Phosphorylated CpxR Restricts Production of the RovA Global Regulator in \textit{Yersinia pseudotuberculosis}

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Abstract

\textbf{Background:} RovA is a global transcriptional regulator of gene expression in pathogenic \textit{Yersinia}. RovA levels are kept in check by a sophisticated layering of distinct transcriptional and post-transcriptional regulatory mechanisms. In the enteropathogen \textit{Y. pseudotuberculosis}, we have previously reported that the extracytoplasmic stress sensing CpxA-CpxR two-component regulatory system modulates rovA expression.

\textbf{Methodology/Principal Findings:} In this study, we characterized CpxR phosphorylation (CpxR$\rightarrow$P) \textit{in vitro}, and determined that phosphorylation was necessary for CpxR to efficiently bind to the PCR-amplified upstream regulatory region of rovA. The precise CpxR$\rightarrow$P binding site was mapped by a nuclease protection assay and directed mutagenesis confirmed that in \textit{vivo} binding to the rovA promoter inhibits transcription. Reduced RovA production was most pronounced following CpxR$\rightarrow$P accumulation in the \textit{Yersinia} cytoplasm during chronic Cpx pathway activation and by the indiscriminate phosphodonor action of acetyl phosphate.

\textbf{Conclusions/Significance:} Cpx pathway activation restricts levels of the RoV global regulator. The regulatory influence of CpxR$\rightarrow$P must therefore extend well beyond periplasmic quality control in the \textit{Yersinia} envelope, to include genes involved in environmental survival and pathogenicity.

Introduction

Extracytoplasmic stress (ECS) has a deleterious effect on bacterial fitness, since it impacts on cell envelope integrity, protein folding and function. Bacteria have consequently evolved multiple signaling pathways to counter these situations [1]. One of these is a two-component regulatory system (TCRS) composed of CpxA and CpxR (for conjugative plasmid expression). In response to diverse stresses (such as high pH, detergents, EDTA, altered membrane lipid composition and bacterial adhesion to surfaces) that ultimately leads to protein misfolding in the periplasm, the Cpx pathway induces synthesis of periplasmic protein folding and degradation factors that aid in the (re)folding of extracytoplasmic proteins and protein complexes [2,3,4].

Spanning the inner membrane, CpxA is a histidine kinase possessing three distinct activities in \textit{E. coli} – autokinase, CpxR kinase and CpxR phosphatase activity [5]. In the absence of inducing signal, CpxA activity is subdued by the binding in the periplasm of the accessory factor CpxP [6]. In recognition of ECS signals however, CpxP is titrated away from CpxA and is targeted for degradation by the DegP serine protease [7,8]. This enables CpxA to become auto-phosphorylated presumably at the conserved histidine residue at position 249 [5]. This permits the phosphate to be relayed to the CpxR response regulator that, based on homology to other response regulators, would occur on the aspartate residue at position 51 [9]. At least in \textit{E. coli}, CpxR$\rightarrow$P is then capable of up- or down-regulating the transcription of many genes that function to alleviate the effects of ECS [2,3,4]. Once this has occurred, status quo is restored by the phosphatase activity of CpxA, which dephosphorylates CpxR [5]. Thus, a consequence of generating CpxA phosphatase deficient \textit{E. coli}, as would result from a full length \textit{cpxA} deletion mutant or so-called gain-of-function mutants (designated \textit{cpxA}$^{\ast}$), would be to accumulate CpxR$\rightarrow$P [5,10,11,12,13,14]. In these situations, acetyl phosphate (acetyl$\rightarrow$P), a small molecular weight phosphodonor, can also elevate CpxR$\rightarrow$P levels [5,15,16,17]. This CpxA-independent phosphorylation of CpxR by acetyl$\rightarrow$P is potentially a global signal reflecting the status of bacterial growth and central metabolism [18].

Homologues to components of the Cpx pathway exist in a number of clinically important bacterial pathogens. We have...
recently studied the function of the Cpx TCRS in the enteropathogen Yersinia pseudotuberculosis [19,20], a bacterium causing self-limiting gastroenteritis in infected individuals [21]. Intriguingly, loss of CpxA in this bacterium caused a down-regulation of several determinants known to be important in the pathogenesis of disease in mouse infection models; most notably, the plasmid-encoded Ysc-Yop type III secretion system (T3SS), an integral outer membrane adhesin, termed invasin, and RovA, a member of the MarR/SlyA family of transcriptional regulators [19,20]. These studies provided the first indications that CpxR–P could act as a negative transcriptional regulator of Y. pseudotuberculosis virulence factors. The fact that we could demonstrate in vitro CpxR–P binding to inv and rva promoters supported this view, although typical consensus CpxR–P binding sites in these regions were never located [20]. Moreover, it was assumed from this earlier work that Y. pseudotuberculosis lacking CpxA phosphatase activity must accumulate CpxR–P, serving as the fundamental basis for transcriptional repression of virulence gene expression in this mutant background [19,20]. However, this critical notion is unproven.

RovA was first identified in Yersinia because it was required for transcription of the inv gene encoding for invasin [22,23]. Whole-genome analyses has since indicated a global regulatory role for RovA in pathogenic Yersinia [24,25,26]. Additional virulence strategies among the RovA regulon include the prominent pH 6 antigen chaperone-usher system, the Ysc-Yop T3SS, and other uncharacterized potential secretion systems. To regulate these virulence determinants, RovA levels are therefore tightly controlled by multiple pathways in accordance with the prevailing environmental growth conditions. Dynamic regulatory mechanisms are obviously paramount for RovA to impart significant global control on virulence gene transcription in Yersinia. Thus, this study therefore seeks to substantiate the contribution of the Cpx TCRS to control of RovA-dependent virulence gene regulation in Yersinia.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids can be viewed in Table S1. We consistently used Y. pseudotuberculosis YPIII/pIB102 (serotype III) as the parental strain. pIB102 is a virulence plasmid encoding for the Ysc-Yop T3SS and is a variant of cryptic pIB1. It differs only by a kanamycin resistance cartridge inserted into the yscR gene, which does not reduce the pathogenicity of YPIII/pIB1 in mouse models [27]. The strain also harbors an internal duplication within phoP located on the chromosome. This is predicted to generate an inactive truncated variant of the PhoP response regulator that impairs bacteria growth and survival inside macrophages [28]. Bacteria were normally cultivated in Luria-Bertani (LB) agar or broth at either 26°C (Y. pseudotuberculosis) or 37°C (E. coli) with aeration. When examining transcription and translation from rvaO and inv, bacteria were grown at both 26°C and 37°C in LB broth to late stationary phase. These conditions were selected on the basis that reports from other laboratories have clearly established the peak rvaO and inv expression occurs in bacteria grown at low temperature to stationary phase [22,24]. Where required, antibiotics were added at the final concentrations of carbenicillin (Cb; 100 µg per ml), kanamycin (Km; 50 µg per ml), Trimethoprim (Tp; 10 µg per ml) and chloramphenicol (Cm; 25 µg per ml).

Mutant construction

To construct individual in-frame deletions, site-directed amino acid substitutions and nucleotide ‘shuffle’ mutations, we applied the standard overlap PCR technique using the relevant primer combinations listed in Table S2. To facilitate the sequencing process (performed by MWG Biotech AG, Ebersberg, Germany), all amplified fragments were initially cloned into pCR®-TOP-TOPO TA (Invitrogen AB, Stockholm, Sweden). Confirmed fragments were then lifted into the mutagenesis vector, pDM4. These mutagenesis constructs were then conjugated by E. coli S17-1/pMOS into Y. pseudotuberculosis. Mutated alleles were initially introduced into the recipient genome by a single cross-over event. A second single cross-over event followed to secure the complete allelic exchange, which was initially screened for on the basis of sacB-dependent sucrose sensitivity [19,29]. The presence of the mutated allele in the Y. pseudotuberculosis genome was then confirmed by diagnostic PCR and sequence analysis of the amplified regions flanking the mutation.

