Crosstalk between the lipopolysaccharide and phospholipid pathways during outer membrane biogenesis in *Escherichia coli*

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The outer membrane of gram-negative bacteria is composed of phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet. LPS is an endotoxin that elicits a strong immune response from humans, and its biosynthesis is in part regulated via degradation of LpxC (EC 3.5.1.108) and WaaA (EC 2.4.99.12/13) enzymes by the protease FtsH (EC 3.4.24.-). Because the synthetic pathways for both molecules are complex, in addition to being produced in strict ratios, we developed a computational model to interrogate the regulatory mechanisms involved. Our model findings indicate that the catalytic activity of LpxK (EC 2.7.1.130) appears to be dependent on the concentration of unsaturated fatty acids. This is biologically important because it assists in maintaining LPS/phospholipids homeostasis. Further crosstalk between the phospholipid and LPS biosynthetic pathways was revealed by experimental observations that LpxC is additionally regulated by an unidentified protease whose activity is independent of lipid A disaccharide concentration (the feedback source for FtsH-mediated LpxC regulation) but could be induced in vitro by palmitic acid. Further experimental analysis provided evidence on the rationale for WaaA regulation. Overexpression of *waaA* resulted in increased levels of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) sugar in membrane extracts, whereas Kdo and heptose levels were not elevated in LPS. This implies that uncontrolled production of WaaA does not increase the LPS production rate but rather reglycosylates lipid A precursors. Overall, the findings of this work provide previously unidentified insights into the complex biogenesis of the *Escherichia coli* outer membrane.

lipopolysaccharide | fatty acids | computational model | bacterial membrane regulation

The outer membrane of gram-negative bacteria is decorated with a potent endotoxin (called lipid A), which plays a significant role in bacterial pathogenicity and immune evasion (1). It also acts as a physical barrier protecting the cell from chemical attack and represents a significant obstacle for the effective delivery of numerous antimicrobial agents (2, 3). The outer membrane is composed of phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet (4). Phospholipids consist of a glycerol molecule, a phosphate group, and two fatty acid moieties (except for cardiolipins) (5) (see reviews (5, 6) and *SI Appendix* for the biosynthesis and regulation of phospholipids). LPS, on the other hand, contains three distinct components: lipid A, core oligosaccharides, and O-antigen (7, 8). Lipid A is the sole essential component of LPS, and its biosynthesis involves nine enzyme-catalyzed reactions (8). The lipid A pathway has been widely investigated, and we recently produced a pathway model that incorporates all of the known regulatory mechanisms (9). Briefly, the first reaction step catalyzed by LpxA is highly unfavorable, which makes the proceeding enzyme, LpxC, the first committed enzyme (10). LpxC is regulated by the protease FtsH (11, 12), and we recently postulated that the negative feedback signal arises from lipid A disaccharide, the substrate for LpxK (9). Furthermore, FtsH regulates WaaA (formerly called KdtA), an enzyme downstream of LpxC (13). The exact rationale for WaaA regulation remains unknown.

A wealth of research exists for either LPS or phospholipids biosynthesis; however, our current understanding on the crosstalk between both pathways is limited at the moment. Because both pathways are synchronized to ensure a proper balance of membrane components (11, 14), studies underpinning the underlying mechanisms would appear valuable. There are a number of experimental findings that indicate the existence of strong links between both biosynthetic pathways (11, 15, 16). Thus, in the context of outer membrane biogenesis, the role involving phospholipids cannot be ignored in the study of LPS regulation. Furthermore, during membrane synthesis, ∼20 million molecules of fatty acids are synthesized in *Escherichia coli* (8). Yu et al. (17) reconstituted an in vitro steady-state kinetic system of fatty acid biosynthesis using purified enzymes and observed that the maximum fatty acid production rate obtainable was 100 μM/min. This production rate falls far below the amount of fatty acids required by a cell in vivo [if one assumes a cell volume of 6.7×10^{-16} L (18) and a generation time of 30 min (19)]. Therefore, to test the consistency of reported in vitro parameters and investigate the role of the biosynthetic enzymes on fatty acids turnover rate, a "systems" approach is necessary. Similarly, ever since the regulation of WaaA by FtsH was first reported (13), no

Significance

This work examines the relationship between bacterial phospholipid biosynthesis and lipopolysaccharides (LPS) regulation. Because LPS is a potent endotoxin in addition to being essential for the survival of gram-negative bacteria, our experimental findings are of importance to the fields of microbiology, immunology, and drug design. In addition, the computational aspect of this work represents an in-depth kinetic model comprising 81 chemical reactions; hence, computational and systems biologists would find our work useful. Furthermore, recent interests in the field of biofuel production by bacteria also imply that our study can help elucidate mechanisms for increased saturated or unsaturated fatty acids synthesis. Consequently, our work appeals to a broad range of disciplines.

