Inhibition of Acetyl Phosphate-dependent Transcription by an Acetylatable Lysine on RNA Polymerase*

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Background: Phosphorylation and acetylation are ubiquitous post-translational modifications of bacterial proteins.
Results: Glucose-induced cpxP transcription requires acetyl phosphate. This activity is inhibited by lysine 291 of the RNA polymerase α subunit, which becomes acetylated under inhibitory conditions.
Conclusion: Phosphorylation and acetylation co-regulate the cpxP promoter.
Significance: Central metabolism is implicated in the regulation of the stress-responsive promoter cpxP.

The ability of bacteria to adapt to environmental changes has allowed these organisms to thrive in all parts of the globe. By monitoring their extracellular and intracellular environments, bacteria assure their most appropriate response for each environment. Post-translational modification of proteins is one mechanism by which cells respond to their changing environments. Here, we report that two post-translational modifications regulate transcription of the extracytoplasmic stress-responsive promoter cpxP: (i) acetyl phosphate-dependent phosphorylation of the response regulator CpxR and (ii) acetyl coenzyme A-dependent acetylation of the α subunit of RNA polymerase. Together, these two post-translational modifications fine-tune cpxP transcription in response to changes in the intracellular environment.

The response regulator CpxR and its cognate sensor kinase CpxA constitute the two-component signal transduction pathway CpxAR (Fig. 1A). This signal pathway is highly conserved in Enterobacteriaceae and can be found in other proteobacteria. In Escherichia coli, the CpxAR pathway regulates transcription of at least 50 genes (1, 2). The CpxAR pathway also has been implicated in regulation of some virulence factors. In enteropathogenic E. coli and in Legionella pneumophila, the pathway regulates the type IV bundle-forming pilus and some components of type IV secretion, respectively (reviewed in Ref. 3). In Haemophilus ducreyi, it regulates proteins involved in resistance to phagocytosis, affecting the ability of H. ducreyi to infect humans (4).

In response to certain extracytoplasmic signals (e.g. alkaline pH, outer and inner membrane perturbation, surface attachment, or misfolded proteins) (reviewed in Ref. 3), CpxA is thought to autophosphorylate on a conserved histidine residue (His-248), using ATP as its phosphoryl donor. Phosphorylated CpxA then donates its phosphoryl group to a conserved aspartate residue (Asp-51) of CpxR. The latter phosphorylation event regulates CpxR-dependent transcription (5–7) (Fig. 1A).

In the absence of such extracytoplasmic cues (for example, during growth in tryptone broth buffered at pH 7), CpxA functions as an inhibitor of the Cpx pathway. This inhibition is thought to be accomplished by the removal of phosphoryl groups from phospho-CpxR (Fig. 1B) (7, 8), an activity that has been demonstrated in vitro (9, 10). In vivo, the existence of this activity is supported by the apparent accumulation of phospho-CpxR by cpxA mutants of Yersinia pseudotuberculosis (11).

Under such conditions (Fig. 1B), acetyl phosphate (AcP), the intermediate of the Pta-AckA pathway, has been proposed to be the phosphoryl donor to CpxR (5, 12). AcP is often used to phosphorylate response regulators in vitro and much evidence now exists to support the hypothesis that this process contributes to in vivo activation of a subset of those response regulators (reviewed in Ref. 12). For example, the response regulator RcsB can be induced to activate capsule biosynthesis and repress flagellar biogenesis in an AcP-dependent manner (13). Although many behaviors associated with disruption of the Pta-AckA pathway correlate with AcP concentrations, some do not (reviewed in Ref. 12). One such behavior is the CpxA-independent induction of cpxP transcription that occurs when glucose is added to tryptone-based growth media (8, 14, 15). If a behavior strictly depends on AcP to donate its phosphoryl group to a response regulator, then an ackA mutant, which accumulates AcP (16), should elicit behavior opposite to that exhibited by a pta mutant, which cannot synthesize AcP (16). Thus, if glucose-induced cpxP transcription depended strictly on AcP, then an ackA mutant should respond more robustly to glucose than its wild-type (WT) parent, whereas the pta (or pta ackA) mutant should respond more poorly. Although the latter is true (14), the former is not (8); ackA mutants do not elicit the predicted robust response, even though purified CpxR readily autophosphorylates in the presence of AcP (6, 11). This puz-
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FIGURE 1. The two-component response regulator CpxR can become activated by two independent mechanisms. A, activation by the cytoplasmic membrane sensor kinase CpxA. Upon activation by diverse extracytoplasmic signals, CpxA autophosphorylates on a conserved histidine residue, using ATP as its phosphoryl donor. Phospho-CpxA then acts as a phosphoryl donor to the response regulator CpxR, which autophosphorylates on the conserved aspartate residue (Asp-51). By homology to OmpR, phosphorylation of Asp-51 of CpxR is predicted to induce a conformational change that promotes DNA binding, which is predicted to expose its DNA-binding domain, which promotes binding to its target genes (2, 6). B, activation by AcP. In the absence of extracytoplasmic stimuli, CpxA functions as a net phosphatase, removing inorganic phosphate (P_i) from CpxR-P. Under these conditions, CpxR can become phosphorylated using as its phosphoryl donor AcP, the intermediate of the Pta-AckA pathway (5, 8). Phosphotransacetylase (Pta) converts acetyl-coenzyme A (AcCoA) to AcP, whereas acetate kinase (AckA) converts AcP to acetate (44). Bent arrow, cpxP promoter. +1, transcription initiation site.

zling observation could mean that AcP is not the in vivo phosphoryl donor to CpxR. Alternatively, glucose-induced cpxP transcription might be inhibited in an ackA mutant (8).

We previously proposed that glucose-induced cpxP transcription is regulated by a reversible acetylation event mediated by the protein acetyltransferase YfiQ (also known as Pka and PatZ in E. coli) and the protein deacetylase CobB. We also presented evidence that nearly 30 lysine residues on three different RNAP subunits are acetylated in a glucose- and YfiQ-dependent manner. One of these acetylated lysines (the surface-exposed Lys-298 of the α subunit) is required for glucose-induced cpxP transcription (15).

In addition to YfiQ and Lys-298, glucose-induced cpxP transcription also requires CpxR (8), a member of the OmpR/PhoB family of DNA-binding response regulators (reviewed in Ref. 17) that does not appear to be an acetylation target (15). On the basis of OmpR homology, phosphorylation of Asp-51 of CpxR is predicted to induce a conformational change that promotes gene regulation (17). Indeed, Asp-51 is required for AcP-dependent phosphorylation of CpxR purified from Y. pseudotuberculosis (11) and for CpxA-induced cpxP transcription (18).

In this report, we investigated the role of CpxR phosphorylation in glucose-induced cpxP transcription. We provide evidence that, in addition to YfiQ and Lys-298 of α, glucose-induced cpxP transcription requires CpxR. We further demonstrate that the unexpectedly low level of cpxP transcription exhibited by glucose-exposed ackA mutant cells (8) requires Lys-291, a surface-exposed residue on the carboxyl-terminal domain of α (αCTD) that becomes acetylated when ackA mutants are grown in the presence of glucose. Taken together, these observations are consistent with a model in which AcP-dependent phosphorylation of CpxR is required for glucose-induced activation of cpxP transcription, a process that appears to be modulated by differential acetylation of RNAP.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Bacteriophage—All bacterial strains used in this study are listed in Table 1. Derivatives were constructed by generalized transduction with P1kc, as described previously (19). The transcriptional fusion PcpxP-lacZ, carried by λPcpxP as described previously (14), was a generous gift from Thomas Silhavy (Princeton University, Princeton, NJ). Construction of monolysogens was performed and verified as described previously (20, 21).