Western blotting

Protein derived from lysates of the bacterial pellet was fractionated by 12% (for invasin) or 15% (for RovA) SDS-PAGE. Protein was then transferred to Schleicher and Schuell Protran nitrocellulose (GE Healthcare) using a Hoefer semi-dry transfer assembly. Proteins of interest were bound with specific rabbit polyclonal antibodies that were a gift from Petra Dersch (anti-RovA), Hans Wolf-Watz (anti-invasin), Thomas Silhavy (anti-MBP-CpxR) or Shu-ichi Nakayama (anti-CpxR). These were then detected with an anti-rabbit monoclonal antibody conjugated with horse radish peroxidase (GE Healthcare) and a homemade chemiluminescent solution.

nlpE cloning and expression

The primer combination for PCR amplification of Y. pseudotuberculosis nlpE is listed in Table S2. The DNA fragment was cloned into pBAD18 [30] using EcoRI/XbaI restriction to generate pJF027. This placed nlpE expression under arabinose control. The nlpE allele from E. coli cloned under arabinose control in pBAD18 (termed pND18) was a gift from Thomas Silhavy. These two constructs along with the vector control were electroporated into Yersinia. To induce nlpE expression, overnight bacterial cultures were used to seed fresh growth medium supplemented with 0.2% (w/v) L-arabinose and appropriate antibiotic selection.

Cloning, expression and purification of CpxR variants

Alleles encoding CpxR wild type and the CpxRΔnlpE and CpxRΔnlpEΔnlpA variants were amplified with gene specific primers (Table S2) using template DNA derived from parental Y. pseudotuberculosis or the respective mutants and cloned with NdeI and XhoI in front of the IPTG inducible promoter of pET22b(−). These expression plasmids were maintained in E. coli BL21(DE3) plyS allowing for the IPTG-inducible expression of individual CpxR variants fused to a C-terminal His6 tag. Recombinant protein was purified as previously described [20].

Electrophoretic mobility shift assay (EMSA)

32P end-labeled forward primers listed in Table S2 were used for the PCR amplification of the promoter regions of cpxR–P, rvaO, ppdA, ail, ail-like, inv, pccA and psaA. The amplified DNA fragments were purified by agarose gel electrophoresis. Purified CpxR was mixed with the DNA fragments (~2 to 5 nM) in a 20 µL reaction volume containing 20 mM Tris-HCl pH 7.0, 30 mM Acetyl phosphate, 125 mM KCl, 10 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.23 mg per ml BSA and 5 µg per ml sonicated salmon sperm DNA. BSA corresponding to the highest CpxR concentration (1.5 µM) was used as a negative control, and about
7 fold excess of unlabeled PCR amplified DNA was used in a competition reaction. After incubation at 30°C for 1 h and addition of the DNA dye solution (40% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol), the mixture was loaded directly onto a pre-run 5% polyacrylamide gel. Gel electrophoresis was performed at 1 x TBE at 100 V for 2 h at 4°C. After gel drying, signals were detected by autoradiography with a Storm 860 PhosphorImager (Molecular Dynamics).

DNPase 1 footprinting assay

The primers pE-rovAfo3, pE-ppiAfo and pcpXR for were radioactively labeled with 32P using γ32P-ATP (Perkin Elmer) and T4 polynucleotide kinase (Fermentas). The labeled pE-rovAfo3, pE-ppiAfo and pcpXR were paired with unlabeled pE-rovAfo3, pE-ppiAfo and pcpXR respectively and used to PCR amplify promoter regions of rovA (314 bp), ppiA (276 bp) and the divergent cpxR-cpxP (245 bp). For a control, the labeled pcpXR was paired with unlabeled pcpXR for the PCR amplification of an internal region of cpxR (389 bp). Subsequently, 1.5 nM of the amplified DNA fragments and 0, 50, 100, 200 and 400 nM acetyl-32P phosphorylated CpxRwt::His6 were mixed in a 40 µL reaction containing 25 mM Hepes (pH 8), 100 mM potassium glutamate, 0.5 mg/mL BSA. After incubation of the reaction mixture at room temperature for 10 minutes, 1 µL DNase 1 (0.0005 mg/mL; Sigma) was added and the reaction was left for 80 seconds at 37°C. The DNase 1 digestion was stopped by addition of phenol/chloroform and 200 µL DNase 1 STOP (0.4 M sodium acetate [pH 5], 2.5 mM EDTA, 10 ng/mL herring sperm DNA), and then the DNA was precipitated using ethanol. Samples were analyzed on a 7% denaturing polyacrylamide gel and visualized with a Storm 860 PhosphorImager (Molecular Dynamics).

Visualization of phosphorylated CpxR (CpxR-P)

Purified CpxR variants labeled with acetyl-32P were mixed with 2 µL loading buffer (0.02% [v/v] Bromophenol blue, 20% [v/v] Glycerol). For analysis, samples were either fractionated on a SDS-12% PAGE or a Manganese(II)-Phos-tagTM (33 µM) 12.5% acrylamide gel. Purified CpxR-P was detected by the Pro-Q® Diamond phosphoprotein gel staining method as described by the manufacturer (Invitrogen). To confirm equal loading, subsequent detection of total protein present on the gel was revealed by SYPRO® Ruby staining (Invitrogen). In both cases, fluororescent output was recorded using a Fluor-S® Multimager (BioRad) and band intensity quantified with Quantity One® quantitation software version 4.2.3 (BioRad). The presence of recombinant purified CpxR-P or that which is produced endogenously and present in bacterial lysates was also assessed by coupling Manganese(II)-Phos-tagTM acrylamide gel fractionation to immunoblottting with monospecific polyclonal anti-CpxR antisera. The Manganese(II)-Phos-tagTM approach is an affinity based system for the recognition and separation of anionic substrates, such as phosphorylated proteins, during polyacrylamide electrophoresis [31]. For example, characteristic separation patterns for phosphoprotein isoforms can be generated according to the number and size of the phosphate group. As a result, it later proved to be routinely applicable to in vitro and in vivo visualization of TCRS response regulator aspartate phosphorylation [32]. Samples for this analysis were generated from pelleted bacteria vigorously resuspended in 1.2 M formic acid preceding a very brief incubation at room temperature. Prior to fractionation, lysates were solubilized by addition of 4 x loading buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 4% BME, and 0.02% Bromophenol Blue) and neutralized by the addition of minuscule amounts of 3 M NaOH.

Biophysical analysis of intact and digested CpxR-P

The reduction of CpxR-P using sodium borohydride was performed as described previously [33,34]. Before the reaction, CpxR-P was desalted using C18 micro columns and dried by vacuum centrifugation [35,36]. Controls were performed using unphosphorylated CpxR under identical conditions.

Intact mass determination of CpxR and CpxR-P utilized purified recombinant CpxR, and this CpxR phosphorylated with acetyl-32P. Duplicate samples of both phosphorylated and non-phosphorylated CpxR were then desalted on homemade C18 columns [35,36]. For analysis by ESI-MS, the proteins were dissolved in 50% (v/v) acetonitrile containing 0.5% (v/v) formic acid. ESI-MS spectra of intact CpxR and CpxR-P were acquired by ESI-MS in the positive ion mode using a Q-Tof ultima mass spectrometer (Waters, Manchester, UK). Spectra were acquired off-line using nano spray capillaries (Q-tof) from Proxeon (Odense, Denmark) and deconvoluted using the MassLynx 4.0 software.

Peptides of CpxR and CpxR-P were prepared using trypsin and pepstatin. Protein was desalted using C18 micro columns and dried by vacuum centrifugation [35,36]. In-solution digestion using trypsin was performed for 40 min at 37°C in 10 µl of fresh 50% ammonium bicarbonate containing 10 ng/µl of sequencing grade trypsin (Promega Biotech AB, Nacka, Sweden). In-solution digestion using pepstatin (pepsin from porcine gastric mucosa, Sigma Life Sciences) was performed for 40 min at 37°C in 20 µl of 5% (v/v) formic acid containing 20 ng/µl of pepstatin. An alternative protocol, in-solution digestion using pepstatin was performed for 10 min at 37°C in 20 µl of 5% (v/v) formic acid containing 100 ng/µl of pepstatin. The generated peptides were then purified using C18 micro-columns [35,36] loaded with Poros R3 C18 material (Applied Biosystems, Stockholm, Sweden) and dried by vacuum centrifugation. In addition, enrichment and purification of phosphorylated peptides was performed by affinity chromatography on titanium dioxides as described [37,38,39].

