An acid-tolerance response system protecting exponentially growing *Escherichia coli*

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The ability to grow at moderate acidic conditions (pH 4.0–5.0) is important to *Escherichia coli* colonization of the host’s intestine. Several regulatory systems are known to control acid resistance in *E. coli*, enabling the bacteria to survive under acidic conditions without growth. Here, we characterize an acid-tolerance response (ATR) system and its regulatory circuit, required for *E. coli* exponential growth at pH 4.2. A two-component system CpxRA directly senses acidification through protonation of CpxA periplasmic histidine residues, and upregulates the *fabA* and *fabB* genes, leading to increased production of unsaturated fatty acids. Changes in lipid composition decrease membrane fluidity, F0F1-ATPase activity, and improve intracellular pH homeostasis. The ATR system is important for *E. coli* survival in the mouse intestine and for production of higher level of 3-hydroxypropionate during fermentation. Furthermore, this ATR system appears to be conserved in other Gram-negative bacteria.
enteric bacteria such as *Escherichia coli* (*E. coli*) and *Salmonella* can colonize and cause disease in the human intestinal tract. They have to combat acidic environments during the whole process of invading the host. With pH values as low as 1.5–2.5, the stomach has been recognized as a natural antibiotic barrier. With their passage into the small intestine, *E. coli* cells will encounter a less acidic environment (pH 4.0–6.0) with the presence of organic acids produced by the normal intestinal flora.

*E. coli* has developed variable acidic stress response systems, including the acid resistance (AR) systems response to extreme acid stress and the acid tolerance response (ATR) system towards mild and moderate acid stress. Up to now, five AR systems, AR1–AR5, are reported. The AR1 system is activated by alternative o factor (RpoS) and cAMP receptor protein (CRP). Due to the involvement of CRP, the AR1 system is repressed by glucose. The AR2–AR5 systems are all dependent on a specific extracellular amino acid, and consist of an antipporter as well as a decarboxylase enzyme that is usually induced by low pH and extracellular amino acid, except that AR3 can be induced at acidic pH in the absence of glutamate. They confer acid resistance by consumption of intracellular protons in amino acid decarboxylation reaction to produce a less acidic internal pH, using glutamate, arginine, lysine and ornithine as their corresponding substrates, respectively. All five AR systems can protect stationary phase cells from the extreme acidity and prolong survival, while only AR2 and AR3 were reported to function during the exponential phase.

Among AR systems, AR2 is by far the most effective and the most complex. The glutamate decarboxylase isoforms, GadA and GadB, and the glutamate/γ-aminobutyric acid antipporter GadC are key components of AR2, and their regulation relies on the action of over 20 proteins and 3 small noncoding RNAs, including two-component systems EvgAS and PhoPQ; regulatory proteins RpoS, GadE, RcsB, GadX, GadW and HNS; protease ClpXP and Lon; and small RNAs DsrA, GadY and GcvB, which together form a regulatory network with high level of complexity (for a review, see refs. 5,7). The periplasmic chaperons HdeAB and their cytoplasmic counterpart Hsp31, which assist the refolding of denatured proteins during the acid stress, are also induced as part of the AR2 regulon.

The ATR system, though poorly understood, is induced by exposing *E. coli* cells to moderate acid stress (pH 4.5–5.8), and will protect cells from a subsequent challenge of extreme acid pH (pH 2.0–3.0). ATR can be activated during adaptation at mild acidic pH by the regulators Fur and PhoPQ in exponential phase cells and by RpoS and OmpR in stationary phase cells, but the stationary phase cells are much more tolerant to acid than the log phase cells.

Benefited from the complicated AR and ATR systems, *E. coli* can survive without growth for several hours at pH 2.0–3.5. Meanwhile, levels of the UFAs, including palmitoleic acid (C16:1) and oleic acid (C18:1), were increased by 3.83- and 1.66-fold; meanwhile, the levels of palmitic acid (C16:0) and stearic acid (C18:0) were reduced. These fatty acids shown in Fig. 1b represented more than 96% of total fatty acids in *E. coli* cells, consistent with those results reported previously.

