Effects of Lipopolysaccharide Core Sugar Deficiency on Colanic Acid Biosynthesis in Escherichia coli

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ABSTRACT

When 10 Escherichia coli mutant strains with defects in lipopolysaccharide (LPS) core biosynthesis were grown on agar medium at 30°C, four of them, the ΔwaaF, ΔwaaG, ΔwaaP, and ΔwaaB strains, formed mucoid colonies, while the other six, the ΔwaaU, ΔwaaaR, ΔwaaO, ΔwaaC, ΔwaaQ, and ΔwaaY strains, did not. Using light microscopy with tannin mordant staining, the presence of exopolysaccharide around the cells of the mutants that formed mucoid colonies could be discerned. The ΔwaaF mutant produced the largest amounts of exopolysaccharide, regardless of whether it was grown on agar or in liquid medium. The exopolysaccharide was isolated from the liquid growth medium of ΔwaaF cells, hydrolyzed, and analyzed by high-performance liquid chromatography with an ion-exchange column, and the results indicated that the exopolysaccharide was consistent with colanic acid. When the key genes related to the biosynthesis of colanic acid, i.e., wza, wzb, wzc, and wcaA, were deleted in the ΔwaaF background, the exopolysaccharide could not be produced any more, further confirming that it was colanic acid. Colanic acid could not be produced in strains in which the waaC, waaF, waaQ, and ΔwaaY genes were deleted, suggesting that a change of lipopolysaccharide structure in ΔwaaF cells might be sensed by the RcsCDB phosphorelay system, leading to the production of colanic acid. The results demonstrate that E. coli cells can activate colanic acid production through the RcsCDB phosphorelay system in response to a structural deficiency of lipopolysaccharide.

IMPORTANCE

Lipopolysaccharide and colanic acid are important forms of exopolysaccharide for Escherichia coli cells. Their metabolism and biological significance have been investigated, but their interrelation with the cell stress response process is not understood. This study demonstrates, for the first time, that E. coli cells can activate colanic acid production through the RcsCDB phosphorelay system in response to a structural change of lipopolysaccharide, suggesting that bacterial cells can monitor the outer membrane integrity, which is essential for cell survival and damage repair.

Bacteria produce a wide range of exopolysaccharides, which have specific monomer compositions, substituent decorations, and biosynthetic pathways. Genes involved in biosynthesis of these exopolysaccharides are often clustered in specific loci within the genome and encode glycosyltransferases, polymerizing and branching enzymes, and enzymes responsible for addition of substituents (1). Escherichia coli K-12 cells contain several gene clusters related to biosynthesis of polysaccharides (2–7), such as lipopolysaccharide (LPS) and colanic acid (CA) (Fig. 1). Understanding the fundamental processes involved in exopolysaccharide biosynthesis and the regulation of these processes is important.

As a major molecule in the outer membrane of E. coli K-12 cells (8, 9), LPS consists of a hydrophobic lipid A domain (8, 10) and a nonrepeating core oligosaccharide. The core oligosaccharide typically contains residues of 3-deoxy-D-manno-octulosonic acid (Kdo), heptose (Hep), glucose (Glc), galactose (Gal), and phosphate substituents, and these constituents are synthesized by enzymes encoded by the waa gene cluster (Fig. 1A). WaaC and WaaF sequentially add Hep to Kdo2-lipid A. WaaP and WaaY add phosphate groups to the first and second Hep residues. WaaQ adds the third Hep to the second Hep residue (11, 12). WaaG adds the first Glc residue to the second Hep residue (13). WaaB adds a Gal residue to the first Glc. WaaO and WaaR add the second and third Glc residues to the first Glc. WaaU adds the fourth Hep to the third Glc (14). Single deletions of the genes encoding the enzymes for addition of the core oligosaccharide of LPS in E. coli W3110 resulted in different LPS structures (Fig. 1A) (15). The core oligosaccharide of the ΔwaaY strain was devoid of phosphate on the second Hep (11). In the ΔwaaQ strain, the third Hep was lost and WaaY activity was impaired, while in the ΔwaaP strain the phosphoryl substituents on the first Hep were not seen and activities of WaaQ and WaaY were obviated (11).

