

Signal Integration by the Two-Component Signal Transduction Response Regulator CpxR[∇]

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The CpxAR two-component signal transduction system in *Escherichia coli* and other pathogens senses diverse envelope stresses and promotes the transcription of a variety of genes that remedy these stresses. An important member of the CpxAR regulon is *cpxP*. The CpxA-dependent transcription of *cpxP* has been linked to stresses such as misfolded proteins and alkaline pH. It also has been proposed that acetyl phosphate, the intermediate of the phosphotransacetylase (Pta)-acetate kinase (AckA) pathway, can activate the transcription of *cpxP* in a CpxA-independent manner by donating its phosphoryl group to CpxR. We tested this hypothesis by measuring the transcription of *cpxP* using mutants with mutations in the CpxAR pathway, mutants with mutations in the Pta-AckA pathway, and mutants with a combination of both types of mutations. From this epistasis analysis, we learned that CpxR integrates diverse stimuli. The stimuli that originate in the envelope depend on CpxA, while those associated with growth and central metabolism depend on the Pta-AckA pathway. While CpxR could receive a phosphoryl group from acetyl phosphate, this global signal was not the primary trigger for CpxR activation associated with the Pta-AckA pathway. On the strength of these results, we contend that the interactions between central metabolism and signal transduction can be quite complex and that successful investigations of such interactions must include a complete epistatic analysis.

A common mechanism used by bacteria to sense changes in their environment is the two-component signal transduction (2CST) pathway. As the name implies, the simplest of these pathways consists of two components: a sensor kinase (SK) and a response regulator (RR). The SK is a histidine kinase that autophosphorylates a conserved histidine residue using ATP as its phosphoryl donor. The phosphorylated SK can then donate the phosphoryl group to its cognate RR, an aspartyl kinase that autophosphorylates a conserved aspartate residue. Many SKs possess both kinase and phospho-aspartate phosphatase activities. For some SKs, the phosphatase activity may be the critical function in vivo (for reviews, see references 21, 27, 56, 61, and 62).

CpxA/CpxR is an autogenously regulated 2CST pathway (Fig. 1A). CpxA, an SK with autokinase, phosphotransfer, and phospho-CpxR phosphatase activities (18, 42), is located in the cytoplasmic membrane, where it senses diverse signals, including alkaline pH, altered membrane lipid composition, interaction with hydrophobic surfaces, and misfolded pilin subunits, as well as exposure to copper, detergents, and EDTA. In response, CpxA autophosphorylates and donates its phosphoryl group to CpxR, the cognate RR. When phosphorylated, this transcription factor controls part of the envelope stress response system, pilus assembly, type III secretion, motility and chemotaxis, adherence, and biofilm development. Furthermore, the Cpx pathway is required for invasion of host cells in diverse pathogenic bacteria, including *Escherichia coli* (enterohemorrhagic *E. coli* and uropathogenic *E. coli*), *Salmonella*

enterica serovar Typhimurium, *S. enterica* serovar Typhi, *Shigella sonnei*, *Yersinia enterocolitica*, and *Legionella pneumophila* (for reviews, see references 15, 16, 43, and 46–48). A recent study demonstrated that *Xenorhabdus nematophilus* requires the Cpx pathway both to colonize its nematode host (*Steinernema carpocapsae*) and to kill larvae of the tobacco hornworm (*Manduca sexta*). Thus, it appears that the Cpx pathway functions generally in the establishment of bacterium-host associations (20).

The Cpx pathway includes at least two more upstream components. The first additional component, CpxP, was identified as an alkaline-induced member of the Cpx regulon (9). This periplasmic chaperone binds to the periplasmic domain of CpxA and inhibits its autokinase activity (18, 44, 45). It appears that CpxP binds to specific features of certain misfolded proteins, making them available to the ATP-independent periplasmic protease DegP (7, 14, 23, 29). The second component, the outer membrane lipoprotein NlpE, activates CpxA when it is overexpressed (54), and it is required for activation of CpxA in response to adherence to hydrophobic surfaces (36). Since adhesion requires NlpE but not CpxP and since stresses that require CpxP do not require NlpE (9, 14, 36), CpxA must integrate information from at least these two distinct stress response pathways (14, 44). Furthermore, it is likely that additional upstream components exist (44). For example, CpxA- and CpxR-dependent resistance to copper exposure requires neither CpxP nor NlpE (66).