Author contributions: A.E. and J.G. designed research; A.E., C.H., and J.G. performed research; S.S.A. contributed new reagents/analytic tools; A.E., S.S.A., and J.G. analyzed data; and A.E. and J.G. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: Our model has been deposited in the BioModels database and assigned the identifier MODEL1601080000.

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study has investigated the underlying regulatory mechanism to date. This would also appear important because under wild-type conditions, WaaA catalyzes a step that is required for the endotoxic activity of lipid A (20).

In this work, we present a detailed picture of the crosstalk between the LPS and phospholipids biosynthetic machinery. Our work involves a computational kinetic model spanning 81 chemical reactions and involving 90 chemical species. Additionally, we used a series of *E. coli* fatty acid biosynthesis mutants to investigate the effect of substrate flux into the saturated and unsaturated fatty acid pathway on LpxC stability. Our complete model agrees qualitatively with published datasets and with our own experiments. Our results imply that the catalytic activation of LpxK is dependent on unsaturated fatty acids. Furthermore, our experimental investigations have implicated a secondary protease involved in LpxC regulation. Finally, we have provided experimental evidence to explain the rationale for WaaA regulation.

Results

LPS/Phospholipids Model Construction. We developed a computational metabolic model that incorporates genetic regulation and several feedback sources (Fig. 1). The required parameters were mostly derived from experimental literature, whereas others were estimated from published data. We modeled the interactions between substrates and enzymes under steady-state conditions using Michaelis–Menten and mass action kinetics. Our initial set of parameters were unable to reproduce experimentally-observed pathway regulatory behavior. This was solely due to a limitation in the kinetic parameters of LpxK, as described below. In addition, we made minor modifications to FabZ, FabB, and FabI parameters for fitting purposes. A full description of the model architecture, model construction, and parameters' estimation is presented in *SI Appendix*.

Model Findings Indicate the Catalytic Activity of LpxK is Dependent on Unsaturated Fatty Acids. It was previously reported in Ray and Raetz (16) that the catalytic activity of LpxK is dependent on phospholipids. Phospholipids in *E. coli* contain a combination of saturated and unsaturated fatty acids; however, our model indicates that the LpxK catalytic activation is driven solely by unsaturated fatty acids. When we initially assumed in our model that all fatty acids (i.e., both saturated and unsaturated fatty acids) present in phospholipids could catalytically activate LpxK, the model findings deviated substantially from experimental results. For instance, inhibiting either FabA or FabZ in our model had no effect on LpxC stability, whereas LpxC is rapidly degraded under both conditions in vivo (Fig. 2*A* and ref. 15, respectively). From our observations, there were sufficient levels of saturated fatty acids to catalytically activate LpxK, which prevented an accumulation of lipid A disaccharide, the feedback source for LpxC degradation. However, when the LpxK activation signal arose solely from unsaturated fatty acids, our model agreed qualitatively with all our experimental results.

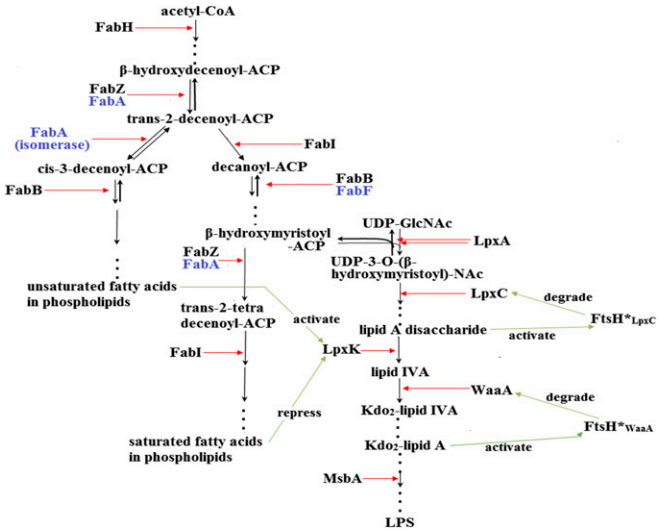

Fig. 1. Model of the *E. coli* LPS and phospholipids biosynthesis pathway. Green arrows represent pathway interactions that were derived from our current and previous work (9). A detailed model schematic is presented in *SI Appendix*.