E. coli CA is made from a repeat unit of Glc, Gal, fucose (Fuc), and glucuronic acid (GlcA) (16), and its biosynthesis is related to the wca cluster of 20 genes (Fig. 1B) (17, 18). The genes for synthesis of the fucose nucleoside-sugar precursors are localized within the CA gene cluster. The manB and manC genes are directly involved in the biosynthesis mechanism of GDP-mannose, which...
is converted via a three-step pathway to yield GDP-fucose. The wcaI gene is located with the fucose-synthesizing genes, so WcaI is a putative glycosyltransferase involved in transfer of the Fuc unit. The genes for the synthesis of the other nucleotide-sugar precursors can be found dispersed in the genome. The stepwise assembly of the repeat unit is initiated via the action of WcaJ, which transfers the first Glc unit to the C55 lipid carrier. The next sugar monomers are transferred by WcaA, WcaC, WcaE, or WcaL. The order of the synthesis steps is not completely clarified and was proposed based mainly on sequence similarities of these putative enzymes to enzymes in other bacterial polysaccharide pathways, not on biochemical evidence. The structural similarity found for WcaL suggests an involvement in transfer of a Gal or GlcA unit (1, 17). Wza, Wzb, and Wzc play important roles in polymerization and translocation of CA from the inner membrane to the cell surface (1). Wza and Wzc span the cell envelope, while the protein tyrosine phosphatase Wzb controls the phosphorylation state of Wzc (19).

Expression of the wca operon is regulated by the RcsCDB phosphorelay system (20–24). The RcsCDB phosphorelay system includes the transmembrane sensor kinase RcsC, the inner membrane protein RcsD, and the response regulator RcsB; the accessory coregulator RcsA and the membrane-bound lipoprotein RcsF are also involved (24, 25). Signals are normally transduced by RcsF exposed on the cell surface to RcsC (26); RcsC autophosphorylates and transfers phosphate to RcsD and then to the cytoplasmic RcsB protein (17). RcsB, either alone or as a dimer with RcsA, activates transcription at the single promoter upstream of the wca operon for maximal CA expression. RcsA availability is normally limited because this protein is rapidly degraded by the temperature-sensitive ATP-dependent protease Lon (27). RcsC and RcsD transfer the phosphate group to RcsB to modulate gene expression in the following three possible ways: (i) RcsC is autophosphorylated and interacts with RcsD, (ii) RcsC is autophosphorylated and forms a complex with a nonphosphorylated RcsC protein, or (iii) RcsD is autophosphorylated and interacts with a nonphosphorylated RcsD protein (28–30).

Normally, CA is not produced when E. coli is grown as planktonic cultures, and its biosynthesis is dependent on damage to the cell envelope structure, osmotic shock, or growth temperature (23, 31–37). Both CA and LPS are synthesized in the cytosol, assembled at the outer surface of the inner membrane, and subsequently transported beyond the outer membrane (8), and they play important roles in biofilm formation (9, 38). Outer membrane integrity is monitored by the stress response system, which initiates damage repair pathways. This process is essential for cell survival (8). In recent years, the metabolism and biological significance of LPS and CA have been investigated, but the interrelation between LPS and CA in the stress response process is not yet clear.

Under certain conditions, CA can attach to LPS through a covalent linkage (39). To study the interrelation between LPS and CA, we investigated CA production in 11 E. coli K-12 strains that synthesize different LPS species. Four E. coli LPS core mutants, the ΔwaaF, ΔwaaG, ΔwaaP, and ΔwaaB strains, produced CA, but the other six LPS core mutants, the ΔwaaU, ΔwaaR, ΔwaaO, ΔwaaC, ΔwaaQ, and ΔwaaY strains, did not. ΔwaaF cells produced the largest amounts of CA. The results suggest that E. coli...
K-12 cells activate CA production through the RcsCDB phosphorelay system in response to LPS structural deficiency.

MATERIALS AND METHODS

Construction of E. coli mutant strains and growth conditions. The E. coli strains used in this study are listed in Table 1. They were usually grown in LB medium (5 g/liter yeast extract, 10 g/liter tryptone, and 10 g/liter NaCl) or M9 medium (17.1 g/liter Na2HPO4 12H2O, 3 g/liter KH2PO4, 4 g/liter glucose, 1 g/liter NH4Cl, 0.5 g/liter NaCl, 0.24 g/liter MgSO4, and 0.011 g/liter CaCl2). When necessary, 100 mg/liter ampicillin or 50 mg/liter kanamycin was added.