Like many RRs, purified CpxR can accept a phosphoryl group from acetyl phosphate (acetyl-P) (37, 42). Acetyl-P, the high-energy intermediate of the phosphotransacetylase (Pta)-acetate kinase (AckA) pathway (Fig. 1B), has a larger ΔG° of hydrolysis (-43.3 kJ/mol) than ATP (-30.5 kJ/mol in complex with Mg^{2+}). Thus, acetyl-P stores more energy than ATP,

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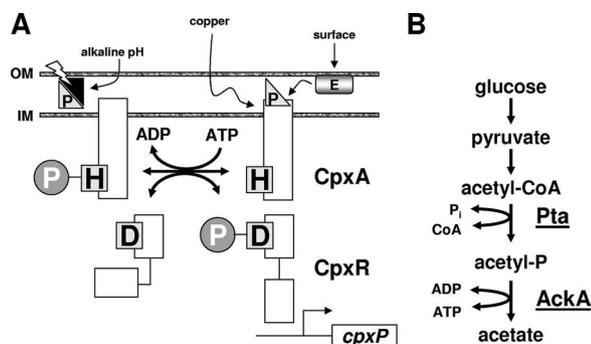


FIG. 1. Pta-AckA pathway and Cpx two-component signal transduction pathway. (A) Schematic diagram of the Cpx pathway. OM, outer membrane; IM, inner or cytoplasmic membrane; H, site of autophosphorylation of the histidine kinase CpxA; D, site of autophosphorylation of the cognate response regulator CpxR; bent arrow, transcription initiation site of the CpxR target gene, *cpxP*. CpxP (P), a periplasmic chaperone (gray triangle), inhibits the activity of CpxA. CpxP binds to a substrate (black triangle). The CpxP-substrate complex is degraded by the ATP-independent periplasmic protease DegP (thunderbolt). Released from CpxP, CpxA can autophosphorylate using ATP as its phosphoryl donor. Phospho-CpxA then acts as a phosphoryl donor to CpxR. Phosphorylation exposes the CpxR DNA-binding domain (57), which binds its target genes, including *cpxP*. CpxA also possesses phosphatase activity, which removes phosphoryl groups from phospho-CpxR in the form of P_i . (B) Schematic diagram of the Pta-AckA pathway that converts acetyl-CoA to acetate via an acetyl-P intermediate.

which forms the basis for its pivotal role in global signaling (for a review, see reference 63). Reversible *in vivo*, the Pta-AckA pathway interconverts coenzyme A (CoA), ATP, and acetate with acetyl-CoA, ADP, and inorganic phosphate (P_i). This reversibility permits both acetyl-CoA synthesis (acetate activation) and acetate evolution (acetogenesis). During acetogenesis, Pta synthesizes acetyl-P and CoA from acetyl-CoA and P_i , while AckA generates ATP from acetyl-P and ADP. Simultaneously, AckA produces acetate, which cells freely excrete into the environment. Thus, the steady-state concentration of acetyl-P depends on the rate of its formation catalyzed by Pta and the rate of its degradation catalyzed by AckA (for reviews, see references 49 and 63).

Acetogenesis has several key functions. It recycles CoA, facilitating glycolytic flux and hence rapid growth in the presence of an excess of a preferred carbon source (e.g., glucose or pyruvate) (for reviews, see references 49 and 63). This function also can reinitiate stalled tricarboxylic acid (TCA) cycle function, providing CoA to convert α -ketoglutarate to succinyl-CoA (17). Critically, acetogenesis provides the majority of ATP in the absence of robust TCA cycle activity (49, 63). However, the conversion of acetyl-CoA to acetate and ATP often does not go to completion; *E. coli* cells can and do maintain a significant pool of acetyl-P (26, 32, 41). This pool serves the cell in two distinct and equally important ways. First, acetyl-P serves as a storage molecule for carbon (C), phosphate (OPO₃), and energy in the form of its high-energy C—OPO₃ bond (34). Second, acetyl-P can serve as a signaling molecule (for a review, see reference 63).