As a consequence, the ratio of unsaturated to saturated fatty acids increased from 0.11 at pH 7.0 to 0.32 at pH 4.2. Comparable to this result in *E. coli*, a shift in the unsaturated/saturated ratio in response to acid stress was also observed in *Streptococcus mutants*.

We also found that the expression of two essential genes required for UFAs biosynthesis, *fabA* and *fabB* (*Fig. 1c*), was significantly upregulated under the acidic condition since their protein and mRNA levels were elevated (Fig. 1d, e). A previous study showed that overexpression of *fabA* and *fabB* increased UFA contents in *E. coli*. Therefore, it is plausible that overexpression of *fabA* and *fabB* may allow *E. coli* to grow under pH 4.2. To test this hypothesis, we cloned *fabA* and *fabB* into vector pTrcHis2B, and introduced them into BW25113 wild-type strain. The strain carrying empty vector presented a CFU ratio of 0.26 ± 0.03, similar with that of BW25113 wild-type strain, while either strain with *fabA* or *fabB* showed much higher acid tolerance at pH 4.2 (*Fig. 1f*).

In agreement with this result, strains harboring a temperature-sensitive mutant of *fabA* or *fabB* gene became much more susceptible to acid at 42 °C, whereas the wild-type strain showed
Fig. 1 Improved acid tolerance of exponentially growing E. coli caused by increased production of unsaturated fatty acids. a Growth of E. coli BW25113 strain at different pH. The strain was grown to $3 \times 10^8$ CFU mL$^{-1}$ in minimal medium at pH 7.0, and transferred into the same medium at pH 7.0 and pH 4.2 ($n = 3$ biologically independent samples). CFU ratio = CFU at pH 4.2/CFU at pH 7.0. b Membrane lipid composition of BW25113 strain after acidic challenge at pH 4.2 for 1 h. The compositions were determined by GC-MS, and fatty acid content is given as the relative peak area (peak area of one fatty acid/total peak area) × 100% ($n = 3$ biologically independent samples). c Biosynthetic pathway of unsaturated fatty acids, in which FabA and FabB, 3-hydroxyacyl-ACP dehydratase/isomerase and β-ketoacyl-ACP synthase play essential roles. d Relative mRNA level of fabA and fabB in BW25113 strain after 1 h of exposure to pH 7.0 and 4.2 determined by qRT-PCR ($n = 2$ biologically independent samples with three technical repeats). e Tolerance of BW25113 strain carrying empty vector pTrcHis2B, pfabA or pfabB, respectively, after acidic challenge at pH 4.2 for 1 h ($n = 3$ biologically independent samples). f Tolerance of BW25113 strain, temperature-sensitive FabA and FabB mutants after acidic challenge at pH 4.2 at 30 or 42 °C for 0.5 h ($n = 3$ biologically independent samples). Error bars, mean ± standard error of mean (SEM). Two-tailed Student’s $t$ tests were performed to determine the statistical significance for two group comparisons. The source data are provided as a Source Data file.
We mapped the transcription starts of fabA and fabB using RACE (rapid amplification of cDNA ends) experiment. As was previously reported, for fabA gene, two transcription initiation sites were detected under growth at pH 7.0: S1 positively regulated by FadR32 and S2 negatively regulated by FabR 33. Interestingly, when the cell growth was at pH 4.2, our results indicated that the fabA gene has a third transcription initiation site located at the 203 bp upstream of the start codon, the S3 (summarized in Fig. 3a). For fabB gene, the transcription was initiated 37 bp upstream of the start codon in both conditions (summarized in Fig. 3b), consistent with previous reports34.

qRT-PCR result showed that the mRNA level of fabA and fabB was upregulated by NlpE overexpression in a fabR fadR double mutant strain, indicating that they had no obvious effect on fabA and fabB gene expression under CpxRA-dependent activation (Supplementary Fig. 2).