E. coli W3110 and the ΔwaaF strain (40) were used as background strains (41, 42). Mutants WR001 and WR002 were constructed from W3110 and the ΔwaaF strain, respectively, by deleting wza, wzb, and wzc from the chromosome. The wza, wzb, and wzc genes are contiguous, and there is a stem-loop transcriptional attenuator (4) between wzb and wzc; therefore, they were all deleted together. Briefly, the upstream fragment of this gene cluster was PCR amplified using the primers wzb2-A-F and wzb2-A-R, and the downstream fragment of the cluster was PCR amplified using the primers wzc1-C-F and wzc1-C-R. A DNA fragment containing the kanamycin resistance gene, cre-loxP-kan, was amplified from pDTW201 by using the primers Cre-loxP-kan-U and Cre-loxP-kan-D. The three fragments were then ligated together to form plasmid pBS-kan-wzb2cA, carrying the knockout fragment kan-wzb2cA. Plasmid pKD46 was first transformed into W3110 or the ΔwaaF strain, and then the knockout fragment kan-wzb2cA was amplified and transformed into the cells. The wza, wzb, and wzc genes were deleted from the chromosome through recombination catalyzed by the lambda Red system expressed by pKD46.

To delete the rcsB gene from the chromosome of the ΔwaaF strain, upstream and downstream fragments of rcsB were PCR amplified by using the primer pairs rcsB1-U-F/rcsB1-U-R and rcsB2-D-F/rcsB2-D-R, respectively.

These two fragments were linked with the kanamycin resistance gene, frt/kan, into the plasmid pBluescript II SK(+). The DNA segment was then PCR amplified using the primers rcsB1-U-F and rcsB2-D-R and transformed into ΔwaaF cells containing the plasmid pKD46 by electroporation, resulting in the replacement of rcsB with kan in the chromosome of the ΔwaaF strain. Similarly, the rcsA, rcsC, rcsD, or rcsF gene in the chromosome of the ΔwaaF strain was replaced by kan. Primers rcsA1-U-F and rcsA1-D-R were used for the deletion of rcsA, rcsC1-U-R and rcsC1-D-R were used for deletion of rcsC, and rcsF1-U-F and rcsF1-D-R were used for deletion of rcsF (42).

The correct transformants were selected by growing cells on LB plates containing kanamycin, and they were cured of the plasmid pKD46 by growing the cells at 42°C. Next, the plasmid pCP20 was transferred into the cells, and FLP recombinase was expressed to remove the kan gene inserted in the chromosome. The strains were then cured of the plasmid pCP20 by growing cells at 42°C, resulting in the WR001, WR002, ΔwaaF ΔrcsB ΔwaaF ΔrcsC ΔwaaF ΔrcsD strain (Table 1). The successful insertion and deletion of kan in these mutants were confirmed by PCR analysis. There were no selection markers left on the chromosomes, and thus the strains could grow in medium without the selective pressure of antibiotics. All primers used in this study are listed in Table 2.

Identification of exopolysaccharides of E. coli mutant strains. Exopolysaccharides produced by E. coli cells were identified by tannin mordant staining and electron microscopy by use of a previously published method (43), with minor modifications. Bacteria were grown on M9 agar medium at 22°C for 5 days, 30°C for 2 days, or 37°C for 1 day. Cells were fixed on slides by smearing and drying and then treated with fuchsin solution for 3 min. Fuchsin solution is a mixture composed of 0.3 g basic fuchsin, 10 ml 95% ethanol, and 90 ml 5% phenol. Next, mordant solution was added for 3 min and then washed with distilled water. Mordant solution is a freshly prepared mixture containing 2 volumes of 0.3 g/liter