In the early 1990s, acetyl-P was proposed to act as a global signal by donating its phosphoryl group to a subset of the RR (33, 59). For many years this hypothesis remained unproven.

Recently, however, we used epistasis analysis to obtain definitive genetic support for this hypothesis. By combining mutations in the Pta-AckA pathway with mutations in the Rcs 2CST pathway and then comparing the phenotypes of the resultant double mutants to those of the parental single mutants, we were able to conclude that acetyl-P acts via RcsB to activate genes for encapsulation and to repress genes for flagellation (19). This well-characterized DNA-binding RR controls about 5% of the *E. coli* genome (for reviews, see references 30 and 39) and as much as 20% of the *S. enterica* genome (58), including many genes involved in virulence. Thus, acetyl-P influences expression of multiple genes through a known global regulator. Next, we carefully measured the intracellular acetyl-P concentration and found that it varies several orders of magnitude in response to the environment, as expected for a global signal. Furthermore, we determined that acetyl-P can reach concentrations as high as 4 to 5 mM in wild-type (WT) cells and 15 to 20 mM in *ackA* mutants. These concentrations are more than sufficient for efficient phosphotransfer to RRs (26). Thus, all the available evidence supports the view that acetyl-P can act as a global signal *in vivo* and that it can do so by directly donating its phosphoryl group to at least one RR.

During the course of our investigations, we noticed that disruption of the Pta-AckA pathway resulted in aberrant CpxR-dependent transcription. This led us to reexamine the relationship between acetyl-P, its pathway, and Cpx signaling. Specifically, we tested the hypothesis that the CpxA-independent response to excess glucose relies on phosphoryl exchange from acetyl-P to CpxR (9, 10). Using epistasis analysis, we showed that growth- and central metabolism-associated, CpxA-independent CpxR activation requires that the Pta-AckA pathway remain intact. We also obtained evidence that this dependence does not require acetyl-P, although under certain circumstances this global signal can indeed enhance CpxR activation. Furthermore, we demonstrated that CpxR integrates both extracellular, CpxA-dependent cues and intracellular, Pta-AckA-dependent cues. Finally, in this paper we emphasize that workers need to perform complete epistasis analyses when they attempt to dissect complex relationships between signal transduction and metabolic pathways.

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 (51). The transcriptional fusion ϕ (*PcpxP'-lacZ*) carried by λ *PcpxP* and described previously (9) was a generous gift from Thomas Silhavy (Princeton University, Princeton, NJ). Construction of monolysogens was performed and verified as described previously (38, 52).

Culture conditions. For strain construction, cells were grown in LBK containing 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, and 0.5% (wt/vol) potassium chloride; LBK plates also contained 1.5% agar. For promoter activity assays, cells were grown in tryptone broth K (TBK) containing 1% (wt/vol) tryptone and 0.5% (wt/vol) potassium chloride. Potassium chloride was used instead of sodium chloride because sodium has a negative effect on the survivability of WT cells at alkaline pH (24). For some experiments, the medium (liquid or solid) was buffered with base and acid (100 mM) at ratios required to achieve the desired pH. Cell growth was monitored spectrophotometrically (DU640; Beckman Instruments, Fullerton, CA) by determining the optical density at 600 nm (OD₆₀₀). Kanamycin (40 μ g/ml), tetracycline (15 μ g/ml), and spectinomycin (100 μ g/ml) were added as needed.

Promoter activity assays. To monitor promoter activity from ϕ (*PcpxP'-lacZ*), cells were grown aerobically with agitation at 250 rpm at 37°C in TBK that was unbuffered or TBK that was buffered either at pH 7.0 (TBKpH7) or at pH 8.0