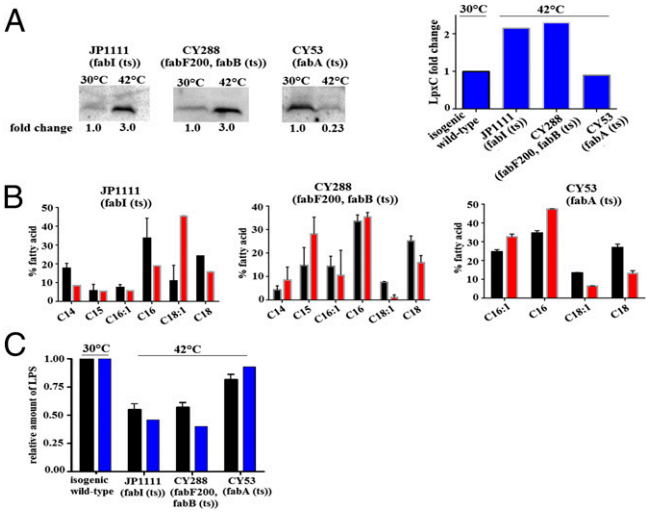

Fig. 2. Role of fatty acid biosynthesis on LpxC regulation. Growth conditions are described in *SI Appendix*. (*A*) Effect of fatty acid biosynthetic enzyme inhibition on LpxC stability. Blue bars on the right represent our computational simulations under steady-state conditions. Here, model enzyme concentrations were reduced arbitrarily to mimic the nonpermissive temperature because there was no available information in the literature on residual enzyme activity in these mutants. We reduced the model concentrations of FabF and FabB by 10- and 20-fold, respectively, to simulate mutant strain CY288. FabI and FabA concentrations were reduced by 100- and 500-fold to simulate strains JP1111 and CY53, respectively. (*B*) Fatty acid composition in strains JP1111, CY288, and CY53. Black and red bars represent growth conditions at 30 °C and 42 °C, respectively. Error bars represent SE of mean. Fatty acids notation are C14, myristic acid; C15, pentadecanoic acid; C16:1, palmitoleic acid; C16, palmitic acid; C18:1, *cis*-vaccenic acid; and C18, stearic acid. (*C*) LPS quantification. LPS was quantified by measuring the amount of Kdo in the bacterial membrane. Black bars represent experimental results, whereas blue bars are simulation results using model conditions as in *A*. Error bars represent SE of mean.

Indeed, it was reported by Ray and Raetz (16) that of all phospholipids species tested, cardiolipins had the most effect at activating LpxK. Although the authors did not provide a rationale for their observation, we suspect their use of bovine heart cardiolipins, which are known to contain at least 94% unsaturated fatty acids of the total fatty acids, were implicated (21). Similarly, cardiolipins of *E. coli* are characterized with more unsaturated fatty acids relative to other phospholipids moieties (22). In this regard, we propose that the activity of LpxK is dependent on the presence of unsaturated fatty acids. This major model adjustment subsequently provided a framework to interpret the experimental results presented below.

Excess Substrate Flux into the Saturated Fatty Acid and LPS Pathway Stimulates LpxC Degradation. We recently reported that lipid A disaccharide is a feedback source for FtsH-mediated LpxC degradation (9); however, other evidence suggests that there may be additional FtsH feedback signals. In particular, it was observed in Ogura et al. (11) that LpxC was highly stabilized in a *fabI* (ts)

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mutant, implying a link between fatty acid and LPS biosynthesis. To understand the role of fatty acids synthesis on LpxC regulation, we analyzed a number of fatty acid biosynthesis mutants.

When *E. coli* strains JP1111 [*fabI* (ts)] and CY288 [*fabF, fabB* (ts)] were grown at the nonpermissive temperature, LpxC was highly stabilized by ∼threefold, whereas LpxC was rapidly degraded in strain CY58 [*fabA* (ts)] mutant (Fig. 2*A*). We next analyzed the saturated and unsaturated fatty acid distribution in these mutants to determine if the regulatory signal arose from lipid A disaccharide or from sources outside of the LPS pathway. In other words, an increment of substrate flux into the saturated fatty acid pathway is an indirect indication of increased flux into the LPS pathway. This is because LPS substrates are derived from the saturated fatty acid pathway arm (Fig. 1).