MALDI-MS of peptides were acquired using a Voyager DE-STR mass spectrometer (AB SIEX, Stockholm, Sweden) in the reflector mode and 2.5 dihydroxybenzoic acid (DHB) solution containing 0.5% (v/v) phosphoric acid as a matrix (2,5 dihydroxybenzoic acid solution G2039A) from Agilent Technologies, Dalco Chromtech AB, Sollentuna, Sweden. LC-MS/MS combined with ESI-TRAP and ETD-TRAP was performed using a HCT ultra ETD II mass spectrometer from Bruker linked to Easy nano LC from Proxeon. Spectra were acquired using the enhanced scanning mode covering a mass range from m/z 200 to m/z 1300.

Analysis of gene transcription by Reverse Transcription (RT)-PCR

Patterns detailing the isolation of total RNA, the reverse transcription of mRNA into cDNA and its use as template for subsequent PCR amplification with the gene specific primers listed in Table S2 are described in detail elsewhere [19,20].

Results

Phosphorylation of the CpxR residue Asp51 by acetyl-32P

Phosphorylation of response regulators is thought to stimulate structural changes leading to formation of functional dimers – a prerequisite for efficient binding to target DNA. Thus, to determine if CpxR-P truly mediates repression of Yersinia virulence, we first wanted to characterize CpxR phosphorylation
in vitro using a high energy phosphate donor, acetyl-P. We initially determined the minimal concentration of acetyl-P needed to sufficiently in vitro phosphorylate CpxR. Purified CpxRwt::His6 was incubated with increasing concentrations of acetyl-P. Aliquots were then fractionated on Manganese(II)-Phos-tag™ 12.5% acrylamide gels to monitor the extent of CpxR-P. As little as 6.25 mM acetyl-P was sufficient to phosphorylate the majority of CpxR (Figure 1). Moreover, the maximal achievable amount of CpxR-P was reached in the presence of 25 mM acetyl-P (Figure 1). Hence, in vitro phosphorylation of CpxR by acetyl-P is a robust method for generating a high proportion of CpxR-P molecules.

On the basis of homology to the OmpR/PhoB response regulator family, the conserved aspartate at position 51 (Asp51) is a characteristic of the OmpR/PhoB family [41] (data not shown). C-terminally His6-tagged fusions of CpxRwt and the two CpxRD51A and CpxRM199A variants were then purified by affinity chromatography from *E. coli*. Acetyl-P was used to investigate which of these purified proteins could still be phosphorylated. The Manganese(II)-Phos-tag™ acrylamide system was used to visualize phosphorylated protein. Only CpxRwt::His6 and CpxRM199A::His6 were deemed to be phosphorylated in *vitro* by acetyl-P (Figure 2B). Given that CpxR2312::His6 exhibited altered mobility in the Manganese(II)-Phos-tag™ acrylamide gel, we also confirmed the absence of phosphorylation using conventional SDS-12%-PAGE followed by visualization with the independent Pro-Q™ Diamond phosphoprotein gel stain technique, while total protein (lower panel) was visualized by the SYPRO® Ruby staining method. In B, samples were separated in a conventional SDS 12% polyacrylamide gel. Phosphorylated protein (upper panel) was specifically detected by the Pro-Q™ Diamond phosphoprotein gel stain technique, while total protein (lower panel) was visualized by the SYPRO® Ruby staining method. Phosphorylated CpxR is indicated by a double asterisk and unphosphorylated CpxR by a single asterisk.

It is possible that substitution at Asp51 may cause an extreme allosteric effect that prevents CpxR phosphorylation at an alternative site. As phosphorylation on Asp residues is considered to be unstable, our initial approach had the goal to reduce phosphorylated Asp51 of CpxR-P using sodium borohydride to a stable homoserine [33,34] and to confirm this conversion by mass spectrometry. However, the yield of this reaction was very low so the approach was not continued. Instead, in *vitro* phosphorylation of CpxR was confirmed by intact mass determinations using ESI-MS (Figure S1). These experiments showed that the major product of *in vitro* phosphorylation of CpxR carried only one phospho

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**Table 1. Summary of mass determination of CpxR and CpxR-P by ESI-MS.**

| Sample | Experimental mass | Theoretical mass |
|--------|-------------------|-----------------|
| CpxR   | 27561 ± 4         | 27560 (unmodified) |
|        | 27564 ± 3         |                 |
|        | 27556 ± 4         |                 |
| CpxR-P  | 27640 ± 3         | 27640 (singularly phosphorylated) |
|        | 27641 ± 2         |                 |

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Having established that CpxR-P carries only one phospho-group that is likely to be at Asp51, we now wanted to gain insight into the role of CpxR-P in virulence gene regulation in *Yersinia*. In a previous study, we noted that the expression of genes encoding the potential adhesins All (AIL – YPTB2867) and Ail-like (YPTB2113), as well as the confirmed adhesins pH6 antigen (psaA) and invasin (invC), are all influenced by loss of CpxA [20]. We made use of the purified CpxRwt and CpxRD51A His6-tagged variants derived from *Yersinia* CpxR and performed an EMSA to examine if CpxR-P bound to radiolabeled PCR-amplified DNA control regions upstream of these *Yersinia* virulence genes. All regions of amplified target DNA included a minimum of ~300 bp group (Table 1). This is consistent with the model that Asp51 is the principal phosphorylation site of CpxR. In addition, the intact mass determination showed that CpxR-P was sufficiently stable to be analyzed without converting Asp51 to homoserine by sodium borohydride reduction.

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**Figure 1. *In vitro* phosphorylation of CpxR by acetyl-P.** Wild type CpxR (CpxRwt::His6) was purified by metal affinity chromatography. Purified protein (1.25 μM) was incubated in sequentially increasing concentrations of acetyl-P (AP). Samples were fractionated on a Manganese(II)-Phos-tag™ 12.5% acrylamide gel and then visualized by the SYPRO® Ruby staining method. Lanes: a, no addition of AP; b, 6.25 mM AP; c, 12.5 mM AP; d, 25 mM AP; e, 50 mM AP; f, 100 mM AP. Phosphorylated CpxR is indicated by a double asterisk and unphosphorylated CpxR by a single asterisk. doi:10.1371/journal.pone.0023314.g001

**Figure 2. Phosphorylation of the CpxR residue Asp51.** Wild type (CpxRwt::His6) and defined variants with the amino acid substitutions Asp51Ala (CpxR2312::His6) and Met199Ala (CpxR2312::His6) were purified by metal affinity chromatography. Purified proteins (1.25 μM) were incubated in the presence of 50 mM acetyl-P (AP) (+). In A, samples were fractionated by a Manganese(II)-Phos-tag™ 12.5% acrylamide gel and then stained by the SYPRO® Ruby staining method. In B, samples were separated in a conventional SDS 12% polyacrylamide gel. Phosphorylated protein (upper panel) was specifically detected by the Pro-Q™ Diamond phosphoprotein gel stain technique, while total protein (lower panel) was visualized by the SYPRO® Ruby staining method. Phosphorylated CpxR is indicated by a double asterisk and unphosphorylated CpxR by a single asterisk. The reason for the enhanced mobility of CpxR2312::His6 when fractionated by Manganese(II)-Phos-tag™ acrylamide gel (Panel A) is uncertain. doi:10.1371/journal.pone.0023314.g002
containing 1.5 single stranded DNA. EMSAs were further controlled by a reaction specificity was aided by the constant presence of BSA and non-specific performances in the presence of the phosphodonor acetyl-

ail.

ail, amounts of CpxR

ail retardation of the hot template (the unbound signal at the bottom bound to all available free DNA resulting in the complete background binding noise and are not believed to represent bona fide binding targets of CpxR cases, the extent of mobility shifted DNA was dependent on the available CpxR

ail which excess ‘cold’ DNA template competed with ‘hot’ DNA for each amplified PCR fragment is given in parentheses. Where indicated, the purified CpxR-His tagged variants were incubated with ‘hot’ DNA templates at the concentrations of 0.75 and 1.5 μM. All reactions were performed in the presence of the phosphodonor acetyl—P and binding specificity was aided by the constant presence of BSA and non-specific single stranded DNA. EMSAs were further controlled by a reaction containing 1.5 μM BSA instead of CpxR or competition reactions in which excess ‘cold’ DNA template competed with ‘hot’ DNA for available CpxR—P. The electrophoretic mobility of the ‘hot’ DNA fragments in the absence of bound protein is highlighted by an arrow, while DNA-CpxR—P complexes are signified with an asterisk (*). In some cases, the extent of mobility shifted DNA was dependent on the CpxR—P concentration. Two asterisks (**) indicate residual non-specific background binding noise and are not believed to represent bona fide binding targets of CpxR—P.

