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Cross-talk suppression between the CpxA–CpxR and EnvZ–OmpR two-component systems in *E. coli*

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Summary

Many bacteria possess large numbers of two-component signalling systems, which are composed of histidine kinase-response regulator pairs. The high level of sequence similarity between some systems raises the possibility of undesired cross-talk between a histidine kinase and a non-cognate response regulator. Although molecular specificity ensures that phospho-transfer occurs primarily between correct partners, even a low level of inappropriate cross-talk could lead to unacceptable levels of noise or interference in signal transduction. To explore mechanisms that provide insulation against such interference, we have examined cross-talk between the histidine kinase CpxA and non-cognate response regulator OmpR in *Escherichia coli*. Our results show that there are two mechanisms that suppress cross-talk between these two proteins, which depend on the corresponding cognate partners CpxR and EnvZ and on the bifunctional nature of the histidine kinases CpxA and EnvZ. When cross-talk is detectable, we find it is independent of CpxA stimulus. We also show that cross-talk suppression leads to mutational robustness, i.e. it masks the effects of mutations that would otherwise lead to increased cross-talk. The mechanisms that provide insulation against interference described here may be applicable to many other two-component systems.

Introduction

Two-component signalling is one of the major modes of signal transduction that bacteria use to sense and respond to the environment. The two components that characterize these systems consist of a histidine kinase and a response regulator, which are usually associated with input (signal) detection and output control respectively. Signal transduction sets the level of response regulator phosphorylation through histidine kinase autophosphorylation, phospho-transfer to the cognate response regulator, and response regulator dephosphorylation by the histidine kinase (see, e.g. Stock *et al*., 1989; 2000; Hoch and Silhavy, 1995). Homology searches of sequenced genomes suggest that many bacteria possess considerable numbers of two-component systems (Koretke *et al*., 2000; Alm *et al*., 2006; Mascher *et al*., 2006; Galperin and Nikolskaya, 2007). On average, the number per species grows rapidly with genome size, and is over 100 in several bacteria (Ulrich *et al*., 2005; Alm *et al*., 2006; Galperin and Nikolskaya, 2007). The presence of large numbers of homologous signalling systems raises the possibility of cross-talk between otherwise distinct two-component systems through phosphotransfer from a histidine kinase to a non-cognate response regulator. Indeed, many histidine kinases can phosphorylate non-cognate response regulators *in vitro*, although the phosphorylation rates are generally much slower than the corresponding rate for phosphorylating the cognate response regulator (Ninfa *et al*., 1988; Igo *et al*., 1989; Fisher *et al*., 1996; Grimshaw *et al*., 1998; Skerker *et al*., 2005; Skerker *et al*., 2008) (reviewed in Laub and Goulian, 2007). The possibility that such cross-phosphorylation or cross-talk might provide a mechanism for complex processing of multiple signals has been recognized for some time (Stock *et al*., 1989; Wanner, 1992; Hellingwerf *et al*., 1995; Hellingwerf, 2005). In a few cases, there is evidence supporting the existence of cross-talk *in vivo*, which may play an important regulatory role. However, on the whole, there are relatively few reports of cross-talk between two-component systems, despite the large numbers of studies on two-component signalling in diverse bacteria and despite specific attempts to look for cross-talk (reviewed in Laub and Goulian, 2007).

This high level of specificity in two-component signalling, which favours cognate partners, is almost certainly due to the molecular specificity associated with interacting domains in the histidine kinase and cognate response regulator (Laub and Goulian, 2007; Skerker *et al*., 2008). However, at least for pairs of two-component systems that share extensive homology in these domains, there may be sufficient overlap to allow some level of interaction and cross-phosphorylation. While relatively weak cross-talk may be unlikely to play an important regulatory role, it is

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potentially a source of background noise, which could interfere with proper signal transduction. In this case, one might expect such two-component systems to possess design features that provide additional protection or insulation against inappropriate cross-phosphorylation to ensure faithful information transfer.

To study the mechanisms that provide insulation between two-component systems, we examined cross-talk between the EnvZ–OmpR and CpxA–CpxR systems in *Escherichia coli*. We chose this pair because they share considerable sequence similarity and both are reasonably well-characterized. In addition, a cytoplasmic fragment of CpxA has previously been shown to cross-talk to OmpR *in vitro* (Skerker *et al*., 2005). The histidine kinase CpxA can be stimulated by conditions associated with cell-envelope stress, e.g. by some misfolded periplasmic proteins or from certain chemical and physical perturbations of the environment. The response regulator CpxR regulates a variety of genes, some of which are secreted proteins that help to fold or degrade misfolded proteins in the periplasm (Raivio, 2005; Ruiz and Silhavy, 2005; Dorel *et al*., 2006). The biological significance of the EnvZ–OmpR system is less well-understood. The histidine kinase EnvZ is often described as an osmosensor, although the connection between osmolarity and EnvZ stimulation is not understood. In *E. coli*, the response regulator OmpR controls transcription of a diverse set of genes, including the genes for the outer membrane porins OmpC and OmpF (Forst and Roberts, 1994; Hoch and Silhavy, 1995; Slauch and Silhavy, 1996; Egger *et al*., 1997).

We examined the ability of CpxA to cross-talk to OmpR in various mutants by inferring relative levels of OmpR phosphorylation from porin transcription and by imaging OmpR–YFP colocalization with porin promoters in live cells. Our results suggest that there are two mechanisms that provide protection or insulation against OmpR phosphorylation by CpxA, which are mediated by EnvZ and CpxR, the cognate partners of the cross-talking pair. We also show that the insulation leads to mutational buffering, i.e. it strongly masks the effects of mutations in *cpxA* that would otherwise lead to increased cross-talk from CpxA to OmpR.

Results

Cross-talk from CpxA to OmpR in the absence of EnvZ and CpxR

To explore cross-talk from CpxA to OmpR, we used strains that contained chromosomal operon fusions of *yfp* to *ompF* and *cfp* to *ompC* (Batchelor and Goulian, 2003; Batchelor *et al*., 2004). Fluorescence of yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) were taken as measures of *ompF* and *ompC* expression respectively. Low levels of OmpR-P activate transcription of *ompF*, while high levels of OmpR-P repress *ompF* and activate *ompC* (Slauch and Silhavy, 1989; Forst *et al*., 1990; Russo and Silhavy, 1991; Lan and Igo, 1998) (Fig. 1). Thus, YFP and CFP fluorescence serve as useful measures to infer changes in OmpR phosphorylation at