| Table 1 | Strains and plasmids used in this work |
|-----------------|-----------------|-----------------|
| Strain or plasmid | Description | Source or reference |
| Wild-type strain | E. coli W3110 | ATCC |
| ΔwaaU mutant | ΔwaaU | 40 |
| ΔwaaR mutant | ΔwaaR | 40 |
| ΔwaaO mutant | ΔwaaO | 40 |
| ΔwaaG mutant | ΔwaaG | 40 |
| ΔwaaF mutant | ΔwaaF | 40 |
| ΔwaaC mutant | ΔwaaC | 40 |
| ΔwaaP mutant | ΔwaaP | 40 |
| ΔwaaQ mutant | ΔwaaQ | 40 |
| ΔwaaY mutant | ΔwaaY | 40 |
| ΔwaaB mutant | ΔwaaB | 40 |
| WR001 | Δwza-wzb-wzc-wcaA::kan | This work |
| WR002 | ΔwaaF Δwza-wzb-wzc-wcaA::kan | This work |
| ΔwaaF ΔrcsA mutant | ΔwaaF ΔrcsA::kan | This work |
| ΔwaaF ΔrcsB mutant | ΔwaaF ΔrcsB::kan | This work |
| ΔwaaF ΔrcsC mutant | ΔwaaF ΔrcsC::kan | This work |
| ΔwaaF ΔrcsD mutant | ΔwaaF ΔrcsD::kan | This work |
| ΔwaaF ΔrcsF mutant | ΔwaaF ΔrcsF::kan | This work |
| pKD46 | P_waa::pEX(Rep(Ts)) AmpR | 42 |
| pKD13 | pCP20 | 42 |
| pDTW201 | Derived from pBluescript II SK(+) by inserting the loxP-kan-loxP segment | 15 |
| pBS-kan-wzb2cA | Plasmid for deleting wza, wzb, and wzc genes; Kan' Amp' | This work |
| pBS-kan-rcsB | Plasmid for deleting rcsB gene; Kan' Amp' | This work |
| pBlueScript II SK(+) | Cloning vector; CoE1 lacZ Amp' | Stratagene |
FeCl₃, 2 volumes of 1.5 g/liter tannins, and 5 volumes of a 2-g/liter saturated solution of potassium aluminum sulfate. For counterstaining, 1% methylene blue was used for 30 s prior to examination of the samples under an oil immersion lens. The cells were stained red and the exopolysaccharides blue.

E. coli K-12 wild-type strain W3110 and its mutant ΔwaaF strain were grown on M9 agar plates at 37°C for 24 h. Bacterial colonies were harvested and washed twice with 10 mM phosphate-buffered saline (PBS), pH 7.2. The cells were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide vapor and washed twice with 10 mM PBS (pH 7.2). Cell samples were placed on grids, contrast stained with uranyl acetate, and analyzed by transmission electron microscopy (TEM) (model H-7650; Hitachi, Japan) at an accelerating voltage of 80 kV.

**Purification of CA produced by E. coli cells.** CA was purified from E. coli cells according to a previously published procedure (44), with minor modifications. Since the yeast extract used in LB medium contains monosaccharides (45) which could interfere with CA analysis, M9 medium was chosen for culture of E. coli cells. Cells were grown in M9 medium at 30°C for 2 days with shaking at 200 rpm and harvested by centrifugation at 18,000 × g for 45 min. The supernatant was collected and mixed with 3 volumes of ice-cold anhydrous ethanol. After precipitating at 4°C for 5 h, the solid product was resuspended in water to a concentration of 100 mg/mL. The mixture was dried by use of N₂, and the mixture was collected and dissolved in 1 ml distilled water, treated with 0.2 M trisodium citrate and heated at 100°C for 5 min. After cooling, 100 μL of 1 M hydroxylamine (1.5 g/liter) was added to the sample, and the absorbance was measured at 526 nm. M9 liquid medium was used as a control, and a standard curve was made using different concentrations of glucuronic acid. The numbers of cells used to determine the levels of CA production were normalized by serial dilution and plating.

**RESULTS**

Some E. coli LPS core mutant cells secrete large amounts of mucoid exopolysaccharide. Previously, we investigated the influence of the LPS core oligosaccharide on membrane stability, permeability, biofilm formation, and autoaggregation by using a whole set of single-gene deletion mutants of E. coli W3110 (40). When the strains were grown on solid M9 medium at 30°C for 2 days, E. coli W3110 and most of the mutants showed rough colonies, consistent with their LPS structures, which contain no O-antigen repeats. However, the ΔwaaG, ΔwaaF, ΔwaaP, and ΔwaaB mutants formed mucoid colonies. Mucoid colonies are usually associated with an excessive production of exopolysaccharide; therefore, colonies of E. coli W3110 and the 10 mutant strains were shown to be mucoid and observed by microscopy. A blueish mucoid substance was observed around the cells of the ΔwaaG, ΔwaaF, ΔwaaP, and ΔwaaB strains but not those of W3110 and the other mutants (Fig. 2). Exopolysaccharide production was detected for the ΔwaaG, ΔwaaF, ΔwaaP, and ΔwaaB strains when these mutants were grown on solid M9 medium at 30°C, leading to the mucoid colonies. Exopolysaccharide production was not detected for cells complemented with waaG,
waaF, waaP, and waaB, respectively (data not shown), suggesting that these genes are somehow associated with the biosynthesis of CA.

