The DNA-binding domain of the *Escherichia coli* CpxR two-component response regulator is constitutively active and cannot be fully attenuated by fused adjacent heterologous regulatory domains

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Two-component systems (TCS) based on a sensor histidine kinase and a phosphorylated cognate target regulator allow rapid responses to environmental changes. TCS are highly evolutionarily conserved, though in only a few cases are the inducing signals understood. This study focuses on the *Escherichia coli* CpxR response regulator that responds to periplasmic and outer-membrane stress. N-terminal deletion mutations have been isolated that render the transcription factor constitutively active, indicating that the N terminus functions, in part, to keep the C-terminal winged-helix DNA-binding effector domain in an inactive state. Analysis of truncations spanning the CpxR interdomain region revealed that mutants retaining the $\alpha 5$ helix significantly augment activation. Hybrid proteins obtained by fusing the CpxR effector domain to structurally similar heterologous N-terminal regulatory domains, or even GFP, failed to restore repression to the C-terminal domain. These findings shed light on the mechanism of CpxR effector domain activation and on the investigation of constitutive mutants obtained by truncation in other TCS.

INTRODUCTION

The ability to rapidly detect environmental changes and to couple appropriate responses to these captured cues provides bacteria with an undeniable selective advantage. Two-component systems (TCS) serve as a highly evolutionarily conserved stimulus–response coupling mechanism (Parkinson & Kofoid, 1992). Hundreds of such systems have been described in Eubacteria and Archaea, as well as a few eukaryotic organisms (Volz, 1995).

The simplest prototypical TCS consists of two proteins: a transmembrane kinase and a cytoplasmic response regulator. In response to an external signal, the sensor kinase autophosphorylates a cytoplasmic histidine residue and subsequently transfers the phosphate to a conserved aspartate residue on the multidomain response regulator. The response regulator is most often composed of two domains and aspartate phosphorylation within the N-terminal regulatory (or receiver) domain results in the activation of the associated C-terminal effector (or output) domain (Cho *et al.*, 2001; Goudreau & Stock, 1998; Stock *et al.*, 2000).

The N-terminal regulatory domains share a conserved CheY-like domain of approximately 125 aa. The three-dimensional

structure of this domain has been solved for several response regulators and the common feature is a doubly wound five-stranded α/β fold (Baikalov *et al.*, 1996; Madhusudan *et al.*, 1997). Amino acids that form the hydrophobic core are conserved and it is reasonable to assume that the overall domain fold is similar for most response regulators (Hoch & Varughese, 2001). Several highly conserved residues define an acidic pocket that includes the phosphorylation target aspartate side chain (Cho *et al.*, 2001; Stock *et al.*, 1995). Mutagenesis studies have demonstrated the key role of this conserved aspartate. Substitution of glutamate for aspartate mimics activation and results in constitutive forms of CheY (Bourret *et al.*, 1990), RcsB (Gupte *et al.*, 1997), OmpR (Lan & Igo, 1998), NtrC (Klose *et al.*, 1993) and yeast Skn7p (Lan & Igo, 1998). In contrast, substitution of asparagine or alanine for the conserved aspartate of RcsB, NtrC and CpxR blocks activation *in vivo* (Gupte *et al.*, 1997; Klose *et al.*, 1993; DiGiuseppe & Silhavy, 2003).

The effector domains usually bind DNA and activate or repress transcription of target genes, though there are exceptions (Galperin *et al.*, 2001; Stock *et al.*, 1995; West & Stock, 2001). Effector domains are classified into subfamilies according to their DNA-binding motif. The more common motifs include the helix–turn–helix (HTH) and the winged-helix–turn–helix families (Kenney, 2002). In addition, the LytTR DNA-binding domain is widely distributed among

Abbreviations: MBP, maltose-binding protein; TCS, two-component system(s).

TCS of Gram-positive bacteria (Nikolskaya & Galperin, 2002).

The precise mechanism of coupled communication between the regulatory domain and the effector domain is largely unknown, but may be mediated in part by a flexible interdomain region of variable length (Eldridge *et al.*, 2002; Mattison *et al.*, 2002; Walthers *et al.*, 2003). The consequence of phosphorylation for some response regulators is a relief of the inhibition imposed on the effector domain by the regulatory domain (Cho *et al.*, 2001), whereas in other response regulators phosphorylation activates the response regulator by triggering its oligomerization (Tsung *et al.*, 1989; Walthers *et al.*, 2003).

