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The *Escherichia coli* Envelope Stress Sensor CpxA Responds to Changes in Lipid Bilayer Properties

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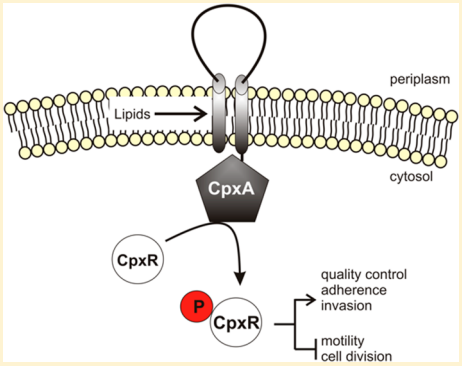
Supporting Information

ABSTRACT: The Cpx stress response system is induced by various environmental and cellular stimuli. It is also activated in *Escherichia coli* strains lacking the major phospholipid, phosphatidylethanolamine (PE). However, it is not known whether CpxA directly senses changes in the lipid bilayer or the presence of misfolded proteins due to the lack of PE in their membranes. To address this question, we used an *in vitro* reconstitution system and vesicles with different lipid compositions to track modulations in the activity of CpxA in different lipid bilayers. Moreover, the Cpx response was validated *in vivo* by monitoring expression of a P*cpxP-gfp* reporter in lipid-engineered strains of *E. coli*. Our combined data indicate that CpxA responds specifically to different lipid compositions.



The Cpx stress response system is a two-component signal transduction pathway in numerous Gram-negative bacteria and of fundamental importance to maintaining cell envelope integrity.[1−4] It consists of an inner membrane sensor kinase CpxA, a cytoplasmic response regulator CpxR, and a periplasmic accessory CpxP protein.[5−7] The sensor kinase CpxA is composed of a periplasmic domain, two transmembrane (TM) helices, and a large conserved cytosolic domain.[8−10] It receives information about the status of the cell envelope through its sensory domain and transmits this signal via its cytosolic domain to the response regulator CpxR. Upon detection of stress signals, CpxA autophosphorylates and transfers its posphoryl group to CpxR, which induces gene clusters responsible for protein secretion, cell envelope biogenesis, and quality control in the envelope.[11−14] In the absence of stress-inducing cues, CpxA acts as a phosphatase and deactivates CpxR activity. CpxP functions as an accessory protein. Under noninducing conditions, CpxP inhibits CpxA autophosphorylation by directly interacting with the periplasmic domain of CpxA.[15−18] In the presence of an inducing cue, CpxP is titrated away and CpxA phosphorylation abilities are activated. Thus, CpxA possesses three distinct catalytic activities: (i) autophosphorylation, (ii) CpxR phosphorylation (phosphotransfer activity), and (iii) CpxR-P dephosphorylation.[15,19]

The Cpx system itself is activated by a wide range of stimuli such as elevated pH,[20] indole,[21,22] salt,[15,18] ethanol/butanol,[23] CO,[24] depletion of the YidC insertase,[25] inhibition of the Lol lipoprotein transfer system,[26] induction of L-forms,[27] overproduction of cell envelope proteins (e.g., NlpE),[28−31] and membrane-active antimicrobial peptides.[32] Because all these stimuli could result in the accumulation of misfolded cell envelope proteins, it has been postulated that misfolded proteins might act as a general trigger of the Cpx pathway.[33] Several studies have also revealed that the Cpx system may specifically respond to perturbations of the cytoplasmic membrane.[34,35] It was shown for instance that the Cpx system is activated in a phosphatidylserine synthase deficient (Δ*pssA*) *Escherichia coli* strain (called AD93).[35] AD93 lacks the membrane component phosphatidylethanolamine (PE)[36] and served as an *in vivo* model system for analyzing the role of PE in the function and stability of membrane proteins and membrane-dependent processes, such as signal transduction[37] and substrate transport.[38] It was found that in strain AD93 membrane protein transporters are unfolded.[39,40] Thus, it is unclear from these studies whether misfolded proteins or changes in the lipid composition activate the sensor kinase CpxA.

To address this question, we investigated the phosphorylation activities of CpxA in different lipid environments using a

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previously established *in vitro* model that allows functional testing of CpxA activity in different lipid environments. The *in vitro* data were supported by *in vivo* assays. Taken together, these data revealed a crucial effect of surface charge and most likely membrane thickness on the Cpx response.

