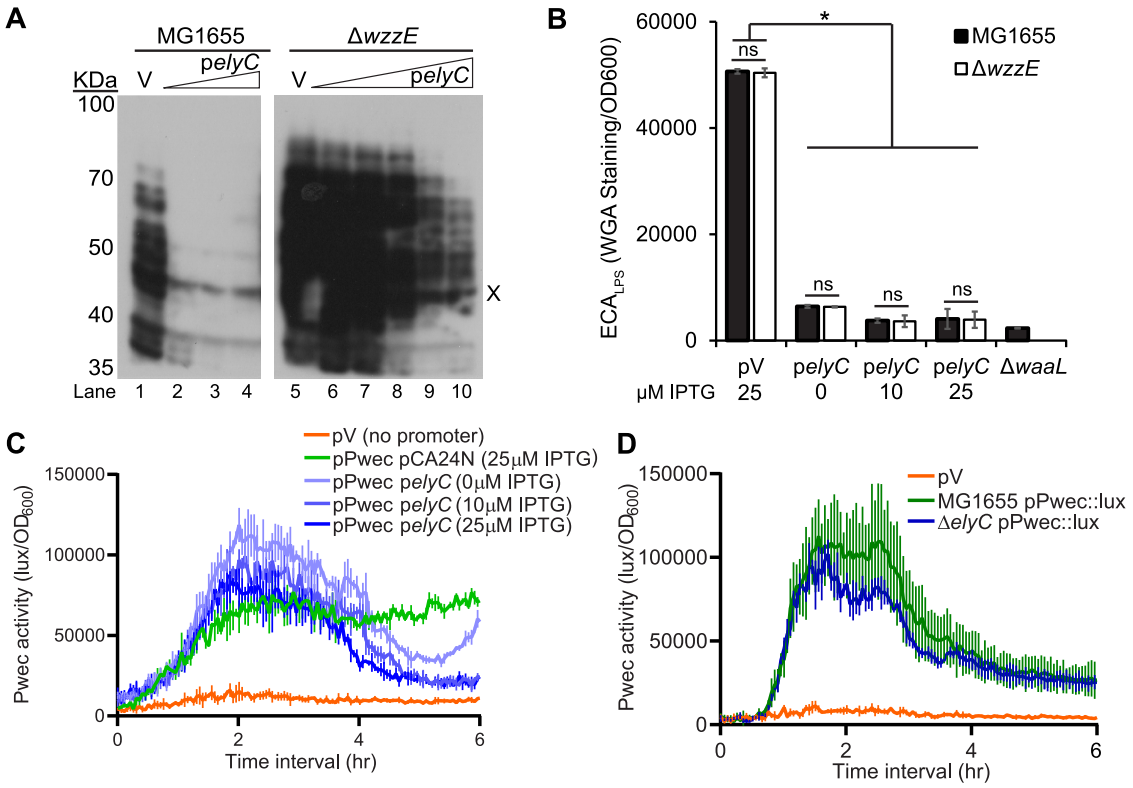

FIG 3 ElyC regulates ECA_{PG} production posttranscriptionally. (A to C) *elyC* was overexpressed from the IPTG-inducible pCA24N vector in the indicated strains. (A) Triangles indicate increasing overexpression of *elyC*. In wild-type cells, even low-level overexpression of *elyC* greatly decreased ECA levels; however, less of a decrease was observed in the Δ*wzzE* strain. The nonspecific "X" band serves as a loading control. "V" samples indicate strains with empty pCA24N induced with 100 μM IPTG. Lanes 2 to 4 are induced with 0, 10, and 25 μM IPTG, respectively. Lanes 6 to 10 are induced with 0, 10, 25, 50, and 100 μM IPTG, respectively. (B) ECA_{LPS} quantification was performed by WGA staining, indicated by fluorescence relative to OD_{600}. The Δ*waaL* strain acts as a negative control. Overexpression of *elyC* reduced ECA_{LPS} levels, but no difference was observed between the wild-type strain and the Δ*wzzE* strain. (C) A bacterial luciferase reporter was used to assay the activity of the P_{wec} promoter, the promoter for the *wec* operon containing genes for ECA biosynthesis. Despite the decrease in ECA levels observed, overexpression of *elyC* did not decrease P_{wec} activity. (D) P_{wec} activity was assayed as in panel C. No increase in P_{wec} activity was observed in the Δ*elyC* strain compared to the wild-type strain. Quantitative data are shown as the mean from three biological replicates ± SEM. *, $P < 0.05$ by the nonparametric Mann-Whitney test; ns, $P > 0.05$ by the Mann-Whitney test.