A handful of TCS have been studied in detail. The *Escherichia coli* CpxA/R TCS monitors outer-membrane stress and proper protein folding in the periplasmic space (for recent reviews see Raivio, 2005; Ruiz & Silhavy, 2005; and references therein). In the absence of envelope protein misfolding, the sensor kinase CpxA is maintained in an inactive state by the periplasmic inhibitor molecule CpxP. Envelope protein misfolding or pilus assembly is predicted to lead to relief of CpxP inhibition and activation of phosphotransfer between CpxA and CpxR. Phosphorylated CpxR is thought to regulate the expression of numerous genes, possibly more than 100, whose products include molecular chaperones and proteases (De Wulf *et al.*, 2002; DiGiuseppe & Silhavy, 2004; Raivio & Silhavy, 2001). In addition to protein misfolding or pilus assembly, a number of conditions have been demonstrated to induce the Cpx response. These include elevated pH and altered membrane composition (Hernday *et al.*, 2004; Raivio & Silhavy, 2001; Ruiz & Silhavy, 2005).

In this work, we examined the *E. coli* CpxR as a model system and studied the effect of N-terminal truncations on cognate target promoter transcription. We also analysed inhibition by the N-terminal domain by asking whether structurally similar heterologous regulatory domains were interchangeable in this system using predictions from a few existing cases where functional hybrid response regulators have been reported. Surprisingly, our hybrid CpxR proteins were all constitutively active. This study has revealed clues to CpxR activation per se and sheds light on simple genetic strategies to dissect other uncharacterized TCS response regulators *in vivo* by molecular resection.

METHODS

Bacterial strains. Bacterial strains used in this study are described in Table 1.

Media and chemicals. All strains were grown in Luria–Bertani (LB) broth or on LB agar at 37 °C. Where necessary, the plates were supplemented with ampicillin, kanamycin and tetracycline at a final concentration of 100, 50 and 12.5 µg ml⁻¹, respectively.

Plasmid constructions and cloning strategies. Primers used to clone the genes encoding CpxR and their mutant derivatives are listed in Table 2. The 651 and 699 bp fragments encoding wild-type (wt) RcsB and CpxR were amplified from *E. coli* MC4100 chromosomal DNA using PCR and Platinum *Pfx* polymerase (Invitrogen). Primers were designed to incorporate upstream *EcoRI* and downstream *BamHI* restriction sites.

The point mutations RcsB D56E and D56N were engineered by overlap fusion PCR (QuikChange; Stratagene).

N-terminal truncation mutations (CpxR 100, 117 and 134) were generated by appropriate PCR primers that incorporated an upstream *EcoRI* site together with an ATG start codon. The numbers indicate the number of amino acids truncated from the N terminus of CpxR (SWISS-PROT accession no. P16244). The insertion sites of a methionine start codon for each of the truncations are indicated in Fig. 3.

The PCR products were digested with *EcoRI* and *BamHI* and subcloned into the arabinose-inducible pBAD24 vector cut with the same enzymes (Guzman *et al.*, 1995). The pBAD24 vector used had been previously modified by site-specific mutagenesis to remove the second upstream *BamHI* site in the pBAD24 promoter without affecting promoter function (D. Ang & W. L. Kelley, unpublished).

Plasmid constructions for the chimeric fusion of heterologous N-terminal sequence to CpxR coding sequences were performed using conveniently modified vectors where unique junction sequence restriction sites had been introduced by overlap fusion PCR. pBAD-CpxR was first modified by introduction of either a unique *PmeI* site (pBAD-*cpxR* 100 vector) or a *KpnI* site (pBAD-*cpxR* 117 vector) at the point of the fusion junction. Plasmids were cleaved with *NdeI*/*PmeI* and ligated with appropriately cleaved PCR products to yield the chimeras of the CpxR 100 series or, alternatively, plasmids were first cleaved with *KpnI*, flush-repaired with T4 DNA polymerase, recut with *NdeI* and then ligated with appropriately cleaved PCR products to yield chimeras of the CpxR 117 series. Primers used to amplify the *Staphylococcus aureus vicR* coding sequences from *S. aureus* N315 chromosomal DNA (Kuroda *et al.*, 2001) are also listed in Table 2. The amplicons were then cloned by a cohesive/blunt strategy into the pBAD-*cpxR* 100 and pBAD-*cpxR* 117 vectors. The same cloning strategy was used to create the expression vectors encoding the RcsB-CpxR chimeras. The GFP-CpxR chimeras were created by ligation of an *NcoI*–*EcoRI* fragment of the CMV-5' EGFP