When strain JP1111 was grown at the nonpermissive temperature, the total proportion of unsaturated fatty acids was substantially increased (Fig. 2*B*). Clearly, inhibition of FabI, which catalyzes the first committed step in saturated fatty acid synthesis (23), enabled the isomerase activity of FabA to divert substrates toward unsaturated fatty acid synthesis. The observed increment in unsaturated fatty acid is indicative of a sufficient reduction of substrate influx into the LPS pathway. This was further confirmed from LPS quantification assay described below. Therefore, LpxC stability in strain JP1111 is as a result of decreased levels of lipid A disaccharide. It should be noted that C16:1 levels were the same in both wild-type and *fabI* mutant, which suggests that the rate of elongation of C16:1 to C18:1 by FabF is dependent on substrate availability (Fig. 2*B*).

Similarly, we analyzed fatty acid distribution in strain CY288. This strain had a lesion in the *fabF* gene in addition to having a temperature-sensitive FabB protein (24). When grown at 30 °C, the fatty acid profile of the cells were characterized with significant levels of medium-chain fatty acids (Fig. 2*B*). This partial inhibition of fatty acid elongation is a result of the lesion in FabF (24), although cells remain viable due to a functional FabB. However, when grown at 42 °C, we observed a further increment in medium-chain fatty acids (especially pentadecanoic acid, an odd-chain fatty acid) and reduction in long-chain fatty acid moieties (Fig. 2*B*). Although increased levels of saturated fatty acids were generally observed at 42 °C, these were mainly odd-chain fatty acids. Odd-chain fatty acyl-ACPs can be used for phospholipids production (25) but are unsuitable substrates for LPS synthesis due to a strict requirement of LpxA for β-hydroxymyristoyl–ACP (26). Therefore, these metabolites would not be expected to be shunted down the LPS pathway. Consequently, LpxC stability in this strain can be attributed to low levels of the lipid A disaccharide. Again, this was confirmed by LPS quantification, as described below.

Furthermore, we observed a concomitant decrease in both long-chain saturated and unsaturated fatty acids (stearic and *cis*-vaccenic acids) in strain CY53 when grown at the nonpermissive temperature (Fig. 2*B*). The only reasonable explanation from the pathway schematic in Fig. 1 is that the rate of synthesis of *trans*2-decenoyl–ACP was reduced, given FabA is the major dehydratase involved in this step (23). As a result, limited substrates would be diverted into both saturated and unsaturated arms of the pathway, resulting in reduced palmitoyl-ACP and palmitoleoyl-ACP concentrations. Increased competition for low levels of palmitoyl-ACP and palmitoleoyl-ACP by phospholipid acyltransferases (PlsB and PlsC) (27, 28) would further ensure that only a small proportion of these acyl pools are elongated to stearic and *cis*-vaccenic acids by FabB and FabF, respectively (29). Although substrate flux into the saturated fatty acid pathway was generally reduced, inhibition of FabA resulted in increased LpxC degradation due to an accumulation of lipid A disaccharide. Accumulation of lipid A disaccharide would occur under this condition because FabA plays a major role in the dehydration of β-hydroxymyristoyl–ACP due to a higher protein copy number (30) and similar catalytic activities with FabZ (23). In other words, a loss of FabA activity would increase the concentration of β-hydroxymyristoyl–ACP, which is then shunted into the LPS pathway.

As a confirmation that strain CY53 grown at 42 °C was characterized with sufficient substrate flux into the LPS pathway, the concentration of LPS was only slightly decreased in comparison with those grown at 30 °C (Fig. 2*C*). In contrast, we observed a reduction in LPS levels of ∼50% in both strains JP1111 and CY288 when grown at the nonpermissive temperature, which supports the idea that LPS synthesis is impeded as a consequence of low substrate availability.

Together, our findings indicate that the FtsH-mediated LpxC degradation signal arises solely from lipid A disaccharide under fatty acid inhibition conditions.

Proteolytic Regulation of LpxC in an *ftsH* Knockout Mutant. Having established from above that excess flux of metabolites into the saturated fatty acid pathway enhances LpxC proteolysis, we examined the likelihood of saturated fatty acids commonly found in membrane phospholipids to directly impact LpxC instability. We observed that in vitro, the addition of 10 and 20 mM of palmitic acid to wild-type (W3110) *E. coli* cell lysates for 10 min resulted in decreased LpxC levels by ∼30% and 57%, respectively