CpxR protein directly binds to the fabA and fabB promoters. To verify the putative CpxR recognition sites, two mutant strains were constructed by site-directed mutagenesis, in which the CpxR binding box on fabA or fabB promoter region was replaced by CATCT-(5 nt)-CATCT sequence, and expression of fabA and fabB was determined. Both western blot and qRT-PCR results
showed that the activating effect of acid stress on expression of fabA and fabB was completely eliminated by substitution of CpxR site (Fig. 3c, d). Moreover, the acid tolerance of these mutants significantly decreased (Fig. 3e).

The function of CpxR site was further confirmed in vitro through gel-shift assay and DNase I footprinting analysis using purified His6-CpxR protein and 211- and 219-bp DNA fragments corresponding to the promoter regions of fabA and fabB, respectively. The purified His6-CpxR protein could shift those two DNA fragments (Fig. 3f), and protect the fabA promoter at the −287 to −255 region (numbering from the start codon) and the fabB promoter at the −119 to −94 region in the noncoding strand containing the putative CpxR binding site (Fig. 3g). All these results demonstrate that the CpxR protein enhances transcription of fabA and fabB by direct binding to their promoters.

**CpxRA is directly activated by acidic environments.** As a sensor histidine kinase in two-component system, CpxA spans the cell membrane and exposes its sensor domain into the periplasm. When CpxA detects a specific signal, it autophosphorylates and then transports the phosphate group to its cognate regulator CpxR, enabling the regulatory activity of CpxR. Without inducing signal, CpxA acts as a phosphatase to maintain CpxR in an inactive state.35,36.

To monitor the expression and phosphorylation level of CpxA protein, we constructed a strain carrying chromosomal His6-tagged CpxA. As shown in Fig. 4a, acid shock significantly enhanced the amount of CpxA-His6 protein in exponentially growing cells. More importantly, increased level of phospho-CpxA, the active form of CpxA, was detected at pH 4.2 (Fig. 4a).

To demonstrate direct activation of CpxRA upon exposure to acidic pH, we used a reconstituted proteoliposome system,
where a different internal and external pH can be stably maintained (Fig. 4b). The CpxA-His6 protein was purified from membranes and reconstituted into vesicles mainly in the inside-out orientation using the detergent-mediated method as described[37]. As shown in Fig. 4c, lowering the pH inside vesicles from 7.0 to 4.2 greatly enhanced the amount of phospho-CpxA. The phosphoryl group was transferred to regulator CpxR when the pH inside vesicles was 4.2, while phosphorylated CpxR could be hardly detected with neutral lumen pH (Fig. 4c) or without the sensor kinase CpxA. These
results suggested that CpxA is capable of activating CpxR upon direct exposure to acidic environments.

To further verify the sensitivity of CpxA periplasmic domain to acidification, a chromosomal PhoQ-CpxA fusion strain was constructed, in which the sensor domain of PhoQ (amino acids 43–193) was replaced by the periplasmic domain of CpxA (amino acids 28–164) (Fig. 4d). PhoPQ is a known two-component system that can sense the acidic pH, Mg^{2+} depletion and antimicrobial peptides, and activate transcription of genes including rstA and slyB. Western blot results showed that both low pH and overexpression of NlpE promoted expression of RstA and SlyB in strain containing the PhoQ-CpxA fusion protein, while only acidic environments increased the protein level of RstA and SlyB in wild-type strain (Fig. 4e). On the other hand, when the CpxA periplasmic domain was replaced with that from another kinase AtoS which senses the presence of acetate, neither could the protein level of FabA-His_6 and FabB-His_6 be enhanced by acidic conditions in vivo, nor could the CpxR protein be phosphorylated at pH 4.2 in reconstituted proteoliposome (Supplementary Fig. 3), indicating that CpxA periplasmic domain is sensitive to acidification. All these results collectively demonstrated that CpxA system is activated by direct exposure of CpxA periplasmic domain to acidic environments.