EXPERIMENTAL PROCEDURES

Strains and Plasmid Construction. Bacterial strains and plasmids used in this study are listed in Table S1 of the Supporting Information. All generated *E. coli* strains were derived from AD93,[41] a strain lacking *pssA* and therefore unable to synthesize phosphatidylethanolamine (PE). This strain was supplemented with plasmids carrying a combination of lipid glycosyltransferase genes[42] from *Acholeplasma laidlawii* or *Arabidopsis thaliana* and named after the major lipid in each strain (GlcDAG, GlcGlcDAG, GalDAG, and GalGalDAG) (Figure S1 of the Supporting Information).

The P*cpxP-gfp* reporter plasmid was prepared by cloning a 230 bp fragment encoding the *cpxP* promoter region into a pFU34[43] vector with the ATG initiation codon. BamHI and SalI restriction sites were used for the insertion of the *cpxP* gene into a pFU34 vector resulting in a pKT10E construct. Afterward, the ampicillin resistance cassette of pKT10E was replaced with the tetracycline cassette from pFU168 using XhoI and SacI restriction sites. The resulting construct was named pEL17E.

Preparation of Engineered *E. coli* Membrane Lipids. Lipid-engineered *E. coli* strains (AD93, GlcDAG, GlcGlcDAG, GalDAG, and GalGalDAG) were grown in 500 mL of 5× LB medium under selective conditions at 30 °C on a rotatory shaker. Cells were harvested by centrifugation (3200*g* for 20 min at 4 °C), and cell pellets were washed once with PBS. Total membrane lipids were extracted from cell pellets by stirring in a 2:1 (v/v) chloroform/methanol mixture, followed by centrifugation (2853*g* for 20 min at 4 °C). KCl/KH_2PO_4 buffer (0.12 M) was added to the extract and the mixture kept at room temperature for 30 min. Chloroform and water phases were separated by centrifugation (2853*g* for 20 min at 4 °C). The chloroform phase was concentrated under N_2 gas. The extracted lipid mixture was applied on a silica gel column as described in ref 44. Briefly, nonpolar lipids and free fatty acids were eluted using 150 mL of chloroform. Polar membrane lipids were eluted with 50 mL of methanol. Lipids were concentrated under N_2 and resolubilized in a 2:1 (v/v) chloroform/methanol mixture. Membrane lipids were analyzed by TLC and quantified as described previously.[42] The protein:lipid ratio (w/w) was estimated to be 1:10000, which is a considerable enrichment of lipids compared to *E. coli* cells in which the ratio is approximately 1:30.

Purification of CpxA-His6 and 6His-CpxR. CpxA-His6 and 6His-CpxR were prepared from BL21(DE3) <pLysS/pI3cpxA> and BL21(DE3) <pLysS/pI4cpxR> as described previously.[15] The amount of protein was determined by the BCA protein assay (Pierce) according to the manufacturer's instructions.

Preparation of Proteoliposomes. Purified CpxA-His6 was incorporated into liposomes using a modified Biobeads-based protocol.[15] Briefly, purified *E. coli* (WT) phospholipids (Avanti Polar Lipids), phospholipids purified from lipid-engineered *E. coli* strains (see above), or synthetic phospholipids (Avanti Polar Lipids), namely, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,1′2,2′-tetraoleoyl cardiolipin (TOCL), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine (16:1 PE), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (16:1 PC), and 1′,3′-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (14:0 CL), were mixed with a PE:PG:CL ratio of 70:20:10. Lipid mixtures were dried under a stream of N_2. Pellets were slowly redissolved in 50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, and 0.47% (v/v) Triton X-100 over a period of 2 h, yielding detergent-destabilized liposomes. Purified CpxA-His6 was added to these liposomes with a lipid:protein ratio of 100:1 (w/w) and Bio-Beads SM-2 (Bio-Rad) with a bead:detergent ratio of 10:1 (w/w). Mixtures were gently stirred at 4 °C overnight. The next morning, fresh additional Bio-Beads were added, and mixtures were stirred for an additional 2 h. Proteoliposome solutions were withdrawn by a pipet and collected by centrifugation for 30 min at 200000*g*. Finally, pellets were suspended in 50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, and 2 mM dithiothreitol. All proteoliposomes were analyzed for relative CpxA-His6 content. Therefore, CpxA-His6-containing proteoliposomes were separated by 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), stained with Coomassie Blue, and quantified using the Molecular Imager Fx (Quantity one, Bio-Rad). Different amounts of BSA were used as standards on the same gel. The efficiency of incorporation of CpxA-His6 into liposomes was determined to be ∼40% (data not shown) with a 90% inside-out orientation of CpxA-His6.