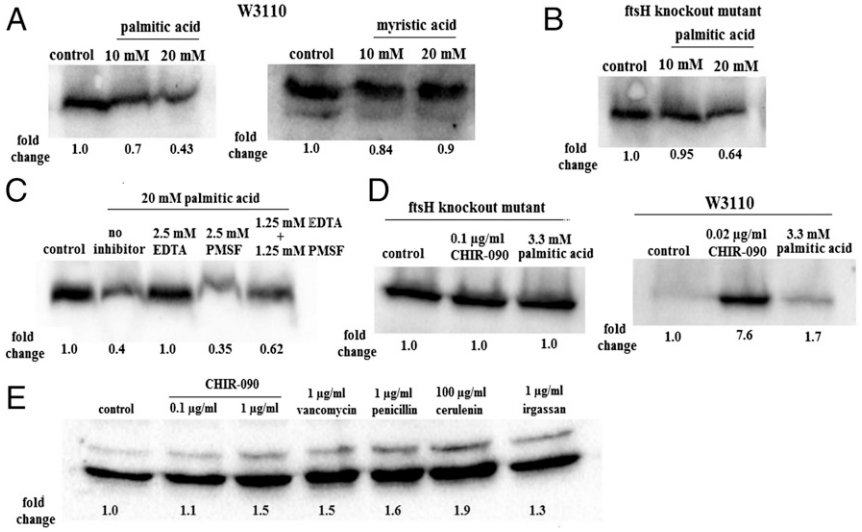

Fig. 3. LpxC proteolysis in an *ftsH* knockout mutant. Assay conditions are described in *SI Appendix*. (*A*) Effect of fatty acids on LpxC stability in vitro. (*B*) Effect of palmitic acid on LpxC stability in *ftsH* knockout cells in vitro. (*C*) Palmitic acid-induced LpxC degradation occurs via a metalloprotease in vitro. Some samples contained EDTA and PMSF to inhibit metalloproteases and serine proteases, respectively. (*D*) Effect of increased palmitoyl-ACP and palmitoyl-CoA concentrations on LpxC stability in vivo. (*E*) LpxC is less degraded in vivo in *ftsH* knockout cells in the presence of antibiotics that affect membrane synthesis/stability.

(Fig. 3*A*). This dose-dependent phenomenon was not observed when myristic acid, a shorter-chain fatty acid, was used. Next, we treated cell lysates of an *ftsH* knockout mutant with palmitic acid and observed similar results (Fig. 3*B*). This therefore indicates that under our in vitro conditions, an unidentified protease contributed toward LpxC degradation. Furthermore, proteolysis was inhibited in the presence of EDTA, which indicates the protease belonged to the class of metalloprotease, similar to FtsH (Fig. 3*C*).

However, under normal physiological conditions, LpxC and palmitic acid are localized in different cellular compartments (cytoplasm and bacterial membrane, respectively) and may not be in direct contact with one another, as it was in our in vitro assays. Because palmitic acid exists in the cytoplasm in the forms of palmitoyl-CoA and palmitoyl-ACP (6), we investigated the effect of both forms of palmitic acid on LpxC stability. Palmitic acid is known to be actively transported across the membrane by FadL (31) and immediately converted to palmitoyl-CoA by FadD (32). Interestingly, when we added palmitic acid to the growth medium of wild-type *E. coli* (W3110), the levels of LpxC were elevated by 1.7-fold (Fig. 3*D*). This observation is readily explainable from prior results and our results presented in Fig. 2. Palmitoyl-CoA induces a strong inhibitory effect on FabI with a K_i value of about 3 μM (33). Due to the inhibition of FabI, LpxC would be stabilized (Fig. 2*A*). Thus, elevated cellular palmitoyl-CoA concentrations have an opposite effect on LpxC stability than the free-form of palmitic acid. On the other hand, under in vivo conditions, inhibiting substrate flux into the LPS pathway will increase the cellular concentration of palmitoyl-ACP (34). This is because when LPS substrate influx is inhibited, it leads to elevated levels of β-hydroxymyristoyl–ACP, which is subsequently dehydrated by FabZ and shunted toward the production of palmitoyl-ACP. We treated cells with sublethal concentrations of an LpxC inhibitor (CHIR-090) to reduce substrate flux into the LPS pathway. Under this condition, we observed LpxC elevation of 7.6-fold in wild-type cells (Fig. 3*D*). This stability is best explained by reduced level of lipid A disaccharide production. Therefore, increment in cellular palmitoyl-ACP concentration has an opposite effect on LpxC stability than the free-form of palmitic acid. However, the presence of palmitic acid or sublethal concentration of CHIR-090 in the culture medium had no effect on LpxC stability in *ftsH* knockout mutant cells (Fig. 3*D*). Together, these findings suggest that the direct interaction of LpxC with long-chain free-fatty acids (i.e., fatty acids not bound to ACP or CoA) facilitated FtsH-independent LpxC proteolysis, as observed in our in vitro assays.