doi:10.1371/journal.pone.0023314.g003 up to 40 bp downstream of the translational start codon. Our experiments were controlled by incorporating DNA encompassing the cpxR/cpxP divergent promoter and the ppiA promoter, both of which are known to bind CpxR—P either from Y. pseudotuberculosis [20] or E. coli [12,17,42]. We also included the rovA gene encoding the transcriptional activator of invasin [22,23], and the psaE gene, which encodes a regulator for pH6 antigen expression [43]. As anticipated from data obtained in our earlier study [20], in vitro phosphorylated CpxRwt bound the promoters of inv and rovA, as well as those known CpxR—P regulon members ppiA and cpxR/cpxP (Figure 3). Interestingly, at least two distinct migration shifts of rovA, ppiA and cpxR/cpxP template was observed when using different concentrations of CpxR—P. This could suggest that these particular promoters contain multiple CpxR—P binding sites of differential affinity so that several CpxR molecules bind cooperatively. This would be reminiscent of how CpxR—P is thought to bind the promoter of csgD involved in curli biogenesis in E. coli [44]. Here, we also demonstrate for the first time a mobility shift of DNA specific to the promoter regions of psaA and psaE. Crucially, identical unlabeled (‘cold’) template DNA could compete for CpxR—P binding. Although the degree of successful competition varied for each template, the addition of cold DNA did reduce the amount of radiolabeled (‘hot’) cpxR/P, ppiA, rovA, inv, psaA and psaE template DNA being retarded in migration during electrophoresis (Figure 3). Quite possibly the ail (YPBT2867) promoter also represents a target of CpxR—P considering that the higher CpxR—P concentration (1.5 μM) bound to all available free DNA resulting in the complete retardation of the hot template (the unbound signal at the bottom of the gel disappears). This is distinct from the reduced amount of ail-like (YPBT2113) promoter template shifted by equivalent amounts of CpxR—P. In this case, significant signal representing free unbound ail-like hot template still exists at the bottom of the gel (Figure 3). In fact, this degree of ‘non-specific’ CpxR—P binding was also observed in the negative control represented by an internal fragment of cpxR encompassing the nucleotides +32 through to +420 downstream of the translational start (Figure 3). Finally, binding by the non-phosphorylated CpxRΔ51A variant was not observed in our assay conditions as evidenced by the absence of a migration shift for any hot DNA template (Figure 3). Taken together, this demonstrates that CpxR phosphorylation at position 51 is required for direct and efficient binding of CpxR—P to the rovA, inv, psaA and psaE (and possibly also the ail) virulence gene finally, binding by the non-phosphorylated CpxRΔ51A variant was not observed in our assay conditions as evidenced by the absence of a migration shift for any hot DNA template (Figure 3). Taken together, this demonstrates that CpxR phosphorylation at position 51 is required for direct and efficient binding of CpxR—P to the rovA, inv, psaA and psaE (and possibly also the ail) virulence gene

Figure 3. Phosphorylation-dependent binding of CpxR to DNA upstream of Yersinia virulence genes. Mobility shift assays were performed with purified CpxRwt::His6 and the non-phosphorylated mutant CpxRΔ51A::His6. Target DNAs were radiolabeled PCR fragments harboring the regulatory regions of cpxR/cpxP, ppiA, rovA, inv, ail (YPBT2867), ail-like (YPBT2113), psaA and psaE. An internal fragment of the cpxR gene was used as a negative control. The approximate size of each amplified PCR fragment is given in parentheses. Where indicated, the purified CpxR-His tagged variants were incubated with ‘hot’ DNA templates at the concentrations of 0.75 and 1.5 μM. All reactions were performed in the presence of the phosphodonor acetyl—P and binding specificity was aided by the constant presence of BSA and non-specific single stranded DNA. EMSAs were further controlled by a reaction containing 1.5 μM BSA instead of CpxR or competition reactions in which excess ‘cold’ DNA template competed with ‘hot’ DNA for available CpxR—P. The electrophoretic mobility of the ‘hot’ DNA fragments in the absence of bound protein is highlighted by an arrow, while DNA-CpxR—P complexes are signified with an asterisk (*). In some cases, the extent of mobility shifted DNA was dependent on the CpxR—P concentration. Two asterisks (**) indicate residual non-specific background binding noise and are not believed to represent bona fide binding targets of CpxR—P.

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Our experiments were controlled by incorporating DNA encompassing the cpxR/cpxP divergent promoter and the ppiA promoter, both of which are known to bind CpxR—P either from Y. pseudotuberculosis [20] or E. coli [12,17,42]. We also included the rovA gene encoding the transcriptional activator of invasin [22,23], and the psaE gene, which encodes a regulator for pH6 antigen expression [43]. As anticipated from data obtained in our earlier study [20], in vitro phosphorylated CpxRwt bound the promoters of inv and rovA, as well as those known CpxR—P regulon members ppiA and cpxR/cpxP (Figure 3). Interestingly, at least two distinct migration shifts of rovA, ppiA and cpxR/cpxP template was observed when using different concentrations of CpxR—P. This could suggest that these particular promoters contain multiple CpxR—P binding sites of differential affinity so that several CpxR molecules bind cooperatively. This would be reminiscent of how CpxR—P is thought to bind the promoter of csgD involved in curli biogenesis in E. coli [44]. Here, we also demonstrate for the first time a mobility shift of DNA specific to the promoter regions of psaA and psaE. Crucially, identical unlabeled (‘cold’) template DNA could compete for CpxR—P binding. Although the degree of successful competition varied for each template, the addition of cold DNA did reduce the amount of radiolabeled (‘hot’) cpxR/P, ppiA, rovA, inv, psaA and psaE template DNA being retarded in migration during electrophoresis (Figure 3). Quite possibly the ail (YPBT2867) promoter also represents a target of CpxR—P considering that the higher CpxR—P concentration (1.5 μM) bound to all available free DNA resulting in the complete retardation of the hot template (the unbound signal at the bottom of the gel disappears). This is distinct from the reduced amount of ail-like (YPBT2113) promoter template shifted by equivalent amounts of CpxR—P. In this case, significant signal representing free unbound ail-like hot template still exists at the bottom of the gel (Figure 3). In fact, this degree of ‘non-specific’ CpxR—P binding was also observed in the negative control represented by an internal fragment of cpxR encompassing the nucleotides +32 through to +420 downstream of the translational start (Figure 3). Finally, binding by the non-phosphorylated CpxRΔ51A variant was not observed in our assay conditions as evidenced by the absence of a migration shift for any hot DNA template (Figure 3). Taken together, this demonstrates that CpxR phosphorylation at position 51 is required for direct and efficient binding of CpxR—P to the rovA, inv, psaA and psaE (and possibly also the ail) virulence gene...
promoters. On the other hand, modulation of expression of the Ail-like homologue (YPTB2113) by CpxR–P is apparently not via direct binding, but must presumably involve at least one other unknown regulatory intermediate.

Mapping the DNA binding site of CpxR–P in the rovA promoter

RovA is a global regulator of Yersinia gene expression, being responsible for fine-tuning expression of a multitude of genes involved in general housekeeping, environmental survival and pathogenicity [24, 25, 26]. Levels of RovA need to be strictly controlled; dissecting these control mechanisms will therefore benefit our general understanding of how this pathogenic bacterium responds and adapts to its prevailing environment. In order to determine how CpxR–P controls the levels of rovA transcription, we performed a nuclease protection (footprinting) analysis to map the DNA binding site of CpxR–P in the rovA promoter. A protected sequence of the sense strand estimated to be 3′-gggtgctaagaattctagaaatctagtaaattgac-5′ was identified within the P2 promoter region of rovA (Figure 4). We could also identify protected regions in the sense strand of ppiA promoter (5′-ttctgtaagatattagtaagattaagttgggt-3′) and the divergent cpxR-cpxP promoter (5′-gctagggcatgtaaagctga-3′) (Figure 4). As expected, a control sequence residing downstream of the cpxR start codon was not protected from DNase1 digestion. In E. coli, the consensus CpxR–P DNA binding sequence is represented by 5′-GTAAA(N)4–8GTAAA-3′ [4, 42]. We manually inspected these DNase1 protected sequences for consensus CpxR–P DNA binding motifs. A poorly conserved putative CpxR–P binding site may lie in the protected region derived from the rovA promoter, while more obviously conserved consensus CpxR–P binding motifs were observed in the protected sequence upstream of ppiA and between cpxP/cpxR (Figure 4).