Culture Conditions—For strain construction, cells were grown in LB containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) sodium chloride; LB plates also contained 1.5% agar. For promoter activity assays, cells were grown in TB7, which contains 1% (w/v) tryptone buffered at pH 7.0 with potassium phosphate (100 mM). Transformation was performed using transformation and storage solution, as previously described (22). Cell growth was monitored spectrophotometrically (DU640; Beckman Instruments, Fullerton, CA) by determining the optical density at 600 nm (A_600). Kanamycin (40 μg/ml), tetracycline (15 μg/ml), spectinomycin (100 μg/ml), chloramphenicol (25 μg/ml), and ampicillin (100 μg/ml) were added to growth media when needed. Five μM isopropyl β-D-thiogalactopyranoside (IPTG) was added to...
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induce gene expression from plasmid vectors, unless otherwise mentioned.

Promoter Activity Assays—To monitor promoter activity from $\Phi$(PcpxP-lacZ), cells were grown aerobically at 37 °C with agitation at 250 rpm in TB7 for 8 h. At regular intervals, 50-μl aliquots were harvested and added to 50 μl of All-in-One β-galactosidase reagent (Pierce Biochemical). β-Galactosidase activity was determined quantitatively using a microtiter format, as described previously (23). Promoter activity was plotted versus $A_{600}^\text{social}$; however, only the last time point is shown. Each experiment included three independent measurements unless otherwise mentioned. All experiments were performed at least twice.

Site-directed Mutagenesis—Site-directed mutagenesis of CpxR and α were conducted using a QuikChange® II XL Site-directed Mutagenesis Kit (Stratagene) in accordance with the manufacturer’s instructions.

Western Immunoblot Analysis—Western immunoblot analysis was conducted on 1-ml samples of cell culture harvested after 7.5 h incubation at 37 °C with aeration. Samples were treated as reported previously (18). Rabbit polyclonal antibody raised against an MBP-CpxR fusion protein was used at a 1:30,000 dilution into 5% milk solution. Both the anti-α (NeoClone Biotechnology) and anti-His6 (Cell Signaling) antibodies were used at a 1:2,000 dilution. Detection was achieved with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies. The blot was incubated with the primary antibody overnight at 4 °C and then with the secondary antibody for 1 h at room temperature.

CpxR Cloning, Expression, and Purification—The WT cpxR allele was amplified from pCA24n-CpxR using primers cpxRFNdeI and cpxRRHindIII. The resulting amplicon was ligated into pET1.2, using the CloneJET™ PCR Cloning Kit (Fermentas). The ligated product was cut out of pET1.2 with NdeI and HindIII, gel-extracted, ligated into pET28 downstream of the IPTG-inducible promoter, and fused to a C-terminal His6 tag. The resulting plasmid was named pbPL001. For purification, expression of CpxR was carried out in BL21 cells transformed with pbPL001. An overnight culture of the transformants was used to inoculate 1 liter of LB at an initial $A_{600}$ of 0.05. The culture was incubated at 37 °C with shaking at 250 rpm. Once the $A_{600}$ reached 0.6–2.5 μM IPTG was added to the culture to induce CpxR expression. After 3 h of induction, the cell pellet was collected by centrifugation at 4 °C and stored at −20 °C overnight.

Cell lysis was done by resuspending the pellet with 10 ml of BugBuster® (Novagen) and 10 μl of Lysonase™ (Novagen). The resuspended cell pellet was incubated for 30 min at room temperature with gentle shaking. Following this room temperature incubation, 30 ml of pH 8.0 resuspension buffer (50 mM Na2HPO4, 1.4 mM NaCl, 20 mM imidazole, 0.1% Tween 20, 5% ethanol, and 15 mM β-mercaptoethanol) was added to the resuspended pellet. The cell debris was pelleted by centrifugation at 15,000 × g, at 4 °C for 30 min. The supernatant was loaded onto a 500 μl of TALON® Metal Affinity Resin (Clontech) column, washed with 10 ml of wash buffer (50 mM Na2HPO4, 0.3 mM NaCl, 30 mM imidazole, 0.1% Tween 20, and 0.5% ethanol) buffered at pH 8.0. CpxR elution was performed in a stepwise manner by flowing 500 μl of wash buffer with increasing imidazole concentration from 35 to 85 mM in 5 mM increments. Fractions 6–11 were collected, combined, and dialyzed overnight at 4 °C into storage buffer (10 mM Tris-HCl, pH 8.0, 100 mM KCl, 50% glycerol, 10 mM MgCl2, 0.1 mM EDTA, and 1 mM DTT). The dialyzed protein was aliquoted and stored at −80 °C for future use.

In Vitro Phosphorylation—In vitro phosphorylation was carried out by incubating lithium potassium AcP (Sigma) with purified His-tagged CpxR at 30 °C for 15 min in buffer containing (40 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 40 mM KCl, and 1 mM DTT).

Detection of Phosphorylated CpxR—Whether from cell lysates or from in vitro phosphorylation, phosphorylated CpxR was detected by first separating phosphorylated from nonphosphorylated CpxR using zinc(II) Phos-Tag™ SDS-PAGE (10% acrylamide (29:1), 350 mM Tris, pH 6.8, 0.1% SDS, 75 μM Phos-Tag and 150 μM Zn(NO3)2) (NARD Institute LTD). Purified protein was detected by staining the gel with SimplyBlue™ (Invitrogen), whereas protein from cell lysate was visualized by Western immunoblot with anti-His6 antibody (Cell Signaling). Cell lysis for Phos-Tag analysis was done at 4 °C with 2× SDS loading buffer. Prior to transfer of the protein onto the membrane, the gel was incubated at room temperature with gentle shaking for 15 min in Towbin buffer containing 1 mM EDTA to chelate the zinc and then for another 15 min in only Towbin buffer.

Immunoprecipitation—For immunoprecipitation, 50 ml of buffered TB cultures were collected after 7.5 h of incubation at 37 °C, pelleted by centrifugation, resuspended in 5 ml of TE buffer, and lysed by sonication. 1 ml of lysate was used for immunoprecipitation of RNAP with anti-RNAP β mouse monoclonal antibodies (NeoClone Biotechnology) and rotated overnight at 4 °C. Protein G-Sepharose® (Sigma) was used to pull down antibodies from cell lysates. Pulled down beads were washed three times with TE buffer and once with wash buffer containing 100 mM NaCl and 50 mM Tris-HCl pH 7.2. Loading buffer was added directly to the beads and samples were heated to 95 °C for 5 min. Samples were resolved by SDS-PAGE and stained with NOVEX® Colloidal Blue Stain (Invitrogen), according to the manufacturer’s instructions.