Table 1. Bacterial strains used in this study

Strain	Relevant genotype	Source or reference
MC4100	F ⁻ <i>araD139</i> Δ(<i>argF-lac</i>) <i>U169 rpsL150</i> (Str ^r) <i>relA1 flbB5301 deoC1 ptsF25 rbsR</i>	Silhavy <i>et al.</i> (1984)
PAD292	MC4100 λRS88 [<i>cpxP'</i> - <i>lacZ</i> ⁺] <i>cpxR1::spc</i>	DiGiuseppe & Silhavy (2003)
SP559	MC4100 λRS88 [<i>degP'</i> - <i>lacZ</i> ⁺] <i>cpxR1::spc</i>	DiGiuseppe & Silhavy (2003)
SG20810	MC4100 <i>lon</i> ⁺ <i>rscC10::Δkan wcaB10::lac-Mu-imm</i> ²	Painbeni <i>et al.</i> (1993)

Table 2. Primers used in this study

Name	Sequence (5'–3')
RcsB-forward PCR	CGGGAATTCCATATGAACAATATGAACGTAATTATTG
RcsB-reverse PCR	GCGGATCCTTAGTCTTTATCTGCCGGACTTAAGGTCAC
RcsB-D56E-1	CATGTGTTGATTACCGAGCTCTCCATGCCTGGC
RcsB-D56E-2	GCCAGGCATGGAGAGCTCGGTAATCAACACATG
RcsB-D56N-1	CATGTGTTGATTACCAACCTCTCCATGCCTGGC
RcsB-D56N-2	GCCAGGCATGGAGAGGTTGGTAATCAACACATG
RcsB100- <i>HincII</i> -Rev	ATCGGTTCGGTGACCTTGTTTAACTGTCAACCACGATCCCTTCGATATCCAGA
RcsB117- <i>StuI</i> -Rev	TCCGGGGTAAATTTCTTCCCGGGAGGCCTCTGCAGCGCGCGAGAGC
CpxR-forward PCR	CGGAATCCCATATGAATAAAATCCTGTTAGTTGATG
CpxR-reverse PCR	GCGGATCCTCATGAAGCAGAAACCATCAGATAGCCGCG
CpxR- <i>SmaI</i> - <i>PmeI</i> Fwd	GCTGGGCGCAGATGACTATCTCCCCGGGTTAAACCGTTTAATGATCGTGAGCT
CpxR- <i>StuI</i> - <i>KpnI</i> Fwd	CACGTATTTCGCGCGATCCTGAGGCCTGGTACCGGTCGCACTGGAGCGAGCAACA
CpxR- <i>StuI</i> - <i>KpnI</i> Rev	TGTTGCTCGTCCAGTTCGACCGGTACCAGGCCTCAGGATCGCGCGAATACGTG
CpxR- <i>SmaI</i> - <i>PmeI</i> Rev	AGCTCACGATCATTAAACGGTTTAAACCGGGGAGATAGTCATCTGCGCCCAGC
CpxR100-forward PCR	CGGAATCCCATATGAAACCGTTTAATGATCGTGAGCTGG
CpxR117-forward PCR	CGGAATCCCATATGCGGTCGCACTGGAGCGAGC
CpxR134-forward PCR	CGGAATCCCATATGCTGGAAGTTGATGCCTTAGTG
VicR-start- <i>KpnI</i>	GGGGTACCAAGGAGGAACACATATGCAATGGCTAGAAAAGTTGTTGTAG
VicR117-Rev- <i>StuI</i>	AAAGGCCTTAAGTTCGCTTTCACACGTG
VicR100-Rev- <i>XmnI</i>	AAGACAAGAATTCGTTCCATAGTCATCTGCACCTAGTTC

expression vector (Tapparel *et al.*, 2003) with *EcoRI*–*NcoI* fragments of pBAD-*CpxR* 100, 117 and 134. All constructs were verified by sequencing using appropriate primers.

β -Galactosidase assays. Single colonies were inoculated into 3 ml cultures of LB broth supplemented with ampicillin ($50 \mu\text{g } \mu\text{l}^{-1}$) or carbenicillin ($50 \mu\text{g } \mu\text{l}^{-1}$) overnight at 37°C with aeration. On the next day, cultures were subcultured 1:100 into fresh LB broth-carbenicillin, supplemented with 0.5% arabinose and grown for an additional 2.5 h at 37°C with aeration. β -Galactosidase assays were performed as described by Miller (1992). Each experiment was repeated a minimum of three times using independent single colony isolates.