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Reference or source
PAD282	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>) <i>U169 rpsL150</i> (Str ^r) <i>relA1 fthD5301 deoC1</i> λ (<i>PcpxP'-lacZ</i>)	14
PAD292	PAD282 <i>cpxR1::spc</i>	14
PAD348	PAD282 <i>cpxA::cam</i>	P. DiGiuseppe and T. Silhavy (Princeton University)
AJW2790	PAD282 <i>ackA::kan</i>	(P1)AJW1939 \rightarrow PAD282 (Km ^r)
AJW2791	PAD282 Δ (<i>ackA pta</i>):: <i>cam</i>	(P1)EPB6 \rightarrow PAD282 (Cm ^r)
AJW2793	PAD282 <i>cpxR1::spc</i> Δ (<i>ackA pta</i>):: <i>cam</i>	(P1)EPB6 \rightarrow PAD292 (Cm ^r)
AJW2794	PAD282 <i>cpxA::cam ackA::kan</i>	(P1)AJW1939 \rightarrow PAD348 (Km ^r)
AJW2920	PAD282 <i>cpxA::cam</i> Δ (<i>ackA pta hisJ hisP dhu</i>) <i>zej-223-Tn10</i>	(P1)AJW2013 \rightarrow PAD348 (Tc ^r /Ace ⁻)
AJW2964	PAD282 <i>cpxA::cam pta::TnphoA'-3</i>	(P1)CP760 \rightarrow PAD348 (Km ^r)
AJW1939	<i>ackA::kan</i>	28
AJW2013	Δ (<i>ackA pta hisJ hisP dhu</i>) <i>zej-223-Tn10</i>	64
CP760	<i>pta::TnphoA'-3</i>	40
EPB6	Δ (<i>ackA pta</i>):: <i>cam</i>	Mark Goulian (University of Pennsylvania)

(TBKpH8). At regular intervals, 50- μ l aliquots were harvested and added to 50 μ l of All-in-One β -galactosidase reagent (Pierce Biochemical). β -Galactosidase activity was determined quantitatively using a microtiter format as described previously (5). Promoter activity was plotted versus OD₆₀₀. For some experiments, only the peak activity is shown below. Each experiment included three independent measurements and was repeated at least once.

pH sensitivity assays. Cells were grown aerobically with agitation at 250 rpm at 37°C in LBK buffered at pH 7.0 (LBKpH7) until either mid-exponential phase or early stationary phase, harvested, serially diluted in LBKpH7, and plated onto LBK plates buffered at the specified pH. The plates were incubated overnight at 37°C, the colonies were counted, and the colony diameters were measured.

RESULTS

Disruption of the Pta-AckA pathway diminishes Cpx signaling. Linkage between acetyl-P and several 2CST pathways has been proposed previously (for a review, see reference 63). Only a few studies, however, have provided definitive evidence for this linkage in vivo (19, 50). As part of our efforts to map the impact of acetyl-P on the entire network of 2CST pathways in *E. coli*, we reexplored the relationship between acetyl-P, its pathway, and the 2CST pathway CpxAR.

It is estimated that phospho-CpxR controls more than 100 *E. coli* genes (13). Of these, the *cpxP* promoter (*PcpxP*) is the most sensitive (14), and its activity depends almost exclusively on CpxR (9). Therefore, to monitor the phosphorylation state of CpxR, we measured β -galactosidase activity from λ (*PcpxP'-lacZ*) (9) carried by the hybrid bacteriophage λ RS88 and present as a monolysogen in the *lattB* site of the chromosome of WT cells (strain PAD282) and isogenic mutants with only *ackA* deleted (strain AJW2790), with both *ackA* and *pta* deleted (strain AJW2791), with only *cpxA* deleted (strain PAD348), and with only *cpxR* deleted (strain PAD292) (14) (Table 1). The latter deletion has a polar effect on the downstream gene *cpxA*; thus, the resultant strain lacks both CpxR and CpxA (10).

We grew the resulting monolysogens in TBK at 37°C, harvested cells at regular intervals, and compared their growth characteristics and β -galactosidase activities. Mutants lacking CpxA or CpxR exhibited growth characteristics indistinguishable from those of their WT parent. In contrast, mutants lacking AckA or both AckA and Pta grew more slowly but to a similar density (Fig. 2A), as reported previously (64). Because of the difference in the growth rate, all data obtained subsequently were plotted versus OD₆₀₀.

WT cells exhibited two peaks of *PcpxP* activity; the first and

smaller peak occurred in mid-exponential phase (OD₆₀₀, ~0.5) and reached about 13,000 Miller units, while the second and larger peak corresponded with entry into stationary phase (OD₆₀₀, ~1.0) and reached about 46,000 Miller units (Fig. 2B).

