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A connector-like factor, CacA, links RssB/RpoS and the CpxR/CpxA two-component system in *Salmonella*

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Abstract

Background: Bacteria integrate numerous environmental stimuli when generating cellular responses. Increasing numbers of examples describe how one two-component system (TCS) responds to signals detected by the sensor of another TCS. However, the molecular mechanisms underlying this phenomenon remain poorly defined.

Results: Here, we report a connector-like factor that affects the activity of the CpxR/CpxA two-component system in *Salmonella enterica* serovar Typhimurium. We isolated a clone that induced the expression of a *cpxP-lac* gene fusion from a high-copy-number plasmid pool of random *Salmonella* genomic fragments. A 63-amino acid protein, CacA, was responsible for the CpxA/CpxR-dependent activation of the *cpxP* gene. The CpxR-activated genes *cpxP* and *spy* exhibited approximately 30% and 50% reductions in transcription, respectively, in a clean *cacA* deletion mutant strain in comparison to wild-type. From 33 response regulator (RR) deletion mutants, we identified that the RssB regulator represses *cacA* transcription. Substitution mutations in a conserved -10 region harboring the RNA polymerase recognition sequence, which is well conserved with a known RpoS -10 region consensus sequence, rendered the *cacA* promoter RpoS-independent. The CacA-mediated induction of *cpxP* transcription was affected in a *trxA* deletion mutant, which encodes thioredoxin 1, suggesting a role for cysteine thiol-disulfide exchange(s) in CacA-dependent Cpx activation.

Conclusions: We identified CacA as an activator of the CpxR/CpxA system in the plasmid clone. We propose that CacA may integrate the regulatory status of RssB/RpoS into the CpxR/CpxA system. Future investigations are necessary to thoroughly elucidate how CacA activates the CpxR/CpxA system.

Keywords: Two-component system, Connector, Network, RssB, RpoS, CacA, CpxR/CpxA

Background

The two-component system (TCS) is one of the most ubiquitous signal transduction systems in bacteria [1]. A prototypical TCS harbors a sensor histidine kinase (HK), which is often integrated into the inner membrane, and a response regulator (RR), which is predominantly a cytoplasmic DNA-binding transcription factor. In the presence of a specific activating signal, the sensor HK is autophosphorylated, and a phosphoryl group is subsequently transferred to a conserved aspartate residue in its cognate RR, thus changing gene expression patterns and cell physiology. Each TCS responds to specific environmental

signals but elude identification even in the well-investigated organisms *Escherichia coli* and *Salmonella*. Due to the high levels of sequence and structure similarity among different TCSs, cross-talk (i.e., phosphotransfer from a HK to its non-cognate RR) may occur in at least some circumstances. However, cross-talk is extremely rare due to the kinetic preference of a sensor HK for its cognate RR [2] and their phosphatase activities [3].

To date, several small proteins connecting TCSs have been reported in *Salmonella* and *E. coli* [4,5]. For example, the 85-amino acid PmrD protein, which is transcriptionally induced by the PhoP/PhoQ system under low Mg²⁺ conditions, binds to the phosphorylated form of the regulator PmrA and hinders its dephosphorylation by the cognate sensor PmrB [6]. Therefore, expression of PmrA-activated genes, some of which are responsible

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for polymixin B resistance and iron resistance in *Salmonella*, is induced even in the absence of an Fe³⁺ signal [7]. The small anti-adaptor proteins IraP and IraM, which promote the stability of the stationary phase sigma S factor (RpoS) of RNA polymerase by hindering an RR (RssB), are also transcriptionally activated by the PhoP/PhoQ system in response to low Mg²⁺ conditions in *Salmonella* [8] and *E. coli* [9], respectively. In contrast to these cytosolic connectors, the small inner membrane proteins SafA (B1500) [10] and MzrA [11] were identified as signal transducers between two TCSs by targeting downstream sensor HKs. SafA elicits a response from the PhoQ sensor to the PhoP regulator even under high Mg²⁺ conditions when the EvgS1 mutant protein [12] induces the EvgA-activated *safA* gene constitutively [10]. Alternatively, MzrA interacts with the EnvZ sensor to control OmpR-regulated gene transcription when *mzrA* expression is induced in a constitutively activated CpxA* mutant background [13] in *E. coli*. The membrane peptide MgrB [14,15], which corresponds to a single TCS, communicates the activation status of the PhoP regulator to its cognate sensor PhoQ in *E. coli* and *Salmonella* [15]. In contrast, the unique membrane peptide PmrR mediates the feedback control of the PmrA/PmrB system indirectly in *Salmonella* [16].

The CpxR/CpxA system regulates pilus assembly, adherence, and biofilm development in response to envelope stress and is required for host cell invasion in several species, including pathogenic *E. coli* and *Salmonella* [17]. The periplasmic chaperone CpxP binds to both the CpxA periplasmic domain and to certain misfolded proteins, which are degraded by the periplasmic protease DegP, therefore integrating information about their turnover status to the kinase activity of CpxA [18-20]. The outer membrane lipoprotein NlpE activates the CpxA protein upon its overexpression [21] and is required for CpxA protein activation after adhering to hydrophobic surfaces [22]. Additional upstream components have been proposed to integrate other stresses in a process that is independent of the CpxP and NlpE pathways [17,23]. For example, the CpxR/CpxA system confers a copper resistance phenotype even in CpxP and NlpE mutants [24]. Notably, *nlpE* (*cutF* or STM0241) is a pseudogene in *Salmonella* [25].

Here, we aimed to identify candidate connector genes that may integrate the signals of other systems. We identified a small protein as a novel connector-like factor from screening high copy plasmid clones that could affect the CpxR/CpxA system status.

Results

Identification of a plasmid clone that activates *cpxP* transcription

To conduct a genetic screen for novel connector proteins acting on the CpxR/CpxA system, we constructed

a strain harboring a *cpxP-lac* transcriptional fusion in *Salmonella*. The *cpxP* gene was chosen as a readout of the activation status of the CpxR/CpxA system because it is likely directly regulated exclusively by this system, unlike other CpxR-activated genes that are also controlled by envelope stress-responsive systems [26-28]. The *lacZY* genes were inserted after the *cpxP* stop codon to ensure that the CpxP protein retained the ability to repress the CpxR/CpxA system. Then, *Salmonella* chromosomal DNA was partially digested with *Sau3AI* and ligated with the high-copy-number plasmid pUC19 (digested with *Bam*HI) to generate a DNA fragment library. Of approximately 10,000 *cpxP-lac* *Salmonella* transformants, a plasmid clone termed pWN1 yielded stable blue colonies on LB plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) and ampicillin and was isolated four times. The blue color of the pWN1 strain was due to elevated *cpxP-lac* fusion expression. We demonstrated that this strain exhibit ~8-fold higher β -galactosidase activity than the same strain harboring the vector control or the plasmid clone pUC19-R1 that was randomly selected during the screening as a negative control (Figure 1A). Sequence analysis revealed that pWN1 harbors only the intact STM1852 open reading frame (ORF), which appeared to encode a 63-amino acid protein with no homology to any protein of known function, as well as the 3' region of STM1851 and the 5' region of *pphA* (Figure 1B). Expression of STM1852 from tetracycline- (Figure 1C) or L-arabinose- (Figure 1D) inducible promoters recapitulated the increase in the β -galactosidase activity observed in the *cpxP-lac* strain, supporting the hypothesis that STM1852 was affecting *cpxP* transcription in the pWN1 plasmid. Here, we named STM1852 "Cpx activating connector-like factor A", or CacA.