LC-MS/MS Analysis Using an LTQ Orbitrap Mass Spectrometer and Identification of Lysine Acetylation Sites—The α, β, and β' bands were excised and subjected to tryptic digestion, as described previously (24). Tryptic peptides were separated and measured online by ESI-mass spectrometry using a nanoACQUITY UPLC™ system (Waters, Milford, MA) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA). A trap column (Symmetry® C18, 5 μm, 180 μm inner diameter × 20 mm, Waters) was used for desalting. Elution was performed onto an analytical column (BEH130 C18, 1.7 μm, 100 μm inner diameter × 100 mm, Waters) by a binary gradient of buffers A (0.1% (v/v) acetic acid) and B (99.9% (v/v) acetonitrile, 0.1% (v/v) acetic acid) over a period of 80 min with a flow rate of 400 nl/min. The Orbitrap XL was operated in data-dependent MS/MS mode using the lockmass option for real time recalibration.

3 A. Boulanger-Castaing and D. M. Hinton, personal communication.
Proteins were identified by searching all MS/MS spectra in “dta” format against an E. coli database (extracted from the Uni-
prot-KB database) using Sorcerer™-SEQUEST (Sequest version 2.7 rev. 11, Thermo Electron including Scaffold_3_00_05,
Proteome Software Inc., Portland, OR). The Sequest search was
carried out considering the following parameters: a parent ion mass tolerance of 10 ppm and fragment ion mass tolerances of
1.00 Da. Up to two tryptic miscleavages was allowed. Methio-
nine oxidation (+15.99492 Da), cysteine carbamidomethyla-
tion (+57,021465 Da), and lysine acetylation (+42,010571 Da)
were set as variable modifications. Proteins were identified by at
least two peptides applying a stringent SEQUEST filter. Sequest
identifications required at least $C_n$ scores of greater than 0.10
and XCorr scores of greater than 1.9, 2.2, 3.3, and 3.8 for singly,
doubly, triply, and quadruply charged peptides. Acetylated pep-
tides that passed these filter criteria were examined manually
and accepted only when $b$ or $y$ ions confirmed the acetyla-
tion site.

### TABLE 1
Strains and plasmid used in this study

| Strain   | Relevant Characteristic                                                                 | Source/Reference                      |
|----------|----------------------------------------------------------------------------------------|---------------------------------------|
| PAD282   | F’ araD139 Δ(argF-lac)U169 rpsL150(Str') relA1 flhD5301 deoC1 Λφ(PropxP-lacZ)          | (18)                                  |
| PAD292   | PAD282 cpxR::spc (spectinomycin insertion in cpxR with polar effect on cpxA)           | (18)                                  |
| PAD348   | PAD282 cpxA::cam                                                                       | DiGuiseppa & Silhavy (Princeton       |
| AJW2794  | PAD282 cpxA::cam ackA::kan                                                             | University)                           |
| AJW3827  | PAD292 ackA::kan                                                                       | P1: AJW1939 → PAD292 (Km<sup>R</sup>) |
| AJW3875  | PAD292 Δ(ackA pta hisJ hisP dhu) zej223::Tn10                                        | P1: AJW2013 → PAD292 (Tc<sup>R</sup>/Ace-) |
| AJW3167  | PAD282 ΔcpxA::kan                                                                      | P1: JW3882 → PAD282 (Km<sup>R</sup>)  |
| AJW3994  | PAD282 ΔackA::frt                                                                      | P1: JW2293 → PAD282, followed by removal of antibiotic marker |
| AJW4303  | AJW3994 ΔtmcA::kan                                                                     | P1: JW2459 (50) → AJW3994            |
| AJW4304  | AJW3994 ΔypeA::kan                                                                     | P1: JW2427 (50) → AJW3994            |
| AJW4305  | AJW3994 ΔyedL::kan                                                                     | P1: JW1917 (50) → AJW3994            |
| AJW4306  | AJW3994 Δaat::kan                                                                      | P1: JW0868 (50) → AJW3994            |
| AJW4307  | AJW3994 ΔhybS::kan                                                                     | P1: JW3125 (50) → AJW3994            |
| AJW4308  | AJW3994 ΔyafP::kan                                                                     | P1: JW0224 (50) → AJW3994            |
| AJW4309  | AJW3994 ΔyilD::kan                                                                     | P1: JW3859 (50) → AJW3994            |
| AJW4310  | AJW3994 ΔrimJ::kan                                                                     | P1: JW1053 (50) → AJW3994            |
| AJW4311  | AJW3994 ΔrimL::kan                                                                     | P1: JW1423 (50) → AJW3994            |
| AJW4312  | AJW3994 ΔyiaC::kan                                                                     | P1: JW3519 (50) → AJW3994            |
| AJW4313  | AJW3994 ΔyihQ::kan                                                                     | P1: JW4269 (50) → AJW3994            |
| AJW4314  | AJW3994 ΔspeG::kan                                                                     | P1: JW1576 (50) → AJW3994            |
| AJW4315  | AJW3994 ΔargA::kan                                                                     | P1: JW2786 (50) → AJW3994            |
| AJW4316  | AJW3994 ΔrimL::kan                                                                     | P1: JW4335 (50) → AJW3994            |
| AJW4317  | AJW3994 ΔhyhY::kan                                                                     | P1: JW3405 (50) → AJW3994            |
| AJW4318  | AJW3994 ΔphnO::kan                                                                     | P1: JW4054 (50) → AJW3994            |
| AJW4319  | AJW3994 ΔyjdJ::kan                                                                     | P1: JW4086 (50) → AJW3994            |
| AJW4320  | AJW3994 ΔyjaB::kan                                                                     | P1: JW3972 (50) → AJW3994            |
| AJW4321  | AJW3994 ΔwecD::kan                                                                     | P1: JW5597 (50) → AJW3994            |
| AJW4322  | AJW3994 ΔyjgM::kan                                                                     | P1: JW5758 (50) → AJW3994            |
| AJW4333  | AJW3994 ΔynoC::kan                                                                     | P1: JW5233 (50) → AJW3994            |
| AJW4867  | PAD282 ΔcpxA::kan ΔackA::frt                                                          | P1: JW3882 → AJW3994 (Km<sup>R</sup>) |
| AJW1939  | ackA::kan                                                                              | (46)                                  |
| AJW2013  | Δ(ackA pta hisJ hisP dhu) zej223::Tn10                                                  | (50)                                  |
| JW2293   | ΔackA::kan                                                                              | (50)                                  |
| JW3882   | ΔcpxA::kan                                                                              | (50)                                  |
RESULTS

Glucose-induced cpxP Transcription Requires Asp-51, the Conserved Phosphoryl Acceptor Residue of CpxR—We recently proposed that glucose-induced cpxP transcription requires Lys-298 on the surface of the αCTD and seems to be regulated by a reversible acetylation event mediated by the acetyltransferase YfiQ and the deacetylase CobB (15). The knowledge that glucose-induced cpxP transcription does not require the sensor kinase CpxA, but requires the cognate response regulator CpxR (8) led us to re-investigate the role of CpxR in this response.