Western blot analysis. One millilitre of exponential-phase subculture used for the β -galactosidase assay (see above) was harvested by centrifugation, resuspended and sonicated in 75 μl SDS-PAGE gel electrophoresis sample buffer (63 mM Tris/HCl, pH 6.8, 15% glycerol, 3.7% SDS, 68 mM β -mercaptoethanol, 0.045% bromophenol blue). The OD_{600} was used to normalize the number of cells per millilitre and to adjust the appropriate volume of each sample loaded and electrophoresed on 12.5% SDS-PAGE gels. The proteins were either directly stained with Coomassie brilliant blue or transferred to nitrocellulose, stained with acidic Ponceau S and subjected to Western blot analysis. Rabbit polyclonal antibody raised against a maltose-binding protein (MBP)-*CpxR* hybrid (Raivio & Silhavy, 1997) was used at a 1:30 000 dilution and revealed with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad). Enhanced chemiluminescence reagents were purchased from Pierce and used according to the manufacturer's instructions. Films were scanned and processed with Image Analysis software (Image Quant TL V2003-01; Amersham).

RESULTS

N-terminal truncation of *CpxR* constitutively activates transcription of a *cpxP-lacZ* reporter

In several response regulators, the N-terminal domain has been shown to exert inhibition on the C-terminal domain by

blocking DNA binding site accessibility, protein–protein contacts or oligomerization interfaces. Precise truncation of a response regulator regulatory domain may therefore serve as a simple strategy to test whether a given output domain is repressed and, if so, to predictably produce useful genetic variants for controlled analysis of downstream target promoters *in vivo* (Parkinson & Kofoid, 1992). We therefore asked whether N-terminal truncations of the *CpxR* regulatory domain were stably expressed and functional for transcriptional activation of cognate promoter reporters in a well defined system.

For our experiments, we exploited the tightly controlled pBAD24 arabinose-inducible system for controlled expression of mutant response regulators. With this approach, the transcriptional activation properties of the regulators can be examined in the absence of the need for the cognate sensor kinase or even detailed knowledge of upstream inducing signals.

Arabinose-inducible plasmids encoding three N-terminal deletions of *CpxR* were constructed and are illustrated in Fig. 1(a). The deletion end points were chosen to correspond to retention of the regulator domain $\alpha 5$ helix (*CpxR* 100), the linker domain (*CpxR* 117) or only the effector winged-helix DNA-binding domain (*CpxR* 134). The exact position of the deletion was chosen using alignment with CheY and the well characterized OmpR (Mattison *et al.*, 2002) and PhoB (Allen *et al.*, 2001) response regulators.

Plasmids encoding wt *cpxR*, the three N-terminal truncations or empty control vector alone were transformed into the strain PAD292 that contains the *cpxP-lacZ* reporter fusion. The promoter of *cpxP* monitors activated *CpxR* and

Fig. 1. Schematic representation of N-terminal truncation mutants (a) and of CpxR hybrid proteins containing various heterologous N-terminal regulatory domains (b). The dual domain structure is represented by dotted rectangles and is separated by a black linker region. The N-terminal regulatory domain is indicated as Reg and the C-terminal effector domain is indicated as Eff. The $\alpha 5$ helix and linker region are shown as H $\alpha 5$ and L, respectively. The position of the conserved Asp within the acidic pocket of the regulatory domain is indicated by an asterisk. (a) Each deletion mutant was named according to the number of amino acids truncated from the N terminus of CpxR (SWISS-PROT accession no. P16244). (b) Two series of chimeric proteins (heterologous sequence shown in black) were generated and are shown as CpxR-100 or CpxR-117 series. The two series differ in the origin of the $\alpha 5$ helix sequence. The entire coding sequence of GFP was also fused in-frame to the N terminus of each CpxR-100 or CpxR-117 construct. The length of the resulting chimeras (aa) is indicated.

is not known to be influenced by other TCS (Danese & Silhavy, 1998; DiGiuseppe & Silhavy, 2003; Otto & Silhavy, 2002). PAD292 also contains a *cpxR1::spc* mutation so that all measured CpxR activity was exclusively plasmid-derived. Transformants were then tested for their response profile as a function of arabinose inducer. The results are shown in Fig. 2.

We found that upon induction with arabinose the truncation CpxR 100 activated the *cpxP-lacZ* reporter approximately 200-fold more than CpxR wt and approximately four- or sixfold more efficiently than truncations CpxR 117 or CpxR 134, respectively (Fig. 2a). Steady-state protein levels were examined by immunoblotting of normalized whole-cell extracts using CpxR-MBP antiserum (Fig. 2b). Since the truncation mutants theoretically could lack some

of the epitopes recognized by the polyclonal anti-MBP-CpxR serum, CpxR wt and CpxR 100, 117 and 134 derivatives were all C-terminally tagged with a triple HA epitope and steady-state expression levels were compared using both monoclonal anti-HA and polyclonal anti-MBP-CpxR serum. We found that the two antibodies recognized CpxR wt, CpxR 100 and CpxR 117 at comparable levels, whereas detection of CpxR 134 was significantly underestimated when probed with the anti-MBP-CpxR serum (data not shown). When taking into consideration adjustments for relative protein expression levels (Fig. 2b), we concluded that the observed relative differences in reporter activity were significant and therefore most probably due to enhanced constitutive activation. This result shows that the C-terminal effector DNA-binding domain of CpxR is strongly constitutively active and that the elevated *cpxP-lacZ*