CacA-mediated *cpxP* activation is dependent on the CpxR/CpxA system

The results described above demonstrated that *cpxP* transcription was induced when CacA was expressed from a high-copy-number plasmid or from a heterologous promoter in an inducer-dependent manner. Next, we compared the β -galactosidase activities of the *cpxP-lac* fusion from *cpxR* and *cpxA* mutant strains harboring pBAD18-*cacA* to an isogenic *cpxR⁺A⁺* strain containing the same plasmid (Figure 1D). We determined that CacA acts upstream of the CpxR/CpxA system because the activities of the *cpxR* and *cpxA* mutant strains expressing CacA were comparable to that of the isogenic *cpxR⁺A⁺* strain with vector (i.e., pBAD18) alone (Figure 1D). This was further supported by the observation that another CpxR-activated gene, *spy*, was induced by CacA protein overexpression (Figure 1C). Moreover, CacA likely acts on the CpxR/CpxA system specifically

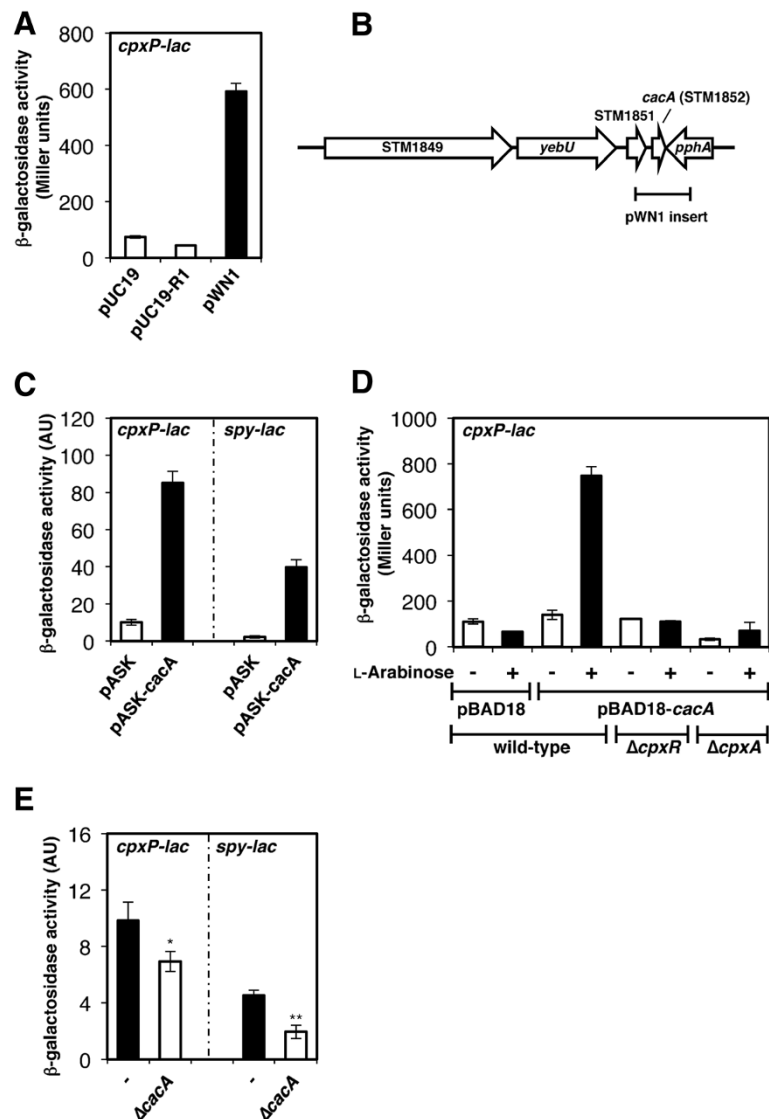


Figure 1 The identification of a novel connector-like factor, CacA. **A.** β -galactosidase activity from a *cpxP-lac* transcriptional fusion expressed in the wild-type strain (AK1052) harboring pUC19, pUC19-R1, and pWN1. Bacteria were grown for 4 h in LB before β -galactosidase activity was measured (Miller units). The data correspond to the means of two independent experiments performed in duplicate, and the error bars represent standard deviations. **B.** A genetic map of the *cacA* (STM1852) locus in *Salmonella*. Each arrow indicates a gene and its orientation in the chromosome. The chromosomal location corresponding to the inserted DNA fragment of the pWN1 plasmid clone is indicated by a horizontal bar. **C.** β -galactosidase activity from *cpxP-lac* or *spy-lac* transcriptional fusions in a wild-type (AK1052 or AK1053) strain harboring pASK or pASK-*cacA*. Bacteria were grown for 2 h in LB in the presence of 0.2 μ g/ml anhydrotetracycline (ATc) before β -galactosidase activity was measured (arbitrary units) as described [42]. The data correspond to the means of three independent experiments performed in duplicate, and the error bars represent standard deviations. **D.** β -galactosidase activity from a *cpxP-lac* transcriptional fusion in the wild-type strain (AK1052) harboring pBAD18 or pBAD18-*cacA* and the $\Delta cpxR$ mutant (AK1061) and $\Delta cpxA$ mutant (AK1062) strains harboring pBAD18-*cacA*. Bacteria were grown for 4 h in LB in the presence (+) or absence (-) of 5 mM L-arabinose before β -galactosidase activity was measured (Miller units). The data correspond to the means of two independent experiments performed in duplicate, and the error bars represent standard deviations. **E.** β -galactosidase activity from *cpxP-lac* or *spy-lac* transcriptional fusions in a wild-type strain (-; AK1052 or AK1053) and a $\Delta cacA$ mutant strain (AK1075 or AK1076). Bacteria were grown for 4 h in N-minimal medium, pH 7.7 with 10 μ M Mg²⁺ before β -galactosidase activity was measured (arbitrary units) as described [42]. The data correspond to the means of three independent experiments performed in duplicate, and the error bars represent standard deviations. Single and double asterisks indicate $p < 0.05$ and $p < 0.01$, respectively, using an unpaired t test for analysis.