We first asked whether this behavior requires phospho-CpxR. To address this question, we attempted to complement the cpxR1 mutant allele in a strain that carries the transcriptional fusion (strain PAD 292; Table 1). cpxR1 is polar on the downstream gene cpxA; thus, this strain expresses neither CpxR nor CpxA (5). Complementation was attempted by transformation with either plasmid-borne WT cpxR (pCA24n-cpxR; Table 1) or plasmid-borne cpxRD51A (pCA24n-cpxRD51A; Table 1). The latter encodes a mutant protein that lacks the conserved aspartyl residue that serves as the phosphoacceptor. Both alleles were expressed from an IPTG-inducible promoter.

We grew the resultant transformants in the absence or presence of 0.4% glucose and measured β-galactosidase activity as a reporter of cpxP promoter function. The transformants that expressed WT CpxR responded to glucose. In contrast, the transformants that expressed CpxRD51A did not (Fig. 2A, left).

| Plasmid        | Relevant Characteristics                                      | Source/Reference |
|----------------|-------------------------------------------------------------|------------------|
| pCA24n         | Control plasmid (Cm<sup>R</sup>)                            | (52)             |
| pCA24n-cpxR    | Plasmid expressing 6xHis-CpxR under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pCA24n-cpxRD51A| Plasmid expressing 6xHis-CpxRD51A under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | This Study       |
| pBPL001        | pET-28 derivative expressing 6xHis-CpxR under the control of an IPTG-inducible promoter (Km<sup>R</sup>) | This Study       |
| pCA24n-yfiQ    | Plasmids expressing 6xHis-yfiQ under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pCA24n-yiiD    | Plasmids expressing 6xHis-yiiD under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pCA24n-atoB    | Plasmids expressing 6xHis-atoB under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pCA24n-argA    | Plasmids expressing 6xHis-argA under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pCA24n-elaA    | Plasmids expressing 6xHis-elaA under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pCA24n-ylgM    | Plasmids expressing 6xHis-ylgM under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pCA24n-yjaB    | Plasmids expressing 6xHis-yjaB under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pCA24n-yhhY    | Plasmids expressing 6xHis-yhhY under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pCA24n-rimJ    | Plasmids expressing 6xHis-rimJ under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pCA24n-yydJ    | Plasmids expressing 6xHis-yid under the control of an IPTG-inducible promoter (Cm<sup>R</sup>) | (52)             |
| pREII          | Plasmid expressing WT rpoA or alanine substitution derivatives (Ap<sup>R</sup>) | (35)             |
| pREII-rpoAK10A | pREII derivative expressing rpoAK10A                        | This Study       |
| pREII-rpoAK25A | pREII derivative expressing rpoAK25A                        | This Study       |
| pREII-rpoAK95A | pREII derivative expressing rpoAK95A                        | This Study       |
| pREII-rpoAK271A| pREII derivative expressing rpoAK271A                       | (35)             |
| pREII-rpoAK291A| pREII derivative expressing rpoAK291A                       | (35)             |
| pREII-rpoAK297A| pREII derivative expressing rpoAK297A                       | (35)             |
| pREII-rpoAK298A| pREII derivative expressing rpoAK298A                       | (35)             |
| pREII-rpoAK304A| pREII derivative expressing rpoAK304A                       | (35)             |
| pREII-rpoAK291R| pREII derivative expressing rpoAK291R                       | This Study       |
| pREII-rpoAK291Q| pREII derivative expressing rpoAK291Q                       | This Study       |
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FIGURE 2. The glucose response requires Asp-51 and AcP. A, λcpxP lysogens of the cpxR1 mutant (strain PAD292), the cpxR1 ackA mutant (strain AJW3827), and the cpxR1 ackA pta mutant (strain AJW3875) were transformed with plasmids pCA24n (vector), pCA24n-cpxR (WT), or pCA24n-cpxR-D51A (D51A). Transforms were grown at 37 °C with shaking in TB7 (open bars), or the same medium supplemented with 0.4% glucose (closed bars). Cells were harvested at regular intervals and A600 and β-galactosidase activity were measured. Only the values at the last time point are shown. The bars indicate the means of triplicate independent cultures, and the error bars indicate the S.D. Inset, Western immunoblot analysis of steady state levels of plasmid-expressed CpxR and CpxR-D51A from whole cell lysates of PAD292 using polyclonal antibody generated against MBP-CpxR fusion protein (gift from Thomas Silhavy). Endogenous MBP serves as a loading control. B, histogram representing the fold-change in cpxP transcription observed by exposure to glucose. The fold-change was calculated dividing the β-galactosidase activity measured in the presence of glucose by the β-galactosidase activity measured in the absence of glucose from A.

steady state levels of the WT and mutant forms of CpxR proteins were similar (Fig. 2A, inset), the lack of cpxP transcription by cells that express the mutant protein cannot be explained by a difference in protein expression levels. Instead, this lack of response likely resulted from the inability of the mutant protein to become phosphorylated (Fig. 3B). We therefore conclude that the response to glucose by cpxP requires Asp-51 and propose that this response requires phosphorylation of CpxR.

The Response to Glucose Requires Acetyl Phosphate—Because the response to glucose occurs independently of the cognate sensor CpxA, but seems to require phospho-CpxR, we sought the source of the phosphoryl group elsewhere. One alternative source of phosphoryl groups is AcP (12, 16), should respond to the addition of glucose in a Glucose-Independent manner, whereas the cpxR1 ackA triple mutant (strain AJW3875; Table 1), which accumulates glucose regardless of Asp-51. Indeed, the cpxR1 ackA double mutant (strain AJW3875; Table 1), which cannot synthesize AcP (12, 16), should not respond to exogenous glucose regardless of the status of Asp-51. Indeed, the cpxR1 ackA double mutant responded to glucose in a Asp-51-dependent manner. In contrast, we predicted that the cpxR1 pta ackA triple mutant (strain AJW3875; Table 1), which cannot synthesize AcP (12, 16), would not respond to exogenous glucose regardless of the status of Asp-51. Indeed, the cpxR1 pta ackA triple mutant did not respond to glucose (Fig. 2A, middle and right, respectively). Similar results were obtained when we used oligo-mediated recombineering (25, 26) to alter the endogenous cpxR allele, such that it encodes the CpxRD51A mutant (data not shown). Thus, in the absence of CpxA and in the presence of glucose, cpxP transcription requires both Asp-51 and AcP.

In Vivo Phospho-CpxR Status Correlates with AcP but Not with cpxP Transcription—The simplest explanation for the requirement of both AcP and Asp-D51 is that the CpxA-independent, glucose-induced cpxP transcription requires AcP-dependent phosphorylation of CpxR. Despite this requirement for AcP, the AcP-accumulating ackA mutant failed to promote the predicted proportional glucose-induced increase in cpxP transcription, eliciting an ~3-fold instead of the ~14-fold increase exhibited by its WT parent (Fig. 2B). This behavior, displayed by cells that overexpress CpxR, is almost identical to the behavior of cells that express CpxR endogenously (8). To understand why an ackA mutant strain that accumulates high levels of AcP responds less robustly to glucose than its AckA + parent, we assessed whether in vivo levels of phospho-CpxR correlate with those of AcP in strains WT or mutant for the Pta-AckA pathway.