background greatly decreased ECA levels (Fig. 3A, lanes 1 to 4). In a Δ*wzzE* mutant, a smaller decrease in ECA levels was observed at high IPTG concentrations (lanes 5 to 10). As with our previous results, this suggests that the removal of *wzzE* changes ElyC's effect on ECA levels. To assay the effect of *elyC* overexpression on ECA_{LPS} in the wild-type and Δ*wzzE* strains, we performed WGA staining and observed a decrease in ECA_{LPS} levels that was similar in the two backgrounds (Fig. 3B). We observed similar results when *elyC* was overexpressed in MC4100 (a Lac^- strain) (Fig. S3B) and when *elyC* was overexpressed from a pBAD33 plasmid (Fig. S3C and D). Overall, these results demonstrate that overexpressing *elyC* decreases ECA levels and ElyC is responsible for inhibiting the production of ECA_{PG} and, to some extent, ECA production overall.

We then asked whether *elyC* affects ECA levels on a transcriptional or posttranscriptional level. We constructed a reporter by cloning the promoter region of the *wec* operon into a promoterless pJW15 vector that harbors a bacterial luciferase operon (Fig. S5) (80, 81). Using this reporter, we observed no consistent decrease in P_{wec} activity with *elyC* overexpression, despite the decrease in ECA levels (Fig. 3C). We also checked the effect of *elyC* deletion on P_{wec} activity and found no increase the P_{wec} reporter activity in this strain (Fig. 3D). Overall, we observed no indication that ElyC regulates ECA levels in a transcriptional manner, making posttranscriptional regulation most likely.