because expression of *CacA* did not affect genes under the direct control of other TCSs (data not shown).

cacA transcription is activated by RpoS but repressed by RssB

Next, we asked whether the *cacA* gene might be regulated by an undefined upstream TCS. To examine candidate TCSs that could potentially affect *cacA* transcription, we constructed a strain with a *cacA* promoter-*lac* fusion 1 (i.e., P_{cacA} -*lac* 1) at the *pgtP* locus on the *Salmonella* chromosome. Then, 33 RR mutant stocks were independently transduced into the P_{cacA} -*lac* 1 strain by phage P22. Whereas most RR mutants exerted minor or no effects on transcription from the *cacA* promoter (data not shown, Figure 2A), the *rssB* mutant exhibited a ~1.5-fold increase in *cacA* promoter activity (Figure 2A). Because RssB is the adaptor protein that recruits RpoS to the ClpXP protease, we examined the effect of a $\Delta rpoS$ mutant on transcription from the *cacA* promoter. As expected, the *rpoS* gene was required for *cacA* expression (Figures 2A and 2B). Consistent with these observations, an alignment of the *cacA* promoter regions from *Salmonella* and its related enteric species revealed a conserved sequence that is present in an RpoS-dependent consensus -10 region sequence (**CTAcacT** from -13 to -7) [29] (Figure 3A).

Moreover, although the location of the predicted -10 region correlates well with a transcription start site (TSS) determined by a genome-scale precise mapping of TSSs that covered 78% of the *Salmonella* ORFs [30], no obvious typical -35 region sequence exists upstream of

the -10 nucleotides (Figure 3A). We mutated this -10 sequence from TCCTACACT to TCGTACACT (-14C/G), ACGTACACT (-16T/A-14C/G), or TCCTTCACA (-12A/T -8T/A) and analyzed their effects on *cacA* transcription (Figures 3B and 3C). In the $\Delta rpoS$ mutant, the β -galactosidase activity of the *cacA* promoter was approximately 1/3 of wild-type levels (Figure 3C). However, the β -galactosidase activities from the *cacA* promoter containing -14C/G or -16T/A -14C/G substitutions were not affected by the $\Delta rpoS$ mutation after 4 h of growth in LB, indicating that these substitution mutations rendered the *cacA* promoter RpoS-independent (Figure 3C). Conversely, when the essential nucleotides -12A and -8T of the canonical -10 region sequence, which permits recognition by both RpoD and RpoS, were mutated, *cacA* promoter activity was abolished independent of RpoS presence (Figure 3C). Taken together, these results demonstrated that the activation of the *cacA* promoter is dependent on the -10 region sequence, which harbors an RpoS recognition site.

Transcription of the CpxR-activated genes *cpxP* and *spy* is attenuated in a *cacA* mutant

Because RpoS activates *cacA* expression, we assessed whether a *cacA* deletion mutation would affect transcription of the CpxA/CpxR-dependent *cpxP* and *spy* genes in low Mg^{2+} , the conditions under which the PhoQ/PhoP-activated IraP prevents the RssB/ClpXP-mediated degradation of RpoS, even at log phase [8]. We determined that *CacA* participates in CpxA/CpxR system activation because *cpxP* and *spy* expression levels

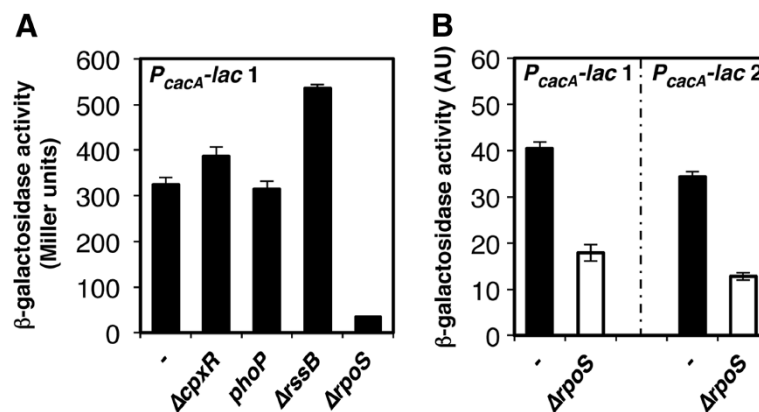


Figure 2 Transcription of the *cacA* gene is activated by RpoS but repressed by RssB. **A.** β -galactosidase activity from a P_{cacA} -*lac* transcriptional fusion 1 in the wild-type (-; AK1056), $\Delta cpxR$ mutant (AK1063), *phoP* mutant (AK1064), $\Delta rssB$ mutant (AK1065), and $\Delta rpoS$ mutant (AK1066) strains. Bacteria were grown for 4 h in LB before β -galactosidase activity was measured (Miller units). The data correspond to the means of two independent experiments performed in duplicate, and the error bars represent standard deviations. **B.** β -galactosidase activity from P_{cacA} -*lac* transcriptional fusion 1 or 2 in a wild-type strain (-; AK1056 or AK1067) and a $\Delta rpoS$ mutant strain (AK1059 or AK1071). Note that the P_{cacA} -*lac* 1 strain contains a DNA fragment encompassing the 3' region (80 bp) of STM1851 and the intergenic region (110 bp) between STM1851 and *cacA*, whereas the P_{cacA} -*lac* 2 strain harbors only the intergenic region (110 bp) between STM1851 and *cacA* preceding the *lacZ* gene (See **Methods**). Bacteria were grown for 4 h in LB before β -galactosidase activity was measured (arbitrary units) as described [42]. The data correspond to the means of three independent experiments performed in duplicate, and the error bars represent standard deviations. The data in the panels **A** and **B** were obtained using two different methods.