Fig. 2. Transcriptional activation by the CpxR N-terminal truncation mutants. (a) Strain PAD292, which contains a *cpxP-lacZ* reporter gene, was transformed with plasmids encoding the indicated CpxR derivatives, or vector alone. β -Galactosidase assays were performed as described in Methods in the absence (bars not visible on graph) or presence (dark grey bars) of 0.5% arabinose. Values for a typical experiment are indicated. The observed differences in relative activity were reproducible in multiple independent experiments. (b) Western blot analysis of relative CpxR wt and CpxR truncation mutant protein expression. Normalized whole-cell lysates from cultures used for activity assays depicted in panel (a) (lanes 1–4) were prepared as described in Methods. Arrows indicate the position of endogenous MBP (note polyclonal antiserum was raised to an MBP-CpxR fusion), CpxR and the truncation mutants. The relative levels of CpxR, CpxR 100, CpxR 117 and CpxR 134 are indicated below the figure with CpxR wt set equal to 1. *The CpxR 134 expression level is underestimated by this method (see text for details).

reporter activity observed with the CpxR 100 derivative compared to the other two truncations is dependent upon the presence of the linked regulatory domain $\alpha 5$ helix. We conclude that the receiver domain of wt CpxR normally exerts tight negative control over the highly active effector DNA-binding domain and that interdomain residues can modulate the activated state.

To determine whether the observed reporter gene activation by CpxR truncations was indeed specific for regulons under CpxR control, the same plasmids were tested in strain SP559 containing the *degP-lacZ* fusion that also responds to activated CpxR. When we monitored reporter output in the presence or absence of arabinose inducer, we observed that CpxR 100 was five times more active relative to CpxR wt and approximately 2.5–3.5 times more active than CpxR 117 and CpxR 134, respectively (data not shown). Control immunoblot analysis of steady-state protein levels confirmed that the truncations were all stably expressed in SP559 and were similar to those levels observed in PAD292 (data not shown). We conclude that the observed relative activity levels were therefore most probably due to differences in the affinity of the CpxR winged-helix DNA-binding domain for the two tested CpxR target promoters. When the plasmids encoding *cpxR* truncations were tested in strain SG20810 containing the *wca-lacZ* reporter, which is not a known member of the *cpxR* regulon (De Wulf *et al.*, 2002), we observed no specific reporter induction under conditions where a control cognate response regulator (*rcsB* D56E) strongly induced the reporter (data not shown). We conclude from these results that the constitutive CpxR truncation mutants retain their specificity for promoters included in the CpxR regulon and that they do not act by generally affecting transcription.

Conserved core and acidic pocket residues in response regulator domains

Regulatory domains of TCS response regulators are highly conserved throughout bacterial species and several critical residues that comprise the acidic pocket with the site of sensor-kinase-directed phosphorylation are nearly universally conserved (Hoch & Varughese, 2001). A ClustalW analysis of the regulatory domains of CpxR, VicR and RcsB (heterologous domains used in our study; see below) was performed and results were compared to the location of the known secondary structure elements of *E. coli* CheY, PhoB and OmpR, and *Bacillus subtilis* YycF and PhoP. The alignment is depicted in Fig. 3.

Despite a low degree of overall amino acid sequence identity, clusters of highly similar amino acids that comprise β -sheets $\beta 1$, $\beta 3$, $\beta 4$ and $\beta 5$ were clearly evident in the alignment (Fig. 3, amino acids boxed in grey). In addition, conservative amino acid substitutions were found distributed throughout the entire domain, including residues within all five α helices. When the position of the similar substitutions was fitted to the known structure of CheY, most conserved residues defined the buried domain interior (data not shown). On the basis of this alignment and other structural considerations, we reasoned, as have others, that response regulator regulatory domains could share both common activation and perhaps common repression mechanisms.