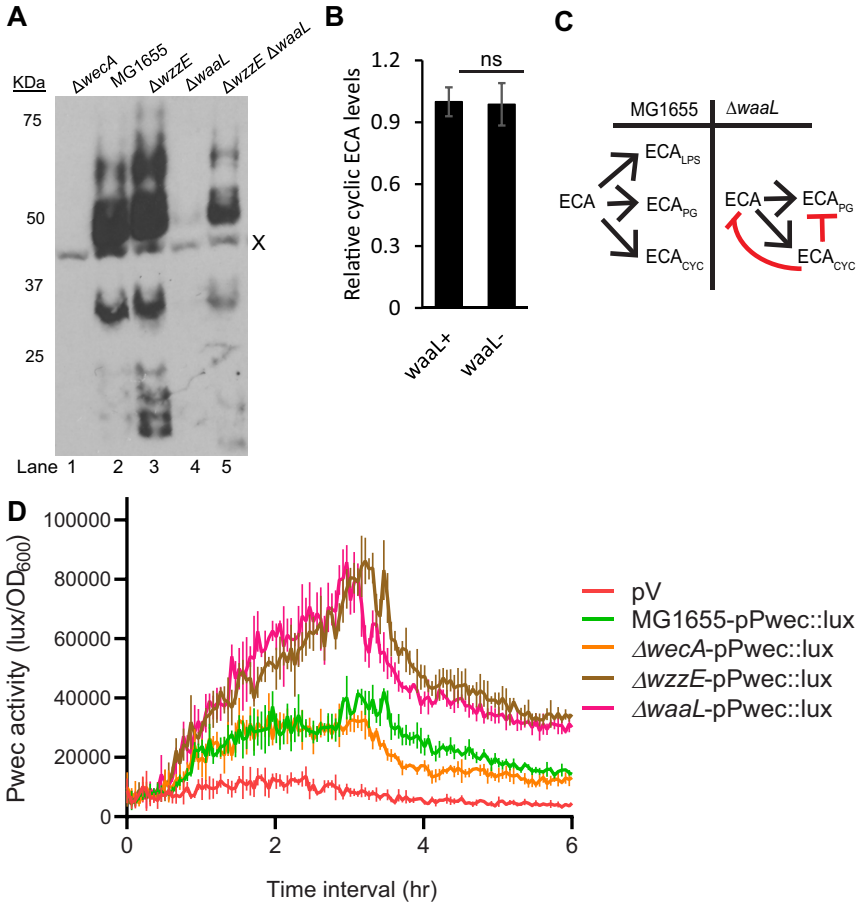

FIG 4 ECA_{CYC} is involved in feedback regulation of ECA_{PG} levels. (A) The level of the linear forms of ECA was assayed in the indicated strains by immunoblotting. The Δ*wecA* strain serves as a negative control for ECA. The nonspecific "X" band serves as a loading control. Much less ECA_{PG} is observed in the Δ*waaL* strain compared to linear ECA levels in the wild-type strain. However, in the Δ*wzzE* Δ*waaL* strain, ECA_{PG} levels return to near-wild-type levels. (B) ECA_{CYC} levels were compared in Δ*wecH*::kan and Δ*waaL* Δ*wecH*::kan strains by MALDI-TOF. WecH is responsible for nonstoichiometric acetylation of ECA. The levels of ECA_{CYC} are comparable between the strains. (C) Model for ECA_{PG} and ECA_{CYC} levels with loss of ECA_{LPS} is shown. In wild-type cells, the three forms of ECA are produced at an appropriate ratio. When Δ*waaL* is deleted and ECA_{LPS} is lost, the remaining ECA is funneled into ECA_{PG} and ECA_{CYC}. However, ECA_{CYC} levels are maintained at a consistent level and ECA_{PG} levels are decreased, suggesting ECA_{CYC} levels are measured to provide regulation of ECA_{PG} production and ECA levels overall (red arrows). (D) Activity of the P_{wec} promoter was assayed by luciferase assay in the indicated strains. P_{wec} activity does not correlate with changes in ECA levels, suggesting that the effect of ECA_{CYC} on ECA levels is posttranscriptional. Quantitative data are shown as the mean from three biological replicates ± SEM. ns, $P > 0.05$ by the Mann-Whitney test.

ECA_{CYC} acts with ElyC to regulate ECA_{PG} production. Throughout our experiments, we observed that the effect of ElyC on ECA_{PG} levels was less in the absence of *wzzE*. This led us to ask whether WzzE or ECA_{CYC} was playing a role in the pathway through which ElyC regulated ECA_{PG} levels. To differentiate between the effects of WzzE and ECA_{CYC} on this pathway, we utilized the previous observation that levels of linear ECA are very low in a Δ*waaL* mutant (36). We have confirmed this effect: there is much less ECA detectable by immunoblotting in Δ*waaL* cells than in wild-type cells or a Δ*wzzE* mutant (Fig. 4A; lane 4 compared to lanes 2 and 3). However, in the Δ*wzzE* Δ*waaL* mutant the ECA levels return to near-wild-type levels (lane 5). Our initial explanation was that there was much more ECA_{LPS} than ECA_{PG} present and that the excess ECA freed by removing ECA_{LPS} was funneled into ECA_{CYC}, which is not detectable by immunoblotting. However, this did not fit well with our observations of overexpressing *elyC* in a Δ*wzzE* background (Fig. 3A and B), where we observed a large decrease in ECA_{LPS} levels but a relatively small decrease in ECA levels overall. Therefore, we purified ECA_{CYC} and