Autophosphorylation and Phosphotransfer Activity Assays. Autophosphorylation assays were performed with CpxA-containing proteoliposomes in phosphorylation buffer [50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 2 mM DTT, 50 mM KCl, and 5 mM $MgCl_2$] containing 40 μM [γ-^{32}P]ATP (1.2 Ci/mmol) (PerkinElmer) as described previously.[15] To determine the whole phosphorylation cascade, CpxA-His6-containing proteoliposomes and purified 6His-CpxR were mixed in a ratio of 1:8 and incubated in phosphorylation buffer with 100 μM [γ-^{32}P]ATP (0.48 Ci/mmol) for 30 min at room temperature. Then, ADP was added to shift CpxA activities toward dephosphorylation. Samples were collected continuously over the next 30 min. Afterward, samples were separated by SDS−PAGE and quantified by autoradiography as described previously.[15] For phosphotransfer assays, CpxA-containing proteoliposomes resuspended in buffer A [25 mM HEPES (pH 7.5), 0.1 M NaCl, and 0.1 M KCl] were mixed with purified 6His-CpxR in a molar ratio of 1:8. The reaction was started by addition of 0.2 mM ATP, and samples were collected at various time points. The reaction was stopped by adding SDS sample buffer. Phosphoproteins were separated by Mn-Phos-tag acrylamide gel electrophoresis,[45] stained with Coomassie Blue, and analyzed by densitometry using ImageJ (EMBL). To directly compare our results with previous results, a native *E. coli* (WT) lipid mixture of commercial origin (Avanti) was used.

Promoter Activity Assays for Lipid-Engineered *E. coli* Strains. Lipid-engineered *E. coli* strains (AD93, GlcDAG, GlcGlcDAG, GalDAG, and GalGalDAG) transformed with the P*cpxP-gfp* reporter plasmid were grown under selective conditions in 2× LB medium at 30 °C on a rotatory shaker (180 rpm) and harvested during the exponential phase of growth. GFP fluorescence was determined as described previously.[46] Triplicate measurements were performed for each assay, and values were normalized to the amount of CpxA and TetR per cell (both determined by immunoblotting).

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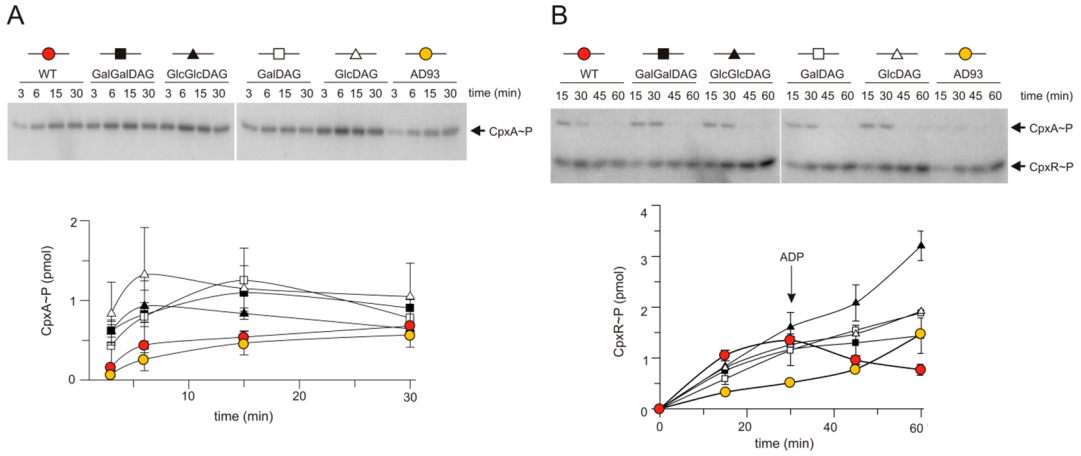