Interestingly, when we monitored the in vivo LpxC degradation rate in *ftsH* knockout cells that were treated with a higher concentration of CHIR-090 or other antibiotics that affected the bacterial membrane, we observed an increase in LpxC concentration ranging from 1.3- to 1.9-fold (Fig. 3*E*). This indicates that in untreated cells, residual LpxC proteolysis occurred, and thus, confirms the presence of an additional regulatory protease. Additionally, these results also indicate that LpxC regulation is crucial to the bacterial cells, which must maintain the desired protein levels irrespective of an accumulation of lipid A disaccharide, or presence/absence of a functional FtsH protease.

WaaA Regulation. In addition to LpxC, WaaA regulation is also crucial to cellular viability (*SI Appendix*, Fig. S5). Using computational simulations, we recently postulated that the regulation of WaaA may help prevent reglycosylation of lipid precursors rather than regulating the rate of LPS synthesis (9). In other words, the regulatory role is "qualitative" rather than "quantitative." To clarify these assumptions, we overexpressed *waaA* and quantified LPS using two approaches: first, by quantifying the amount of Kdo in crude membrane extracts, and second, by quantifying the levels of Kdo and heptose from extracted LPS. Under *waaA* overexpression conditions, the levels of Kdo in crude membrane extracts were elevated by ~20% (Fig. 4*A*). However, an increment in Kdo may not indicate increased LPS level under *waaA* overexpression. This is because the role of WaaA is to add two Kdo residues to its LPS lipid precursor (lipid IV_A) (35); therefore, the possibility still exists that overproduction of WaaA may result in multiple additions of Kdo to lipid IV_A (i.e., reglycosylation) or, alternatively, Kdo may be added to other lipid acceptors (other than lipid IV_A) in the membrane (35, 36). This also indicates that quantification of LPS using the Kdo assay may be inappropriate under *waaA* overexpression conditions. To further clarify these possibilities, we reduced substrate flux into the LPS pathway using 1/2 MIC of CHIR-090. The rationale for this was that by reducing the influx of substrates, the LPS synthesis rate would be dependent on substrate availability and not WaaA concentration. Similarly, overexpression of *waaA* under this condition led to a 20% increase in Kdo levels in comparison with cells that were solely treated with CHIR-090 (Fig. 4*A*). These findings indicate that the rate of glycosylation by WaaA is independent of lipid IV_A availability.

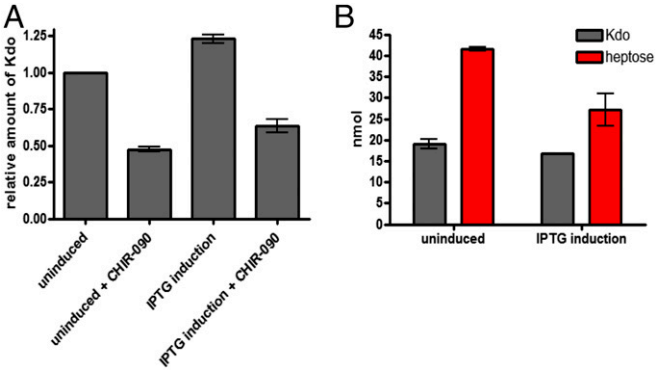

Fig. 4. WaaA regulation. Assay conditions are described in *SI Appendix*, and error bars represent SE of mean. (*A*) Kdo concentration under *waaA* overexpression in membrane extracts. (*B*) Kdo and heptose quantification from extracted LPS under *waaA* overexpression.

Furthermore, it was essential to investigate if the reglycosylated lipid IV_A or other alternate lipids (i.e., lipids other than lipid IV_A) that could be glycosylated by WaaA are being consumed for LPS synthesis. Consequently, we quantified the concentration of both Kdo and heptose sugars present in extracted LPS. Heptose sugar is primarily used in the synthesis of core oligosaccharides and is usually present in a 2:1 ratio with Kdo in wild-type cells (20). Under *waaA* overexpression, Kdo and heptose levels from extracted LPS were both decreased by ~12% and 34%, respectively (Fig. 4*B*). Together, these results suggest that unregulated WaaA production results in multiple glycosylation of lipid precursors that mostly accumulate within the membrane and are poor substrates for LPS synthesis. However, it does appear that a small proportion of these reglycosylated products are consumed for LPS production, given the observed ratio of Kdo and heptose from extracted LPS (Fig. 4*B*).