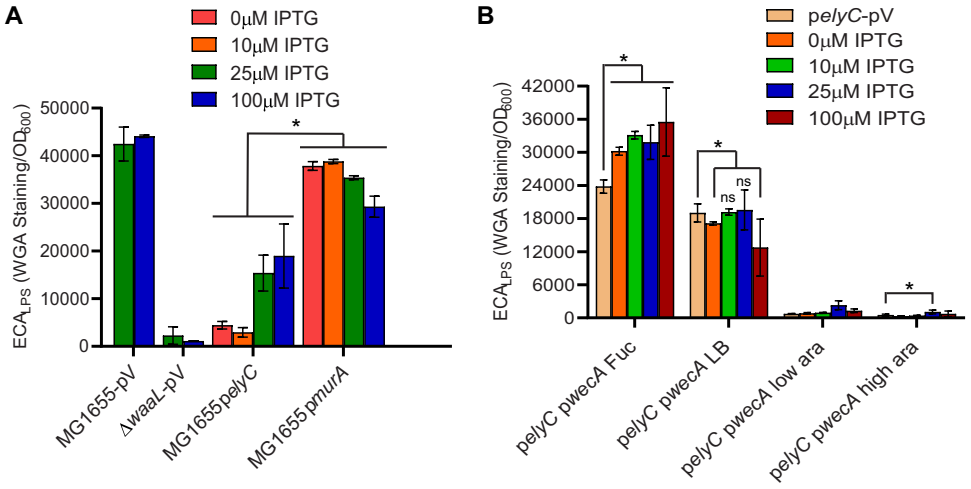

FIG 5 ElyC regulates ECA levels independently of Und-P availability. ECA_{LPS} levels were assayed by WGA staining. Data are shown as fluorescence relative to OD_{600}. (A) *elyC* or *murA*, the first gene in peptidoglycan biosynthesis, was overexpressed from the IPTG-inducible pCA24N vector. Overexpression of *murA* increases the utilization of Und-P by the peptidoglycan biosynthesis pathway. The effect of *murA* overexpression on ECA_{LPS} levels was smaller than that of *elyC*. (B) *elyC* was overexpressed from the arabinose-inducible and fucose-repressible pBAD33(K) plasmid. *wecA* was overexpressed from the IPTG-inducible pCA24N vector. Overexpression of *wecA* increases the utilization of Und-P by the ECA biosynthesis pathway. *wecA* overexpression increases production of ECA_{LPS} in the absence of *elyC* overexpression. However, *wecA* overexpression does not suppress the decrease in ECA_{LPS} levels when *elyC* is overexpressed. Fuc, 0.05% fucose; LB, no inducer or repressor; low ara, 0.02% arabinose; high ara, 0.2% arabinose. Data are shown as the mean from three biological replicates ± SEM. *, $P < 0.05$ by the nonparametric Mann-Whitney test; ns, $P > 0.05$ by the Mann-Whitney test.

quantified the ECA_{CYC} levels through matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectroscopy (Fig. S6). We found no effect of Δ*waaL* on cyclic ECA levels (Fig. 4B and Fig. S6). Thus, while the ECA_{CYC} levels remained constant, the total amount of ECA was decreased in the Δ*waaL* strain. That a free pool of ECA caused by the removal of ECA_{LPS} leads to decreased linear ECA levels and steady ECA_{CYC} levels demonstrates that ECA_{CYC} plays a role in regulating ECA_{PG} biosynthesis (Fig. 4C). Thus, ECA_{CYC} is involved in regulation of ECA_{PG} production. Then, we measured P_{wec} activity in Δ*wecA*, Δ*wzzE*, and Δ*waaL* strains to determine whether the regulation is transcriptional or posttranscriptional. We found that any changes in reporter activity in the mutants did not correlate with the changes in the amounts of ECA observed (Fig. 4A and D). Thus, ECA_{CYC} has a role in controlling ECA_{PG} production that appears to be through posttranscriptional regulation. Importantly, the levels of ECA are similar between the Δ*elyC* and Δ*waaL* Δ*elyC* strains (Fig. 2B), and the effect of ElyC on ECA_{PG} levels is much less in the absence of *wzzE* (Fig. 2B and Fig. 3A). These data demonstrate that ElyC and ECA_{CYC} act together in this regulatory pathway.

Und-P allocation is not responsible for the effect of ElyC on ECA levels. Previous reports have shown that the overexpression of the gene responsible for Und-P synthesis or the gene responsible for the first step in peptidoglycan biosynthesis relieved peptidoglycan stress in a Δ*elyC* strain (28, 82). This led to the suggestion that ElyC balances Und-P use between pathways (28). It is not possible for the effect of ElyC specifically on ECA_{PG} to be caused by Und-P allocation. However, it is possible that Und-P allocation is responsible for the effect on total ECA levels.

Therefore, we asked whether the effect of ElyC on overall ECA biosynthesis was due to Und-P allocation. We compared the effect of overexpressed *murA*, the first committed step for peptidoglycan biosynthesis (61), with that of the overexpression of *elyC* to determine whether they phenocopy each other. Although overexpression of *murA* does cause a decrease in the abundance of ECA_{LPS}, it is much less than that caused by *elyC* (Fig. 5A). Both *murA* and *elyC* decrease levels of linear ECA observed through immunoblotting (Fig. S4B). This result suggests that increasing the competition for substrates may not be