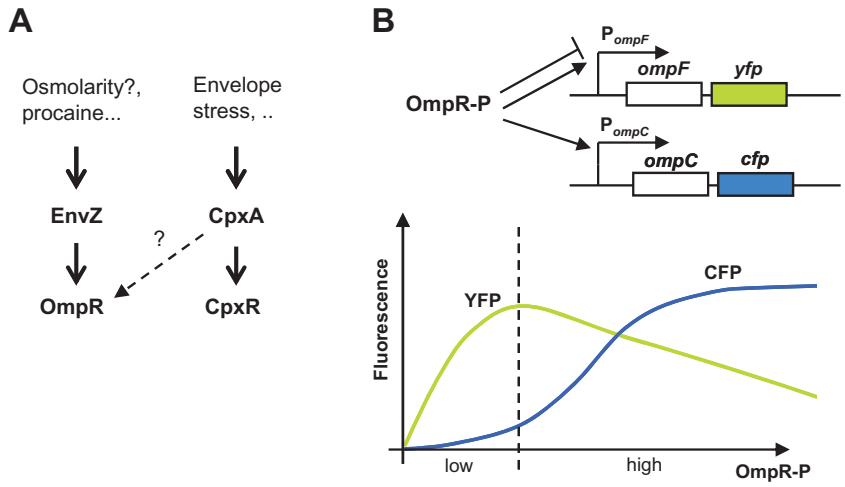


Fig. 1. Schematic of the EnvZ–OmpR and CpxA–CpxR two-component systems and porin transcriptional reporters.
A. The histidine kinases CpxA and EnvZ control the phosphorylation states of the response regulators CpxR and OmpR respectively. The dashed line denotes potential cross-phosphorylation (cross-talk) from CpxA to OmpR. CpxA can be stimulated by perturbations to the cell envelope arising from, for example, misfolding of some proteins in the periplasm. EnvZ is reputed to be an osmosensor, although the connection between EnvZ activation and extracellular osmolarity is not understood. In addition, EnvZ can be stimulated by the lipophilic compound procaine.
B. Strains containing chromosomal operon fusions of *yfp* to *ompF* and *cfp* to *ompC* provide a readout of porin transcription. A low level of OmpR-P leads to activation of *ompF* transcription while a high level leads to repression of *ompF* and activation of *ompC*.

low and high OmpR-P levels respectively. The CpxA–CpxR system also regulates *ompF* and *ompC* transcription (Batchelor *et al*., 2005), which could in principle make it difficult to interpret observations of porin transcription in terms of phosphorylation of OmpR by CpxA. However, this is only an issue for CpxR$^+$ strains and, as discussed below, we do not observe cross-talk in such strains. We further address this through *in vivo* studies of OmpR–YFP binding to the *ompF* promoter described below.

For our growth conditions, *ompF* expression is high and *ompC* expression is relatively low in wild-type strains, which is consistent with intermediate levels of OmpR-P (Slauch and Silhavy, 1989; Forst *et al*., 1990; Russo and Silhavy, 1991; Lan and Igo, 1998). In the absence of EnvZ, OmpR phosphorylation is greatly decreased, resulting in significantly lower *ompF* and *ompC* transcription (Fig. 2 and data not shown). Disrupting the *cpxRcpxA* operon in addition to *envZ* results in a further decrease in *ompF* transcription, although the effect is slight. However, when CpxA expression is restored to wild-type levels, there is a significant increase in *ompF* transcription. As OmpR expression levels are the same in the *envZ*$^-$ *cpxR*$^-$ *cpxA*$^-$ and *envZ*$^-$ *cpxR*$^-$ *cpxA*$^+$ strains (Fig. 2, lanes 3 and 4), the increase in transcription is not due to an increase in OmpR. Furthermore, the increase is absent when the conserved histidine at position 248 in CpxA is replaced with an alanine, suggesting that CpxA autophosphorylation is required (Fig. 2). We also note that transcription of *ompF* is not detectable in strains lacking OmpR (Fig. S1). Taken together, these data suggest that there is cross-talk from CpxA to OmpR when the proteins are expressed at wild-type levels in an *envZ*$^-$ *cpxR*$^-$ strain.

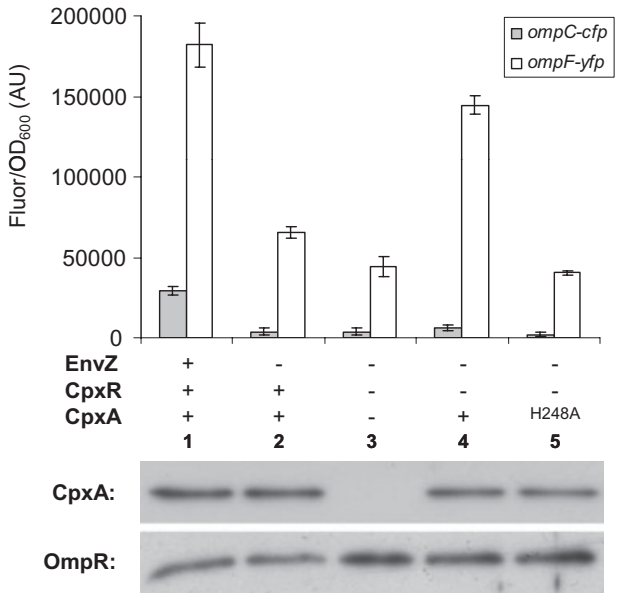


Fig. 2. CpxA increases *ompF* expression in the absence of EnvZ and CpxR. CFP and YFP fluorescence (in arbitrary units), normalized by OD_{600}, of cultures of MDG147 (wild-type) (lane 1), EPB30 (*envZ*$^-$) (lane 2), AFS198 (*envZ*$^-$ *cpxR*$^-$ *cpxA*$^-$) (lane 3), AFS197 (*envZ*$^-$ *cpxR*$^-$ *cpxA*$^+$) (lane 4) and AFS199 (*envZ*$^-$ *cpxR*$^-$ *cpxAH248A*) (lane 5), which possess *ompF–yfp* and *ompC–cfp* operon fusions. Each lane also shows Western blots with anti-CpxA and anti-OmpR antibodies for the corresponding strain. Cultures were grown in glycerol minimal medium as described in *Experimental procedures*. The normalized fluorescence values are averages of measurements from three independent cultures and error bars indicate standard deviations.

When CpxA was expressed above wild-type levels in an *envZ*$^-$ *cpxR*$^-$ strain, we observed a further decrease in *ompF* transcription (data not shown) and a corresponding increase in *ompC* transcription (Fig. 3, lanes 2–4), which are consistent with high levels of OmpR phosphorylation. Over this range of CpxA overexpression, OmpR levels remained relatively constant, showing a twofold increase at most (Fig. S2). In addition, *ompC* transcription is not detectable when CpxA is overexpressed in *ompR*$^-$ strains (Fig. S1).

EnvZ suppresses cross-talk from CpxA to OmpR

The cross-talk described above was observed in strains that lacked both EnvZ and CpxR. When we performed similar experiments in *envZ*$^+$ *cpxR*$^-$ strains, we no longer observed a CpxA-dependent increase in *ompC* expression, even when CpxA was strongly overexpressed (Fig. 3, compare lanes 2–4 and 6–8). This was not due to a decrease in OmpR levels in the *cpxR*$^-$ *cpxA*$^+$ strain; OmpR levels were comparable to or slightly higher than wild-type (Fig. S2). These results suggest that EnvZ suppresses cross-talk from CpxA to OmpR. Note that for the highest level of CpxA overexpression in an *envZ*$^-$ strain, *ompC* expression is comparable to that of the *envZ*$^+$ *cpxA*$^-$ strain (Fig. 3, lanes 4 and 5). One might therefore expect that overexpression of CpxA in an *envZ*$^+$ strain would result in even higher levels of OmpR-P and thus give higher levels of *ompC* expression. However, we instead found that this condition gave essentially the same level of *ompC* expression as that of the *envZ*$^+$ *cpxA*$^-$ strain (Fig. 3, lanes 5 and 8). This is not due to saturation of the *ompC* promoter because higher levels of expression can be reached with increased stimulation of EnvZ with procaine (data not shown) or with a constitutively active mutant of EnvZ (kinase+ phosphatase–) (Hsing *et al*., 1998) (Fig. 3, lane 12).