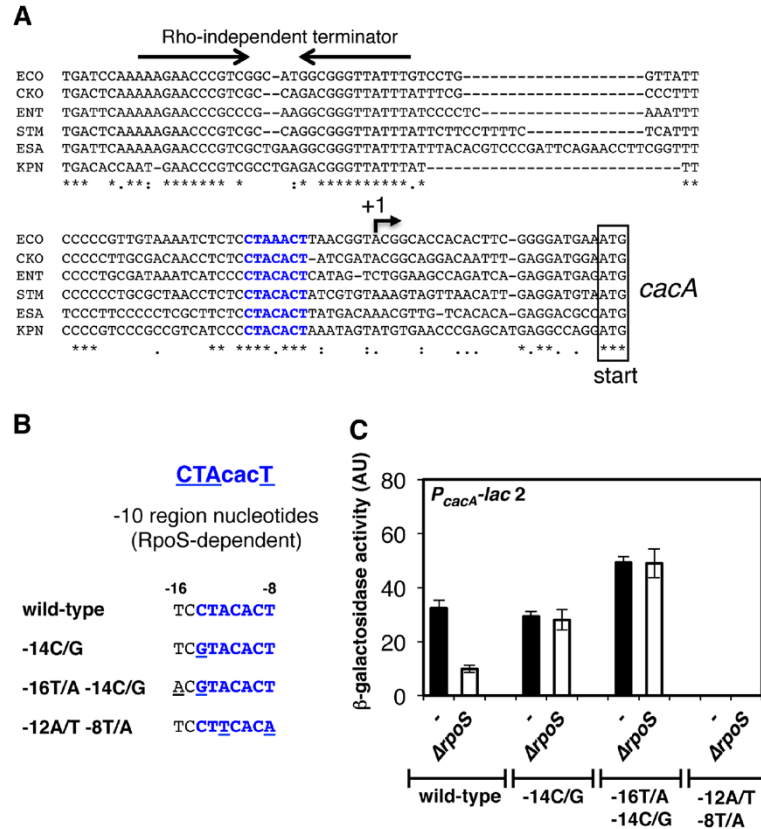


Figure 3 The *cacA* promoter harbors a conserved -10 region sequence that is crucial for RpoS-dependent regulation. **A**. Alignment of the DNA sequences of the intergenic region between the *cacA*-coding region and its upstream ORF (STM1851) in *E. coli* (ECO), *C. koseri* (CKO), *Enterobacter* sp. 638 (ENT), *S. enterica* serovar Typhimurium LT2 (STM), *Klebsiella pneumoniae* (KPN), and *C. sakazakii* (ESA). Asterisks correspond to nucleotides that are conserved in all listed species. Twin dots and single dots indicate conservative and semiconservative substitutions, respectively. The -10 region sequence is marked in bold blue letters. The bent arrow indicates the transcription start site (TSS) of the *cacA* transcript, as determined by a recent report [30] (designated position +1). The inverted arrows indicate predicted Rho-independent terminator sequences. The initiation codons for the *cacA* gene are boxed. **B**. Designated mutations in the *cacA* promoter. The -10 region sequence (**CTAcacT** from -13 to -7) [29] represents a consensus sequence that is recognized by RpoS. The -10 region sequence of the *cacA* promoter is highlighted in blue. The numbers shown above the wild-type sequence are the positions relative to the *cacA* TSS [30]. The substituted nucleotides (-14C/G, -16T/A -14C/G, and -12A/T -8T/A) are underlined. **C**. β -galactosidase activity from a *P_{cacA-lac 2}* transcriptional fusion 2 in the wild-type (-; AK1067), Δ rpoS mutant (AK1071), -14C/G *cacA* promoter mutant (AK1068), Δ rpoS -14C/G *cacA* promoter mutant (AK1072), -16T/A -14C/G *cacA* promoter mutant (AK1069), Δ rpoS -16T/A -14C/G *cacA* promoter mutant (AK1073), -12A/T -8T/A *cacA* promoter mutant (AK1070), and Δ rpoS -12A/T -8T/A *cacA* promoter mutant (AK1074) strains. Bacteria were grown for 4 h in LB before β -galactosidase activity was measured (arbitrary units) as described [42]. The data correspond to the means of three independent experiments performed in duplicate, and the error bars represent standard deviations.

were reduced by approximately 30% and 50%, respectively, in the *cacA* deletion mutant compared with wild-type (Figure 1E).

Thioredoxin 1 is required for the CacA-mediated activation of the CpxR/CpxA system

Pull-down experiment of the Glutathione S Transferase (GST)-CacA fusion protein recovered the GroEL and thioredoxin 1 (TrxA) proteins, suggesting that they interact directly with CacA (data not shown). Because GroEL has been shown to associate with proteins that are overexpressed, we did not investigate its role further. Instead, we focused on the effect of TrxA on the CacA-

mediated activation of the CpxR/CpxA system because CacA orthologs contain four conserved cysteine residues (Figure 4A) and because TrxA catalyzes thiol disulfide redox reactions in a variety of substrate proteins [31]. We investigated TrxC, another thioredoxin, and TrxB, which participates in the regeneration of reduced TrxA and TrxC [31], as controls. Whereas mutations in *trxA*, *trxB*, and *trxC* did not affect *cpxP* transcription in strains harboring vector alone, the *trxA* mutant expressing CacA significantly decreased the levels of *cpxP* transcription compared to wild-type expressing CacA (Figure 4B). These results indicate that TrxA is required for the CacA-mediated activation of the CpxR/