In the absence of prior knowledge of activator signals or even downstream targets of TCS, several laboratories have

Fig. 3. ClustalW alignment (www.ebi.ac.uk/clustalw) of CpxR N-terminal amino acids 1–137 together with the corresponding N-terminal regions of *E. coli* RcsB, OmpR, PhoB and CheY, *B. subtilis* YycF and PhoP, and *S. aureus* VicR. Strongly conserved residues contributing to the formation of the acidic pocket are indicated in dark grey; other conserved residues, including those contributing to the hydrophobic core, are shown in light grey. Black arrows indicate the location of domain swapping in chimeric protein constructions as well as the positions of the CpxR N-terminal truncation mutants. For each response regulator, the homology score (www.ebi.ac.uk/clustalw) with the entire amino acid sequence of CpxR is indicated in parentheses. The sequence data were obtained from SWISS-PROT (<http://us.expasy.org>) under accession numbers P16244 (CpxR), P37478 (YycF), P13792 (PhoP), Q7A8E1 (N315-VicR), P08402 (PhoB), P03025 (OmpR), P14374 (RcsB) and P06143 (CheY). CheY and PhoB α (double underline) and β (single underline) secondary structure limits are indicated as well as the linker (boxed) region of PhoB (Allen *et al.*, 2001) and OmpR (Mattison *et al.*, 2002), and the predicted location of the CpxR $\alpha 5$ helix and its linker region. Arrows over PhoB indicate published active truncations of this protein. Ch1 refers to the fusion junction of functional CheY-PhoB chimeras (Allen *et al.*, 2001), * refers to the fusion point of two functional reciprocal chimeras created by domain exchange between *B. subtilis* PhoP and YycF (Howell *et al.*, 2003) and RRB denotes the location of the junction in a functional OmpR-PhoB chimera (Walthers *et al.*, 2003).

suggested or described the use of hybrid response regulator proteins as tools to dissect activation by a known stimulus, or to dissect the underlying mechanisms of interdomain signalling in a response regulator evoked by sensor kinase phosphorylation (Allen *et al.*, 2001; Howell *et al.*, 2003; Walthers *et al.*, 2003). This strategy rests, in part, on the presumption that a high level of predicted structural similarity between receiver domains exists and therefore effector domain repression and interdomain signalling could be preserved in the hybrid. The generality of this approach is unclear since too few examples exist to date.

Fusion of heterologous response regulator receiver domains with CpxR effector domain

Our results presented above show that the CpxR effector DNA-binding domain is efficiently repressed by its N-terminal receiver domain. Thus, CpxR belongs to that class of two-component effectors where native signal capture and receiver domain phosphorylation by the sensor kinase most likely relieves inhibition. In light of the limited studies mentioned above, the question naturally arises whether CpxR effector domain repression can be achieved by substituting structurally similar response regulator receiver

domains, or whether the CpxR receiver domain has specifically evolved non-interchangeable repressor mechanisms. The ability to produce functional exchanges that have been reported may be fortuitous with the limited number of TCS systems employed.

To test whether functional chimeras could be constructed with CpxR, we used as a guide published examples of fusion points engineered with YycF/PhoP, PhoB/OmpR and CheY/PhoB that are indicated for reference in Fig. 3 and its legend. Both PhoB and OmpR regulatory domains have been shown to require their cognate linker region to produce functional chimeras when fused to heterologous effector domains (Walthers *et al.*, 2003). Since we wanted to test the ability of response regulator receiver domains to repress our highly active CpxR 100 derivative that possesses its own $\alpha 5$ helix and linker region, we chose not to test fusion of PhoB and OmpR to avoid $\alpha 5$ helix and linker domain duplication. Instead, we simply asked if any TCS N-terminal domain, per se, could impart repression when grafted to CpxR. We chose VicR, which is the *S. aureus* orthologue of *B. subtilis* YycF, and RcsB. VicR has a higher predicted homology score (www.ebi.ac.uk/clustalw) than either PhoB or OmpR when compared to CpxR, although all are TCS response regulators of the winged-helix DNA-binding domain motif family. RcsB is predicted to be less similar and belongs to the helix–turn–helix TCS family. The full-length green fluorescent protein (GFP) that has been widely used in many organisms was also fused to CpxR to observe if an unrelated stably folded domain could impart steric inhibition.

In the first series of constructs, hereafter referred to as the ‘100 series’, the $\alpha 5$ helix was encoded by *cpxR*, whereas in the second series of constructs, hereafter referred to as the ‘117 series’, the $\alpha 5$ helix was derived from the fused regulatory domain or was entirely absent (GFP) (Fig. 1b). The precise choice of fusion site with or without the cognate $\alpha 5$ helix was in part determined by prior studies that first described the importance of this helix in maintaining proper interdomain communication in hybrid response regulators (see Fig. 3 for details) (Allen *et al.*, 2001; Howell *et al.*, 2003). All plasmids were transformed into *cpxP-lacZ* reporter strain PAD292 and their activity was measured in the presence or absence of arabinose inducer. The results are shown in Fig. 4.