CpxR suppresses cross-talk from CpxA to OmpR

The previous results suggest that in *cpxR*$^-$ cells, EnvZ suppresses cross-talk from CpxA to OmpR. We similarly looked at the effect of CpxR in *envZ*$^-$ cells. Expression of *ompF* is significantly lower in *envZ*$^-$ *cpxR*$^+$ cells when compared with *envZ*$^-$ *cpxR*$^-$ cells (Fig. 2, lanes 2 and 4).

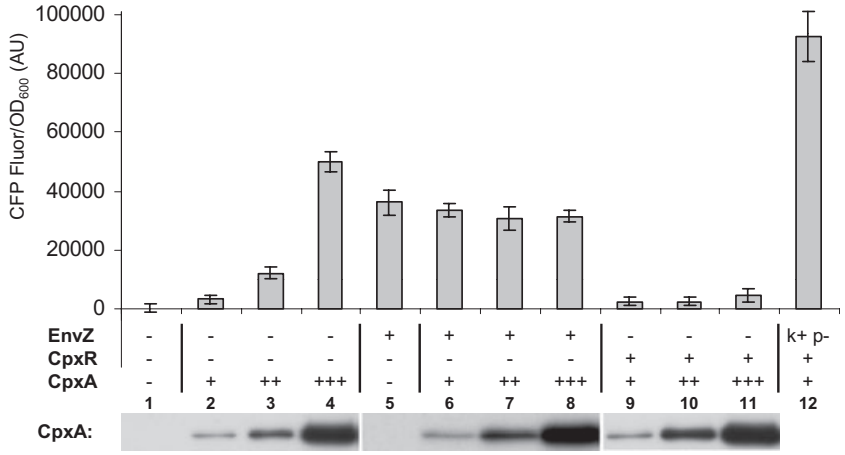


Fig. 3. CpxA overexpression increases *ompC* expression only in the absence of both EnvZ and CpxR. CFP fluorescence (in arbitrary units), normalized by OD_{600}, of cultures of *ompC–cfp* operon fusion strains AFS198 (*envZ*$^-$ *cpxR*$^-$ *cpxA*$^-$) (lane 1), AFS197 (*envZ*$^-$ *cpxR*$^-$) (lanes 2–4), AFS201 (*cpxR*$^-$ *cpxA*$^-$) (lane 5), AFS200 (*cpxR*$^-$) (lanes 6–8), AFS202 (*envZ*$^-$) (lanes 9–11) and EAL10 (*envZ964* K+ P–) (lane 12). The allele *envZ964* encodes a kinase+ phosphatase– variant of EnvZ (Hsing *et al*., 1998). For lanes 3–4, 7–8 and 10–11, CpxA expression was increased by supplementing the growth medium with 5 or 10 μM IPTG. Western blots with anti-CpxA antibodies are shown for all strains except EAL10. Cultures were grown in glycerol minimal medium as described in *Experimental procedures*. The normalized fluorescence values are averages of measurements from three independent cultures and error bars indicate standard deviations.

Furthermore, while CpxA overexpression in *envZ*$^-$ *cpxR*$^-$ strains resulted in a strong increase in *ompC* expression, the effect was abolished in an *envZ*$^-$ *cpxR*$^+$ strain for the same range of CpxA expression (Fig. 3, compare lanes 2–4 and 9–11). The fact that CpxR lowers expression of both *ompF* and *ompC* in *envZ*$^-$ cells suggests that the effect is not due to direct action of CpxR-P at the porin promoters, which causes repression of *ompF* and activation of *ompC* (Batchelor *et al*., 2005), and is instead due to suppression of cross-talk from CpxA to OmpR. Note that for our growth conditions the Cpx system is not strongly stimulated and does not have an observable effect on porin transcription when compared with a *cpxR*$^-$ *cpxA*$^-$ strain (Batchelor *et al*., 2005). We also verified that the suppression by CpxR was not due to a change in OmpR levels in the *envZ*$^-$ background; OmpR levels are comparable in the *cpxR*$^+$ and *cpxR*$^-$ strains for the same range of CpxA expression (Fig. 2 and Fig. S2).

Based on homology with OmpR, the aspartate at position 51 in CpxR has been identified as the probable site of phosphorylation (DiGiuseppe and Silhavy, 2003). CpxRD51A, which contains an aspartate-to-alanine substitution at this position, shows a CpxR-null phenotype in measurements of transcription of CpxR-regulated genes (DiGiuseppe and Silhavy, 2003). When we tested *envZ*$^-$ strains containing a *cpxRD51A* allele, we observed CpxA-dependent cross-talk that was similar to that of an *envZ*$^-$ *cpxR*$^-$ strain (data not shown). This suggests that CpxR must be phosphorylatable to suppress cross-talk from CpxA to OmpR.

CpxA cross-talk increases OmpR–YFP binding to DNA in vivo

Porins are controlled by a complex regulatory network, with multiple proteins and small RNAs regulating their expression (Pratt *et al*., 1996; Liu and Ferenci, 2001; Chen *et al*., 2004; Batchelor *et al*., 2005; Guillier *et al*., 2006; Johansen *et al*., 2006; Papenfort *et al*., 2006; Rhodius *et al*., 2006; Valentin-Hansen *et al*., 2007). The EnvZ–OmpR system is an essential part of this network as *ompF* and *ompC* are not transcribed in the absence of OmpR phosphorylation. Nevertheless, it is possible that the changes in *ompF* and *ompC* expression described above are not due to an increase in OmpR phosphorylation by CpxA and are instead due to the interaction of CpxA with other cellular components that affect porin expression. Therefore, to provide more direct evidence of cross-talk from CpxA to OmpR, we used a previously developed method for imaging the binding of an OmpR–YFP translational fusion to DNA in live cells (Batchelor and Goulian, 2006). This method is based on the clustering *in vivo* of a multicopy plasmid containing the *ompF* promoter, pP_{ompF}, which contains multiple OmpR binding sites. OmpR–YFP binding to the plasmid DNA results in distinct spots of fluorescence, which colocalize with plasmid clusters. Changes in OmpR binding can be quantified by measuring the changes in fluorescence intensity of these spots (Batchelor and Goulian, 2006).