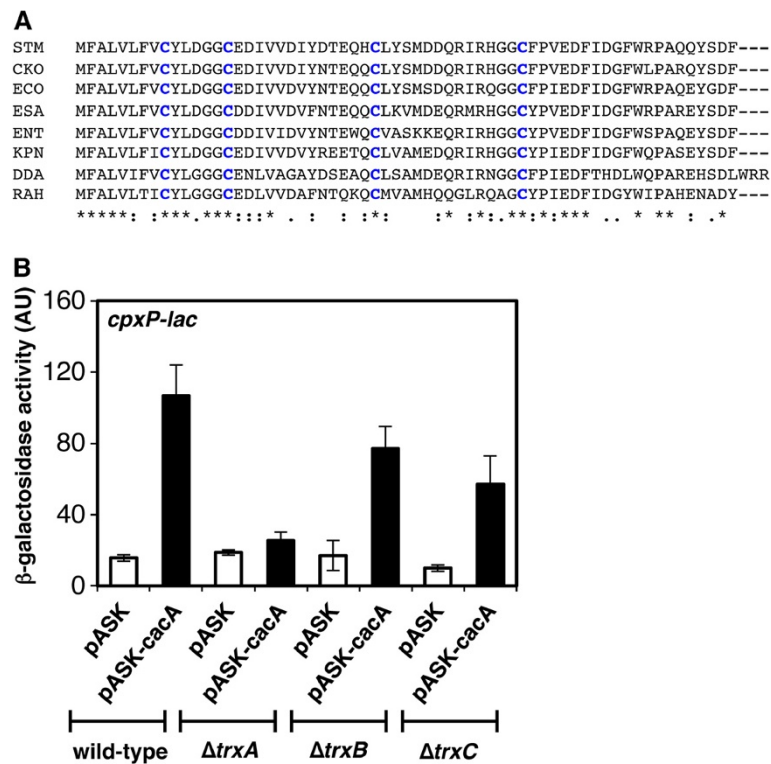


Figure 4 The CacA-dependent activation of the CpxR/CpxA requires functional thioredoxin 1. **A**. Alignment of the amino acid sequences of the CacA protein of *S. enterica* serovar Typhimurium LT2 (STM), *C. koseri* (CKO), *E. coli* (ECO), *C. sakazakii* (ESA), *Enterobacter* sp. 638 (ENT), *Klebsiella pneumoniae* (KPN), *D. dadantii* Ech703 (DDA), and *Rahnella* sp. Y9602 (RAH). Conserved cysteine residues are marked in bold blue letters. Asterisks indicate amino acids that are conserved in all listed species. Twin dots and single dots indicate conservative and semiconservative substitutions, respectively. **B**. β-galactosidase activity from a *cpxP-lac* transcriptional fusion in the wild-type (AK1052), Δ*trxA* mutant (AK1080), Δ*trxB* mutant (AK1081), and Δ*trxC* mutant (AK1082) strains harboring plasmids pASK or pASK-*cacA*. Bacteria were grown for 2 h in LB in the presence of 0.2 μg/ml ATc before β-galactosidase activity was measured (arbitrary units) as described [42]. The data correspond to the means of three independent experiments performed in duplicate, and the error bars represent standard deviations.

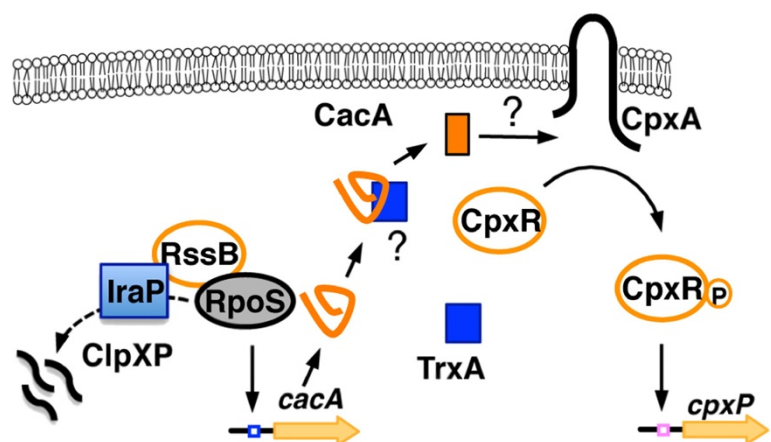


Figure 5 A model for the regulatory interactions between RssB/RpoS and the CpxR/CpxA system. RpoS accumulates during stationary phase and log phase, when the small anti-adopter protein IraP inhibits the RssB/ClpXP-mediated degradation of RpoS in low Mg²⁺ conditions [8]. RpoS induces expression of CacA, which stimulates the CpxR/CpxA system thus activating *cpxP* transcription. TrxA functionally associates with CacA-mediated Cpx induction.

CpxA system. This suggests that cysteine thiol-disulfide exchanges participate in CacA-dependent Cpx activation.

Discussion

We identified CacA, encoded on a plasmid clone, as a novel connector-like factor that activated the CpxR/CpxA system from screening a library of high-copy-number plasmids containing various *Salmonella* chromosomal DNA fragments. CacA appears to exclusively act on the CpxR/CpxA system because a similar induction was not observed in other TCS reporter strains with the same clone. This observation was not just an artifact of CacA overexpression or from its expression driven by a heterologous promoter because deleting this gene revealed a moderate decrease in transcription of the *cpxP* and *spy* genes, which are directly regulated by the CpxR/CpxA system. Moreover, the activation of the *cacA* gene promoter is, at least in part, dependent on RpoS, the stability of which is subject to RssB/ClpXP-mediated processability and the -10 region sequence. Taken together, we hypothesize that CacA may integrate information about the regulatory status of RssB/RpoS into the CpxR/CpxA system (Figure 5). However, future investigations are necessary to fully elucidate the mechanism of CacA-mediated CpxR/CpxA activation.

Several assessments of how the CacA protein activates CpxR-regulated genes were attempted. However, we did not detect a physical association between CacA and the CpxR/CpxA system. For example, no significant interaction was observed between the CacA protein and the CpxR/CpxA system in our bacterial two-hybrid system analyses (data not shown), although we cannot completely dismiss that these proteins do not interact directly. Instead, thioredoxin 1 amino acid sequences were recovered by our pull-down assay. *trxA* inactivation impacted the activation of the CpxR/CpxA system by CacA, which possesses the conserved cysteine residues. This is in contrast to a report that demonstrated that a *dsbD* mutation activated the CpxR/CpxA system in *Vibrio cholerae* [32], where the DsbC-DsbD pathway promotes proper folding of substrate proteins with disulfide bond(s) at the periplasm using the cytoplasmic reducing ability of thioredoxin [33]. Moreover, the cysteine residues of NlpE are critical for activating the CpxR/CpxA system in *E. coli* [34], and a periplasmic LolA derivative with an artificial disulfide bond activates the CpxR/CpxA system [35]. Notably, perturbing the oxidizing environment of the periplasm in *dsbA* and *dsbB* mutants or treating wild-type cells with dithiothreitol (DTT) activated the PhoP/PhoQ system in *E. coli* [36]. Disruption of disulfide bond formation affects this system largely via an additional small protein component, MgrB, and its conserved cysteine residues.

Currently, we cannot exclude the possibility that the interaction between CacA and TrxA is an artifact CacA protein overexpression because TrxA interacts with many proteins, including the RR RcsB [37]. Because we were unable to detect the 63-amino acid CacA protein at native levels, we employed a larger tag or carrier protein in several biochemical experiments, including the pull-down assay. Protein instability likely precludes thorough analysis of small proteins of less than 50 amino acids or so [38]. Notably, deletion of *trxA* did not impact *cpxP* transcription levels in normal growth conditions (e.g., LB medium). More strict conditions need to be tested, as some small proteins accumulated within bacterial cells upon exposure to sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA) [38].

The specificity that TCS connectors exhibit for their targets is likely a key contributing factor in the fidelity of the integration of TCS signals at a post-translational level. In fact, the PmrD connector protein can inhibit the dephosphorylation of phospho-PmrA but not of its closest homolog, the response regulator YgiX [6]. Although recognizing novel connectors in genomic sequences based on their uniqueness is far from trivial, genetic approaches will continue to help elucidate links amongst TCSs.

Conclusions

In this study, we identified the CacA protein as an activator of the CpxR/CpxA system. This factor may be another example of an emerging class of small proteins [39] that function as nodes in the TCS network and function to integrate their signaling pathways in *Salmonella*.

Methods

Bacterial strains, plasmids, primers, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Primers used in this study are listed in Table 2. All *S. enterica* serovar Typhimurium strains are derived from wild-type 14028s and were constructed by phage P22-mediated transduction as previously described [40]. Bacteria were grown at 37°C in N-minimal media [41] buffered with 50 mM Bis-Tris, pH 7.7, and supplemented with 0.1% casamino acids, 38 mM glycerol and 10 µM or 10 mM MgCl₂. *E. coli* DH5 α was used for preparing plasmid DNA. Ampicillin and kanamycin were used at 50 µg/ml, chloramphenicol at 20 µg/ml and tetracycline at 10 µg/ml.