To further elucidate the effect of *waaA* overexpression on the structural stability of the bacterial membrane, we determined the MICs of different antibiotics for cells that had overexpressed *waaA*. Under these conditions, the cells were more susceptible to antimicrobial agents, which implies that the bacterial membrane becomes more permeable under unregulated WaaA conditions (*SI Appendix*, Table S3).

Discussion

In this work, we developed an integrated computational model for the biosynthesis of phospholipids and LPS pathways. One crucial model finding enabled us to pinpoint a key role of unsaturated fatty acids at stimulating LpxK catalysis, whereas on the

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other hand, excessive saturated fatty acids represses its catalytic activity. There are a number of published datasets that supports this notion. First, as mentioned previously, the phospholipids used in Ray and Raetz (16) were bovine heart cardiolipins, which are known to consist almost entirely of unsaturated fatty acids (21). Furthermore, Roy and Coleman (37) observed that when LpxD was inhibited, the specific activity of LpxK was decreased, whereas the protein half-lives of LpxK were the same under normal and LpxD inhibition conditions. Further analysis by the authors indicated that overexpression of LpxD does not increase the specific activity of LpxK. This means that the decrease in LpxK activity is a secondary effect of the altered LpxD. These observations can readily be interpreted from our model. Because β-hydroxymyristoyl–ACP is a substrate used by LpxD, reduced LpxD activity would result in an accumulation of this substrate, which is shunted toward saturated fatty acid production. In return, excess saturated fatty acid would repress the catalytic activation of LpxK.

Because the ratio of unsaturated and saturated fatty acids influences the activation of LpxK, this suggests that LPS synthesis rate is correlated with membrane fluidity. This is due to the fact that unsaturated fatty acids in the membrane increase fluidity, whereas saturated fatty acids decrease fluidity (38). It is probable that under conditions of low membrane fluidity, a reduced LpxK activity may be essential to reduce the amount of LPS produced, which potentially would add an extra permeability barrier and subsequently reduce the influx of metabolites from the external environment. The opposite effect seems to occur under conditions of high membrane fluidity. It was reported previously (39) that *E. coli* grown in environments that had increased membrane fluidity through degradation of LPS led to an induction of PlsB transcription, which would ultimately enhance the production of phospholipids.

There has been some uncertainty as to the role of acyl-ACPs in the regulation of LpxC. In the first scenario, due to the fact that LPS and phospholipids biosynthetic pathways both derive their precursor molecules from β-hydroxymyristoyl–ACP, it was accepted that competition for this common substrate influences the regulation of LpxC (11). In other words, LpxC degradation would help conserve substrates for phospholipids synthesis. This initially seemed reasonable and is supported by findings that FabZ inhibition enhances LpxC degradation (15), and FabZ overexpression results in LpxC stability (11). However, our model and experimental results disagree with this "competition model" regarding β-hydroxymyristoyl–ACP availability. As an example, LpxC would have been expected to be stabilized under conditions of FabA overexpression, which invariably increases substrate flux into the saturated fatty acid pathway (40), and subsequently elevate the pool of β-hydroxymyristoyl–ACP. This should lead to a reduced competition between LpxC and FabZ for substrates. On the contrary, we observed an increased rate of LpxC degradation under this condition (*SI Appendix*, Fig. S4*A*). In support of our model's disagreement with the "competition model," Ogura et al. (11) also observed that LpxC levels were elevated in *fabI* (ts) mutants. Although the authors had initially suggested that increased concentration of *trans*2-tetradecenoyl–ACP may enhance LpxC stability due to the observation that LpxC levels are also being elevated when FabZ is overexpressed, Ogura et al. also admitted the unlikelihood of *trans*2-tetradecenoyl–ACP accumulation under *fabI* (ts) conditions (41). Thus, they were unable to explain the rationale for LpxC stability under FabI inhibition conditions. Our results presented in Fig. 2 indicate that the increased levels of LpxC in *fabI* (ts) mutant occurred due to decreased flux of substrates into the saturated fatty acids and LPS pathways, which ultimately depreciated the lipid A disaccharide concentration.