If CpxA phosphorylates OmpR in *envZ*$^-$ *cpxR*$^-$ strains, then we should be able to detect an increase in binding of OmpR–YFP to porin promoters. We therefore looked

at the effect of CpxA on OmpR–YFP localization in an *envZ*$^-$ *cpxR*$^-$ strain containing the plasmid pP_{ompF}. Whereas *envZ*$^-$ *cpxR*$^-$ *cpxA*$^-$ cells showed a uniform distribution of OmpR–YFP fluorescence, *envZ*$^-$ *cpxR*$^-$ *cpxA*$^+$ cells showed a strong increase in OmpR–YFP localization (Fig. 4B, lanes 2 and 3). This OmpR–YFP localization was not observed in cells expressing the (non-phosphorylatable) mutant CpxAH248A. In addition, *cpxA*$^-$ and *cpxA*$^+$ cells that contained pNull, a plasmid that lacks the *ompF* promoter (and associated OmpR binding sites) but is otherwise identical to pP_{ompF}, showed similar uniform fluorescence. Overexpression of CpxA in *envZ*$^-$ *cpxR*$^-$ cells harbouring pP_{ompF} resulted in a marked increase in fluorescence localization (Fig. 4B). OmpR–YFP localization also increased slightly when CpxA was overexpressed in cells containing pNull, although the effect was always much less than that observed for cells containing pP_{ompF}. Under conditions of high OmpR phosphorylation, OmpR–YFP exhibits weak fluorescent foci even in the absence of a plasmid (Batchelor and Goulian, 2006; E. Libby and M. Goulian, unpubl. obs.). Although we do not understand the origin of this localization, it is likely that this effect accounts for the slight increase in fluorescence localization observed for pNull-containing cells at high levels of CpxA. Taken together, the above results indicate that, in the absence of EnvZ and CpxR, CpxA increases binding of OmpR to the *ompF* promoter, which presumably reflects cross-phosphorylation of OmpR by CpxA.

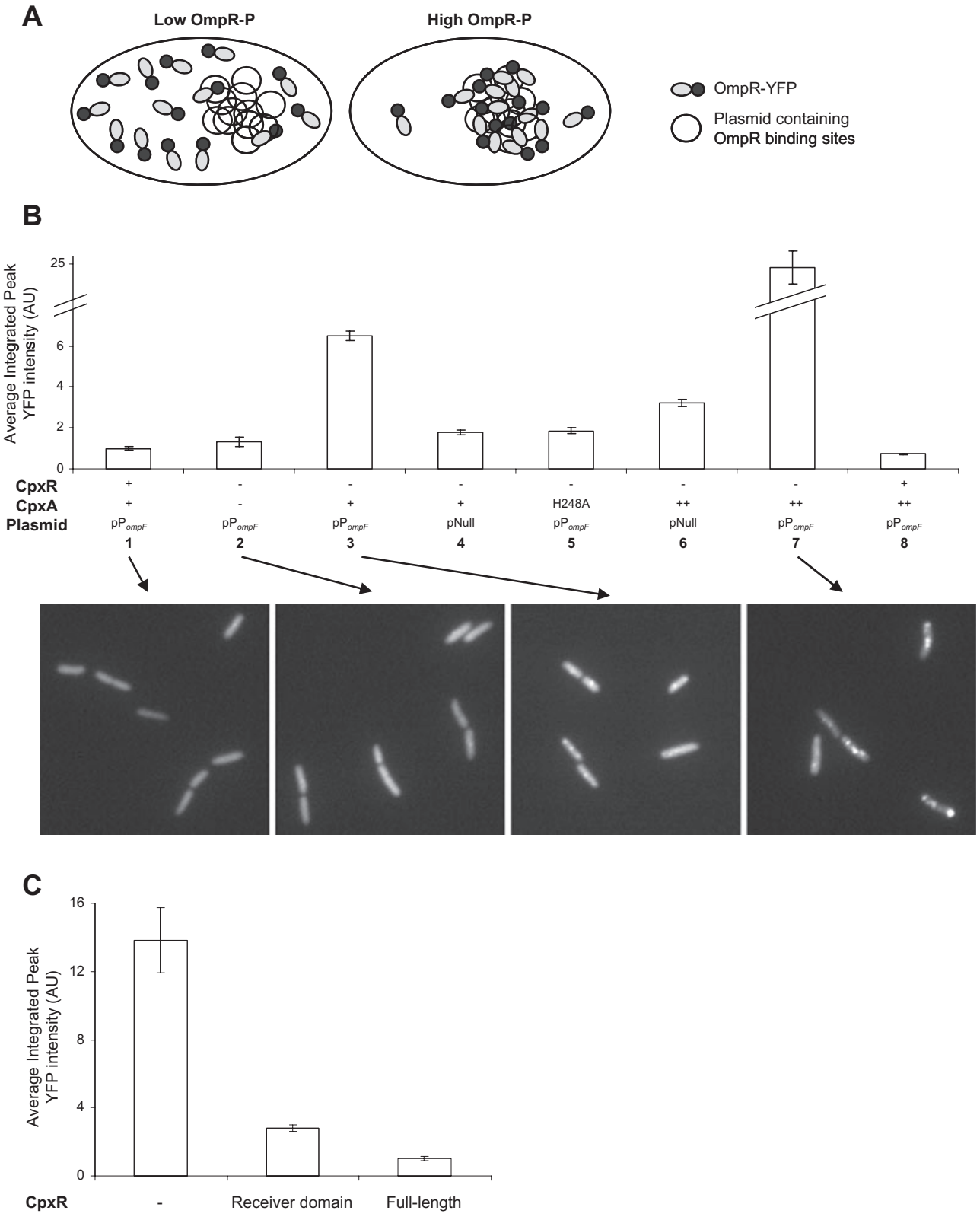
As discussed above, measurements of porin transcription suggest that cross-talk from CpxA to OmpR is suppressed by CpxR. Consistent with this interpretation, OmpR–YFP fluorescence in cells containing the plasmid pP_{ompF} was uniform in *envZ*$^-$ *cpxR*$^+$ cells, in contrast with the fluorescent foci observable in *envZ*$^-$ *cpxR*$^-$ cells (Fig. 4B, lanes 1 and 3). Strikingly, even strong overexpression of CpxA, a condition that gave very bright OmpR–YFP fluorescent foci in an *envZ*$^-$ *cpxR*$^-$ strain, did not show any detectable foci in the *envZ*$^-$ *cpxR*$^+$ strain (Fig. 4B, lanes 7 and 8).

In principle, CpxR could suppress cross-talk by preventing phosphorylated OmpR from binding DNA. However, CpxR has no effect on OmpR–YFP foci in *envZ*$^+$ cells containing pP_{ompF} (data not shown). In addition, intense OmpR–YFP localization is observed in *envZ*$^-$ *cpxR*$^+$ strains grown on pyruvate as the carbon source (data not shown). Growth on pyruvate leads to high levels of acetyl phosphate, which can phosphorylate OmpR (Wanner and Wilmes-Riesenberg, 1992; McCleary and Stock, 1994; Hsing and Silhavy, 1997; Matsubara and Mizuno, 1999; Wolfe, 2005). We also tested a CpxR variant consisting only of the receiver domain (i.e. lacking the DNA-binding domain). Expression of the CpxR receiver domain did not complement a *cpxR* deletion in measurements of transcription of the CpxR-regulated gene *cpxP* (data not shown), as expected. However, when we tested the effect of this receiver domain on OmpR–YFP fluorescent foci, cells showed a strong decrease in foci intensity, which was comparable to the decrease in cells expressing full-length CpxR (Fig. 4C). These observations make it unlikely that CpxR interferes with binding of phosphorylated OmpR and suggest that CpxR suppresses cross-phosphorylation of OmpR by CpxA.