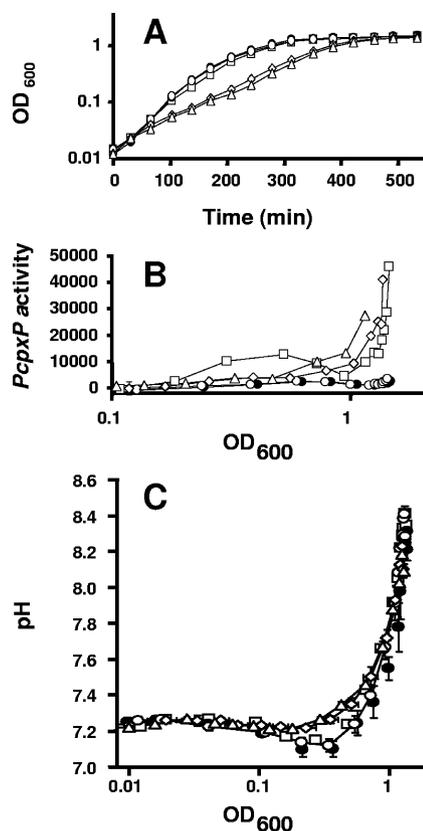


FIG. 2. Disruption of the Pta-AckA pathway diminishes Cpx signaling. WT cells (strain PAD348) (\square) and the isogenic *cpxA* (strain PAD348) (\circ), *cpxR* (strain PAD292) (\bullet), *ackA pta* (strain AJW2791) (\triangle), and *ackA* (strain AJW2790) (\diamond) mutants were lysogenized with a λ RS88 derivative that carried the λ (*PcpxP'-lacZ*) transcriptional fusion. The resultant lysogens were grown with aeration in TBK at 37°C and harvested at regular intervals. The growth was monitored (A), the β -galactosidase activity expressed in Miller units was plotted against OD₆₀₀ to standardize for growth rate differences (B), and the culture pH was plotted against the OD₆₀₀ (C). The values are the means \pm standard deviations of triplicate independent cultures.

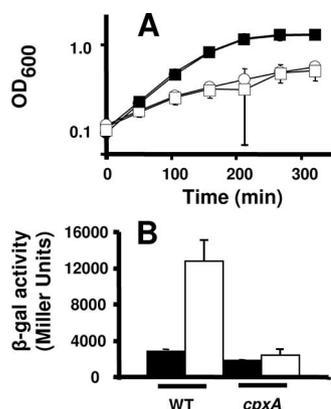


FIG. 3. Cpx response to alkaline pH requires CpxA. $\lambda PcpP$ lysogens of WT cells (strain PAD282) (squares) or the isogenic *cpxA* mutant (strain PAD348) (circles) were grown with aeration at 37°C in TBKpH7 (filled symbols and filled bars) or TBKpH8 (open symbols and open bars) and harvested at regular intervals. Growth was monitored (OD₆₀₀) (A), and the β -galactosidase (β -gal) activity was plotted against OD₆₀₀ (B). Only the final β -galactosidase values are shown. The values are the means and standard deviations of triplicate independent cultures.

Both behaviors depended upon CpxA and CpxR; both *cpxA* and *cpxR* mutants exhibited low activity throughout growth. Intriguingly, *ackA* and *ackA pta* mutants had a hybrid profile; they exhibited little activity during exponential growth but displayed substantial activity during entry into stationary phase. The existence of two activity peaks led us to speculate whether there were two distinct stimuli, one associated with exponential growth and one associated with entry into stationary phase.

Stationary-phase behavior is a CpxA-dependent response to alkaline pH. *PcpP* transcription has been reported to become activated in response to both elevated pH (9, 35) and entry into stationary phase (11, 14). Because TBK is composed primarily of amino acids and because consumption of the amino acids by cells produces ammonia (40, 64), we expected that the culture pH would rise and that the increased pH would lead to increased *PcpP* transcription. To test this hypothesis, we monitored the pH of the culture medium and found that all five strains behaved almost identically (Fig. 2C). Each strain dramatically alkalinized its environment during the transition from exponential growth to stationary phase. In each case, the maximum pH reached by the culture was about 8.4. During mid-exponential growth, the *cpxA* and *cpxR* mutants and their WT parent slightly acidified their environments before alkalinizing them. The *ackA* and *ackA pta* mutants, in contrast, did not do this. This behavior can be attributed to the substantially reduced ability of mutants that lack a functional Pta-AckA pathway to generate and excrete acetate (40, 64).