To monitor phospho-CpxR, we took advantage of a previously reported method used for the detection of phosphorylated proteins (27, 28), including bacterial response regulators (11, 29). This method uses a dinuclear metal complex (i.e. 1,3-bis[bis(pyridin-2-yl-methyl)amino]propan-2-olato d zinc (II)) that has affinity for phosphomonoester diions, such as the asparyl phosphate of response regulators. When included in an SDS-PAGE, this phosphate-binding tag (Phos-Tag) slows the migration of the phosphorylated protein, allowing it to be distinguished from the nonphosphorylated protein by mobility shift.

To verify the report that AcP can function in vitro as a phosphoryl donor to CpxR (6) and to optimize the assay, we incubated purified His6-CpxR with AcP, resolved the proteins by Phos-Tag SDS-PAGE and stained the gel with SimplyBlue (Invitrogen). We detected an AcP-dependent shift in CpxR migration that disappeared when the sample was exposed to 95 °C for 15 min, a condition that induces hydrolysis of phosphorylated aspartyl residues (Fig. 3A).

To test the hypothesis that glucose induces cpxP transcription by increasing phospho-CpxR concentration, we assessed the cpxR1 mutant (strain PAD292) transformed with plasmids that expressed either His6-CpxR or His6-CpxR D51A. We grew the transformants in the absence or presence of 0.4% glucose, harvested cells as the cultures entered stationary phase, separated the cell lysates by Phos-Tag SDS-PAGE, and performed a Western immunoblot analysis with anti-His6 antibody. Consistent with the idea that glucose-induced cpxP transcription
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![Detection of phosphorylated CpxR](image)

**FIGURE 3. Detection of phosphorylated CpxR.** A, purified His$_6$-CpxR was incubated with increasing concentrations of AcP at 30 °C for 15 min and then resolved on a Phos-Tag SDS-PAGE. A fraction of the in vitro phosphorylation reaction carried out with 20 mM AcP was incubated at 95 °C for 15 min to promote hydrolysis of the phosphoryl group. The protein was detected by Coomassie Brilliant Blue. B, lacP lysogens of the cpxR1 mutant (strain PAD292) transformed with plasmids pCA24n (vector), pCA24n-cpxR (WT), or pCA24n-cpxR-D51A (D51A) were grown in TB7 in the absence or presence of 0.4% glucose at 37 °C with shaking. 50 mM IPTG was added to the cultures to induce CpxR expression after cultures reached an A$_{600}$ of 0.6. After 3 h of induction, cell lysates were collected and resolved by Phos-Tag SDS-PAGE before and after incubation at 95 °C for 15 min. CpxR was visualized by Western immunoblot with anti-His$_6$ antibody. C, lacP lysogens of the cpxR1 ackA mutant (strain AJW3827), and the cpxR1 ackA pta mutant (strain AJW3875) transformed with the plasmid pCA24n-cpxR were grown in TB7 in the absence or presence of 0.4% glucose at 37 °C with shaking. After cultures reached an A$_{600}$ of 0.6, 50 mM IPTG was added to the cultures to induce CpxR expression. After 3 h of induction, cell lysates were collected and resolved by Phos-Tag SDS-PAGE. CpxR was visualized by Western immunoblot with anti-His$_6$ antibody. D, lacP lysogens of the WT cpxR mutant (strain PAD282), the cpxA mutant (strain PAD348), and the cpxR1 mutant (strain PAD292) were grown in TB7 in the absence and presence of 0.4% glucose at 37 °C with shaking for 7.5 h. Cell lysate was collected and resolved by Phos-Tag SDS-PAGE. Endogenous WT CpxR was detected using polyclonal antibody generated against the MBP-CpxR fusion protein. Note that MBP is catabolite-repressed. Therefore, its expression is inhibited in the presence of glucose.

To facilitate detection of shifted phospho-CpxR, the aforementioned Phos-Tag experiments were performed with cells that overexpressed His$_6$-CpxR. Because we were aware that overexpression of His-tagged proteins often causes artifacts, we performed a similar experiment with WT and cpxA mutant cells that expressed WT CpxR from the native locus (PAD282 and PAD348, respectively, Table 1). In response to glucose, the phosphorylated fraction of native CpxR increased substantially, regardless of the status of CpxA (Fig. 3D). On the basis of these experiments, we conclude that glucose promotes an AcP-mediated phosphorylation of CpxR that is independent of the sensor kinase CpxA.

**Disruption of the Pta-AckA Pathway Alters the Acetylation Profile of RNA Polymerase**—The observation that AcP acts as the primary phosphoryl donor to CpxR (under the tested growth conditions) raises the following question: why does the cpxA ackA mutant respond less robustly to glucose than its WT parent? We previously hypothesized that disruption of the Pta-AckA pathway could lead to the accumulation of an inhibitor of glucose-induced cpxP transcription (8). For several reasons, we considered the possibility that AccCoA could function as this inhibitor, perhaps by acting as an acetyl-donor. First, the concentration of AccCoA has been shown to rapidly increase when *E. coli* cells are exposed to glucose (30) and growth in the presence of 0.4% glucose causes a general increase in protein acetylation (15). Second, pta mutants excrete large amounts of pyruvate and lactate in an AccCoA-sensitive manner (31), suggesting that disruption of the Pta-AckA pathway causes a metabolic imbalance that could affect the AccCoA pool. Third, cpxP transcription increases when cells are exposed to glucose concentrations up to 0.4%, but decreases progressively in the presence of larger amounts, e.g. 4.0% (Fig. 4), an observation that supports the hypothesis that cpxP transcription is sensitive to central metabolic imbalances. Because AccCoA can provide acetyl groups for protein acetylation (reviewed in Refs. 32–34), because exposure of *E. coli* to 0.4% glucose promotes acetylation of multiple lysines on multiple subunits of RNAP, and because one of those lysines (Lys-298 of α) is required for glucose-induced cpxP transcription (15), we hypothesized that disruption of the Pta-AckA pathway could shift the pattern of RNAP acetylation and that this altered acetylation profile could affect cpxP transcription.

To determine whether disruption of the Pta-AckA pathway alters the RNAP acetylation profile, we grew cpxA ackA mutant cells (strain AJW 2794, Table 1) in the presence or absence of glucose, purified RNAP subunits β, β’, and α by immunoprecipitation (data not shown), and applied liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis using an
Orbitrap XL mass spectrometer to detect lysine acetylation sites of these RNAP subunits. From cells grown in the presence of glucose, we mapped 19 acetylation sites to $\beta$, of which 12 were detected in at least two biological replicates. We mapped an additional 6 acetylation sites to $\beta'$, of which 4 were detected in two biological replicates (Table 2, supplemental Table S2, A–D). Finally, we mapped 7 acetylation sites to $\beta$, of which 4 were detected in two biological replicates (Table 3, Fig. 5, supplemental Table S3, A–D). None of the reproducibly acetylated $\alpha$ sites was acetylated when $ackA$ cells were grown in the absence of glucose (Tables 2 and 3, supplemental Table S3, E–G). In support of our hypothesis that disruption of the Pta-AckA pathway could alter the RNAP acetylation profile, most $\alpha$ lysines acetylated in the glucose-grown $ackA$ mutant were not acetylated in the WT parent and vice versa (compare Table 2 with Table S2, A–D). We were particularly intrigued by the lack of acetylated Lys-298 in glucose-exposed $ackA$ mutant cells and wondered if that could explain their weak response to glucose. Alternatively, acetylation of one of the other lysines might inhibit glucose-induced cpxP transcription.