Based on the analysis of these ‘footprints’, we used sited-directed mutagenesis to shuffle the order of the nucleotides predicted to compose the CpxR–P binding sites upstream of ppiA, cpxR (and cpxP) and rovA (Figure 5A). Since the CpxR–P binding site upstream of rovA might overlap with the −35 box of the P2

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**Figure 4. Mapping the CpxR–P DNA binding site upstream of rovA by nuclease protection (footprinting) analysis.** DNase I footprinting assays were performed to investigate the binding of CpxR–P to a region within the rovA, ppiA and cpxR-cpxP promoters. These 314 base pair (bp), 276 bp and 245 bp fragments respectively, were labeled on the sense strand before being incubated with CpxR–P at the following final concentrations: 50 nM, lane c; 100 nM, lane d; 200 nM, lane e; 400 nM, lane f. The absence of CpxR–P in lanes a and b is indicated by ‘–’. Reactions were resolved by denaturing PAGE and analyzed with a Molecular Dynamics PhosphorImager. Labeled pBR322 DNA digested with MspI (New England Biolabs) was used as a size marker (lane a). An estimation of the protected sequence is given on the right hand side of the panels. Based upon the E. coli consensus sequence of 5′-GTATA(N)4–8GTATA-3′, a putative CpxR–P consensus binding site is highlighted in a gray box. A labeled internal fragment (389 bp) within the cpxR open reading frame served as a non-protected control.

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promoter [45], we generated two scramble mutants in this region. The first was designated Mt 1, which included alteration of the −35 box sequence. In contrast, the second mutant (Mt 2) left the −35 box sequence intact. Significantly, an EMSA revealed a clear reduction in the retardation of PCR amplified fragments of the mutagenized DNA by CpxR, (Figure 5B; designated 'Mt'). Collectively, these data indicate that the sequence 5'-ACAAA(-N)5ACAAA-3' overlapping with the −35 region within promoter P2, a second mutation ('Mt 2') was performed in which the −35 region was left untouched. (B) Mobility shift assays with purified CpxR<sub>wt</sub>::His<sub>6</sub> as outlined in the legend to Figure 3 were performed on radiolabeled amplified DNA from these mutated templates. Once again, the electrophoretic mobility of the 'hot' DNA fragments in the absence of bound protein is highlighted by an arrow, while DNA-CpxR<sub>−P</sub> complexes are signified with a single asterisk (*)

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Figure 5. Site-directed mutagenesis of the CpxR<sub>−P</sub> DNA binding site upstream of rovA. (A) As identified by DNase 1 footprinting (see Figure 4), the potential CpxR<sub>−P</sub> binding site sequence and position is shown relative to each ATG start codon within the regulatory regions of rovA and ppiA and the divergent cpxR/cpxP promoter (designated as 'Wt'). Site-directed mutagenesis was performed to 'shuffle' the nucleotide sequence of each potential CpxR<sub>−P</sub> binding site (designated as 'Mt'). Since the initial mutation in the rovA sequence ('Mt 1') would potentially disrupt RNA polymerase binding because of an altered −35 region within promoter P2, a second mutation ('Mt 2') was performed in which the −35 region was left untouched. (B) Mobility shift assays with purified CpxR<sub>wt</sub>::His<sub>6</sub> as outlined in the legend to Figure 3 were performed on radiolabeled amplified DNA from these mutated templates. Once again, the electrophoretic mobility of the 'hot' DNA fragments in the absence of bound protein is highlighted by an arrow, while DNA-CpxR<sub>−P</sub> complexes are signified with a single asterisk (*)

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In vivo accumulation of CpxR<sub>−P</sub> in the Yersinia cytoplasm reduces levels of RovA

We predict that binding of CpxR<sub>−P</sub> to the rovA promoter restricts transcription. Therefore, elevating available CpxR<sub>−P</sub> in the Yersinia cytoplasm in turn should reduce detectable RovA levels. To test this, we took advantage of work performed in E. coli, whereby phosphatase deficient CpxA<sub>T253P</sub> encoded by the allelic variant designated cpxA<sup>101</sup>* generally lead to a constitutively active Cpx pathway [5,14]. Although never directly tested, such a mutant should accumulate unusually high levels of CpxR<sub>−P</sub>. To test this in Yersinia, we constructed an in <i>cis</i> cpxA<sup>101</sup>* mutation in <i>Y. pseudotuberculosis</i>. We then utilized the Manganese II-Phos-tag<sup>TM</sup> acrylamide gel system to measure <i>in vivo</i> levels of CpxR<sub>−P</sub>. Formic
therefore reverse the build-up of CpxR, function D in the accumulated CpxR on a Manganese(II)-Phos-tagTM 12.5% acrylamide gel and blotted with bacteria were lysed with formic acid and samples rapidly fractionated was used to measure doi:10.1371/journal.pone.0023314.g006 double asterisk (**) indicates phosphorylated CpxR accumulated in the The single asterisk (*) reflects non-phosphorylated CpxR, while the YPIII51/pIB102; mutated cpxR encoding CpxR51A, YPII52/pIB102; mutated cpxR encoding CpxR51A, YPII56/pIB102; cpxR null mutant, YPII08/pIB102. The single asterisk (*) reflects non-phosphorylated CpxR, while the double asterisk (**) indicates phosphorylated CpxR accumulated in the Yersinia cytoplasm. doi:10.1371/journal.pone.0023314.g006 acid-lysed bacteria grown in LB broth to early stationary phase (OD600 in the range of 0.85 to 0.95) at 26°C were fractionated on a Manganese(II)-Phos-tagTM 12.5% acrylamide gel and blotted with affinity purified anti-CpxR antiserum. Strains: parent, YPIII/pIB102; ackA, pta null mutant, YPII69/pIB102; cpxA null mutant, YPII07/pIB102; cpxA, ackA, pta null mutant, YPII49/pIB102; cpxA101* encoding CpxR51, YPII15/pIB102; cpxA101*, ackA, pta null mutant, YPII74/pIB102; mutated cpxR encoding CpxR51A, YPII52/pIB102; mutated cpxR encoding CpxR51A, YPII56/pIB102; cpxR null mutant, YPII08/pIB102. The single asterisk (*) reflects non-phosphorylated CpxR, while the double asterisk (**) indicates phosphorylated CpxR accumulated in the Yersinia cytoplasm.

Elevated levels of the NlpE lipoprotein reduce invasin and RovA levels

Examination of the Cpx response in the absence of a functioning CpxA protein is artificial. We therefore wanted to examine the repressive effect of CpxR–P on RovA production following activation of the Cpx response in which the signal transduction cascade is still intact. Simulating natural Cpx TCRS responsiveness has been achieved by the over-production of the E. coli NlpE (NlpEE.c) lipoprotein [50,51]. We therefore transformed parent and CpxR defective Yersinia with pBAD18 derivatives encoding E. coli nlpE (termed pND10) or Y. pseudotuberculosis nlpE (NlpEYP – pJF027) under the control of an arabinose inducible promoter. Native NlpEYP shares less than 50% identity at the amino acid level with NlpEE.c. Therefore, we considered that native NlpE could possibly be a better inducer of Cpx pathway activation in Yersinia. Bacteria were grown in the presence or absence of arabinose, and production of RovA and invasin was examined. Over-production of either NlpEE.c or NlpEYP in parental Yersinia caused a reduction in RovA and invasin production that was dependent on functional CpxR, because neither lipoprotein caused this decrease in the ΔcpxR null mutant (Figure S3B). Interestingly, NlpEYP appeared to restrict RovA and invasin production to a greater degree. These data reinforce how Cpx pathway activation and the presence of CpxR–P can restrict virulence factor production in Y. pseudotuberculosis.