Figure 1. Autophosphorylation and phosphotransfer activities of the reconstituted sensor kinase CpxA are affected in different lipid environments. Displayed are averages ± the standard error of the mean from three different experiments. (A) To determine CpxA autophosphorylation activity, CpxA-His6 (1 μM) was reconstituted in liposomes derived from *E. coli* (WT) and the different lipid-engineered *E. coli* strains (GalGalDAG, GlcGlcDAG, GalDAG, GlcDAG, and AD93). At different time points, CpxA autophosphorylation activity was visualized (top) and the amount of [^{32}P]CpxA was quantified using the PhosphoImager and [γ-^{32}P] ATP as a standard (bottom). (B) Phosphorylation assay for the CpxA/CpxR signal transduction cascade performed as described in Experimental Procedures. CpxA-His6 (0.5 μM) was reconstituted with the indicated lipids and 6His-CpxR (4 μM) in the presence of 50 mM KCl. The reaction was started by the addition of 100 μM [γ-^{32}P]ATP, and samples were taken at the indicated time points before and after the addition of 1 mM ADP (top). The amount of [^{32}P]CpxR was quantified with a PhosphoImager using [γ-^{32}P]ATP as a standard (bottom).

RESULTS AND DISCUSSION

Phosphatidylethanolamine Is Required for CpxA Function. Previously, it has been reported that the Cpx two-component system is activated in *E. coli* lacking the major phospholipid PE.[35] However, it is unknown whether the Cpx system directly responds to changing properties of the lipid bilayer. To determine if CpxA is directly influenced by changes in lipid bilayer properties, we incorporated purified CpxA into liposomes derived from purified phospholipids from *E. coli* wild type (WT) or AD93 [*E. coli* strain lacking PE (Figure S1 and Table S2 of the Supporting Information)]. To correlate protein activities derived in a different lipid background, we analyzed the incorporation rates of CpxA in the generated proteoliposomes (PLS). Rates of incorporation of CpxA into phospholipids derived from *E. coli* WT or AD93 were comparable (Figure S2 of the Supporting Information). CpxA activities were then studied *in vitro* according to the method of Fleischer et al.[15] In this method, CpxA autophosphorylation is initiated by addition of [γ-^{32}P]ATP, and the phosphorylation state of CpxA was analyzed after 3, 6, 15, and 30 min (Figure 1A). As displayed in Figure 1A, CpxA autophosphorylation was saturated after 30 min. We noted only a marginal reduction in the level of CpxA autophosphorylation during incorporation of CpxA into liposomes derived from AD93 (Figure 1A, red and gold circles).

Next, the effect of PE depletion on the phosphorylation cascade was tested in the presence of the response regulator CpxR. Therefore, CpxA-containing proteoliposomes (WT-PLS and AD93-PLS) were mixed with purified CpxR in a CpxA:CpxR molar ratio of 1:8. The reaction was started by addition of [γ-^{32}P]ATP, and CpxR phosphorylation was quantitatively analyzed over 30 min (Figure 1B). Afterward, dephosphorylation was enhanced by ADP addition, and dephosphorylation of CpxR-P by CpxA was monitored for a further 30 min (Figure 1B). This time, the curves representing the amount of phosphorylated CpxR differed substantially (in Figure 1B, compare red and gold circles). In the presence of AD93-PLS, the amount of phosphorylated CpxR was reduced because of the reduced starting level of active CpxA-P, and we assume that phosphorylation of CpxR by CpxA is comparable in both lipid backgrounds. Notably, dephosphorylation of CpxR-P by CpxA was hardly detectable in the presence of AD93-PLS after the addition of ADP, indicating a severely limited phosphatase activity of CpxA in AD93-PLS (Figure 1B, gold circles). We therefore conclude that the absence of PE in AD93-PLS affects the dephosphorylation activity of reconstituted CpxA and impairs transduction of the signal from CpxA to CpxR. These data explain the *in vivo* observation of Dowhan and co-workers showing that the Cpx system is activated in the AD93 strain.[35]

CpxA Function Is Influenced by the Surface Charge of the Membrane. As PE is a nonbilayer prone lipid with a zwitterionic headgroup,[47] the mixture of liposomes derived from *E. coli* strain AD93 (lacking PE) is considered to have an increased anionic surface charge and decreased membrane curvature compared to those of the WT. To distinguish between surface charge and curvature, alterations in CpxA function were tested in the presence of lipids with different physicochemical properties. Therefore, CpxA was incorporated into liposomes prepared from lipid-engineered *E. coli* strains containing approximately 50 mol % glucolipids from the mycoplasma *A. laidlawii* (GlcDAG and GlcGlcDAG) or galactolipids from *Ar. thaliana* (GalDAG and GalGalDAG) (Table S2 of the Supporting Information). All of these lipids were neutral (contain no net charge) and either bilayer prone (GlcGlcDAG and GalGalDAG) or nonbilayer prone (GlcDAG and GalDAG).[47] Thus, the anionic surface charge is reduced without affecting the membrane curvature (GlcGlcDAG and GalGalDAG). Alternatively, the anionic surface charge is reduced, and the membrane curvature is restored to the WT level (GlcDAG and GalDAG).