CpxA H52 and H117 are required for sensing of acidic pH. As reported, the histidine and glutamic acid residues were regarded as sensors detecting mild acidic pH because they can change their protonation state upon variation in the surrounding pH. So, we hypothesized that the histidine and glutamate residues in CpxA sensor domain might be required for pH sensing. To test this hypothesis, we constructed a series of plasmids encoding CpxA mutants in which the periplasmic histidine/glutamate residue was replaced with alanine, respectively. Strains expressing the mutant CpxA proteins could express fabA in response to NlpE overexpression normally (Supplementary Fig. 4), indicating that mutations in residues of the periplasmic domain of CpxA protein do not impair the kinase activity of CpxA cytoplasmic domain. As shown in Fig. 4f, only two mutants (H52A and H117A) resulted in the loss of CpxA capability to upregulate fabA expression at pH 4.2, while the other mutations in glutamate residues did not change the expression level of fabA. To confirm that protonation of H52 and H117 is responsible for pH-mediated gene regulation, we also mutated these two residues to other basic amino acids, lysine and arginine, respectively. It was discovered that the substitution of H52 and H117 by lysine and arginine had no effect on the response to acidic pH (Fig. 4g).

Moreover, the strain carrying CpxA variant with a basic amino acid residue at positions 52 or 117 presented a higher level of FabA protein even at pH 7.0, when compared with strain with CpxA H52A or H117A mutant (Supplementary Fig. 5), probably due to the protonation of basic amino acid residue at pH 7.0. These results demonstrate that protonation of residues at positions 52 and 117 is required to maintain the response of CpxA to acidic pH, but the presence of a histidine at those positions is not a specific requirement for activation.

Physiological effects of changes in lipid composition. To learn how changed content in membrane lipid affect E. coli cell functions and physiology, several assays were carried out using cells carrying empty vector as control, pfabA, or pfabB, after acidic challenge at pH 4.2. The membrane fluidity was determined by analyzing the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), which is negatively correlated to fluidity. As shown in Fig. 5a, the strains with overexpression of fabA or fabB presented much less cell membrane fluidity than strain carrying empty vector. The membrane permeability was measured by detecting the leakage of OD_{560} materials (predominantly nucleotides), and there was no significant difference observed despite the varied UFA contents in membrane (Supplementary Fig. 6). F_{0}F_{1}-ATPase spans the cell membrane and transports periplasmic protons to cytoplasm with production of ATP, and lipids are required for its optimal functioning. The activity of F_{0}F_{1}-ATPase was measured, and results suggested that the enhancement of UFAs content repressed its activity (Fig. 5b).

As E. coli has to combat a moderate acidic environment (pH 4.0–6.0) and proliferate in host’s small intestine, we carried out mouse gastrointestinal passage experiment to test the in vivo effect of the UFAs-CpxRA system. BALB/c mice were administered BW25113 wild-type strain or mutant with substituted fabA CpxR binding site, and three independent trials with six mice in each trial were performed for each strain. After 24 h, fecal samples were collected to test the presence of each strain. Out of 18 inoculated mice, the wild-type BW25113 strain was recovered from a total of 8 mice, while the fabA CpxR box mutant was detected in only 2 fecal samples (Fig. 5d). The results between the parental and mutant strains were statistically different (p < 0.05), demonstrating that this ATR system significantly promotes E. coli survival in mouse intestinal lumens.

In bio-production of organic acids, product accumulation acidifies the fermentation broth and inhibits the growth of producing strain. Previously, we had constructed a 3-hydroxypropionate (3HP)-producing E. coli recombinant strain. In shaking flask cultivation, the pH of culture decreased to pH 5.2–5.5 along with the production of 3HP, repressing the further production and cell growth (Fig. 5e). So, the pH of fermentation broth had to be adjusted to 7.0 periodically. Then fabA gene was overexpressed to enhance E. coli tolerance to acidic environments, leading to similar 3HP production and cell growth with and without pH adjustment (Fig. 5e). This result demonstrates the great prospect of UFAs-CpxRA-dependent ATR system in organic acids bio-production.