Stimulation of CpxA does not increase cross-talk

For the growth conditions used in our experiments, transcription of the CpxR-regulated gene *cpxP* is relatively low, suggesting that the Cpx system is not strongly activated. Despite this, we were able to observe cross-talk from CpxA to OmpR in an *envZ*$^-$ *cpxR*$^-$ strain. To test whether activation of CpxA increases cross-talk in an *envZ*$^-$ *cpxR*$^-$ strain, we overexpressed the periplasmic protein NlpE, which is a convenient method for stimulating CpxA (Snyder *et al*., 1995). Overexpression of NlpE dramatically increases *cpxP* transcription in wild-type cells and in the *cpxA* expression construct used in our experiments (Fig. 5A and Fig. S3). Surprisingly, we found that NlpE overexpression did not increase transcription

Fig. 4. In the absence of EnvZ and CpxR, CpxA increases OmpR–YFP association with the *ompF* promoter.
A. Plasmid clustering is used to follow OmpR–YFP binding to DNA. Clustering of plasmids containing the *ompF* promoter produces a high local concentration of OmpR-binding sites. Increased OmpR–YFP phosphorylation results in increased colocalization of OmpR–YFP with these clusters, which is readily detectable in fluorescence images (Batchelor and Goulian, 2006).
B. Average integrated peak YFP intensities and representative YFP fluorescence images of strains expressing OmpR–YFP protein fusions and containing either the plasmid pP_{ompF}, which has the *ompF* promoter and associated OmpR binding sites, or the control plasmid pNull. The strains are AFS176/pP_{ompF} (*envZ*$^-$) (lane 1), AFS218/pP_{ompF} (*envZ*$^-$ *cpxR*$^-$ *cpxA*$^-$) (lane 2), AFS196A/pP_{ompF} (*envZ*$^-$ *cpxR*$^-$ *cpxA*$^+$) (lane 3), AFS196A/pNull (*envZ*$^-$ *cpxR*$^-$ *cpxA*$^+$) (lane 4), AFS219/pP_{ompF} (*envZ*$^-$ *cpxR*$^-$ *cpxAH248A*) (lane 5), AFS196A/pNull (*envZ*$^-$ *cpxR*$^-$ *cpxA*$^+$) (lane 6), AFS196A/pP_{ompF} (*envZ*$^-$ *cpxR*$^-$ *cpxA*$^+$) (lane 7) and AFS221/pP_{ompF} (*envZ*$^-$ *cpxR*$^+$) (lane 8). For lanes 6–8, the CpxA expression was increased by supplementing the media with 10 μM IPTG. Representative YFP fluorescence images of cells are shown for the cultures in lanes 1,2,3 and 7.
C. Average integrated peak YFP intensity for AFS196A containing pP_{ompF} and a compatible control plasmid (pWKS130), plasmid expressing the CpxR receiver domain (pAS81-1), or a plasmid expressing full-length CpxR (pAS81-5). Cultures were grown in glycerol minimal medium as described in *Experimental procedures*. For each measurement, the average integrated peak YFP intensity was computed (see *Experimental procedures* for details) for approximately 100 cells. Bars represent the averages of measurements from three independent cultures and error bars indicate standard deviations.



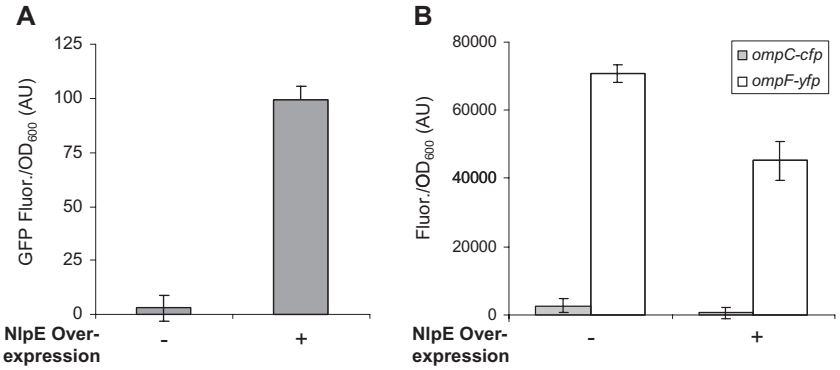


Fig. 5. Stimulation of CpxA does not increase cross-talk.
A. GFP fluorescence (in arbitrary units) normalized by OD_{600} of cultures of the *cpxP′-gfp* reporter strain AFS272 containing either the control plasmid pBAD-HisA or the NlpE overexpression plasmid pND18. The *cpxP* promoter is regulated by CpxR.
B. CFP and YFP fluorescence (in arbitrary units) normalized by OD_{600} of cultures of AFS197 (*envZ*$^-$ *cpxR*$^-$) containing either pBAD-HisA or pND18. AFS197 contains *ompC–cfp* and *ompF–yfp* operon fusions. Cultures were grown to an OD_{600} of 0.1–0.3 in glycerol minimal medium containing ampicillin, diluted 1:10 into the same medium supplemented with 10 mM arabinose and grown back to an OD_{600} of ~0.1–0.3. Bars represent the average of measurements from three independent cultures and error bars indicate standard deviations.

of *ompF* or *ompC* (Fig. 5B). We also found that a constitutively active variant of CpxA that is encoded by the *cpxA24* allele (DiGiuseppe and Silhavy, 2003) showed similar levels of *ompF* and *ompC* transcription as wild-type *cpxA* in *envZ*$^-$ *cpxR*$^-$ cells (data not shown).

CpxR masks the effects of cpxA *mutations on cross-talk to OmpR*

To screen for CpxA mutants that show increased cross-talk to OmpR, we transformed cells containing an *ompC–cfp* operon fusion with a plasmid library expressing *cpxA* in which the 3′ end had been randomly mutated by error-prone pcr. Expression of *ompC* was assessed from images of CFP fluorescence of colonies on agar plates and of individual cells. In the *envZ*$^-$ *cpxR*$^-$ strain, *ompC* expression varied significantly, with many mutants showing considerable increases in CFP fluorescence (Fig. 6). This suggests CpxA mutants that show increased cross-talk from CpxA to OmpR can be readily isolated in *envZ*$^-$ *cpxR*$^-$ cells. In contrast, *envZ*$^-$ *cpxR*$^+$ cells transformed with the same plasmid library showed no significant increases in *ompC* expression (Fig. 6). Thus, these results suggest that CpxR suppresses or masks the effects of CpxA mutants that have increased cross-talk to OmpR.