CpxR–P DNA binding is required for RovA repression in vivo

We have identified a CpxR–P binding site in the regulatory regions of three genes of Yersinia: ptdA, cpxP/cpxR and ackA. We...
have also shown the accumulated CpxR→P levels result in restricted RovA production. Therefore, we wondered whether RovA repression by CpxR→P could be relieved by modulating the CpxR→P binding site within the rovA promoter. The same rovA ‘shuffle’ mutations (Mt 1 and Mt 2) used to identify CpxR→P binding in vitro (see Figure 5) were introduced in cis into the chromosome of the cpa101* mutant of Y. pseudotuberculosis. This background was chosen because the Cpx pathway is constitutively active, CpxR→P accumulation is relatively high and the integrity of the cpxRI transcriptional unit remains intact. Bacteria were grown to early-stationary phase and the extent of RovA production was examined by immunoblot. RovA levels were essentially undetectable in the cpxA101* mutant, YPIII69/pIB102; ΔcpxA null mutant, YPIII07/pIB102; ΔcpxA, ΔackA, pta null mutant, YPIII49/pIB102; cpxA101* encoding CpxR→P, YPIII12/ pIB102; cpxA101*, ΔackA, pta null mutant, YPIII74/pIB102. Molecular weights shown in parentheses are deduced from primary sequence. The single asterisk (*) reflects typical invasin degradation products, while two asterisks (**) indicates a non-specific protein band recognized by the anti-RovA antiserum that also serves as a convenient loading control.

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Figure 7. Cpx pathway activation influences the production of the RovA global regulator. RovA and invasin production was determined from protein isolated from early stationary phase bacteria grown in LB broth at either 26 C or 37 C. Protein was separated on a 12% SDS-PAGE and then identified by immunoblot analysis using polyclonal rabbit antiserum raised against RovA or invasin. Strains: parent, YPIII/pIB102; ΔackA, pta null mutant, YPIII69/pIB102; ΔcpxA null mutant, YPIII07/pIB102; ΔcpxA, ΔackA, pta null mutant, YPIII49/pIB102; cpxA101* encoding CpxR→P, YPIII12/pIB102; cpxA101*, ΔackA, pta null mutant, YPIII74/pIB102. Molecular weights shown in parentheses are deduced from primary sequence. The single asterisk (*) reflects typical invasin degradation products, while two asterisks (**) indicates a non-specific protein band recognized by the anti-RovA antiserum that also serves as a convenient loading control.

Pathogenic bacteria experience diverse environmental conditions both inside and outside of a host. Multiple regulatory pathways are required to incorporate these diverse signals that then converge to fine tune spatial and temporal virulence gene expression. Herein, we report that the effect of CpxA-CpxR pathway activation in the enteropathogen Y. pseudotuberculosis is two-fold; not only does CpxR→P serve to directly up-regulate the expression of genes encoding for periplasmic quality control factors, but it also down-regulates genes encoding virulence associated determinants – especially the global regulator RovA. In Y. pseudotuberculosis, the CpxA-CpxR TCRS therefore serves to coordinate the maintenance of bacterial envelope integrity with a reduction in virulence gene expression to ensure that vital energy resources are used to maximize the chances of survival in the prevailing environment.

How can CpxR→P both induce and repress the expression of target genes in pathogenic Yersinia? A review of studies on other OmpR/PhoB family members provides some insight into the many assorted possibilities. CpxR→P binding to target DNA may physically recruit the RNA polymerase. This principle is well-established for the phosphate starvation regulon, whereby PhoB→P facilitates specific interactions between RNA polymerase and the pho promoters [52,53]. CpxR→P binding to target DNA may also alter DNA curvature that in turn modulates exposure of additional DNA binding sequences to provide access to secondary transcriptional activators or antagonistic repressors. This is no better illustrated than by the reciprocal interactions of OmpR→P and integration host factor within the regulatory region of the ompF porin gene induced by changes in osmolarity [54,55]. The orientation or the proximity of the CpxR→P binding site relative to the transcriptional start may also influence activation or repression, especially if this influences CpxR→P positioning with respect to the RNA polymerase or the binding sites for other activators or repressors. A strong correlation between genes under heavy CpxR-regulatory influence and the positioning of CpxR→P
binding sites within 100 base pairs upstream of the transcription start supports this notion [3]. Differences in relative binding affinities or the actual number of distinct binding sites within each promoter could also determine the degree of CpxR, P influence. This has been used to explain the inverse regulation of the porin genes ompC and ompF by OmpR, P [56]. It is also plausible that intrinsic promoter activity may influence CpxR, P regulatory control. Indeed, such a mechanism may in part determine whether the RcaC response regulator activates or represses transcription of genes encoding products of cyanobacterial photosynthetic light harvesting antenna in response to ambient light color changes [57]. At this stage the mechanisms of CpxR, P action are unclear, but future investigation will aim to reveal whether any of these complex scenarios dictate how CpxR, P mediates both activation and repression of Yersinia gene expression.

Response regulator activation is thought to first require phosphorylation for the triggering of dimerization that is necessary for target DNA binding [58]. However, it is apparent that among the OmpR/PhoB subfamily, some members can bind DNA in the absence of initial phosphorylation. In fact, non-phosphorylated OmpR is proposed to first bind to DNA as a monomer [34,59]. Phosphorylation then occurs to promote dimerization and facilitate DNA binding by the second monomer [59,60]. The CovR response regulator of virulence and stress survival gene expression in Streptococcus spp. can also bind DNA as a non-phosphorylated monomer, although binding is enhanced by phosphorylation-dependent dimerization [61]. It is also notable that PhoP, a response regulator of Salmonella enterica that senses environmental Mg²⁺, dimerizes and binds DNA independent of phosphorylation [62]. In our own study, the non-phosphorylated CpxRD51A variant was not able to bind DNA under the assay conditions used here. However, our previous results suggested the possibility of weak binding when nonphosphorylated CpxR was used in EMSAs with rovA and inv [20]. Thus, while phosphorylation significantly enhances CpxR binding to target DNA, we are unable to unequivocally conclude that binding cannot occur without it.

Mechanisms of rovA transcriptional regulation are complex (Figure S4). It chiefly involves a thermoregulated auto-amplification loop that overcomes H-NS/YmoA mediated silencing [22,45,63,64]. However, an additional layer of positive regulation might incorporate LeuO, a LysR-like regulator [63], while negative regulation does involve another LysR-type protein, RovM [63,65]. This negative regulatory loop probably senses nutritional status since its output is refined by participation of a carbon storage regulator system controlling rovM expression [66]. In addition, RovA activity is affected post-transcriptionally; temperature-dependent conformational changes render RovA less able to bind to target DNA sequences and to resist proteolysis by endogenous proteases [67]. Our data demonstrates another layer
of RovA control; as a result of sensing ECS, accumulated CpxR–P represses rovA transcription by a mechanism that involves direct binding to the rovA promoter. We will endeavor to pry apart this mechanism in order to understand its mechanism of the Cpx pathway. NlpE lipoprotein over-production, either from E. coli (NlpE<sub>E</sub>) or Y. pseudotuberculosis (NlpE<sub>P</sub>) to stimulate an intact Cpx pathway. NlpE<sub>E</sub> and NlpE<sub>P</sub> are about 49% identical at the amino acid level. Even though both proteins activated the Cpx pathway, NlpE<sub>E</sub> did so to a greater extent. Exactly how the Cpx pathway is induced by NlpE is unknown, but it likely involves sensing of bacterial adhesion to surfaces [70]. Relay of this signal probably involves one or more structural changes in NlpE caused by external environmental stresses imposed during the adhesion process [71,72]. Presumably this is mimicked in part by NlpE over-expression; an activating signal may result from erroneously assembled NlpE no longer transported to the outer membrane by the Lol system [71,73]. Whatever the mechanism, it is striking for its molecular conservation and specificity. Of all the many lipoproteins produced by E. coli, only the over-expression of NlpE and one other – the inner membrane located YaaY of unknown function – caused Cpx pathway activation [73]. Thus, comparative structure-function analyses of NlpE<sub>E</sub> and NlpE<sub>P</sub> could potentially provide important insight into the activating mechanism of the Cpx pathway.