**Overexpression of YfiQ Fails to Induce cpxP Transcription in $ackA$ Mutants**—We previously reported that glucose-induced acetylation of Lys-298 on the aCTD required the acetyltransferase YfiQ (15). To test if the weak cpxP transcription exhib-

### TABLE 2

| Beta (Glucose-exposed) | Replicate 1 | Replicate 2 | Replicate 3 |
|------------------------|-------------|-------------|-------------|
| Total Spectra          | 366         | 382         | 305         |
| (R)EAPGEKTVK H1104(42)DK(E) | 1           | 0           | 0           |
| (R)DEOLK H1104(42)DK(L)  | 1           | 1           | 1           |
| (K)LSGSHK H1104(42)RI(R) | 0           | 0           | 1           |
| (R)KDSK H1104(42)GESTSR(D) | 0           | 1           | 0           |
| (R)AVADVGVYVAH H1104(42)R(G) | 1           | 0           | 0           |
| (R)DIK H1104(42)LGPEITADPYNVEALSK(L) | 1           | 0           | 0           |
| (K)VTKP H1104(42)GETLTMPEEK(L) | 1           | 1           | 1           |
| (R)AIIFGK H1104(42)ASDVK(D) | 1           | 1           | 1           |
| (K)ASDV K H1104(42)DSSLR(V) | 1           | 1           | 1           |
| (R)ALDEEMQ H1104(42)OAK(K) | 1           | 1           | 1           |
| (R)AVLAGGEKE H1104(42)LDK(L) | 2           | 1           | 1           |
| (K)LDK H1104(42)LPK(D) | 1           | 0           | 0           |
| (K)KJPS D1104(42)TQQGDDAPVGLK(L) | 0           | 1           | 1           |
| (R)QPGD H1104(42)MAGR(P) | 1           | 0           | 0           |
| (R)GHG K H1104(42)VGLSK(L) | 0           | 1           | 1           |
| (K)NAM K H1104(42)QQGEVAK(L) | 2           | 1           | 2           |
| (K)QQGEVAK K H1104(42)LNEE | 1           | 1           | 1           |
| (R)KJPS D1104(42)GMPATPVPD GAK(E) | 1           | 2           | 2           |
| (K)ELK H1104(42)LGLDFTSGRL(L) | 0           | 1           | 0           |

| Beta’ (Glucose-exposed) | Replicate 1 | Replicate 2 |
|-------------------------|-------------|-------------|
| Total Spectra           | 314         | 248         |
| (K)AQTK H1104(42)TEFDFAK(I) | 1           | 1           |
| (R)QLATTK H1104(42)IAK(K) | 1           | 1           |
| (R)RUEEYK H1104(42)DANGELVAK(A) | 1           | 0           |
| (K)KSL H1104(42)DTTVGR(A) | 1           | 1           |
| (R)AAAEQ V H1104(42)NK(G) | 0           | 1           |
| (K)GHD H1104(42)LSNVK(S) | 1           | 1           |

### TABLE 3

**Acetylated $\alpha$ peptides from $ackA$ cells**

| Peptide | Start | Stop | Acetylated Lysine | Glucose-exposed | Glucose-Naive |
|---------|-------|------|-------------------|----------------|--------------|
|         |       |      |                   | Replicate 1    | Replicate 2 |
|         |       |      |                   | S3C           | S3D          |
|         |       |      |                   | Replicate 1    | Replicate 2 |
|         |       |      |                   | S3E           | S3F          |
|         |       |      |                   | S3G           |              |
|         |       |      |                   | Replicate 1    | Replicate 2 |
|         |       |      |                   | S3E           | S3F          |
|         |       |      |                   | S3G           |              |
| (m)QQGTVFELK(P) | 1 | 10 | 1 | 3 | 1 | 0 |
| (m)MQGTVFELKPL(P) | 1 | 12 | 8 | 28 | 4 | 3 | 6 |
| (m)QQGTVFELKPL(P) | 1 | 12 | 10 | 0 | 0 | 1 |
| (R)LVDIEQVSHTAK(V) | 13 | 25 | 10 | 20 | 10 | 12 | 13 |
| (R)LVDIEQVSHTAKVTEPLER(G) | 13 | 33 | 0 | 0 | 0 | 2 | 1 |
| (R)LVDIEQVSHTAKVTEPLER(G) | 13 | 33 | 25 | 0 | 0 | 0 |
| (R)VGQKDEVILT LNK(S) | 92 | 104 | 139 | 137 | 153 | 90 | 24 |
| (R)VGQKDEVILT LNK(S) | 92 | 104 | 95 | 2 | 1 | 0 | 0 | 0 |
| (R)TEVEKK(T) | 285 | 291 | 4 | 9 | 7 | 9 | 7 |
| (R)TEVEKKTPNLGK(K) | 285 | 297 | 0 | 1 | 0 | 2 | 4 |
| (R)TEVELKKTPNLGK(K) | 285 | 297 | 291 | 2 | 1 | 0 | 0 | 0 |
| (K)TLNPLGK(S) | 292 | 298 | 297 | 2 | 3 | 0 | 0 | 0 |
| (K)KSLS TEIK(D) | 298 | 304 | 0 | 3 | 1 | 2 | 0 |
| (K)KSLS TEIKVLSR(G) | 298 | 310 | 0 | 0 | 0 | 1 | 3 |
| (K)KSLS TEIKVLSR(G) | 298 | 310 | 298 | 0 | 0 | 0 | 0 |
| (K)KSLS TEIKVLSR(G) | 298 | 310 | 7 | 9 | 8 | 9 | 9 |
| (K)KSLS TEIKVLSR(G) | 298 | 310 | 304 | 1 | 1 | 0 | 0 | 0 |

| Total Spectra | 315 | 486 | 298 | 216 | 176 |

1 Green, unacetylated peptides; red, acetylated peptides.

2 m, oxidized methionine; M, methionine; k, acetylated lysine; K, lysine.

3 Supplemental Table in which the raw data are presented.
ited by the ackA mutant was due to the lack of Lys-298 acetylation, we asked whether YfiQ overexpression could suppress the weak response to glucose by cpxA ackA mutants. Into an ackA cpxA/H9261/H9021 (PcpxP/H11032-lacZ) lysogen (strain AJW4867; Table 1), we introduced a plasmid carrying yfiQ or the vector control (Table 1). We grew the resultant transformants in the absence or presence of 0.4% glucose, monitored β-galactosidase activity, and found that yfiQ overexpression had no effect on the weak glucose response of the cpxA ackA double mutant (Fig. 6).

FIGURE 5. In the glucose-exposed ackA mutant, the α subunit of RNAP is acetylated on Lys-291 and five other lysines. Immunoprecipitated subunits of RNAP were separated by SDS-PAGE. The α bands were excised and tryptically digested as described previously (53). The resulting peptides were analyzed in a LTQ OrbitrapXL mass spectrometer as described under "Materials and Methods." The double- or triple-charged acetyllysine-modified peptides M1(+16)-QGSVTEFLK10(+42)PR, LVDEIQVSTHAK25(+42)VTLEPLER, VQGK95(+42)DEVILTLNK, TEVELLK291(+42)TPNLGK, TPNLGK297(+42)K, and SLTEIK304(+42)DVLASR were detected in the digested α sample as mass peaks of m/z = 725.873, 802.7703, 749.9274, 742.4222, 400.3372, and 687.3862, respectively. A, the Xcorr and ΔCn scores of the six acetylated peptides. B, the corresponding CID MS/MS spectrum for the Lys-291-containing peptide TEVELLK291(+42)TPNLGK. C, complete b and y fragment ion series for the Lys-291-containing peptide TEVELLK291(+42)TPNLGK.