Cross-talk from EnvZ to CpxR

We tested whether cross-talk could be observed in the converse direction, from EnvZ to CpxR, by monitoring

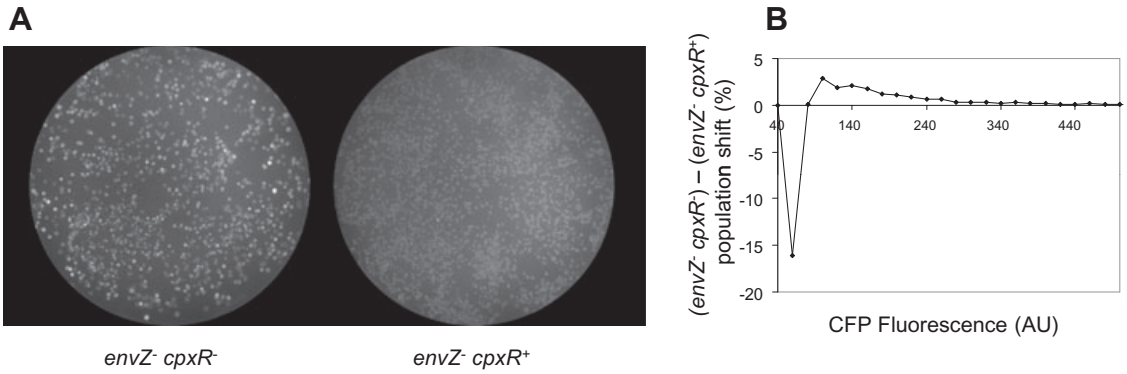


Fig. 6. CpxR masks the effects of mutations in *cpxA*.
A. CFP fluorescence of colonies grown on glycerol minimal medium agar plates. The strains are AFS71 (*envZ*$^-$ *cpxR*$^-$ *cpxA*$^-$) (left image), and AFS70 (*envZ*$^-$ *cpxR*$^+$ *cpxA*$^-$) (right image), expressing a library of CpxA mutants generated by mutating the 3′ end of *cpxA*. Both AFS71 and AFS70 contain *ompC–cfp* operon fusions.
B. The shift in CFP fluorescence distribution measured from single cells re-suspended from plates. The population shift was computed by subtracting the normalized distribution of cellular fluorescence of *envZ*$^-$ *cpxR*$^+$ cells from the corresponding distribution of *envZ*$^-$ *cpxR*$^-$ cells.

transcription of the CpxR-regulated gene, *cpxP* (Fig. S4 and data not shown). We found that *cpxP* transcription increased in the *cpxA*$^-$ *ompR*$^-$ strain but was suppressed by the presence of either CpxA or OmpR, which is similar to our results for CpxA-to-OmpR cross-talk. In addition, just as we found that CpxA stimulation did not affect cross-talk to OmpR, we also found that procaine, a lipophilic compound that activates EnvZ (Taylor *et al*., 1983; Villarejo and Case, 1984; Rampersaud and Inouye, 1991; Batchelor and Goulian, 2006), had no effect on *cpxP* transcription in *cpxA*$^-$ *ompR*$^-$ cells (Fig. S5).

Discussion

The above results suggest there are two mechanisms that protect against cross-talk from CpxA to OmpR. Both mechanisms depend on the cognate partners of the cross-talking pair, CpxR and EnvZ.

Cross-talk to a response regulator in the absence of its histidine kinase has been reported previously (Wanner *et al*., 1988; Stock *et al*., 1989; Amemura *et al*., 1990; Silva *et al*., 1998; Verhamme *et al*., 2002; reviewed in Laub and Goulian, 2007). Many histidine kinases not only phosphorylate but also dephosphorylate their cognate response regulators. The dephosphorylation activity may result from a phosphatase catalytic activity of the histidine kinase or may be due to the ability of the histidine kinase to stimulate an auto-phosphatase activity intrinsic to the response regulator (Stock *et al*., 2000). For simplicity, we will simply use the term phosphatase activity to refer to this histidine kinase-dependent dephosphorylation. It has been suggested that in the absence of stimulus, the phosphatase activity is important for maintaining a two-component system in an off state by preventing inappropriate phosphorylation of the response regulator by other phosphodonors (Stock *et al*., 1989; Wanner, 1992; Alves and Savageau, 2003; Laub and Goulian, 2007). In light of the results described here, it seems likely that this is the case for EnvZ. However, the EnvZ–OmpR system does not simply switch between off and on states. Instead, transcription is regulated in a continuous or graded fashion, which presumably reflects a continuous or graded variation in OmpR-P levels (Batchelor *et al*., 2004). It is possible that under conditions in which EnvZ produces intermediate levels of OmpR-P, another phosphodonor could have a significant effect on OmpR phosphorylation. This is not what we observed for the action of CpxA on OmpR, however. Based on measurements of *ompC* expression (as judged by CFP fluorescence), we did not see any evidence of an increase in OmpR-P levels due to the combined action of EnvZ and CpxA when compared with the levels in an *envZ*$^+$ *cpxA*$^-$ strain (Fig. 3, lanes 5, 4 and 8). In addition, experiments comparing wild-type and *ackA*$^-$ *pta*$^-$ strains suggest that EnvZ has a similar ability to mask the effects of OmpR phosphorylation by the phosphodonor acetyl phosphate (Fig. S6).

A simple model can account for these observations (summarized in Fig. 7A): For conditions in which EnvZ activity leads to intermediate levels of OmpR-P, the model assumes the rates of OmpR phosphorylation and OmpR-P dephosphorylation by EnvZ are both high. At steady state, the balance between the reactions leads to intermediate levels of OmpR-P (Fig. 7A, i). In the absence of EnvZ, if OmpR is phosphorylated by other phospho donors such as CpxA, there is presumably a relatively low rate of OmpR-P dephosphorylation. A low rate of OmpR phosphorylation by CpxA, balanced by this low rate of OmpR-P dephosphorylation, can lead to intermediate levels of OmpR-P at steady state (Fig. 7A, ii). (We have not observed any evidence of CpxA phosphatase activity towards OmpR-P – this point is discussed further below.) When both CpxA and EnvZ are present (but CpxR is still absent), the low OmpR phosphorylation rate by CpxA is negligible compared with that of EnvZ (Fig. 7A, iii). For this reason, cross-talk from CpxA is unobservable when EnvZ is present. Note that this is consistent with *in vitro* studies showing that a cytoplasmic fragment of CpxA phosphorylates OmpR with much slower kinetics compared with a similar fragment of EnvZ (Skerker *et al*., 2005). The same argument should apply for cross-talk from other phosphodonors provided they have relatively low rates of OmpR phosphorylation. Thus, in this model the balance between strong phosphorylation and dephosphorylation activities of EnvZ provides insulation from inappropriate cross-talk, even under conditions that lead to intermediate levels of OmpR-P at steady state.