β-Galactosidase assay

For data presented in Figures 1C, 1E, 2B, 3C, and 4B, a modified kinetic β-galactosidase assay was performed as previously described [42]. *Salmonella* cultures grown in LB overnight were diluted 50 times in fresh medium and

Table 1 Bacterial Strains and Plasmids Used in This Study

Strain or plasmid	Description	Reference or source
S. enterica		
14028s	Wild-type	ATCC
MS7953s	<i>phoP::Tn10</i>	[48]
AK1052	<i>cpxP-lacZ⁺Y⁺</i>	This work
AK1053	<i>spy-lacZ⁺Y⁺</i>	This work
AK1054	<i>pgtP-lacZ⁺Y⁺</i>	This work
AK1055	<i>pgtP-tetA-lacZ⁺Y⁺</i>	This work
AK1056	<i>P_{cacA}-lacZ⁺Y⁺1</i>	This work
AK1007	Δ <i>cpxR::Cm^R</i>	[16]
AK1057	Δ <i>cpxA::Cm^R</i>	This work
AK1058	Δ <i>rssB::Cm^R</i>	This work
AK1059	Δ <i>rpoS::Cm^R</i>	This work
AK1060	Δ <i>cacA::Cm^R</i>	This work
AK1061	<i>cpxP-lacZ⁺Y⁺1</i> Δ <i>cpxR::Cm^R</i>	This work
AK1062	<i>cpxP-lacZ⁺Y⁺1</i> Δ <i>cpxA::Cm^R</i>	This work
AK1063	<i>P_{cacA}-lacZ⁺Y⁺1</i> Δ <i>cpxR::Cm^R</i>	This work
AK1064	<i>P_{cacA}-lacZ⁺Y⁺1</i> <i>phoP::Tn10</i>	This work
AK1065	<i>P_{cacA}-lacZ⁺Y⁺1</i> Δ <i>rssB::Cm^R</i>	This work
AK1066	<i>P_{cacA}-lacZ⁺Y⁺1</i> Δ <i>rpoS::Cm^R</i>	This work
AK1067	<i>P_{cacA}-lacZ⁺Y⁺2</i>	This work
AK1068	<i>P_{cacA-14C/G}-lacZ⁺Y⁺2</i>	This work
AK1069	<i>P_{cacA-16T/A-14C/G}-lacZ⁺Y⁺2</i>	This work
AK1070	<i>P_{cacA-12A/T-8T/A}-lacZ⁺Y⁺2</i>	This work
AK1071	<i>P_{cacA}-lacZ⁺Y⁺2</i> Δ <i>rpoS::Cm^R</i>	This work
AK1072	<i>P_{cacA-14C/G}-lacZ⁺Y⁺2</i> Δ <i>rpoS::Cm^R</i>	This work
AK1073	<i>P_{cacA-16T/A-14C/G}-lacZ⁺Y⁺2</i> Δ <i>rpoS::Cm^R</i>	This work
AK1074	<i>P_{cacA-12A/T-8T/A}-lacZ⁺Y⁺2</i> Δ <i>rpoS::Cm^R</i>	This work
AK1075	Δ <i>cacA</i> <i>cpxP-lacZ⁺Y⁺</i>	This work
AK1076	Δ <i>cacA</i> <i>spy-lacZ⁺Y⁺</i>	This work
AK1077	Δ <i>trxA::Cm^R</i>	This work
AK1078	Δ <i>trxB::Cm^R</i>	This work
AK1079	Δ <i>trxC::Cm^R</i>	This work
AK1080	<i>cpxP-lacZ⁺Y⁺</i> Δ <i>trxA::Cm^R</i>	This work
AK1081	<i>cpxP-lacZ⁺Y⁺</i> Δ <i>trxB::Cm^R</i>	This work
AK1082	<i>cpxP-lacZ⁺Y⁺</i> Δ <i>trxC::Cm^R</i>	This work
E. coli		
DH5a	F ⁻ <i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	[49]
Plasmids		
pUC19	rep _{pMB1} Ap ^R	[50]
pUC19-R1	rep _{pMB1} Ap ^R	This work
pWN1	rep _{pMB1} Ap ^R	This work
pKD3	rep _{R6Kγ} Ap ^R FRT Cm ^R FRT	[45]

Table 1 Bacterial Strains and Plasmids Used in This Study (Continued)

pKD46	rep _{pSC101ts} Ap ^R <i>p_{araBAD} γ β exo</i>	[45]
pCP20	rep _{pSC101ts} Ap ^R Cm ^R <i>cl857λP_R flp</i>	[51]
pCE37	rep _{R6Kγ} Km ^R FRT <i>lacZY_{this}</i>	[44]
pBAD18	rep _{pMB1} Ap ^R <i>p_{araBAD}</i>	[52]
pBAD18-cacA	rep _{pMB1} Ap ^R <i>p_{araBAD} cacA</i>	This work
pASK-IBA3 plus(pASK)	rep _{pMB1} Ap ^R <i>tetR p_{tet}</i>	IBA
pASK-cacA	rep _{pMB1} Ap ^R <i>tetR p_{tet} cacA</i>	This work

grown for 2 h or 4 h at 37°C. Culture aliquot (80 μl) were added to individual wells of a clear 96-well plate containing 20 μl Reporter Lysis buffer (Promega, Madison, WI, USA). Cells were lysed by freezing at -80°C and subsequent thawing at 37°C for ~20 min. One hundred microliters of 1.32 mg/ml 2-Nitrophenyl β-D-galactopyranoside (ONPG, Sigma-Aldrich, St. Louis, MO, USA) in Z-buffer was then added to each well and mixed thoroughly. OD₄₁₅ for each well was read 20 times within 40 min at 25°C using the Model 680 microplate reader (BioRad, Hercules, CA, USA). β-galactosidase activity is reported in arbitrary units [AU] and represents the rate of ONPG conversion (i.e., Velocity, with units of mOD₄₁₅ per minute) divided by the OD₅₉₅ of the bacterial culture at the time of collection. For data presented in Figures 1A, 1D, and 2A, β-galactosidase assays were performed in triplicate, and the activity in Miller units was determined as described [43]. Data correspond to mean values of two or three independent experiments performed in duplicate.