To investigate the effect of temperature on exopolysaccharide formation, the above 11 E. coli strains were grown on solid M9 medium at 22°C and 37°C and detected using tannin mordant staining. After growth of the strains at 37°C for 1 day, a blueish mucoid exopolysaccharide was observed only around ΔwaaF cells, not around the other 10 strains. After growth of the strains at 22°C for 5 days, large amounts of blueish mucoid exopolysaccha-

ride were observed around cells of the ΔwaaG and ΔwaaF mutants and relatively small amounts of exopolysaccharide were observed around W3110, ΔwaaB, ΔwaaC, ΔwaaP, ΔwaaQ, ΔwaaU, and ΔwaaY cells (Fig. 2). The observation of more strains producing the exopolysaccharide at the low temperature suggested that the exopolysaccharide might be CA, as CA production in bacteria has been shown to increase when cells are grown at low temperatures (36, 44, 48).

Since the ΔwaaF strain produced an extremely large amount of exopolysaccharide (Fig. 2), ΔwaaF colonies formed at 30°C were further analyzed by electron microscopy, using wild-type W3110 as a control (Fig. 3). A mucoid substance was found around ΔwaaF cells (Fig. 3B) but not around W3110 cells (Fig. 3A), while flagella were found on W3110 cells (Fig. 3A) but were not observed around ΔwaaF cells (Fig. 3B). This suggests that the LPS structural change in E. coli ΔwaaF might affect the production of exopolysaccharide and flagella on the cell surface.

The exopolysaccharide secreted by the ΔwaaF strain was loosely associated with the bacterial cells (Fig. 3). Since the exopolysaccharide might have been released when the cells were grown in liquid medium, we collected samples for analysis. E. coli W3110 and the 10 LPS core mutant strains (ΔwaaU, ΔwaaR, ΔwaaO, ΔwaaG, ΔwaaF, ΔwaaC, ΔwaaP, ΔwaaQ, ΔwaaY, and ΔwaaB strains) were grown in liquid M9 medium at 18°C, 22°C, 30°C, and 37°C. Exopolysaccharide samples were collected from the culture medium and analyzed (Fig. 4). When cells were grown at 37°C, only very small amounts of exopolysaccharide were secreted

FIG 2 Light microscopic observation of mordant-stained cell colonies of 11 E. coli strains grown on M9 solid medium for 5 days at 22°C, for 2 days at 30°C, or for 1 day at 37°C. Magnification, ×1,000.

FIG 4 Comparison of exopolysaccharide production levels of 11 E. coli strains grown in M9 liquid medium at different temperatures (18°C, 22°C, 30°C, and 37°C) for 2 days. M9 liquid medium was used as a control. Error bars indicate standard deviations for three parallel samples.
by a few strains. However, when cells were grown at 30°C, a significant amount of exopolysaccharide (0.27 mg/liter) was produced by the _E. coli_ waaF strain, while negligible amounts of exopolysaccharide (<0.1 mg/liter) were produced by the other strains. When cells were grown at 22°C, 0.10 mg/liter exopolysaccharide was produced by the waaF strain, while no detectable amount of exopolysaccharide was produced by the other strains. When cells were grown at 18°C, 0.04 mg/liter exopolysaccharide was produced by the waaF strain, 0.01 mg/liter exopolysaccharide was produced by the waaB strain, and no detectable exopolysaccharide was produced by the other strains. Overall, exopolysaccharide production in liquid medium was not as significant as that observed on solid medium. We observed that _E. coli_ waaF cells produced more exopolysaccharide than the other 10 _E. coli_ strains, regardless of whether they were grown in liquid or on solid medium, especially at 30°C. Therefore, we selected the ΔwaaF mutant grown at 30°C for further investigation.