The regulation of LpxC becomes more complicated by our observations that an unidentified protease degrades LpxC. Although our in vitro results provided evidence that palmitic acid enhances LpxC proteolysis via another metalloprotease, this mechanism appears not to reflect the normal in vivo physiological setting (Fig. 3). In fact, an increase in the isoforms of palmitic acid usually found in the cytoplasm (i.e., palmitoyl-ACP and palmitoyl-CoA) stabilized LpxC levels (Fig. 3*D*). Therefore, the observed in vitro results were probably due to a direct interaction of the free-form of palmitic acid with LpxC. A possible implication is that during the synthesis of fatty acids, dissociation of the palmitic acid prosthetic group from its carrier protein (perhaps through the action of thioesterases) triggers LpxC proteolysis. This mechanism may be linked to cellular toxicity, as it is well documented that long-chain free-fatty acids (i.e., fatty acids not bound to ACP or CoA) such as palmitic acid are toxic to cells (42). An alternative explanation to the in vitro results could be due to palmitic acid binding to LpxC. Palmitic acid has also been reported to bind directly to the active site of LpxC in vitro (43). Therefore, the actual LpxC substrate and excess palmitic acid possibly competes for the enzyme active site, in which case, palmitic acid may act as an inhibitor. Proteolysis under this condition could be directed at aberrant LpxC proteins. In support of this, Fuhrer et al. (12) had initially suggested a possible involvement of other classes of proteases in the degradation of nonfunctional LpxC. Irrespective of the in vitro results, residual degradation of LpxC still occurs under FtsH inactivation conditions in vivo. Interestingly, treatment of bacterial cultures with compounds that are expected to reduce the levels of lipid A disaccharide and stabilize LpxC in wild-type cells also led to LpxC stability in *ftsH* knockout mutants, although much higher concentrations of those compounds were required (Fig. 3*E*). However, this does not indicate that lipid A disaccharide is the feedback source under those FtsH inactivation conditions because other antibiotics that directly targeted the membrane structure also resulted in LpxC stability (Fig. 3*E*). Therefore, the regulatory signal for FtsH-independent LpxC proteolysis arises from an unidentified metabolite or pathway. Due to LpxC being localized in the cytoplasm, this unidentified protease would most likely be localized in the cytoplasm or inner membrane. Thus, under conditions of direct LPS attack from the environment (i.e., outer membrane), it is highly plausible that this protease functions in parallel with a separate adapter protein that senses the vulnerability of the outer membrane and translates such information to the protease. Indeed, in a similar pattern, FtsH-mediated LpxC degradation has been reported to be dependent on an adapter protein YciM (44). As a result of LpxC being the first committed step in LPS synthesis, it is intuitively reasonable that under conditions that directly alter the membrane structure, cells must attain the desired level of LPS through LpxC regulation irrespective of the concentration of lipid A disaccharide or presence of an active FtsH protein.

Furthermore, our experimental findings imply that an uncontrolled production of WaaA does not increase LPS level but rather reglycosylates lipid IV_A. In support of our claim, a previous in vitro study (36) observed that under excess concentration of WaaA, there were several unidentified products obtained from lipid IV_A that were higher in molecular weight than Kdo_2- lipid IV_A. Because wild-type *E. coli* usually possesses two Kdo residues, this indicates the glycosylation pattern of lipid IV_A is crucial and tightly regulated. In addition, Reynolds and Raetz (45) had reported previously that the importance of WaaA is mainly to provide the right substrates for LpxL, making its role more of a qualitative one.

In conclusion, we present a model for the biosynthesis of the outer membrane in *E. coli*, which we have used to explain prior published findings and our own experimental results. Our model agrees qualitatively with the presented experimental findings and, to some degree, quantitatively. Our experimental data indicate a strong correlation between membrane fluidity and LpxC degradation. Furthermore, all our results indicate lipid A disaccharide is the sole feedback source for FtsH-mediated LpxC degradation, and WaaA regulation helps to prevent reglycosylation of LPS precursors. Altogether, the findings of this work provide previously unidentified insights into the complex biogenesis of the *E. coli* outer membrane.

Methods

Computational Model Construction and Simulation. We simulated our LPS and phospholipids synthesis model using deterministic methods. Our primary tool was the COPASI software (46), and we assumed a 6.7×10^{-16} liter cell volume (18). Our simulations represented an *E. coli* cell generation under optimal growth conditions, which is 1800 s (19). We have identified previously (9) that using stochastic simulations and accounting for stochasticity would have a negligible effect on the results due to the high copy numbers of all model components, which justified the use of deterministic simulations. A detailed description of our parameters' estimation and model construction is presented in *SI Appendix*.

Experimental Procedures. The procedures used in the preparation of cell extracts, Western blotting, fatty acids, and LPS analyses are described in *SI Appendix*.

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