Remarkably, we found that in the presence of arabinose inducer, all hybrid proteins of the 100 series were constitutively active on the *cpxP-lacZ* promoter reporter when compared to arabinose-induced native CpxR, which shows only slight activity (Fig. 4a). When we next examined the activity of the CpxR 117 series of hybrid constructs we observed a significantly altered activity profile, though qualitatively, the results were congruent with the CpxR100 series. In conclusion, we found that no predicted structurally related receiver domain in the similarity range (approx. 38–40 % aa identity) that had been shown to function for a few published examples could fully restore repression of the

CpxR effector domain whether the fusion point was chosen with or without the native CpxR $\alpha 5$ helix.

Immunoblot analysis of whole-cell extracts using anti-MBP-CpxR antiserum showed that the steady-state expression levels of the hybrid proteins was significantly higher than the steady-state expression levels of the CpxR truncation mutants or the GFP chimera (Fig. 4c and d). This finding was unexpected since truncation of the CpxR regulatory domain alone did not significantly increase protein expression and stability (for example, see CpxR 100 or CpxR 117 expression levels in Fig. 2b). It is therefore probable that the fused heterologous regulatory domain (but not GFP) had a positive effect on hybrid protein expression and/or stability and suggests that uncharacterized determinants within the native N-terminal domain could affect protein levels and turnover. It is also formally possible that strong overexpression per se of the hybrid protein perturbs the mechanism of repression, through, for example, subtle changes in spatial alignment, oligomeric state or transient interaction with other proteins.

Notably, one chimera, VicR-Cpx117 was strongly active, even in the absence of arabinose inducer. This observation may be explained by *cis*-acting DNA sequences that alter normally strong pBAD promoter repression and/or by the presence of cryptic promoter elements that are not under plasmid-encoded *araC* repression. We did not explore the molecular details of this observation further.

The hybrid proteins are constitutively active

Our inability to effectively repress the active CpxR DNA-binding domain with fused heterologous receiver domains that were predicted to preserve the three-dimensional domain fold was unexpected since several groups have reported the successful use of response regulator chimeras that correctly respond to input signal and retain proper interdomain communication (Allen *et al.*, 2001; Howell *et al.*, 2003; Walthers *et al.*, 2003). When we further examined the hybrid proteins where we had additionally substituted glutamate for the conserved aspartate to mimic the phosphorylation active state (i.e. RcsB D/E CpxR 100 and RcsB D/E CpxR 117), an activating mutant of the RcsB response regulator (Gupte *et al.*, 1997), we found no additional significant increased activation compared to the unmodified hybrid alone (RcsB wt CpxR 100 and RcsB wt CpxR 117) (Fig. 5). The same results were obtained for the VicR hybrid proteins (data not shown). Collectively, we conclude that our CpxR hybrids did not retain proper interdomain repression and could not be further derepressed by glutamate modification.

DISCUSSION

In this study, we showed that constitutively active mutants of the CpxR response regulator can be obtained by molecular resection of an inhibitory sequence. This approach to

Fig. 4. Transcriptional activation by the CpxR N-terminal chimeric proteins of the CpxR 100 (a) or CpxR 117 (c) series. For the hybrid proteins, only the relevant origin of the N-terminal domain is indicated. Strain PAD292 was transformed with plasmids encoding the indicated CpxR derivatives or vector alone. β -Galactosidase assays were then performed as described in the legend to Fig. 2 in the absence (light grey bars) or presence (dark grey bars) of 0.5% arabinose. (b, d) Western blot analysis of relative CpxR wt and CpxR chimeric protein expression. Normalized whole-cell lysates from cultures used for activity assays depicted in panels (a) and (c) were prepared in the presence of 0.5% arabinose (lanes 1–4) as described in Methods. The arrow indicates the position of wt CpxR.

produce active variants may be generally feasible for a number of other TCS response regulators provided that adequate precautions are taken to carefully examine the importance of retention, or removal, of the $\alpha 5$ helix and adjoining linker regions. As an example, N-terminal truncated PhoB (like CpxR, a winged-helix protein), lacking the linker region, was shown to transactivate the *phoA* promoter *in vivo*, but lost activation completely when the $\alpha 5$ helix was retained (Allen *et al.*, 2001; Ellison & McCleary, 2000). We report just the opposite effect of the $\alpha 5$ helix with our CpxR truncations. Indeed, we observed significant enhanced transactivation *in vivo* when the CpxR $\alpha 5$ helix was retained.