Identification of the exopolysaccharide secreted by _E. coli_ ΔwaaF cells as CA. Exopolysaccharide was purified from ΔwaaF cells, hydrolyzed, and analyzed by HPLC with ion-exchange columns (Fig. 5). Five peaks were observed in the chromatogram, at 3.3, 9.3, 10.7, 26.1, and 29.1 min. Fucose, glucose, galactose, and glucuronic acid standards processed in parallel also yielded peaks, at 3.3, 9.1, 10.6, and 29.2 min, respectively. Since these four monosaccharide residues in the exopolysaccharide secreted by ΔwaaF cells (Fig. 5) are the major components of CA (Fig. 1B), the results indicated that the exopolysaccharide secreted by ΔwaaF cells was CA. The strong peak at 26.1 min observed in all spectra was raised by the gradient change of the mobile phase used in the analysis.

To verify if the exopolysaccharide secreted by ΔwaaF cells was CA, four contiguous genes (wza, wzb, wzc, and wcaA) of the CA operon were deleted from the chromosomes of W3110 and the ΔwaaF mutant, resulting in _E. coli_ mutant strains WR001 and WR002, respectively. W3110, WR001, the ΔwaaF mutant, and WR002 were grown in liquid medium at 30°C for 48 h, and CA was purified. Although these strains grew well, with optical densities at 600 nm (OD600) of around 2 after 48 h, the levels of CA production in these cells were different (Fig. 6A). CA was produced at 0.006 mg/liter by W3110 cells and at 0.277 mg/liter by ΔwaaF cells, but no CA was produced by WR001 and WR002 cells (Fig. 6A). When the strains were grown on solid medium and stained with tannin mordant, blue mucoid CA was observed only around ΔwaaF cells, not around W3110, WR001, and WR002 cells (Fig. 6B). When the _wca_ operon was damaged by deletion of the _wza_, _wzb_, _wzc_, and _wcaA_ genes, WR001 and WR002 could not produce CA at all, demonstrating that the exopolysaccharide secreted by ΔwaaF cells was CA. The significant difference in CA production in W3110 and ΔwaaF cells suggests that CA production was inhibited in W3110 but activated in ΔwaaF cells; therefore, the structural change of LPS in ΔwaaF cells might be the reason for the high level of CA production.

Production of colanic acid in _E. coli_ ΔwaaF cells is regulated through the RcsCDB phosphorelay system. The RcsCDB phos-

![FIG 5](http://jb.asm.org/) HPLC analysis of hydrolyzed exopolysaccharide isolated from ΔwaaF cells grown in M9 medium at 30°C.

![FIG 6](http://jb.asm.org/) Comparison of CA production levels in _E. coli_ W3110, WR001, the ΔwaaF mutant, and WR002 grown in liquid medium at 30°C for 2 days (A) and microscopic observation of cell colonies grown on solid medium at 30°C for 2 days and stained with mordant (B).
The membrane lipoprotein RcsF senses cell envelope stress and conveys the information to the histidine kinases RcsC and RcsD in the cytoplasmic membrane. To further investigate whether this system is related to the excessive CA production in ΔwaaF cells, double mutant strains (ΔwaaF ΔrcsA, ΔwaaF ΔrcsB, ΔwaaF ΔrcsC, ΔwaaF ΔrcsD, and ΔwaaF ΔrcsF strains) were constructed, and levels of CA production in these strains were determined. In liquid M9 medium, these strains grew as well as the wild-type strain, and their OD₆₀₀ reached about 2.5 after 48 h, but liquid M9 medium, these strains grew as well as the wild-type strain, and their OD₆₀₀ reached about 2.5 after 48 h, but were not detected. The results suggest that the RcsABCD proteins are somehow connected to CA production in E. coli ΔwaaF cells. The LPS structure of Hep-Kdo₂-lipid A in E. coli ΔwaaF cells might stimulate RcsB phosphorylation in an RcsC-independent manner.