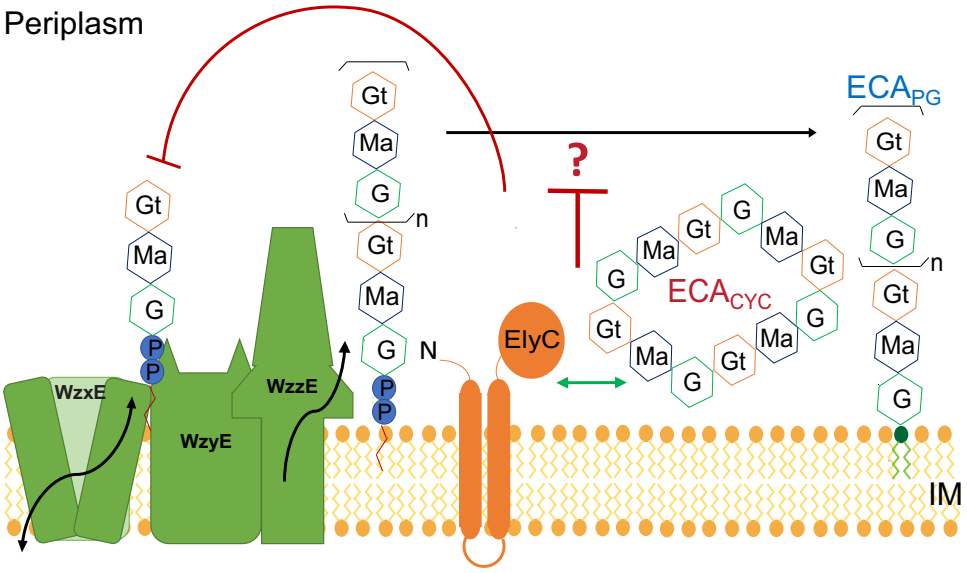

FIG 6 Model for regulation of ECA$_{PG}$ biosynthesis. Our data demonstrate that ElyC and ECA$_{CYC}$ are part of a pathway that regulates the levels of ECA$_{PG}$ and that ElyC can also regulate total ECA levels to a lesser extent. WzxE, WzyE, and WzzE flip completed ECA repeat units on Und-PP across the IM and polymerize the ECA molecule to a regulated chain length. We propose that ElyC and ECA$_{CYC}$ act together to regulate the reaction that removes polymerized ECA from Und-PP and forms phosphoglyceride-linked ECA$_{PG}$. "?" represents the unknown enzymes(s) responsible for this reaction. As ECA$_{CYC}$ is present in the periplasm, its levels can be assessed by ElyC to provide feedback regulation. This assessment may involve a physical interaction.

solely responsible for the effect of ElyC on ECA biosynthesis. If ElyC did act through balancing Und-P utilization, we further reasoned that overexpression of *wecA*, the first gene in ECA biosynthesis, would suppress the effect of *elyC* overexpression on ECA biosynthesis. To determine whether this was true, we overexpressed both *wecA* and *elyC* in the wild-type strain. We observed that overexpression of *wecA* increased production of ECA$_{LPS}$ when *elyC* was not overexpressed (Fig. 5B, fucose samples). However, there was no increase in ECA$_{LPS}$ levels with *wecA* overexpression when *elyC* was induced with low arabinose or high arabinose levels (Fig. 5B). Therefore, the effect of ElyC on total ECA levels is not through allocation of Und-P.

DISCUSSION

In this study, we have elucidated a novel pathway regulating the biosynthesis of ECA$_{PG}$ (Fig. 6). We have provided evidence that ElyC specifically regulates the biosynthesis of ECA$_{PG}$ by acting as an inhibitor under normal physiological conditions. Furthermore, we have revealed that full function of ElyC requires the presence of ECA$_{CYC}$ and that ECA$_{CYC}$ itself can regulate the level of ECA$_{PG}$. The mechanism of regulation by both ElyC and ECA$_{CYC}$ appears to be posttranscriptional.

A number of studies have shown that disruption of intermediate steps in ECA biogenesis leads to isoprenoid carrier stress and peptidoglycan synthesis defects due to the accumulation of ECA synthesis intermediates on Und-P (26–30). In fact, a recent study has biochemically confirmed the accumulation of these intermediates (25). Similar defects have been observed with the disruption of O-antigen or colanic acid biosynthesis (60, 83). It is clear that the stress on peptidoglycan synthesis increases the further down the ECA biosynthesis pathway that the disruption occurs (26, 29, 60). Thus, blocking the pathway after the first sugar is added to Und-P causes very little stress, while blocking the pathway after addition of the next sugar causes cell shape defects, stress response activation, and increased permeability, and blocking the pathway after the addition of the third sugar is lethal (26–29, 30).

The possibility that, if only one form of ECA could be made, disruption of a step in ECA biosynthesis past polymerization would also be lethal led us to our TraDIS

approach to studying ECA_{PG} biosynthesis. Interestingly, we observed the most robust phenotype from increased ECA_{PG} biosynthesis rather than loss of ECA_{PG} biosynthesis, elucidating another route to sequester Und-P. Under these conditions, the transfer of ECA from Und-PP to make ECA_{PG} may become limiting, causing the buildup of polymerized ECA on Und-PP. Although we are analyzing other hits obtained in the TraDIS experiment, it is likely the phenotype of the Δ*elyC* mutant has a more substantial effect than the loss of ECA_{PG} synthesis itself. In this case, our data would suggest that the Und-PP released by the polymerization of ECA combined with new synthesis of Und-P is sufficient for peptidoglycan synthesis. Recent advances in the biochemical analysis of ECA biosynthesis intermediates (25) will make this an interesting area for future investigation.