We find that cross-talk from CpxA to OmpR is blind to the level of CpxA stimulation. Indeed, we observed cross-talk to OmpR without taking steps to stimulate CpxA. In addition, stimulation of CpxA by NlpE overexpression had no effect on cross-talk to OmpR. It is possible that in the absence of CpxR, CpxA is not stimulated by NlpE overexpression. However, we observed similar results for cross-talk from EnvZ to CpxR when we examined the effect of EnvZ stimulation with procaine. These observations may be related to the fact that we do not see any evidence of cross-talk in the phosphatase activity of CpxA or EnvZ (data not shown). In particular, they may indicate that the input stimulus affects the phosphatase activity but not the kinase activity for CpxA and EnvZ. An alternative explanation is that phosphotransfer from CpxA-P to OmpR is rate-limiting and that virtually all of the CpxA in the cell is phosphorylated under steady-state conditions. In this case one would expect cross-talk to be blind to stimulus irrespective of whether stimulus increases the rate of CpxA autophosphorylation or decreases the CpxA phosphatase activity. (This latter point assumes only the

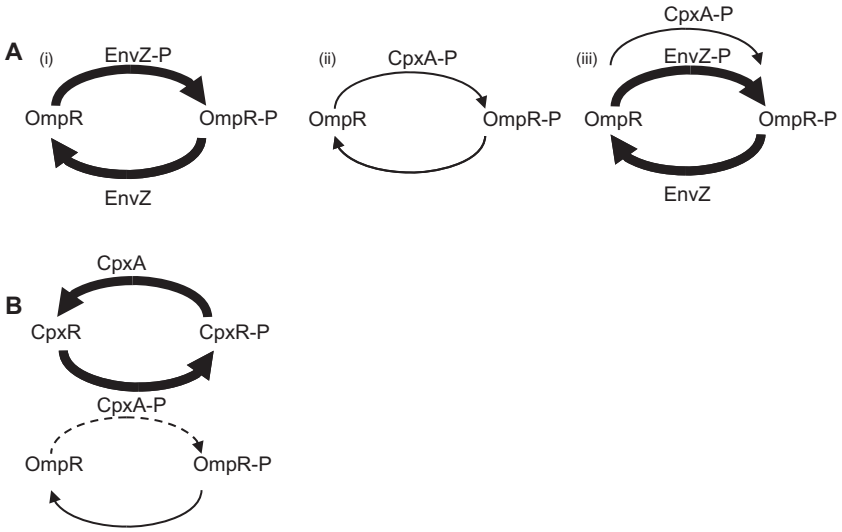


Fig. 7. Proposed mechanisms for cross-talk suppression by EnvZ and CpxR.
A. Suppression by EnvZ. When EnvZ is present and CpxA is absent, a balance between strong EnvZ kinase and phosphatase activities sets the level of OmpR-P (i). In the absence of EnvZ and CpxR, CpxA phosphorylates OmpR with slow kinetics and OmpR-P dephosphorylates (e.g. by hydrolysis) with slow kinetics. At steady state, the balance between slow phosphorylation and dephosphorylation leads to significant levels of OmpR-P (ii). When EnvZ and CpxA are both present, the weak phosphorylation of OmpR by CpxA is negligible compared with the strong phosphorylation by EnvZ (iii).
B. Suppression by CpxR. OmpR and CpxR compete for phosphorylation by CpxA. Even when the steady-state levels of CpxR-P are low, there is a high rate of CpxR phosphorylation by CpxA, which is balanced by a high rate of CpxR-P dephosphorylation by CpxA. This enables CpxR to effectively outcompete OmpR for phosphorylation by CpxA.

unphosphorylated form of CpxA can function as a phosphatase). Note that this explanation would also account for the lack of any observable phosphatase cross-talk.

We did not detect cross-talk from CpxA to OmpR in *envZ*$^-$ strains unless the cells also lacked CpxR. We observed similar results for cross-talk from EnvZ to CpxR. Similar behaviour has also been reported previously for cross-talk between PhoR/PhoB and the ectopically expressed VanS/VanR system in *E. coli* (Silva *et al*., 1998). Our results suggest a model in which CpxR out-competes OmpR for interaction with CpxA (Fig. 7B). If the phosphorylation of CpxR and OmpR by CpxA can be described by standard competitive inhibition, then the ratio of the phosphorylation rates for the two response regulators is the ratio of catalytic efficiencies for the two reactions, which is independent of the total CpxA concentration: $V_{OmpR}/V_{CpxR} = (kcat_{OmpR}/KM_{OmpR})/(kcat_{CpxR}/KM_{CpxR})$. The steady-state concentration of phosphorylated response regulator is determined by the balance of the phosphorylation and dephosphorylation rates. Therefore, the corresponding ratio of the concentrations of phosphorylated response regulators at steady state, [OmpR-P]/[CpxR-P], depends on the details of the dephosphorylation mechanism. If the dephosphorylation reactions were independent of CpxA and followed first order kinetics with rate constants $k_{CpxR\text{-}P}$ and $k_{OmpR\text{-}P}$, then $[OmpR\text{-}P]/[CpxR\text{-}P] = (k_{CpxR\text{-}P}/k_{OmpR\text{-}P}) \times (V_{OmpR}/V_{CpxR})$, which is again independent of CpxA concentration. However, the situation is different when the histidine kinase plays a role in response regulator dephosphorylation. If CpxA mediates dephosphorylation of CpxR-P but not of OmpR-P, then a simple model of the cycle of phosphorylation and dephosphorylation predicts that [CpxR-P] is insensitive to CpxA concentration (Batchelor and Goulian, 2003; and Fig. S7). This implies the ratio [OmpR-P]/[CpxR-P] decreases with decreasing [CpxA]. Thus, cross-talk is kept to a minimum for low histidine kinase concentrations. Interestingly, histidine kinase concentrations appear to be relatively low compared with the concentrations of the cognate response regulators in two-component systems for which the ratio has been measured (Cai and Inouye, 2002; T. Miyashiro and M. Goulian, unpubl. obs.).

Studies of several other pairs of homologous two-component systems suggest that similar insulation mechanisms depending on phosphatase activity and response regulator competition suppress cross-talk between these pairs as well. We observed cross-talk from EnvZ to CpxR (Fig. S4), PhoR to OmpR, and CusS to OmpR and CpxR (data not shown) only when the reciprocal response regulator and histidine kinase were absent. Similar results have also been observed for cross-talk from several different two-component systems to the PhoR/PhoB system (Kim *et al*., 1996; Silva *et al*., 1998; Zhou *et al*., 2005). Thus, the mechanisms described here may quite generally provide insulation between pairs of homologous two-component

systems comprised of bifunctional histidine kinases. In some cases we did not observe cross-talk even when these protective layers were removed. For example, we did not observe cross-talk from RstB to OmpR and from BaeS and PhoR to CpxR under our growth conditions. In these cases, this may indicate that the relevant domains in the histidine kinases and response regulators that make contact during phospho-transfer do not share sufficient similarity. Indeed, molecular specificity is undoubtedly the dominant mechanism for minimizing cross-talk between two-component systems (Skerker *et al*., 2008). However, the molecular determinants of this specificity are not sufficiently well-understood to rank two-component system pairs based on their potential for cross-talk. Nevertheless, for those pairs that are sufficiently similar that cross-phosphorylation can occur, the protective mechanisms described here can provide a significant level of insulation. In effect, these mechanisms amplify or enhance the effects of molecular specificity. Without them, the level of specificity is not sufficient to completely block the effects of cross-talk *in vivo* (e.g. cross-talk from CpxA to OmpR).

These mechanisms for suppressing cross-talk may also have important implications for the evolution of two-component systems. Whether new circuits with new inputs and outputs evolve through co-evolution of histidine kinase-response regulator pairs, or by establishment of new interacting partners, the evolutionary path is likely to be constrained by selection against inappropriate cross-talk with other two-component systems. Mechanisms that suppress cross-talk should partially relax some of these constraints and therefore make it possible for well-insulated two-component systems to emerge in fewer mutational steps. The same argument implies that these mechanisms should make it more difficult to isolate mutants with increased cross-talk. Consistent with this prediction, we observed that in an *envZ*$^-$ *cpxR*$^-$ background, *ompC* expression was highly susceptible to mutations in *cpxA* (Fig. 6). In contrast, the presence of CpxR masked or buffered the effects of this variation so that the same pool of mutated *cpxA* showed no significant variation in *ompC* expression.