The efficiency of incorporation of purified CpxA into liposomes made from these foreign lipids (hereafter named GalGalDAG-PLS, GlcGlcDAG-PLS, GlcDAG-PLS, and GalDAG-PLS) was similar to that of WT-PLS or AD93-PLS

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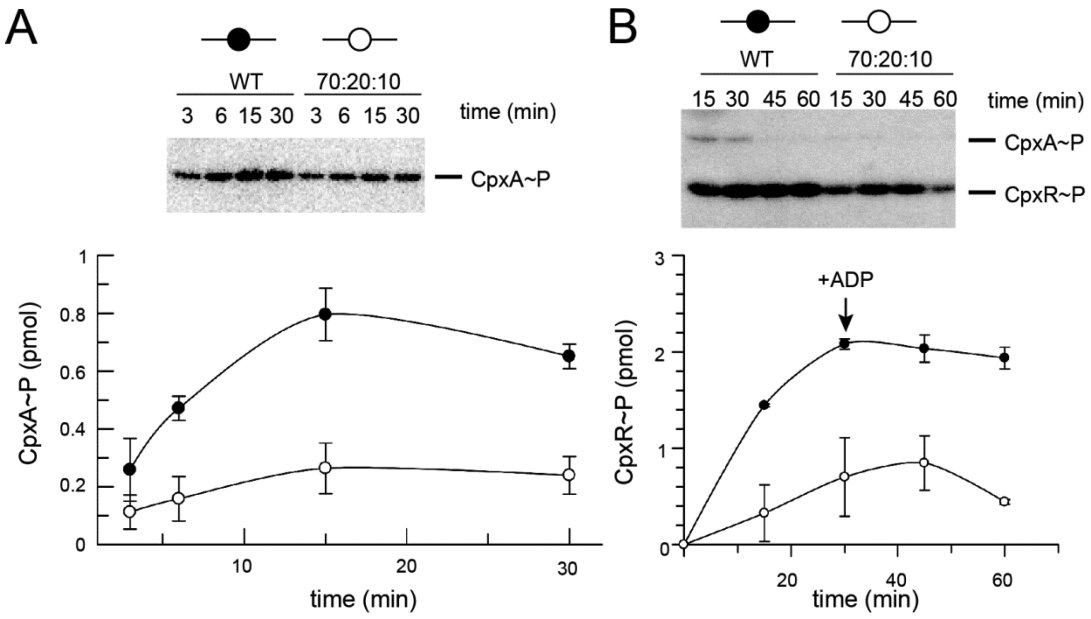

Figure 2. Synthetic DOPE/DOPG/TOCL lipid mixture that affects the autophosphorylation and phosphotransfer activities of reconstituted CpxA. Shown are averages ± the standard error of the mean from three different experiments. (A) Autophosphorylation activity of CpxA was determined as described in the legend of Figure 1A. Therefore, CpxA (1 μM) was reconstituted into liposomes with the indicated DOPE/DOPG/TOCL (mole percent) lipid composition. Samples were analyzed with a PhosphoImager (top), and the amounts of [^{32}P]CpxA were quantified using [γ-^{32}P]ATP as a standard (bottom). (B) The phosphorylation assay for the CpxA/CpxR signal transduction cascade was performed as described in the legend of Figure 1B using CpxA-His6 (0.5 μM) reconstituted into liposomes with the indicated DOPE/DOPG/TOCL (mole percent) lipid composition and 6His-CpxR (4 μM). The amounts of [^{32}P]CpxR were quantified on a PhosphoImager using [γ-^{32}P]ATP as a standard (bottom).

(Figure S2 of the Supporting Information). Also, the average size of the proteoliposomes indicated no significant differences between the proteoliposome mixtures (Figure S3 of the Supporting Information). However, testing the phosphorylation abilities of reconstituted CpxA showed remarkable differences. Autophosphorylation of CpxA in all artificial proteoliposomes mixtures was enhanced compared to that of WT-PLS (Figure 1A). Interestingly, the amount of phosphorylated CpxR was comparable between artificial lipid mixtures and the wild-type lipid mixture (Figure 1B), but again, dephosphorylation of CpxR-P was not efficient in any of the artificial lipid mixtures (Figure 1B, after addition of ADP). As only the surface charge but not the membrane curvature is reduced in all artificial lipid mixtures, we can conclude that CpxA activity is primarily affected by the surface charge.