With the methods of detecting ECA we have employed, we cannot exclude the possibility that the increase in ECA we observe when deleting *elyC* is due to the accumulation of polymerized ECA on Und-P, suggesting Δ*elyC* disrupts ECA_{PG} biosynthesis. In this model, overexpressing *elyC* would decrease the accumulation of ECA on Und-P, leading to lower levels of ECA observed. However, we do not feel this model fits our data well for the following reasons. (i) The ECA species observed in the immunoblot assay appears to be the major form of ECA present in the cells. To our knowledge, polymerized ECA attached to Und-P has never been detected when Und-P-linked ECA biosynthesis intermediates are investigated (25, 45). In fact, loss of the ability to make a full subunit of ECA was required to initially demonstrate that ECA is synthesized on Und-P (45). (ii) If overexpressing *elyC* increases conversion of Und-P-linked ECA to ECA_{PG}, an amount of ECA_{PG} should be produced equal to the amount of Und-P-linked ECA lost, leading to very little change in the amount of detectable linear ECA when *elyC* is overexpressed. (iii) At least some proportion of the increased ECA observed when *elyC* is deleted is surface exposed, and it is unlikely that there is a surface exposure mechanism for Und-P-linked ECA. (iv) Finally, accumulation of Und-P-linked ECA cannot explain the large decrease in linear ECA levels observed when *waaL* is deleted. Deletion of *waaL* prevents the production of ECA_{LPS}, removing one possible route for Und-P-linked ECA to be processed to the final forms of ECA; therefore, deletion of *waaL* would not be expected to decrease levels of Und-P-linked ECA.

The synthesis of ECA overall, and of ECA_{PG} in particular, occurs at the IM, a location that is physically distant from the eventual localization of ECA_{PG} at the cell surface (21). Thus, it would be difficult for the biosynthesis of ECA_{PG} to be directly regulated based on the accumulation of ECA_{PG} on the cell surface. ECA_{CYC} provides an ideal solution to this problem, allowing the biosynthesis of ECA to be assessed using a molecule located in the more accessible periplasm (29). As an IM protein with N-terminal transmembrane domains and a comparatively large periplasmic domain (58), ElyC is well situated to interact physically, as well as genetically, both with the ECA biosynthesis machinery and with ECA_{CYC}. Studies of ElyC's physical interactions are ongoing in our lab.

Given their respective localizations, we favor a model in which ElyC provides feedback regulation for the reaction(s) producing ECA_{PG} based on the levels of ECA_{CYC} present in the periplasm (Fig. 6). In this model, we speculate that ElyC undergoes constant transient interactions with ECA_{CYC} that control the activity or binding capability of ElyC. Thus, when ElyC and ECA_{CYC} interact, ElyC would become functional and inhibit the reaction producing ECA_{PG}, possibly through direct interaction with the protein(s) responsible for synthesizing ECA_{PG}. The inhibition could occur through alteration of activity or of degradation rates of the protein(s) producing ECA_{PG}. The amount or time of interaction between ElyC and ECA_{CYC} would, therefore, control how much ECA_{PG} is produced. Levels of ECA_{CYC} could in this way be constantly monitored to maintain appropriate levels of ECA production, while leaving ECA_{CYC} largely free to perform its functional roles in the cell.

In this model, it would be possible for ElyC and ECA_{CYC} to act alone. However, it is also possible that other members of the regulatory pathway exist. These pathway members would not be in our TraDIS data set if their loss did not cause a large increase

in ECA_{PG} biosynthesis (e.g., their effects are not inhibitory or they are redundant) or if the genes involved are essential due to their roles in other pathways. If there are other pathway members involved to transmit signals to the cytoplasm, the regulation of ECA_{PG} biosynthesis could also occur by controlling the levels of the protein(s) responsible for ECA_{PG} biosynthesis.

Previous work found deletion of *elyC* causes lysis of cells at room temperature in LB medium with 1% NaCl (LB Miller) (28). This lysis occurred due to a severe defect in peptidoglycan biosynthesis, which was attributed to allocation of Und-P between biosynthesis pathways. The peptidoglycan biosynthesis defect was not observed in cells grown at 37°C (28), the temperature at which our experiments were performed. Nevertheless, our results are consistent with the observation of isoprenoid stress inhibiting peptidoglycan synthesis in Δ*elyC* strains. Specifically, we have determined that this stress is due to the overproduction of ECA_{PG} rather than to the initial allocation of Und-P for peptidoglycan biosynthesis. In fact, our data demonstrate that the effect of ElyC overexpression on total ECA levels is epistatic to the allocation of Und-P between biosynthesis pathways (Fig. 5). Thus, the effect of ElyC on Und-P availability for peptidoglycan synthesis is due to its role in regulating the synthesis of ECA_{PG}.