In an effort to provide experimental evidence for a phosphorylation of CpxR on Asp<sub>51</sub>, peptides were generated by fast enzymatic digestion. Commonly used proteases such as trypsin, chymotrypsin, and the endoproteinasises AspN and GluC, were not suitable to generate peptides for a study of Asp<sub>51</sub>. All produced theoretically predicted Asp<sub>51</sub>-containing peptides with masses above 4000, which is relatively high for successful structural analyses by tandem mass spectrometry (MS/MS). Nevertheless, a protocol for fast digestion with trypsin and pepsin was developed. While MALDI-MS and ion trap MS combined with ESI and ETD ionization [74] both confirmed the identity of digested CpxR and CpxR–P with high confidence, no peptides containing phosphorylated Asp<sub>51</sub> or any other phosphorylation sites were detected (data not shown). This was even the case after enrichment of phosphopeptides using titanium dioxide [37,38,39]. Thus, we were unable to analyze phosphorylation of CpxR–P by mass spectrometry. In part, this can be explained by the inability to
generate specific peptides in the optimal mass range between 1200 and 2000 that would facilitate MS analysis.

In this study we have described how CpxR–P accumulation has a direct negative effect on rovA expression in Yersinia. RovA is a global regulator targeting in excess of 60 genes in each of Y. enterocolitica and Y. pestis [24,25]. As several of these genes may represent novel virulence determinants in these bacteria, the direct and indirect impact of Cpx pathway activation on Yersinia pathogenicity can be widespread. We see CpxR–P as an ‘override mechanism’ capable of a quick response to repress virulence gene expression in over-committed, fully-induced bacteria that suddenly find themselves in an unfavorable environment exposed to ECS where virulence factors are no longer useful for their survival – to continue to express them would waste precious energy resources.

Supporting Information

Table S1  Bacterial strains and plasmids used in this study. (RTF)

Table S2  Oligonucleotides used in this study. (RTF)

Figure S1  Mass determination of intact CpxR and CpxR–P by MALDI-MS. The respective CpxR and CpxR–P peaks are indicated along with the experimental mass (Daltons). (TIF)

Figure S2  Growth curves of various Y. pseudotuberculosis bacteria. Overnight cultures of parental and mutant bacteria were subcultured into fresh LB broth (time point 0 hours) and their growth during aerobic culturing with agitation at 26°C was monitored over a period of 9 hours by optical density measurement at 600 nm (A and B). Parent, YPIII/pBI102; YPIII07, ΔcpxA; YPIII08, ΔcpxR; YPIII06, mutated qpxA encoding CpxRM196A; YPIII49, ΔcpxA, ackA, pta null mutant; YPIII51, cpxA101* encoding CpxAR253P; YPIII52, mutated cpxA encoding CpxR253A; YPIII09, ΔackA, pta null mutant; YPIII14, cpxA101* encoding CpxAR253P, YPIII15, mutated cpxA encoding CpxR253A; YPIII113, ΔackA, pta null mutant; YPIII174, cpxA101*, ackA, pta null mutant; YPIII112, qpxA/qpxPΔ601 placed in the parent background; YPIII114, qpxA/qpxPΔ601 1 placed in the parent background; YPIII115, qpxA/qpxPΔ601 2 placed in the parent background; YPIII111, qpxA/qpxPΔ601 1 placed in the parent background; YPIII112, qpxA/qpxPΔ601 1 placed in the cpxA101* background; YPIII110, rovAΔ25/rovAΔ25 placed in the cpxA101* background; YPIII112, rovAΔ25/rovAΔ25 1 placed in the cpxA101* background. The asterisk (*) signifies modest to significant (indicated by a ‘+’ symbol) regulatory links to other positive (green) and negative (red) factors controlling rovA expression [22,23,45,64,65,66,75,76,77]. Recently, H-NS was described to be a part of the CpxR–P regulon [2]. However, our in vitro electrophoretic mobility shift analysis did not reveal any CpxR–P bound to the hns or ymdA promoters (Liu and Francis, unpublished). Not shown in this diagram is the influence of CpxR–P on lom expression [78], but this connection is still relevant given how RovA is subject to proteolysis by the Lon protease [67]. Elevated CpxR–P levels also diminishes efficient T3S and the production of other ‘non-invasin’ adhesins by a mechanism(s) that are not yet understood (red dotted line). (TIF)

Figure S3  CpxR–P DNA binding is required for repression of rovA in vivo. For transcription analysis, crude semi-quantitative RT-PCR was performed on mRNA isolated from Y. pseudotuberculosis. Given the limitations of semi-quantitative RT-PCR, we attempted to increase the robustness of our assay by reverse transcribing mRNA isolated from bacteria grown at 26°C in LB broth to two different growth phases as measured by optical density at 600 nm; the first being an OD600 value between 0.45 to 0.55 (mid-logarithmic phase) and the second being an OD600 measurement between 0.85 to 0.95 (early-stationary phase). This was necessary to control for the altered growth rate of those bacteria expected to accumulate toxic levels of CpxR–P (Figure S2B). Samples were subjected to RT-PCR with primers specific for rovA (plus RT). As an mRNA loading control, we analyzed the transcription of rpoA encoding the σ70-subunit of RNA polymerase, which remained the same regardless of genetic background or phase of growth. To confirm the purity of the RNA isolated, PCRs with rpoA and ms primers were performed on template derived from RT reactions in which the enzyme was intentionally excluded (minus RT). PCR analysis on these samples indicated that the RNA isolation was essentially free of genomic DNA contamination. All images were acquired with a Fluor-S Multimager (Bio-Rad) and analyzed with Quantity One software version 4.2.3 (Bio-Rad). DNA fragment sizes in base pairs are given in parentheses. Strains: parent, YPIII/pBI102; cpxA101* encoding CpxAR253P, YPIII15/pBI102; cpxR/cpxPΔ601 (shuffle mutation of the CpxR–P binding site identified in Figure 4) placed in the cpxA101* background, YPIII1115/pBI102; rovAΔ25 1 placed in cpxA101*, YPIII120/pBI102; rovAΔ25 2 placed in cpxA101*, YPIII121/pBI102. (TIF)

Figure S4  Established and potential mechanisms of Cpx-dependent modulation of rovA and ms expression. The Cpx pathway is a sensor of extracytoplasmic stress (ECS) [1]. However, the role of CpxR–P as a central regulator of Y. pseudotuberculosis pathogenicity is also becoming apparent (this study) [19,20]. CpxR–P levels in the bacterial cytoplasm are manipulated by cognate CpxA kinase and phosphatase activity. This is even further affected by the CpxA-independent phosphorylation of CpxR via second messenger phosphodonor; the levels of which are somehow influenced by an intact AckA/Pta pathway. In turn, CpxR–P binds directly to the rovA and ms promoters repressing transcriptional output (red line). This influence may also be augmented indirectly through as yet unknown (indicated by a dashed line and a '?' symbol) regulatory links to other positive (green) and negative (red) factors controlling rovA and/or ms expression [22,23,45,64,65,66,75,76,77]. Recently, H-NS was described to be a part of the CpxR–P regulon [2]. However, our in vitro electrophoretic mobility shift analysis did not reveal any CpxR–P bound to the hns or ymdA promoters (Liu and Francis, unpublished). Not shown in this diagram is the influence of CpxR–P on lom expression [78], but this connection is still relevant given how RovA is subject to proteolysis by the Lon protease [67]. Elevated CpxR–P levels also diminishes efficient T3S and the production of other ‘non-invasin’ adhesins by a mechanism(s) that are not yet understood (red dotted line). (TIF)

Figure S5  Regulator binding sites in the upstream flanking sequence of rovA. Y. pseudotuberculosis rovA transcription is initiated from two sites (P1 and P2) upstream of the translational start codon (TTG – green) [15]. DNA sequences flanking P1 and P2 serve as binding sites for an array of regulators including H-NS (purple), RovM (dark blue) and RovA (red) [45,65]. We have now shown herein that CpxR–P (brown) also binds to DNA sequences that incorporate the −35 region of the P2 promoter. Based upon the binding site in the −35 region however, CpxR–P is expected to prevent access to the P2 promoter by the RNA polymerase holoenzyme. It is not yet known if or how CpxR–P binding influences the binding of the other rovA DNA-binding regulators. (TIF)

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References

1. Macrìtche DM, Raivo TL (2009) Envelope stress responses. In: Boek A, Currie III R, Kaper JB, Karp PD, Neidhardt FC, et al. (2009) Escherichia coli and Salmonella Cellular and Molecular Biology, http://www.escolarg.org. Washington, DC.: ASM Press.

2. Bury-Mone S, Noman Y, Reymond N, Barbet R, Jacquet E, et al. (2009) Environmental control of invasin expression in Yersinia enterocolitica. Infect Immun 77: 7724–7733.