Strain construction

Strain AK1052, which encodes a transcriptional fusion of *cpxP-lacZY* on the chromosome, was constructed as described [44]. A Cm^R cassette was amplified from pKD3 using the primers 35 and 36 and integrated immediately downstream of the stop codon of the *cpxP* gene on the 14028s chromosome by the one-step gene inactivation method [45]. The junction region of *cpxP* and the Cm^R cassette was amplified from the chromosome and confirmed by direct nucleotide sequencing. After removing the Cm^R cassette, the *lacZY* transcriptional fusion plasmid pCE37 was integrated into the FLP recombination target sequence immediately downstream of the *cpxP* gene by FLP-mediated recombination.

Strain AK1053, which encodes a transcriptional fusion of *spy-lacZY* on the chromosome, was constructed as described [44]. A Cm^R cassette was amplified from pKD3 using the primers 37 and 38 and integrated immediately downstream of the stop codon of the *spy* gene on the 14028s chromosome by the one-step gene inactivation method [45]. The junction region of *spy* and the

Table 2 Primers used in this study

Primers	Sequence (5' → 3')
Primers for strain and plasmid constructions	
35	GTTGAAATTATTGAGTAGTAGCAACTCACGTTACCAGTAACATATGAATATCCTCCTTAG
36	GACAGGGATGGTGTCTATGGAAAGGAAAACAGGGTTGTTAGTGTAGGCTGGAGCTGCTTC
37	CCCGGCCAAGAAGGTAAATGCCTGCTGCGGCAGAATAACATATGAATATCCTCCTTAG
38	TGTCGACAAGACCGCGCTCTTAAATTATGCGGAAAGTTAGTGTAGGATGGAGCTGCTTC
84	ACATAATCAGGACTCACTGCAGCTTGCAGCGCAATAACATATGAATATCCTCCTTAG
85	AATGTCGGCGTCTGTTCCTCCAGGAAGGCTAATCGTTTGTAGTGTAGGCTGGAGCTGCTTC
333	TCCTACACTATCGTGTAAAGTAGTTAACATTGAGGATGTACATATGAATATCCTCCTTAG
336	GTTAGCGCGGATACAATAGCGGTATCAGCGACCGAGGGTTAGTGTAGGCTGGAGCTGCTTC
337	GGAATTCTAACATTGAGGATGTAATGT
338	ACGCGTCGACTTAAAAATCGCTATATTGCT
367	ACCATGCCACTATTGATTAAGCCAGTCAGGGGAGAGAACGTGTAGGCTGGAGCTGCTTC
368	GGCCGGTAAAGCAATTTCCGCTCACTCTCCGTTTGGTCACATATGAATATCCTCCTTAG
393	ATTGCGTGGTCGCGGCTATCTGATGGTTCCGCTTCATGAGTGTAGGCTGGAGCTGCTTC
394	GATAAAAAATCGGCTGCATTCGACAGGCCGATGGTTTTTACATATGAATATCCTCCTTAG
451	ACATAATCAGGACTCACTGCAGCTTGCAGCGCAATAAATCTAATGCGCTGTTAATCACT
452	GTTGTAACGACGCGCCAGTGAATCCGTAATCATGGTCATCAAGCACTGTCTCCTGTT
453	ACATAATCAGGACTCACTGCAGCTTGCAGCGCAATAAATCTCAACGGGGAACATTC
454	GTTGTAACGACGCGCCAGTGAATCCGTAATCATGGTCATTACATCCTCAATGTTAACTA
473	TTGCTAGTTCGGTCAAGGGATCACGGGTAGGAGCCACCTTGTGTAGGCTGGAGCTGCTTC
474	GCCAGTCGACAGACTGGCCTTTTTTTGACAAGGGTACTTACATATGAATATCCTCCTTAG
639	GAGGAATAATAAATGTTCCGCTGGTACTTTTTG
640	TTAAAAATCGCTATATTGCTGCGCAGG
832	ACATAATCAGGACTCACTGCAGCTTGCAGCGCAATAAATCAAAAAGAACCCGTCGCC
833	GCAGGGGGAAATGAGAAAAGGAAGAATAAATAACCCGCTG
834	CTTTTCTCATTTCCCTGCGCTAACCTCTCGTACACTATCGTGTAAAGTAGTTAACATTGAGGATGTA
835	CTTTTCTCATTTCCCTGCGCTAACCTCACGTACACTATCGTGTAAAGTAGTTAACATTGAGGATGTA
836	CTTTTCTCATTTCCCTGCGCTAACCTCTCCTCACAATCGTGTAAAGTAGTTAACATTGAGGATGTA
1160	GCTACACCAACACGCCAGGCTTATTCCTGTGGAGTTATATGTGTAGGCTGGAGCTGCTTC
1161	CCATACAGCGCCTTTGTCATTGACGTATAAAAAGGTATTACATATGAATATCCTCCTTAG
1164	ACAATTCTGCTCATTGTCTGCCAACAACTATGGGGATCTCGTGTAGGCTGGAGCTGCTTC
1165	AGTCGCTTTTTTACTTTTGTACTGATTTGTA AAAACTACATATGAATATCCTCCTTAG
1166	CGCGTAGCGGGACGCTTCCGACGTATTCAGAGGTTAGCTGTGTAGGCTGGAGCTGCTTC
1167	GAGGTGAAAACGGGGCACAAGATGCGCCCGTGGCGTTTACATATGAATATCCTCCTTAG

Cm^R cassette was amplified from the chromosome and confirmed by direct nucleotide sequencing. After removing the Cm^R cassette, the *lacZY* transcriptional fusion plasmid pCE37 was integrated into the FLP recombination target sequence immediately downstream of the *spy* gene by FLP-mediated recombination.

Strain AK1054, which encodes a transcriptional fusion of *pgtP-lacZY* on the chromosome, was constructed as described [44]. A Cm^R cassette was amplified from pKD3 using the primers 84 and 85 and integrated immediately downstream of the stop codon of the *pgtP* gene

on the 14028s chromosome by the one-step gene inactivation method [45]. The junction region of *pgtP* and the Cm^R cassette was amplified from the chromosome and confirmed by direct nucleotide sequencing. After removing the Cm^R cassette, the *lacZY* transcriptional fusion plasmid pCE37 was integrated into the FLP recombination target sequence immediately downstream of the *pgtP* gene by FLP-mediated recombination.