Our data suggest three possible explanations for the more severe phenotype of *elyC* loss at lower temperatures. (i) Disruption or dysregulation of biosynthetic or transport pathways, such as protein secretion, can lead to cold-sensitive phenotypes due to slowing of the pathway at lower temperatures (84). Thus, growth at room temperature might slow ECA synthesis more than peptidoglycan synthesis, leading to increased sequestration of Und-P at lower temperatures. (ii) We have observed the chain length (number of repeat units per molecule) of ECA is less at 30°C than at 37°C (36). Thus, the same amount of ECA repeat units will be made into more final ECA molecules, increasing the amount of Und-P utilized for ECA synthesis at lower temperatures. (iii) Finally, ElyC may have an additional function at room temperature that also diverts Und-P from peptidoglycan synthesis that is not apparent at 37°C. Interestingly, Kouidmi et al. found an increase in periplasmic protein aggregation when Δ*elyC* cells were grown at room temperature that could be suppressed by overproduction of two periplasmic chaperones, DsbG and Spy, leading to restoration of peptidoglycan biosynthesis (69). These data may suggest an additional function for ElyC during growth at low temperatures.

The three forms of ECA are synthesized from a common precursor—polymerized ECA on Und-PP. Clearly, mechanisms are necessary to ensure the proper balance is maintained between the forms of ECA, both to support their proper functions and to avoid stress caused by dysregulation of biosynthetic pathways. WaaL, the O-antigen ligase, attaches both ECA and O-antigen to LPS (43, 85). In smooth strains that produce O-antigen, very little ECA_{LPS} is produced (43, 85, 86), suggesting that the availability of WaaL is a rate-limiting step in the production of ECA_{LPS}. Our results confirm that increasing the levels of WaaL causes more ECA_{LPS} to be produced. Our data further suggest the regulation of production of ECA_{PG} and ECA_{CYC} is dependent on feedback regulation by ElyC based on ECA_{CYC} levels. The lesser effect of this regulatory pathway on ECA_{LPS} may suggest that ECA_{LPS} production is largely a by-product of O-antigen synthesis. In many *Enterobacterales*, O-antigen shares an initial GlcNAc residue with ECA (43, 87, 88), which may lead WaaL to be somewhat promiscuous. Nevertheless, surface exposure of ECA_{LPS} leads to production of ECA antibodies, the consequences of which have not been fully explored (21).

Since the discovery of ECA_{CYC}, there has been longstanding debate about whether ECA_{CYC} has a role in biosynthesis of the other forms of ECA or plays its own functional role within the cell. Previously, we demonstrated ECA_{CYC} plays a role in maintaining the OM permeability barrier (36). In our current work, we show that ECA_{CYC} also plays a role in regulating the synthesis of ECA_{PG}. Thus, our work clearly indicates that ECA_{CYC} plays a dual role in the cell—both necessary for the proper function of the OM permeability barrier and involved in the regulation of ECA synthesis. This can be compared to classic

biosynthetic pathways, such as those for amino acid synthesis, where the product of the pathway, useful in and of itself, also acts to allosterically regulate its own production, maintaining a constant pool of the biosynthetic product (89). Overall, the discovery of the ElyC-ECA_{CYC} pathway controlling ECA_{PG} biosynthesis will provide a foothold in characterization of the mechanism of ECA_{PG} biosynthesis, in understanding the regulation of ECA synthesis under changing environmental conditions, and in investigating both the functional and regulatory role of ECA_{CYC}.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains, plasmids, and primers used in this study are listed in Table S4 in the supplemental material. Strains were grown at 37°C in LB Lennox medium with the necessary antibiotics: kanamycin (25 mg/liter), chloramphenicol (20 mg/liter), and tetracycline (20 mg/liter), unless otherwise noted. IPTG at the indicated concentrations (0 to 100 μM) was used for overexpression from the pCA24N plasmid. L-Arabinose and α-D-fucose at the indicated concentrations were used to induce or repress the P_{BAD} promoter, respectively. The deletion alleles were utilized from the Keio collection (65), unless otherwise indicated. New deletion alleles were constructed using λ-Red recombineering, as has been described previously (90). Mutants were made by P1*vir* transduction (91). Markerless deletion strains were generated by flipping out the kanamycin cassette with Flp recombinase as described previously (90).

Plasmids from the ASKA library were used for overexpression experiments (79). *elyC* with its native ribosome binding site (RBS) was cloned into pBAD33 (92) through HiFi Assembly (New England Biolabs [NEB]) per the manufacturer's protocol using the pBAD33 and *elyC* (o/l pBAD33) primers (Table S4). Subsequently, the chloramphenicol resistance cassette was replaced with the kanamycin resistance cassette from pZS21 (93) using HiFi assembly and the pBAD33-*elyC* and kanR primers (Table S4). The promoter region of the *wec* operon was cloned from −500 to +20 in relation to the start codon of *wecA* upstream of the *luxCDABE* operon in the pJW15 vector (80, 81) using HiFi assembly and the Pwec and pNLP10/JW15 primers (Table S4).