The suppression mechanisms may also make it more difficult to evolve circuits that use cross-regulation as part of their design. However, this does not preclude the possibility of cross-talk among two-component systems. Sufficiently strong interactions between a bifunctional histidine kinase and non-cognate response regulator should result in robust cross-phosphorylation even in the presence of their cognate partners. Indeed, there are at least a few reports of true cross-regulation between two-component systems (Laub and Goulian, 2007). Nevertheless, even in such cases, the cross-talking pair presumably must be protected from cross-phosphorylation with other two-component systems. The mechanisms described here may provide part of this insulation.

Experimental procedures

Growth and media

Liquid cultures were grown at 37°C with aeration in minimal A medium (Miller, 1992) with 0.2% glycerol as the carbon source unless specified otherwise. This medium was used to minimize autofluorescence and maintain low levels of the alternative phosphodonor acetyl phosphate (Wolfe, 2005). Plasmids were maintained by growing with 50 μg ml^{-1} ampicillin. The *lac* promoter was induced using isopropyl-β-D-thiogalactoside (IPTG).

Strains and plasmids

A table of plasmids and strains used in this study are listed in Table S1. P1 transductions were performed using standard procedures (Miller, 1992).

Measurements of CFP, YFP and GFP fluorescence

Liquid cultures were grown to saturation at 37°C with aeration in minimal medium, diluted 1:1000 and grown to an optical density at 600 nm (OD_{600}) of approximately 0.1–0.3. Cultures were cooled in an ice-water slurry and supplemented with streptomycin to a final concentration of 125–250 μg ml^{-1} to inhibit protein synthesis. Samples of 2 ml of culture were warmed to room temperature and measured for fluorescence essentially as described in Batchelor *et al*. (2005) using 434/475 nm, 505/527 nm and 502/512 nm for the excitation/emission wavelengths for CFP, YFP and green fluorescent protein (GFP) measurements respectively. Fluorescence intensity was normalized by OD_{600}, and background fluorescence, measured from cultures of MG1655, was subtracted.

OmpR–YFP fluorescence localization

Cultures were grown to saturation at 37°C with aeration in glycerol minimal medium, diluted 1:1000 and grown in the same medium until they reached an OD_{600} of approximately 0.1–0.3. Cells were immobilized on an agarose gel pad placed between a microscope slide (Corning, Corning, NY) and number 1.5 cover glass (Corning), essentially as described in Batchelor *et al*. (2004), and maintained at 37°C. Agarose gel pads were made by dissolving SeaKem LE Agarose (Cambrex, Rockland, ME) to 1% in glycerol minimal medium. Fluorescence microscopy was performed using an Olympus IX81 microscope enclosed in an insulated chamber maintained at 37°C as described previously (Batchelor and Goulian, 2006). A 1.6× magnification lens was used to increase the apparent cell size within images. For experiments in Fig. 4C, an Andor iXon EM+ DU-897 EMCCD camera was used to acquire images. For cells expressing OmpR–YFP, a differential interference contrast (DIC) image was acquired, followed by a YFP image with an exposure of 750 ms. Image analysis was performed using a modified

version of our own software described previously (Batchelor and Goulian, 2006). For analysis of OmpR–YFP localization, the fluorescence intensity was measured along the largest intensity moment in a 15×15 pixel area centred about the brightest pixel within the cell boundary. The intensity along each direction originating from the brightest pixel was fit to the Gaussian profile of the form: $A \exp(-Bx^2) + C$, where x is the distance from the brightest pixel. The values for A and B, corresponding with the fit that yielded the greater value of C, were used to integrate the Gaussian to full-width half-maximum, resulting in the integrated peak YFP intensity. To account for day-to-day variations in fluorescence due to lamp intensity, alignment, etc., integrated peak intensities for each measurement were normalized by those measured for AFS176/pP_{ompF} on the same day.

Protein quantification

Liquid cultures were grown to saturation at 37°C with aeration in glycerol minimal medium, diluted 1:1000 and grown to an OD_{600} of approximately 0.1–0.3. Cultures were placed in an ice-slurry and supplemented with streptomycin to a final concentration of 125–250 μg ml^{-1} to inhibit protein synthesis. Cultures were centrifuged and pellets were re-suspended in water. Total protein content was assayed using the BCA Protein Assay (Pierce, Rockford, IL). Samples normalized for protein content were analysed on 12% SDS-PAGE gels using standard buffers and conditions (Ausubel *et al*., 1998). Anitbodies for CpxA, EnvZ and OmpR were gifts from T. J. Silhavy (Princeton University, Princeton, NJ). Anti-rabbit IgG conjugated to horseradish peroxidase ECL (GE Healthcare, Piscataway, NJ) was used for secondary antibody. Blots were visualized using substrate from the ECL Plus detection kit and Hyperfilm ECL chemiluminescence film (GE Healthcare). Digital images of the film were acquired using the Epson Expression 1680 scanner with transparency adapter. A Stouffer 21-Step Transparency guide (Stouffer, Mishawaka, IN) was scanned with the film as a standard to determine the intensity range of the scan. Band intensities were quantified using ImageJ (NIH, Bethesda, MD) by integrating the intensity of an area surrounding the band of interest and subtracting the average of the corresponding background values for areas directly above and below the band.

CpxA mutagenesis and measurements

Error-prone PCR was used to generate a library of *cpxA* mutants. The PCR reaction was performed in a total volume of 50 μl with: 3.5 mM $MgCl_2$, 0.25 mM $MnCl_2$, 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP, 2 μM of each primer 5′-GCCGATAACGCCGATCATCC-3′ and 5′-CGGGTTCTAG AAAAGCTGGACGCGG-3′, 25 ng of pAS23, and 5 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The amplified DNA product was digested with RsrII and XbaI and cloned into the same sites in pAS23. The ligation reaction was transformed into either AFS70 or AFS71 and plated on glycerol minimal medium agar plates supplemented with 50 μg ml^{-1} ampicillin. Plates were incubated at 37°C for approximately 48 h. Fluorescence images of plates were acquired using a Nikon D50 camera (Nikon, Melville, NY) connected to the fluorescence illuminator from a Zeiss microscope (Zeiss 2FL fluorescence adapter) with a 100 W mercury lamp, using D436/20 excitation, 455dclp beam splitter, and D480/40 emission filters for CFP (Chroma, Brattleboro, VT). For single cell measurements, cells were re-suspended directly from agar plates into glycerol minimal medium and immobilized on agarose gel pads as described above.

Fluorescence microscopy

Single cell fluorescence measurements were performed using an Olympus IX81 microscope essentially as described previously (Miyashiro and Goulian, 2007a). For GFP, the fluorescence filter set was HQ470/40 excitation, Q495lp beamsplitter, HQ525/50 emission (Chroma, Brattleboro, VT). A DIC image was acquired followed by a CFP or GFP image. Image analysis was performed as described in Miyashiro and Goulian (2007b) using DIC images to construct masks for cell boundaries.

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