The UFAs-CpxRA system functions in diverse bacteria species. Salmonella Typhimurium LT2 and Shigella flexneri 2a str. 2457T are both common enteric pathogens, and their FabA proteins share more than 98% identity with E. coli FabA (Supplementary Fig. 7). To test whether UFAs are required for growth under acidic pH in these bacteria, the empty vector and recombinant plasmid carrying E. coli fabA gene were transformed into Salmonella Typhimurium LT2 and S. flexneri 2a str. 2457T. In acidic challenge tested at pH 5.0, the transformation of empty vector did not affect the acid tolerance of those two strains, but the overexpression of fabA gene elevated those CFU ratios by 2.32- and 1.99-fold, respectively (Fig. 6a). Moreover, mRNA level of fabA and fabB was upregulated by acid challenge in Salmonella, Klebsiella and Pseudomonas strains (Fig. 6b), and the consensus sequence of CpxR site was also found upstream of the fabA and fabB genes in additional bacteria species (Table 1). Additionally, Cronobacter sakazakii clone carrying transposon insertion in cpxR gene was identified as acid-sensitive mutant. All these facts suggest this ATR system functioning in exponential phase is highly conserved across bacteria species.
administration to BALB/c mice. Three independent trials with six mice in each trial were performed for each strain.

**Discussion**

According to the results shown above, we present a previously uncharacterized ATR system as well as its regulatory pathway in exponentially growing *E. coli*. We demonstrated that the two-component system CpxRA can directly sense the acidic environments, and activate transcription of UFAs synthetic genes, resulting in increased UFAs contents in cell membrane lipid and normal growth of *E. coli* at pH 4.2. Our findings greatly enriched our understanding of the networks contributing to bacterial acid resistance, and the mechanisms for stress response governed by the CpxRA system.

As reported, the acid limit for *E. coli* growth is pH 4.0–4.5, and all known AR and ATR systems only prolong survival of *E. coli* cells under acidic conditions, but cannot support growth at pH 4.0–4.5. In our study, overexpression of fabA and fabB genes for UFAs biosynthesis, or activation of the two-component regulatory system CpxRA both restore the growth capability of *E. coli* at pH 4.2. Compared with those previously known AR systems, this growth-conferring ATR system is expected to have more important physiological significance. Firstly, bacteria can grow normally under acidic pH with activation of the CpxRA- and UFAs-dependent system, achieving higher biomass, which may be required for successful pathogenesis and efficient bio-production. Secondly, de novo mutations during DNA duplication play a critical role in bacterial stress resistance development, and the rapid proliferation at low pH provides an opportunity for the evolution of novel AR and ATR systems. Thirdly, functioning of previously known AR systems is subject to more external limitations, such as AR1 is repressed by glucose and AR2–AR5 are dependent on exogenous amino acids, whereas the UFAs-CpxRA system can be activated by acidic pH alone because CpxA is capable of phosphorylating CpxR upon exposure to pH 4.2 in a reconstituted proteoliposome system.
We proved that the transcription of fabA stimulated by CpxRA is required for multiplication of E. coli in mouse intestine, indicating that UFAs-CpxRA system is also related to pathogenesis of E. coli pathovars, as well as those previously known AR systems. While various pathotypes of E. coli colonize and infect different organs, they all have to combat acidic environments during invading the host’s digestive tract. With pH values as low as 2.5–2.5, the stomach has been recognized as a natural antibiotic barrier. Benefited from AR1–AR5 systems, E. coli can survive in the gastric acid for hours. With their passage into the small intestine, E. coli cells will encounter a less acidic environment (pH 4.0–6.0) with the presence of organic acids produced by the normal intestinal flora. As pathogenic E. coli strains must reproduce rapidly to cause disease ultimately, the UFAs-CpxRA system is likely to play a key role. In summary, the successful enteric pathogen must possess two abilities, survival in extreme acidic condition and quick growth in moderate acidic environment. Consequently, this ATR system may be a new target for the development of antimicrobials.