Strain AK1055, which encodes a transcriptional fusion of *tetA-lacZY* on the chromosome, was constructed by the one-step gene inactivation method [45]. The *tetA*

gene was amplified from the MS7953s chromosomal DNA using the primers 451 and 452 and integrated between the *pgtP* gene and the *lacZ* gene in the AK1054 chromosome by the one-step gene inactivation method [45]. Strain AK1056, which harbors a fusion of the *cacA* promoter and *lacZY* genes at the *pgtP* locus, was constructed by a combination of the one-step gene inactivation method and the counterselection method for Tet^s colonies. A PCR fragment containing the *cacA* promoter was amplified from *Salmonella* chromosomal DNA using the primers 453 and 454 and recombined into the chromosome, replacing the *tetA* insertion in the strain AK1055. Strain AK1067, which harbors a fusion between the *cacA* promoter and the *lacZY* gene at the *pgtP* locus, was constructed by a combination of the one-step gene inactivation method and the counterselection method for Tet^s colonies. A PCR fragment containing the *cacA* promoter was amplified from *Salmonella* chromosomal DNA using the primers 832 and 454 and recombined into the chromosome, replacing the *tetA* insertion in the strain AK1055. Strain AK1068, which harbors *lacZY* genes under the control of a mutant *cacA* promoter with a nucleotide substitution (TCCTACACT to TCGTACACT) in the -10 region at the *pgtP* locus, was constructed by a combination of the one-step gene inactivation method and the counterselection method for Tet^s colonies. A PCR fragment containing the mutant *cacA* promoter was amplified from *Salmonella* chromosomal DNA using the primers 832, 833, 834, and 454 by the asymmetric PCR-based synthesis method [46] and recombined into the chromosome, replacing the *tetA* insertion in the strain AK1055. Strain AK1069, which harbors *lacZY* genes under the control of a mutant *cacA* promoter with two nucleotide substitutions (TCCTACACT to ACGTACACT) in the -10 region at the *pgtP* locus, was constructed by a combination of the one-step gene inactivation method and the counterselection method for Tet^s colonies. A PCR fragment containing the mutant *cacA* promoter was amplified from *Salmonella* chromosomal DNA using the primers 832, 833, 835, and 454 by the asymmetric PCR-based synthesis method [46] and recombined into the chromosome, replacing the *tetA* insertion in the strain AK1055. Strain AK1070, which harbors *lacZY* genes under the control of a mutant *cacA* promoter with two nucleotide substitutions (TCCTACACT to TCCTTCACA) in the -10 region at the *pgtP* locus, was constructed by a combination of the one-step gene inactivation method and the counterselection method for Tet^s colonies. A PCR fragment containing the mutant *cacA* promoter was amplified from *Salmonella* chromosomal DNA using the primers 832, 833, 836, and 454 by the asymmetric PCR-based synthesis method [46] and recombined into the chromosome, replacing the *tetA* insertion in the strain AK1055.

Strain AK1057, which harbors a deletion in the *cpxA* coding region, was constructed by the one-step gene inactivation method [45]. A Cm^R cassette was amplified from pKD3 using the primers 393 and 394 and recombined into the 14028s chromosome. Strain AK1058, which harbors a deletion in the *rssB* coding region, was constructed by the one-step gene inactivation method [45]. A Cm^R cassette was amplified from pKD3 using the primers 367 and 368 and recombined into the 14028s chromosome. Strain AK1059, which harbors a deletion in the *rpoS* coding region, was constructed by the one-step gene inactivation method [45]. A Cm^R cassette was amplified from pKD3 using the primers 473 and 474 and recombined into the 14028s chromosome. Strain AK1060, which harbors a deletion in the *cacA* coding region, was constructed by the one-step gene inactivation method [45]. A Cm^R cassette was amplified from pKD3 using the primers 333 and 336 and recombined into the 14028s chromosome. Strain AK1077, which harbors a deletion in the *trxA* coding region, was constructed by the one-step gene inactivation method [45]. A Cm^R cassette was amplified from pKD3 using the primers 1160 and 1161 and recombined into the 14028s chromosome. Strain AK1078, which harbors a deletion in the *trxB* coding region, was constructed by the one-step gene inactivation method [45]. A Cm^R cassette was amplified from pKD3 using the primers 1164 and 1165 and recombined into the 14028s chromosome. Strain AK1079, which harbors a deletion in the *trxC* coding region, was constructed by the one-step gene inactivation method [45]. A Cm^R cassette was amplified from pKD3 using the primers 1166 and 1167 and recombined into the 14028s chromosome.

Plasmid construction

The pBAD18-*cacA* plasmid, encoding the CaxA protein, was constructed by cloning a PCR fragment, generated using the primers 337 and 338 from a pWN1 template, between the *EcoRI* and *BamHI* sites in the pBAD18plasmid. The pASK-*cacA* plasmid, encoding the CaxA protein, was constructed by TA cloning [47] of a PCR fragment, generated using the primers 639 and 640 from a 14028s genomic DNA template, into the pASK-IBA3plus plasmid that had been digested with *SmaI* and T-tailed.

Screening for a gene that activates the CpxR/CpxA system

Chromosomal DNA prepared from an overnight culture of wild-type strain 14028s was digested with *Sau3AI* (0.01 U/μl) for 4 h. The digested DNA was separated on a 0.8% agarose gel, and 0.5–5 kb fragments were collected and ligated to pUC19 plasmid DNA that had been digested with *BamHI* and dephosphorylated by alkaline phosphatase. The ligation mixture was transformed into *E. coli* DH5α, and ampicillin-resistant transformants

were selected. Plasmid DNA was prepared from a pool of ~100,000 transformants and used to transform the strain AK1052. Transformants were serially diluted and spread onto LB plates containing ampicillin and 40 µg/ml X-gal to obtain 1,000 ~ 10,000 colonies per plate. Plasmids were isolated from colonies that developed a blue color on LB plates containing ampicillin and X-gal. These plasmids were reintroduced into AK1052 by electroporation, and four transformants were selected on LB plates containing ampicillin and X-gal. A random single white colony from the same plate was also selected as a negative control.

Abbreviations

ATc: Anhydrotetracycline; AU: Arbitrary units; CKO: *Citrobacter koseri*; DDA: *Dickeya dadantii* Ech703; DTT: Dithiothreitol; ECO: *Escherichia coli*; EDTA: Ethylenediaminetetraacetic acid; ENT: *Enterobacter* sp. 638; ESA: *Cronobacter sakazakii*; GST: Glutathione S Transferase; HK: Histidine kinase; IPTG: Isopropyl-β-D-thiogalactopyranoside; LB: Luria-Bertani; KPN: *Klebsiella pneumoniae*; ONPG: 2-Nitrophenyl β-D-galactopyranoside; ORF: Open reading frame; RAH: *Rahnella* sp. Y9602; RR: Response regulator; SDS: Sodium dodecyl sulfate; STM: *Salmonella enterica* serovar Typhimurium LT2; TCS: Two-component system; TSS: Transcription start site; X-gal: 5-Bromo-4-chloro-3-indolyl-β-D-galactoside.

Competing interest

The authors declare that they have no competing financial interests.

Authors' contributions

AK designed the experiments. AK, HH, WN, HE, KH performed the experiments. AK wrote the manuscript. RU edited the manuscript. All authors read and approved the final manuscript.

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