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Lys-291 on RNAP αCTD Contributes to Transcription Inhibition—That YfiQ overexpression does not promote glucose-induced cpxP transcription in the cpxA ackA mutant background led us to test the alternative hypothesis: that this weak response requires one of the lysines that became acetylated in the absence of ackA. Because the gene that encodes α (rpoA) is essential for bacterial survival, we used a previously reported partial-diploid system (35) to test the hypothesis that one of the other acetylated α lysines inhibits cpxP transcription. Into an ackA cpxA λΦ(PcpxP’-lacZ) lysogen (strain AJW4867; Table 1), we introduced a plasmid carrying yfiQ or the vector control (Table 1). We grew the resultant transformants in the absence or presence of 0.4% glucose, monitored β-galactosidase activity, and found that yfiQ overexpression had no effect on the weak glucose response of the cpxA ackA double mutant (Fig. 6). Lys-291 on RNAP αCTD Contributes to Transcription Inhibition—That YfiQ overexpression does not promote glucose-induced cpxP transcription in the cpxA ackA mutant background led us to test the alternative hypothesis: that this weak response requires one of the lysines that became acetylated in the absence of ackA. Because the gene that encodes α (rpoA) is essential for bacterial survival, we used a previously reported partial-diploid system (35) to test the hypothesis that one of the other acetylated α lysines inhibits cpxP transcription. Into an ackA cpxA λΦ(PcpxP’-lacZ) lysogen that carries the WT rpoA gene in its native location on the chromosome (strain AJW2794), we introduced plasmids carrying either the WT rpoA allele or Lys to Ala point mutation derivative alleles (Table 1) with the goal of determining if any lysine functional group contributes to this phenotype. We grew the resultant transformants in the absence or presence of 0.4% glucose and monitored β-galactosidase activity. cpxA ackA mutant cells that overexpressed the α K291A mutant responded robustly to the presence of glucose. In contrast, we observed little or no response from cpxA ackA mutants that overexpressed WT α, Lys to Ala mutations of the other 4 lysines of the αCTD (Fig. 7A) or Lys to Ala mutations of the 3 acetylated lysines on the α amino-terminal (data not shown).
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We also investigated whether Lys-291 could inhibit 4.0% glucose-induced \( \text{cpxP} \) transcription in \( \text{cpxA} \) mutant cells (strain PAD348) whose Pta-AckA pathway remains intact. Indeed, cells that overexpressed the K291A variant responded more robustly to the presence of 4% glucose than did cells that overexpressed WT \( \text{cpxA} \). In contrast, Lys-291 was not required for Lys-298-dependent, glucose-induced \( \text{cpxP} \) transcription, as \( \text{cpxA} \) mutant cells that overexpressed either WT \( \alpha \) or the K291A mutant responded robustly to 0.4% glucose (Fig. 7C), as reported previously (15). We conclude that inhibition, whether caused by excessive glucose or by disruption of the Pta-AckA pathway, acts through Lys-291.

To determine whether the requirement for Lys-291 could involve acetylation, we took advantage of a genetic strategy commonly used in studies of eukaryotic protein acetylation (36). In this approach, the lysine is converted to either a glutamine or an arginine. The KQ substitution mimics the neutral acetylated lysine, whereas the KR substitution mimics the positively charged unacetylated lysine. Consistent with the hypothesis that Lys-291 acetylation inhibits glucose-induced \( \text{cpxP} \) transcription, \( \text{cpxA} \) mutant cells overexpressing the K291Q mutant variant of \( \alpha \) responded to glucose with less intensity than did cells that overexpressed WT \( \alpha \) or its K291A or K291R mutant variants (Fig. 7C). This decrease in \( \text{cpxP} \) transcription, however, cannot be explained by a decrease in steady state levels of protein (data not shown). These results lend support to the hypothesis that Lys-291 acetylation decreases the response to glucose by \( \text{cpxP} \).

Is an Acetyltransferase Required for the Weak Response to Glucose by \( \text{ackA} \) mutants?—To identify an acetyltransferase that participates in Lys-291-dependent inhibition of \( \text{cpxP} \) transcription, we transformed WT cells with a set of multicopy plasmids; each plasmid expressed one of 22 known or predicted \( \text{E. coli} \) acetyltransferases (Table 1). We grew the resultant transformants in the presence of glucose and IPTG to induce acetyltransferase expression, and measured \( \beta \)-galactosidase activity.

Relative to the WT strain carrying the vector control, overexpression of 8 different acetyltransferases substantially inhibited the glucose response, whereas overexpression of only one increased the response (Fig. 8A and data not shown).

To test if one of these acetyltransferases was required for the weak response to glucose by the \( \text{ackA} \) mutant, we deleted each acetyltransferase from the \( \text{ackA} \) mutant (strain AJW3994) and screened for double mutants whose \( \text{cpxP} \) promoter could respond robustly to glucose. Only the \( \text{ackA} \) \( \text{wecD} \) double exhibited robust \( \text{cpxP} \) transcription (Fig. 8B). However, this expression is unlikely to involve Lys-291 acetylation as it occurred even in the absence of glucose (Fig. 8B, inset). On the basis of these results, we conclude that multiple protein acetyltransferases can inhibit glucose-induced \( \text{cpxP} \) transcription, but that no single acetyltransferase may be required.

DISCUSSION

Phosphorylation and Acetylation Regulate CpxA-independent, Glucose-induced \( \text{cpxP} \) Transcription—We propose that the two high-energy central metabolites, AcP and AcCoA, function together to activate transcription from the \( \text{cpxP} \) promoter. We base this proposal on the following observations. (i) The intracellular pools of both AcP and AcCoA are dynamic. They fluctuate throughout growth and upon exposure to different carbon sources (16, 30). Furthermore, both central metabolites can be used as donors for post-translational modification of proteins: AcP by donating its phosphoryl group for protein phosphorylation, and AcCoA by donating its acetyl group for protein acetylation (12, 32). (ii) Glucose-induced, CpxA-independent \( \text{cpxP} \) transcription requires both the phosphoacceptor site of CpxR (Asp-51) and the ability of cells to synthesize AcP (Fig. 2). Indeed, Phos-Tag mobility shift assays of cell lysates clearly show that AcP functions as the primary phosphoryl donor to CpxR (Fig. 3) under the tested growth conditions. (iii) Glucose-induced, CpxA-independent \( \text{cpxP} \) transcription also requires an acetylation event involving AcCoA, the acetyltransferase YfiQ, and the deacetylase CobB. In addition, glucose-induced \( \text{cpxP} \) transcription involves a lysine (Lys-298) located on the surface of a CTD that is acetylated in a YfiQ- and glucose-dependent manner (15).