**DISCUSSION**

When 10 E. coli mutant strains with defects in LPS core biosynthesis were grown on agar medium at 30°C, four of them, the ΔwaaF, ΔwaaG, ΔwaaP, and ΔwaaB strains, produced CA, while the other six did not. The ΔwaaF mutant produced the largest amount of CA, regardless of whether it was grown on agar or in liquid medium. The RcsCDB phosphorelay system can sense cell envelope stress and regulates more than 100 genes involved in cell division, motility, and biofilm formation (13, 54–56); however, whether it can sense LPS has not been reported previously. To investigate whether the RcsCDB phosphorelay system is related to the excessive CA production in ΔwaaF cells, the rcsA, rcsB, rcsC, rcsD, and rcsF genes were deleted in the ΔwaaF background, resulting in the ΔwaaF ΔrcsA, ΔwaaF ΔrcsB, ΔwaaF ΔrcsC, ΔwaaF ΔrcsD, and ΔwaaF ΔrcsF double mutant strains, respectively. CA production was not observed when the rcsA, rcsB, rcsD, or rcsF gene was deleted from the ΔwaaF strain, but a small amount of CA was produced when the rcsC gene was deleted from this strain, suggesting that the structural change of LPS in ΔwaaF cells might be sensed by the RcsCDB phosphorelay system, leading to the production of CA. RcsF, a surface-exposed lipoprotein, adopts a transmembrane conformation with the lipidated N terminus exposed at the cell surface and the core domain in the periplasm (51); it might sense defects in LPS of the ΔwaaF strain and activate CA production. It has been reported that RcsC and RcsD can transfer a phosphate group to RcsB to modulate gene expression in the following three possible ways: autophosphorylated RcsC interacting with RcsD, autophosphorylated RcsC forming a complex with nonphosphorylated RcsC, and autophosphorylated RcsD interacting with nonphosphorylated RcsD (33, 38). Our results demonstrate that the CA produced in the ΔwaaF mutant is made in an RcsC-independent pathway, since the ΔwaaF ΔrcsC strain produced 1/3 the CA produced by the ΔwaaF strain, while the ΔwaaF ΔrcsD strain did not produce CA. Therefore, we postulate that RcsB phosphorylation might be stimulated through RcsC-RcsD and/or RcsD-RcsD interactions.

The ΔwaaC strain synthesized the shortest LPS with the Gal residue, Kdo₂-lipid A, among the 10 E. coli LPS core mutant strains, but it did not produce CA. The LPS synthesized by the ΔwaaF strain is Hep-Kdo₂-lipid A, suggesting that the 1st Hep is necessary for the RcsCDB phosphorelay system. We propose that RcsF might recognize core-lipid A molecules with the 1st Hep residue to produce RcsD-dependent RcsB phosphorylation. However, further study is required to gain a comprehensive understanding of the interaction between RcsF and the specific core-lipid A structures.

When the strains were grown on solid medium at 37°C, only the ΔwaaF strain produced CA. At 30°C, the ΔwaaF, ΔwaaG, ΔwaaP, and ΔwaaB strains produced CA, and at 22°C, relatively small amounts of CA were observed around most of the 11 strains (Fig. 2). In liquid medium, only the ΔwaaF strain produced a significant amount of CA, and the level of CA production increased when the temperature increased from 18°C to 30°C; however, at 37°C, only a small amount of CA was produced by ΔwaaF cells (Fig. 4). A high temperature was not beneficial for CA production (regardless of growth effects), possibly because the coregulator RcsA can be degraded rapidly at high temperature by Lon, an ATP-dependent protease (48). At 30°C and on solid medium, the ΔwaaF, ΔwaaG, ΔwaaP, and ΔwaaB strains produced CA, while in liquid medium, only the ΔwaaF strain produced CA, possibly because RcsC can be activated when cells are grown on a
solid surface (31). Further investigation is required to understand the detailed effects of temperature on CA production when the LPS structure is changed.

All these analyses revealed that the excessive CA production observed with some LPS defects is a result of multiple factors. The LPS defect in the ΔwaaF strain likely provides a high signal for the RcsCDB phosphorelay system, which make ΔwaaF cells produce a large amount of CA. At the gene level, the CA operon (wca), the RcsCDB system, the rsA gene (encoding an accessory coregulator), and the rcsF gene (encoding a membrane lipoprotein) are all involved. However, further experiments are still needed to explain the function of the whole regulation network.

Apart from the RcsCDB phosphorelay system, the σ28 signaling system can also monitor LPS structural changes in E. coli (57) and trigger a signaling cascade, leading to the upregulation of factors needed to combat the envelope damage. Therefore, a rapid response to structural changes of LPS in the outer membrane may represent a broad strategy for Gram-negative bacteria to monitor their interface with the environment.

ACKNOWLEDGMENT

This study was supported by the National Natural Science Foundation of China (grants 31170069 and 31201290).

FUNDING INFORMATION

This work, including the efforts of Xiaoyuan Wang, was funded by National Natural Science Foundation of China (NSFC) (31170069). This work, including the efforts of Xiaojing Hu, was funded by National Natural Science Foundation of China (NSFC) (31201290).

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