UFAs-CpxRA-dependent ATR system also has potential application in bio-production of organic acids, which are valuable platform chemicals and have been successfully produced by recombinant E. coli strains. However organic acids cause acidification of fermentation broth and inhibit E. coli growth at concentrations far below what is required for economical production. Now large quantity of base titrant are required to raise pH of the media in organic acids production process, and large amounts of acid must be consumed to recover the organic acids in the protonated form after production. If we could construct acid-tolerant strains growing at a pH less than the pKa of the produced acid, the additional consumption of acid and base titrants will be circumvented and the overall production cost will be lowered remarkably. As the UFAs-CpxRA system functions in exponential phase, is not repressed by glucose (the carbon source in most fermentation), and does not need exogenous amino acids, this phenomenon is consistent with that S. mutans cells grown at pH 5.0 had higher UFAs composition and lower proton permeability than those grown at pH 7.0. Moreover, changes in fatty acid composition probably also affect the PTS system and enzyme secretion. Overall, changing membrane fatty acid composition may improve the bacterial ability to adapt to acidic environment and be an important factor in bacterial acid response. In this study, the cyclopropane fatty acid (CFA) could not be detected in exponential phase E. coli cells, although it was regarded as a major factor in acid resistance of stationary phase E. coli. However, UFA can be converted into CFA by CFA synthase, and increased UFAs content will potentially enable the synthesis of CFA in stationary phase.

We demonstrate that the E. coli kinase CpxA is a direct sensor for acidic pH. Our data provide strong evidence that a decrease in pH protonates the histidine residues at positions 52 and 117 in CpxA periplasmic domain, leads to events catalyzed by its cytoplasmic domain, including phosphorylation of CpxA, transfer of phosphoryl group to regulator CpxR, as well as activation of CpxR-dependent gene transcription. As titratable by pH, histidine has been regarded as sensor detecting mild acidic pH, and also plays an essential role in the activation of sensor kinase PhoQ by pH 60. But the molecular details of how histidine senses the low pH signal in PhoQ and CpxA are different. Protonation of residues at positions 52 and 117 is effective to activate CpxA at acidic pH, whereas the imidazole ring of histidine is important in maintaining the response of PhoQ to acidity 60.

Our results evidenced that CpxRA is a key system in the acid stress response of exponentially growing E. coli (Fig. 7), consistent with a previous proteomic analysis highlighting the importance of CpxRA in acid stress. Upon exposure to acidic environments, CpxRA system stimulates the transcription of UFAs synthetic genes, resulting in improved intracellular pH homeostasis. Additionally, CpxRA upregulates some genes involved in cell wall modification, including peptidoglycan (PG) cross-linking proteins YcfS, YcbB and DacC; PG cleaving proteins AmiA, AmiC and Sl61–63. The induction of those genes led to an increase of cross-linking between PG and outer membrane proteins, and an increase of cell wall stability, which may help protecting E. coli cells from acid challenge. That proteomic study also indicated the repression of AR2 system by CpxRA.Because AR2 system is responsible for survival below pH 3.0 and log phase cells with overexpression of AR2 genes are not more acid resistant, the CpxRA-mediated repression of AR2 in exponentially growing cells above pH 3.0 will guarantee that AR2 system is not induced in an inappropriate situation to avoid the metabolic burden. Furthermore, the sensor kinase CpxA was proved to cross-talk with noncognate response regulator OmpR, which itself is involved in the acid stress response of E. coli. All of these data indicate that E. coli has
Bacteria were grown at 37 °C in Luria-Bertani broth (Oxoid) or in E minimal conditions. The identity of this signal still remains a topic of much debate. According to the results in this study, we propose that there must be an activating signal for CpxA under in vivo conditions. The AR2 system. Genes cpxA and cpxR evolved multiple acid response mechanisms as well as precise regulatory circuits, to target specific stress conditions. In many Gram-negative pathogens, the CpxRA system plays an important role in the regulation of virulence factors, including pilus, secreted virulence effectors and type III secretion system. For CpxRA to upregulate expression of these virulence genes, there must be an activating signal for CpxA under in vivo conditions. The identity of this signal still remains a topic of much debate. According to the results in this study, we propose that moderate acidic pH (such as the intestine and macrophage) is the activating signal of CpxA in vivo. As the previous study showed that CpxRA also contributed to bacterial resistance to antimicrobial peptides, a component of the host antimicrobial defense, acidic pH and antimicrobial peptide may have synergistic effect on CpxRA activation. Further study is ongoing to address this hypothesis.