TraDIS sample preparation and analysis. Transposon mutant libraries were constructed from Δ*wzzE* Δ*waaL* and Δ*wecA* Δ*wzzE* Δ*waaL* strains by electroporation of the EZ-Tn*5* <KAN-2>Tnp transposome (Lucigen) as previously described (94). The library in wild-type MG1655 was previously described (94). About 306,000 and 186,000 individual colonies were pooled for the initial transposon library of the Δ*wzzE* Δ*waaL* and Δ*wecA* Δ*wzzE* Δ*waaL* strains, respectively. Liquid LB cultures were grown from the pooled libraries of mutants for 10 generations. DNA was extracted from the pooled libraries before and after growth in liquid medium using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. Next, Illumina DNA fragment libraries were prepared using the TraDIS approach and sequenced on an Illumina HiSeq 2500 sequencer, as has been described previously (64, 94). Data were analyzed and mapped to the *E. coli* K-12 genome NC_000913.3 as has been described previously (94).

Analysis of ECA levels. (i) Immunoblot analysis for linear ECA levels. ECA levels were assayed from overnight cultures, as previously described with slight modifications (36). Specifically, membranes were probed with a rabbit polyclonal anti-ECA antibody at a 1:30,000 dilution (a kind gift from Renato Morona at the University of Adelaide). Goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Prometheus) was used at a 1:100,000 dilution and detected using Prosignal Pico ECL (Prometheus) using Prosignal enhanced chemiluminescence (ECL) blotting film (Prometheus).

(ii) Dot blot for ECA surface exposure. Surface-exposed ECA was detected using a dot blot assay as has been described previously with minor modifications (72). Specifically, the following antibodies were used for detection: anti-ECA (1:5,000), anti-BamD (1:5,000), and anti-RcsF (1:5,000) (95, 96).

(iii) WGA staining for ECA_{LPS} quantification. Standard conditions for WGA staining of ECA_{LPS} were as follows. Two hundred fifty microliters of overnight culture was centrifuged for 3 min at 3,400 × *g* in round-bottom 96-well plates. After removing the supernatant, pelleted cells were washed with 200 μl of 1× phosphate-buffered saline (PBS). After washing, cells were resuspended in 200 μl of 1× PBS with a 1:100 volume of WGA-AF488 (Thermo Fisher Scientific) prepared per the manufacturer's instructions. Samples were incubated in the dark at room temperature for 10 min. Then, cells were washed twice with 200 μl 1× PBS and resuspended in 110 μl 1× PBS. Next, 100 μl of each sample was aliquoted to a black-wall, clear-bottom 96-well plate where the optical density at 600 nm (OD_{600}) and fluorescence at excitement (Ex.) 485 nm and emission (Em.) 519 nm was recorded using a BioTek Synergy H1 plate reader autogained based on sample fluorescence. Fluorescence relative to OD_{600} was calculated.

(iv) Quantification of ECA_{CYC}. For ECA_{CYC} quantification, ECA_{CYC} was purified and subjected to matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry, and the relative abundance of ECA_{CYC} between samples was calculated as has been described previously (29, 36).

P_{wec} reporter assay. Overnight cultures of the indicated strains containing the pJW15-P_{wec} plasmid were subcultured (1:100) into 100 μl of fresh LB broth in a black-wall, clear-bottom 96-well plate. The plate was sealed with a Breathe-Easy sealing membrane (Sigma), and the luminescence and OD_{600} of each strain were measured every 3 min for 6 h using a BioTek Synergy H1 plate reader as previously described (80, 81). Each biological replicate was performed in technical quadruplicate.

Data availability. The sequencing data are available in the Sequence Read Archive database (SRA) (https://www.ncbi.nlm.nih.gov/sra, BioProject ID PRJNA763934).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, JPG file, 1.8 MB.
FIG S2, JPG file, 1.3 MB.
FIG S3, JPG file, 0.1 MB.
FIG S4, JPG file, 0.1 MB.
FIG S5, JPG file, 0.1 MB.
FIG S6, JPG file, 1.1 MB.
TABLE S1, PDF file, 0.1 MB.
TABLE S2, PDF file, 0.1 MB.
TABLE S3, PDF file, 0.1 MB.
TABLE S4, PDF file, 0.2 MB.

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