3. Dañes PN, Murphy CK, Silhavy TJ (1995) Multicopy suppression of cold-sensitive sec mutants. J Bacteriol 179: 831–839.

4. De Wulf P, McGuire AM, Liu X, Lin EC (2002) Genome-wide profiling of promoter recognition by the two-component response regulator CpxR-P in Escherichia coli. J Biol Chem 277: 26562–26651.

5. Raivio TL, Silhavy TJ (1999) Transduction of envelope stress in Escherichia coli by the Cpx two-component system. J Bacteriol 179: 5263–5272.

6. Isaac DD, Pinkner JS, Huhggen SJ, Silhavy TJ (2003) The two-component system is controlled by amplification and feedback inhibition. J Bacteriol 181: 4389–4390.

7. Boelow DR, Raivo TL (2005) Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxX and the periplasmic protease DegP. J Bacteriol 187: 6622–6630.

8. Volč K (1998) Structural conservation in the CheV superfamily. Biochemistry 37: 11741–11753.

9. Oglová J, Dong JM, De Wulf P, Furlong D, Boyd D, et al. (1998) Aberrant cell division and random FruI ring positioning in Escherichia coli cpxA-P mutants. J Biol Chem 273: 38498–38500.

10. De Wulf P, Lin EC (2000) Cpx two-component signal transduction in Escherichia coli. J Bacteriol 182: 1423–1426.

11. De Wulf P, Kwon O, Lin EC (1999) The Cpx envelope stress response is regulated by CpxA-P levels underpinning Cpx phenotypes. J Bacteriol 181: 1798–1815.

12. Danese PN, Snyder WB, Cosma CL, Carlson JH, Silhavy TJ, Snyder WB (1995) Mutational analysis of the Cpx envelope stress response. J Bacteriol 177: 4121–4130.

13. Danese PN, Snyder WB, Cosma CL, Davis LJ, Silhavy TJ (1995) The Cpx two-component signal transduction system in Escherichia coli. Mol Gen Genet 249: 408–418.

14. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.

15. Cosma CL, Danese PN, Carlson JH, Silhavy TJ, Snyder WB (1995) Mutation analysis of the CpxA gene and for virulence. Mol Microbiol 66: 189–205.

16. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.

17. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.

18. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.

19. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.

20. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.

21. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.

22. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.

23. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.

24. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.

25. De Wulf P, Wolf-Watz H (1984) Molecular cloning of the temperature-inducible CpxA gene and for virulence. Mol Microbiol 66: 189–205.
50. Snyder WB, Davis LJ, Danese PN, Cosma GI, Silhavy TJ (1995) Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. J Bacteriol 177: 4216–4223.

51. Sunharalingam P, Spencer H, Gallant CV, Martin NL (2003) Salmonella enterica serovar typhimurium cdhB is growth phase regulated and involved in relaying Cpx-induced signals. J Bacteriol 183: 432–443.

52. Makino K, Amemura M, Kawamoto T, Kimura S, Shimagawa H, et al. (1996) DNA binding of PhoB and its interaction with RNA polymerase. J Mol Biol 259: 15–26.

53. Makino K, Amemura M, Kim SK, Nakata A, Shimagawa H (1995) Role of the sigma 70 subunit of RNA polymerase in transcriptional activation by activator protein PhoB in Escherichia coli. Genes Dev 7: 149–160.

54. Ramani N, Huang L, Freundlich M (1992) In vitro interactions of integration host factor with the ompF promoter-regulatory region of Escherichia coli. Mol Gen Genet 231: 248–253.

55. Slauch JM, Silhavy TJ (1991) cis-acting elements that control cell cycle- and host factor-dependent transcriptional activation of the Escherichia coli OmpR. J Bacteriol 173: 4039–4048.

56. Yoshida T, Qin J, Egger LA, Inouye M (2000) Transcription regulation of ompF and ompC by a single transcription factor, OmpR. J Biol Chem 275: 17114–17123.

57. Li L, Alvey RM, Bezy RP, Kehoe DM (2008) Inverse transcriptional activities during complementary chromatic adaptation are controlled by the response regulator RcsC binding to red and green light-responsive promoters. Mol Microbiol 68: 295–297.

58. Gao R, Stock AM (2010) Molecular strategies for phosphorylation-mediated regulation of response regulator activity. Curr Opin Microbiol 13: 160–167.

59. Rhee JE, Sheng W, Morgan-LK, Nolet R, Liao X, et al. (2008) Amino acids important for DNA recognition by the response regulator OmpR. J Biol Chem 283: 8664–8677.

60. Ames SK, Frankema N, Kenney LJ (1999) C-terminal DNA binding stimulates constitutive expression of OmpR. J Biol Chem 274: 11792–11797.

61. Gusa AA, Gao J, Stringer V, Churchward G, Scott JR (2006) Phosphorylation of the group A Streptococcal CovR response regulator causes dimerization and promoter-specific recruitment by RNA polymerase. J Bacteriol 188: 4620–4626.

62. Perron-Savard P, De Crescenzo G, Le Moual H (2005) Dimerization and DNA binding of the Salmonella enterica PhoP response regulator are phosphorylation independent. Microbiology 151: 3979–3987.

63. Lawrence MB, Miller VL (2007) Comparative analysis of the regulation of sucd from the pathogenic yersiniae. J Bacteriol 189: 5963–5975.

64. Tran HJ, Heroven AK, Windler L, Speter T, Beatrix B, et al. (2003) Analysis of RovA, a transcriptional regulator of Yersinia pseudotuberculosis virulence that acts through antirepression and direct transcriptional activation. J Biol Chem 280: 42423–42432.

65. Heroven AK, Dersch P (2006) RovM, a novel LysR-type regulator of the virulence activator gene rcd, controls cell invasion, virulence and motility of Yersinia pseudotuberculosis. Mol Microbiol 62: 1469–1483.

66. Heroven AK, Bohme K, Rohde M, Dersch P (2006) A Car-type regulatory system, including small non-coding RNAs, regulates the global virulence regulator RovA of Yersinia pseudotuberculosis through RovM. Mol Microbiol 68: 1179–1195.

67. Herbst K, Bujara M, Heroven AK, Onoiz W, Weichert M, et al. (2009) Intracellular thermal sensing controls proteolysis of Yersinia virulence regulator RovA. PLoS Pathog 5: e1000435.

68. Labandeira-Rey M, Brautigam CA, Hansen EJ (2010) Characterization of the CpxRA regulon in Haemophilus ducreyi. Infect Immun 78: 4779–4791.

69. Hirano Y, Hosain MM, Takeda K, Tokuda H, Miki K (2007) Structural studies of the Cpx pathway activator NlpE on the outer membrane of Escherichia coli. Structure 15: 963–976.

70. Yamamoto K, Ishihama A (2005) Transcriptional response of Escherichia coli to external copper. Mol Microbiol 56: 215–227.

71. Misyali H, Tanaka-Masuda K, Matsuyama S, Tokuda H (2004) Effects of lipoprotein overproduction on the induction of DegP (HtrA) involved in quality control in the Escherichia coli periplasm. J Biol Chem 279: 39807–39813.

72. Chi A, Huttenthaler C, Geer LY, Coon JJ, Syka JE, et al. (2007) Analysis of phosphorylation sites on proteins from Saccharomyces cerevisiae by electron transfer dissociation (ETD) mass spectrometry. Proc Natl Acad Sci U S A 104: 2193–2198.

73. Miyadai H, Tabata-Masuda K, Nakamura S, Ishihama A (2004) Detection of DegP (HtrA) expression in Yersinia pseudotuberculosis through competition with RovA and interaction with YmoA. J Bacteriol 186: 5101–5112.

74. Ellison DW, Miller VL (2006) H-NS represses in vivo transcription in Yersinia enterocolitica through competition with RovA and interaction with YmoA. J Bacteriol 188: 2193–2198.

75. Ellison DW, Young B, Nelson K, Miller VL (2003) YnoA negatively regulates expression of invasin from Yersinia enterocolitica. J Bacteriol 185: 7153–7159.

76. Brzostek K, Brzostkowska M, Bukowska I, Karwicka E, Raczkowska A (2007) Structural studies of the Cpx regulon destabilizes the F plasmid transfer activator, TraJ, via the HsLU protease in Escherichia coli. Mol Microbiol 67: 516–527.