The Synthetic Lipid Environment Changes CpxA Function. Next, we analyzed the effect of a synthetic lipid mixture that reflects the native composition of the main phospholipids of the *E. coli* cytoplasmic membrane on CpxA activities. Therefore, CpxA was incorporated into liposomes prepared from 70 mol % DOPE, 20 mol % DOPG, and 10 mol % TOCL (Table S2 of the Supporting Information). The rate of incorporation for this synthetic lipid mixture was comparable to the rates of incorporation of CpxA into the wild-type lipid mixture [38.7 ± 29.8% (Figure S2 of the Supporting Information)].

Interestingly, CpxA incorporated into liposomes derived from the synthetic lipid mixture exhibited a reduced autophosphorylation propensity compared to that of the *E. coli* wild-type lipids (in Figure 2A, compare empty and filled circles). A reduced amount of CpxA-P correlated with a reduced level of phosphorylation of CpxR (Figure 2B). Furthermore, we observed a slightly enhanced dephosphorylation of the phosphorylated CpxR after addition of ADP compared to that of the wild-type lipid mixture (Figure 2B). Because the average acyl chain length is around 16.5 carbons in *E. coli* lipids and 18 carbons in the synthetic oleoyl (18:1*cis*) species, it appears likely that bilayer thickness affects predominantly the autophosphorylation activity of CpxA.

To further validate whether membrane thickness affects CpxA phosphorylation activities, we compared phosphorylation of CpxR by CpxA reconstituted in a synthetic oleoyl (18:1) or palmitoleoyl (16:1) lipid mixture (Figure 3). The level of phosphorylated compared to nonphosphorylated CpxR was reduced for CpxA reconstituted in synthetic oleoyl (18:1) species in comparison with that of CpxA reconstituted in the *E. coli* wild-type lipid mixture. Besides, the ratio of phosphorylated to nonphosphorylated CpxR was significantly increased when CpxA was reconstituted in a synthetic palmitoleoyl (16:1) lipid mixture in comparison with that of CpxA reconstituted in a synthetic oleoyl (18:1) lipid mixture. This finding supports our assumption that bilayer thickness affects the phosphorylation activities of CpxA.

The Cpx System Responds to *in Vivo* Changes in the Lipid Bilayer. Our *in vitro* analyses showed that the reconstituted phosphorylation and dephosphorylation abilities of CpxA are modulated by changes in lipid bilayer properties of proteoliposomes. Changes in the lipid moiety of the cytoplasmic membrane may function as an inducing cue for the activities of CpxA and thus CpxA−CpxR signal transduction. As a logical next step, we analyzed whether changes in the lipid moiety of the cytoplasmic membrane also induce Cpx responses *in vivo* by using different lipid-engineered *E. coli* strains.[42] For this purpose, we analyzed the transcriptional regulation of *cpxP*, which is transcribed by phosphorylated CpxR.[5] We inserted the *cpxP* promoter region (P*cpxP*) into a *gfp* reporter plasmid and monitored in-cell fluorescence. Compared to *E. coli* WT, we observed an increased level of

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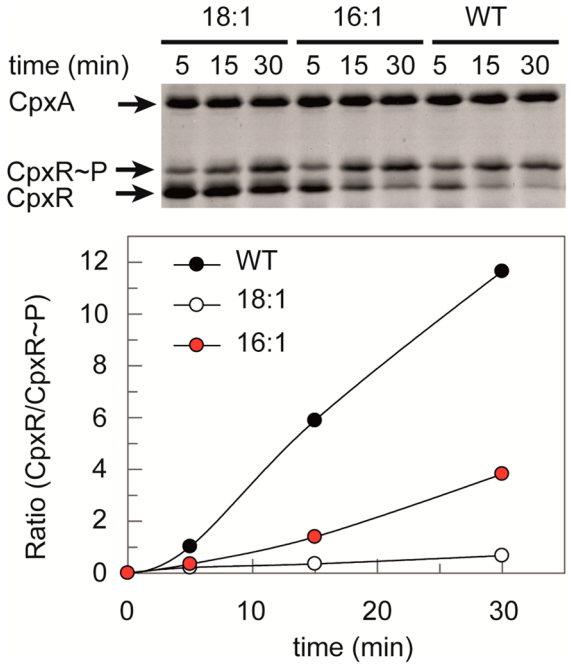