Methods

Bacterial strains and growth conditions. All strains and plasmids used in this study are listed in Supplementary Data 1, and all primers used are listed in Supplementary Data 2. Phage P1 was used for generalized transductions in E. coli. Bacteria were grown at 37 °C in Luria-Bertani broth (Oxoid) or in E minimal medium (0.8 mM MgSO4, 10 mM citric acid, 5.75 mM K2HPO4, 16.7 mM NaH2PO4, 0.5% glucose). When necessary, antibiotics were added at final concentrations of 100 μg mL−1 for ampicillin, 20 μg mL−1 for chloramphenicol or 50 μg mL−1 for kanamycin. E. coli DH5α was used as host for the preparation of plasmid DNA, and E. coli y2123 was used for preparation of suicide vectors. Diaminopimelic acid (50 μg mL−1) was used for the growth of y2123 strain. LB agar containing 10% sucrose was used for sacB gene-based counter selection in allelic exchange experiments. The software ImageJ (version 1.52a) was used to analyze western blot results, and the online version of Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for sequence alignment.

Plasmids were constructed by digesting PCR fragments containing target gene and cloning into corresponding vectors as normal. Derivatives of pTrc-cpxA with nucleotide substitutions were constructed using Q5 Site-Directed Mutagenesis Kit (New England Biolabs) according to the manufacturer’s specifications. All plasmids were confirmed by DNA sequencing. Strains harboring chromosomal epitope-tagged proteins were generated using λ Red recombine system. DNA fragments encoding PhoQ-CpxX fusion or carrying substituted CpxX were generated by joint PCR using primers shown in Supplementary Data 2, and cloned into the suicide vector pRE112. The resulting plasmids were used to mediate the allelic exchange to generate strains with chromosomal PhoQ-CpxX fusion and CpxX site mutation.

In acidic challenge experiments, the strains were grown in E medium (pH 7.0) to an OD600 of 0.6. For strains with plasmid, IPTG was added to final concentration of 0.5 mM at an OD600 of 0.4, and the strain was further grown to an OD600 of 0.6. Then, the cells were harvested and washed twice with fresh E medium (pH 7.0) and inoculated into fresh medium with various pH adjusted, and strains were grown for another 30–60 min before the cells were collected to determine the CFU, membrane lipid composition, mRNA and protein levels.

Quantitative RT-PCR and RACE. Total RNA was isolated from bacterial culture using EASYspin Plus bacterial RNA quick extract kit (Aidlab Biotechnologies, China) according to the manufacturer’s instructions. RNA concentration was determined by spectrophotometry at 260 nm. Removal of genomic DNA and synthesis of cDNA were carried out using PrimeScript RT reagent Kit with gDNA Eraser (Takara). qRT-PCR was conducted using TB Green Premix Ex Taq (Takara) and primers shown in Supplementary Data 2, and the relative difference of mRNA level was calculated using the ΔΔCt method. Two independent biological samples with three technical repeats for each sample were performed for each qRT-PCR analysis.

The RACE experiment was performed using SMARTer RACE cDNA Amplification Kit (Clontech), according to the manufacturer’s instructions. The primers 1128 + 1129 and 1130 + 1131 were used to determine the transcription start sites of fabA and fabB under the NlpE overexpression conditions, respectively.