The presence (CpxR 117) or absence (CpxR 134), of linker sequence alone did not substantially alter activation in our experiments. Thus, the choice of truncation point, amino acid composition, protein–protein interactions and tertiary structure may explain why linkage between the $\alpha 5$ helix and its cognate effector domain led to alternative outcomes in different systems.

Several laboratories have reported the successful use of response regulator chimeras that maintain interdomain communication and respond to input signal (Allen *et al.*, 2001; Howell *et al.*, 2003; Walther *et al.*, 2003). One study

Fig. 5. Transcriptional activation by RcsB wt-, D51E-, D51N-CpxR 100 and -CpxR 117 chimeras. Strain PAD292 was transformed with plasmids encoding the indicated CpxR derivatives or vector alone. β -Galactosidase assays were performed as described in the legend to Fig. 2 in the absence (light grey bars) or presence (dark grey bars) of 0.5% arabinose. (b) Western blot analysis showing relative RcsB-CpxR chimeric protein expression. Normalized whole-cell lysates from cultures used for activity assays depicted in (a) were prepared in the presence of 0.5% arabinose (lanes 1–6) as described in Methods.

of the *B. subtilis* YycF TCS revealed that in response to phosphate starvation, both PhoP-YycF and YycF-PhoP hybrids retained functional interdomain signalling and in each case the linker domain was associated with its cognate effector domain (Howell *et al.*, 2003) (Fig. 3). In the case of CheY-PhoB chimeric proteins, results showed that the $\beta 5/\alpha 5$ loop and $\alpha 5$ helix, in addition to the linker sequence, must belong to PhoB for correct phosphorylation-mediated modulation of transcriptional activation (Allen *et al.*, 2001). In contrast, chimeras formed between OmpR and PhoB showed that correct effector domain regulation by either grafted N terminus required attachment of its cognate interdomain linker (Walthers *et al.*, 2003).

In our study, we had designed our CpxR chimeric expression plasmids using two fusion points to assess the role of the $\alpha 5$ sequence contributed by the N- or C-terminal domain. Despite the use of a fusion site in the CpxR 100 series that was identical to a fully functional fusion reported in the CheY-PhoB study (Allen *et al.*, 2001; marked Ch1 in Fig. 3), we found that all of our CpxR 100 fusions, as well as all CpxR

117 fusions, were strongly expressed and highly active. This discrepancy may be the result of sequence variability within the interdomain region (helix $\alpha 5$ and linker; see Fig. 3). Therefore, analysis of any proper transmission of an activating phosphorylation signal mimic was precluded in our study. Indeed, all tested hybrids with an Asp to Glu substitution at the phosphorylation site (e.g. RcsB D56E/CpxR 117), a modification known to render, for example, native RcsB constitutively active (Gupte *et al.*, 1997) as well as in several other TCS response regulators, did not further increase their activity when compared to the activity of unmodified hybrids. Our finding that N-terminal domain swaps in the CpxR system produce robust constitutive mutants in each case tested nevertheless calls into question the proposed general strategy of interchanging domains to study signalling and output of unknown response regulators (Howell *et al.*, 2003). Until the details of interdomain communication are fully understood, it is unclear whether one can easily and predictably exchange domains even though in alignment they may show overall amino acid similarity and probably share three-dimensional structure.

The demonstration of wide variability in several inter-domain interfaces of response regulators by crystal structure analysis is consistent with this notion (Robinson *et al.*, 2003).

Most response regulators described to date have been placed into one of two classes with regard to the consequence of phosphorylation and the mechanism of activation. In the first class, exemplified by NtrC (composed of three domains) or OmpR (Tsung *et al.*, 1989; Walthers *et al.*, 2003), phosphorylation of the receiver domain activates the response regulator by triggering its oligomerization. In the second class of response regulators, the regulatory domains are thought to act negatively on their effector domains and phosphorylation relieves this inhibition by triggering a conformational change and/or inducing oligomerization as proposed for PhoB (Ellison & McCleary, 2000), Spo0A (Ireton *et al.*, 1993), NarL (Baikalov *et al.*, 1996) and FixJ (Da Re *et al.*, 1994, 1999). Our data indicate that CpxR most probably belongs to this second class and has evolved a particularly strong interdomain inhibition mechanism. Whether truncation of the N-terminal inhibitory domain induces a conformational change necessary for DNA binding, facilitates oligomerization, enhances contact with RNA polymerase or even promotes altered DNA topology in its active state needs further detailed biochemical and structural study. Nevertheless, in light of our findings with CpxR, it is tempting to speculate that simple removal or substitution of a regulatory domain may lead to activation in many cases and as a consequence yield useful genetic variants for the study of response regulator targets *in vivo*.

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