Evidence That AcCoA Inhibits AcP-dependent \( \text{cpxP} \) Transcription—Given that glucose-induced \( \text{cpxP} \) transcription requires AcP to phosphorylate CpxR, we were initially surprised that the AcP-accumulating \( \text{ackA} \) mutant (16) responded to 0.4% glucose with only a modest increase in \( \text{cpxP} \) transcription (Fig. 2B). We previously proposed that this weak response resulted from accumulation of a central metabolic intermediate with inhibitory properties (8). Despite evidence that AcCoA-dependent acetylation contributes to glucose-induced \( \text{cpxP} \) transcription, we considered AcCoA to be an obvious candidate for the inhibitory compound. (i) \( \text{E. coli} \) tightly limits CoA synthesis (37). (ii) The limited supply of CoA would be overwhelmed by exposure to large amounts of glucose (e.g. 4.0%), resulting in a large AcCoA-to-CoA ratio (16, 30). (iii) Disruption of the Pta-AckA pathway also would be expected to put pressure on the limited CoA pool. Although deletion of \( \text{pta} \) or \( \text{ackA} \) does not appear to alter the AcCoA to CoA ratio (16), the deletion of \( \text{pta} \) clearly alters the ratio of excreted central metabolic intermediates: WT cells primarily excrete acetate; in con-
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A Potential Role for Lys-291—The acetylation profile of α isolated from the cpxA ackA mutant differed from the acetylation profile of α isolated from its WT parent (compare Fig. 5 in this report to Fig. 7 of Ref. 8). One α acetylation was common to both strains. In contrast, five acetylations were specific to the cpxA ackA mutant including Lys-291, whereas only one was specific to WT (i.e. Lys-298). Our genetic screen suggests that Lys-291 is definitely involved in weakening the response to 0.4% glucose by the ackA mutant (Fig. 7A) or to 4.0% glucose by its WT parent (Fig. 7B). We currently do not possess definitive evidence that acetylation of Lys-291 causes these behaviors; however, support for such a mechanism is provided by the dramatic reduction in glucose-induced cpxP transcription by the K291Q mutation (Fig. 7C), which is predicted to exhibit properties that mimic acetylated Lys-291 (36). Although the underlying molecular mechanism remains to be determined, it is distinctly possible that Lys-291 acetylation could affect one of the many interactions made by α and, as a result, decrease the stability of the transcription complex. Lys-291 is located on the αCTD helix-hairpin-helix motif, which is reported to contribute to both nucleic acid binding and protein-protein interaction (38). To the best of our knowledge, no evidence exists that Lys-291 makes direct contact with DNA. In contrast, Lys-291 has been reported to make direct contact with activation region 2 of the transcription antiterminator NusA (39). Furthermore, Lys-291 is located near the 287 determinant, an αCTD surface known to make direct contact with the catabolite activator protein (also known as CRP) (reviewed in Ref. 40). Although no direct evidence exists for an αCTD-CpxR interaction, our report that the αCTD is required for glucose-induced cpxP transcription (15) hints that an interaction between these two proteins might exist, perhaps in a manner similar to that reported for OmpR, another member of the winged helix-turn-helix family of response regulators (reviewed in Ref. 41).

Using the well-characterized CRP-αCTD interaction as a guide, we hypothesize that Lys-291 might participate in an interaction between CpxR and the αCTD. If so, then the acetylation of Lys-291 could affect this interaction and therefore impact transcription. Efforts are underway to identify the

were harvested at regular intervals and both $A_{600}$ and β-galactosidase activity were measured. The fold-changes in β-galactosidase activity from cells grown in the presence of glucose relative to those grown in the absence of glucose are reported in the histogram. The bars indicate the means of triplicate independent cultures, and the error bars indicate the S.D. B, β-galactosidase activity of αCTD-lysogens of the cpxA mutant (strain PAD348) transformed with plasmid pREII carrying the WT allele of rpoA, lysine to alanine, lysine to arginine, and lysine to glutamine mutant derivatives of residue 291. Transformants were grown for 7.5 h at 37 °C with shaking in TB7 in the absence (white bars) or presence of 0.4% glucose (black bars). Cells were harvested at regular intervals and both $A_{600}$ and β-galactosidase activity was measured. The bars indicate the means of triplicate independent cultures, and the error bars indicate the S.D.

![FIGURE 7. Lys-291 inhibits cpxP transcription.](image-url)
mechanism by which Lys-291 and its acetylation affect cpxP transcription.

YfiQ Is Not Involved—We found no evidence that YfiQ plays a role in Lys-291-dependent inhibition of cpxP transcription. Although overexpression of YfiQ promoted a small increase in glucose-induced cpxP transcription, it did not alter the weakened response by the ackA mutant (Fig. 6). This weakened response also was unaffected by deletion of yfiQ (data not shown). At present, we do not know how Lys-291 becomes acetylated. Although the overexpression of several acetyltransferases can inhibit cpxP transcription in the WT parent, deletion of any single enzyme did not permit the ackA mutant to respond robustly to glucose (Fig. 8). A simple explanation for these results is that multiple acetyltransferases could acetylate Lys-291. Alternatively, Lys-291 could become acetylated independently of the action of an acetyltransferase. Efforts to distinguish between these two mechanisms are underway.

AcP Functions as a Phosphoryl Donor in Vivo—Much evidence supports the hypothesis that AcP can serve as a phosphoryl donor to two-component response regulators, e.g. CpxR (12, 42, 43). (i) Many purified response regulators autophosphorylate when exposed to AcP (44). (ii) In response to diverse carbon sources and growth phase, the intracellular AcP pool can reach at least 3 mM (16), a concentration that permits efficient in vitro autophosphorylation. (iv) The regulation of about 100 E. coli genes correlates with the status of AcP (46). This regulation depends on response regulators, e.g. RcsB (13).
and CpxR (8); however, this dependence does not extend to the cognate sensor kinases RcsC (13) and CpxA (8). (v) AcP-dependent behaviors have been reported in diverse bacteria, including pathogens (for example, Refs. 47 and 48). (vi) A specificity determinant that limits AcP-dependent autophosphorylation of a response regulator has been identified in Campylobacter (49). Here, we have shown that in vivo phosphorylation of CpxR does not require CpxA. Instead, it requires Pta (Fig. 3). This observation provides the definitive evidence that AcP functions as the primary phosphoryl donor to CpxR, at least under the tested conditions, which were designed to dampen extracytoplasmic stimuli (e.g. alkaline pH) known to activate CpxA kinase activity (8). This observation also fulfills a major prediction made by the hypothesis that AcP can act as a global signal, in vivo evidence that AcP donates its phosphoryl group directly to at least one response regulator.

Finally, we acknowledge the existence of a weak glucose- and AcP-independent signal by the pta ackA double mutant. Currently, we do not know the origin of this weak signal. Perhaps, under the conditions tested, a small percentage of CpxR is phosphorylated by a noncognate sensor kinase and/or another small phospho-donor.

Concluding Remarks—In summary, we propose that AcP can function as a phosphoryl donor to CpxR, both in vitro and in vivo. The output of this phosphorylation event, at the level of the cpxP promoter, seems to be modulated by acetylation of two lysine residues on the surface of RNAP aCTD. Our work suggests that acetylation of Lys-298 somehow contributes to promoter activity, whereas acetylation of Lys-291 dampens this activation.

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