Figure 3. Phospholipid acyl chain lengths affect the phosphotransfer activity of reconstituted CpxA. CpxA (2.5 μM) was reconstituted into liposomes with the indicated DOPE/DOPG/TOCL (mole percent; 70/20/10) lipid composition with a C18 or C16 acyl chain length. CpxA reconstituted in the WT lipid mixture serves as a control. Equimolar CpxR amounts were added, before the reaction was started via addition of ATP (3 μM). Each reaction was stopped at the indicated time points, and phosphoproteins were visualized by 8% Phos-tag PAGE. The amount of phosphorylated and unphosphorylated CpxR protein was determined by densitometry.

expression in all lipid-engineered strains, except for that of GalGalDAG (Figure 4). These observations are consistent with

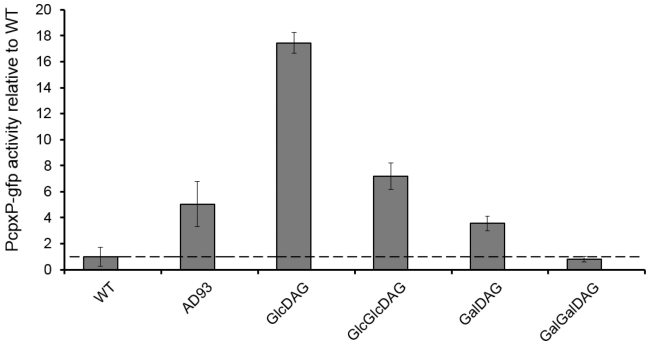

Figure 4. *In vivo* cpx responses in lipid-engineered *E. coli* strains. CpxP expression was monitored in different lipid-engineered *E. coli* strains by measuring the GFP fluorescence from the P*cpxP-gfp* reporter construct. Fluorescence values were normalized to the amount of CpxA and TetR per cell (both determined by Western blotting). The latter accounted for possible differences in copy number of the P*cpxP-gfp* plasmid. The WT value was set to 1.0 (---).

our *in vitro* data, showing that CpxR is not efficiently dephosphorylated (Figure 1B). As a consequence, the Cpx system activates the expression of several target genes, including genes linked to maintenance of cellular integrity and function of the bacterial envelope.[11,14,48] The best-characterized Cpx-regulated proteins are cell envelope chaperones and proteases, such as the disulfide oxidoreductase DsbA, the peptidyl-prolyl-isomerase PpiA, and the bifunctional chaperone/protease DegP. Another candidate is the phosphatidylserine-decarboxylase (Psd), which is upregulated by CpxR-P.[14,48] The *psd* gene catalyzes the decarboxylation of phosphatidylserine to form phosphatidylethanolamine,[49] suggesting a direct linkage of the Cpx system and lipid homeostasis.

CONCLUSION

In this study, we incorporated CpxA into lipid vesicles with varying lipid compositions and monitored changes in the phosphorylation cascade of the Cpx system. Our data show that the Cpx system clearly depends on lipid bilayer properties. In addition, an increase in anionic surface charge and likely bilayer thickness of the vesicles resulted in increased levels of CpxR-P. Accordingly, the Cpx system directly senses changes in the lipid bilayer properties resulting in Cpx system activation. This activation of the Cpx system can affect the transcription of more than 100 genes, including several involved in lipid homeostasis.[11,14,48] Thus, the Cpx system is likely involved in remodeling the lipid composition as a response of sensing changes in the lipid bilayer properties. Altogether, this study is an important step in understanding how bacteria adapt to changes in the lipid bilayer to maintain membrane integrity.

ASSOCIATED CONTENT

Supporting Information

Supplementary Experimental Procedures, Tables S1 and S2, and Figures S1−S3. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00242.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

DO, dioleoyl (18:1*cis*); TO, tetraoleoyl (18:1*cis*); PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; DAG, diacylglycerol; GalDAG, β-galactosyl-DAG; GalGalDAG, α-galactosyl-β-galactosyl-DAG; GlcDAG, α-glucosyl-DAG; GlcGlcDAG, α-glucosyl-α-glucosyl-DAG.

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