Purification of His6-CpxX and CpxX-His6. Purification of His6-CpxX and CpxX-His6 was conducted according to Fletchers et al. E. coli strain BW2513 with pTrc-cpxX or pTrc-cpxA was grown at 37 °C with aeration in LB medium. Gene expression was induced with 0.5 mM IPTG for 3–4 h. Membrane fractions and cytosolic fraction were separated by ultracentrifugation. His6-CpxX was solubilized in cytosolic fraction and purified by Ni-affinity chromatography. Membrane proteins were solubilized with 1% dodecyl-β-D-maltoside (DM), and then CpxX-His6 was also purified by Ni-affinity chromatography.

Preparation of proteoliposomes. Proteoliposomes were reconstructed as previously described with small modification. E. coli phospholipids (Avanti) were dried under a stream of nitrogen, and slowly dissolved in sodium citrate–hydrochloric acid buffer (pH 7.0, 5.0, 4.2) with 10% glycerol (vol/vol) and 0.47% Triton X-100 (vol/vol), respectively. Purified CpxA-His6 was added to the mixture and mixed at room temperature. Bio-Beads SM-2 (Bio-Rad) were added and stirred in a bead/detergent ratio of 10:1(w/v), and the mixture was gently stirred at 4 °C overnight. After 16 h, fresh Bio-Beads were added, and the mixture was stirred for another 6 h. The proteoliposomes were collected by ultracentrifugation. To test autophosphorylation, proteoliposomes were incubated with 300 μmol ATP in physiological buffer (50 mM Tris-HCl pH 7.5, 10% glycerol (vol/vol), 2 mM dithiothreitol (DTT), 50 mM KCl, 5 mM MgCl2) at room temperature for 30 min. 5x SDS sample buffer was loaded to termination reaction. To analyze...
phosphotransfer, purified His6-CpxR was added to this mixture and incubated at room temperature for 20 min. Then samples were ultracentrifuged and the upper phase was collected. 5× SDS sample buffer was loaded to stop the reaction. To detect the phosphorylation level of CpxA and CpxR, all the samples were subjected to 8% PAGE using T4 polynucleotide kinase (New England Biolabs) and a 32P-labeled DNA sequence ladder generated with the same primers using a Maxam and Gilbert protocol44. The phosphorylation level of CpxA and CpxR, all the samples were subjected to 8% PAGE, 7.5% gel (200 mM Tris−HCl, pH 7.5, 1 mM ethylene diamine tetraacetic acid, 5 mM DTT, 10 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, and 5% glycerol. The mixture was subjected directly to 4% TAE-PAGE.

Excitation assays were performed at wavelengths 395 and 475 nm with the emission at 510 nm using FluoroMax-4 Spectrofluorometer. The gene encoding ratiometric pH-sensitive green fluorescence probe was expressed in E. coli using T4 polynucleotide kinase (New England Biolabs) and subsequently diluted. The diluted samples were then plated on LB agar containing 50 μg mL−1 of 100 mM MgCl2, and 0.005 units of DNase I (Fermentas) were added, and the mixture was incubated at room temperature for 2 min. The DNAase I digestion was stopped by phenol treatment, and the DNA was precipitated. Samples were analyzed by 6% polyacrylamide electrophoresis by comparison with a nonphosphorylated forms in two separated bands.

Received: 18 June 2019; Accepted: 5 March 2020; Online published: 20 March 2020

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financially supported by the NSFC (31722001 and 31670089), and Natural Science Foundation of Shandong Province (JQ201707).

**Author contributions**
G.Z. designed the experiments. Y.X., Z.Z., W.T., Y.S., Y.D., B.L., J.W., M.L., Y.W. and S.S. performed the experiments. G.Z., M.X., Y.X. and Q.Q. analyzed the results. G.Z., M.X., Y.X. and Q.Q. wrote the manuscript. All authors edited the manuscript before submission.

**Competing interests**
The authors declare no competing interests.

**Additional information**
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-15350-5.

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**Peer review information** Nature Communications thanks Daniela De Biase, Peter Lund and the other, anonymous, reviewer for their contribution to the peer review of this work. Peer reviewer reports are available.

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