Since the increase in pH occurred concomitant with entry into stationary phase, we performed two different experiments to distinguish between these two distinct potential stimuli. First, we grew cells at 37°C in TBKpH7 or TBKpH8, harvested cells at regular intervals, and monitored their growth and *PcpP* activity. When grown at pH 8.0, *PcpP-lacZ* lysogens of WT cells exhibited about five times more promoter activity than they exhibited when they were grown at pH 7.0. In contrast, *PcpP-lacZ* lysogens of *cpxA* mutant cells exhibited weak

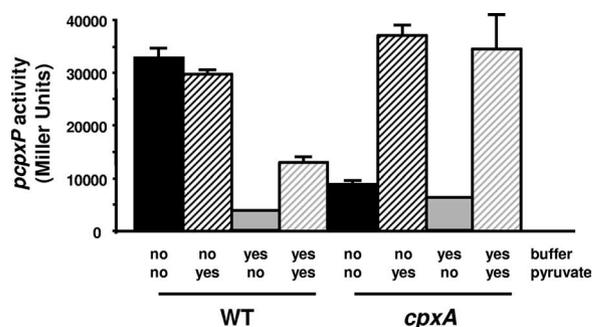


FIG. 4. Cpx response to pyruvate does not require CpxA. $\lambda PcpP$ lysogens of WT cells (strain PAD282) or the isogenic *cpxA* mutant (strain PAD348) were grown with aeration at 37°C in TBK (black bars) or in TBKpH7 (gray bars) either in the absence (filled bars) or in the presence (striped bars) of 0.8% sodium pyruvate. Cells were harvested at regular intervals, and the β -galactosidase activities were plotted against the OD₆₀₀; only the final values are shown. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.

promoter activity regardless of the pH (Fig. 3B). Since both types of cells grew more than twice as slowly at pH 8.0 as at pH 7.0 (Fig. 3A), however, the higher activity at pH 8.0 could have been due to the lower growth rate instead of the higher pH. To differentiate between these two possibilities, we compared *PcpP* activity while controlling for pH. We grew WT cells and *cpxA* mutants at 37°C in either unbuffered TBK or TBKpH7, harvested cells at regular intervals, and monitored their growth and *PcpP* activity (Fig. 4). WT cells exhibited high levels of *PcpP* activity in TBK but not in TBKpH7. In contrast, buffering had no significant effect on the *PcpP* activity exhibited by *cpxA* mutants. These results support the conclusion that pH is the principal stimulus behind the *PcpP* activity exhibited by WT cells and, by extension, the isogenic *ackA* and *ackA pta* mutants. Furthermore, these data lend credence to the reports that the pH response requires CpxA (9, 35).

Growth-dependent Cpx response depends on both CpxA and the Pta-AckA pathway. To determine if the Cpx pathway responds to some growth phase-dependent stimulus, we performed an epistasis analysis. We combined mutations in the Pta-AckA pathway with mutations in the Cpx signaling pathway, grew the cells in TBKpH7, and compared the *PcpP* activities of the double mutants to those of the parental single mutants and to those of the WT (Fig. 5A). WT cells (PAD282) exhibited *PcpP* activity that increased about 12-fold from early exponential growth to entry into stationary phase. Isogenic *cpxA* (PAD348), *ackA* (AJW2790), and *ackA pta* (AJW2791) mutants behaved similarly, but not identically. During early exponential growth, the Pta-AckA pathway mutants exhibited significantly less activity than their WT parent, which displayed less activity than its *cpxA* mutant.

Mutants defective in both pathways, *cpxA ackA* (AJW2794) and *cpxA ackA pta* (AJW2920) mutants, also exhibited growth-dependent behavior. However, they exhibited little or no detectable activity during early exponential growth and upon entry into stationary phase exhibited only about 25% the *PcpP* activity exhibited by their parents and the WT. This outcome was quite similar to the outcome for mutant strains that lacked CpxR, i.e., *cpxR* (PAD292) and *cpxR ackA pta* (AJW2793)

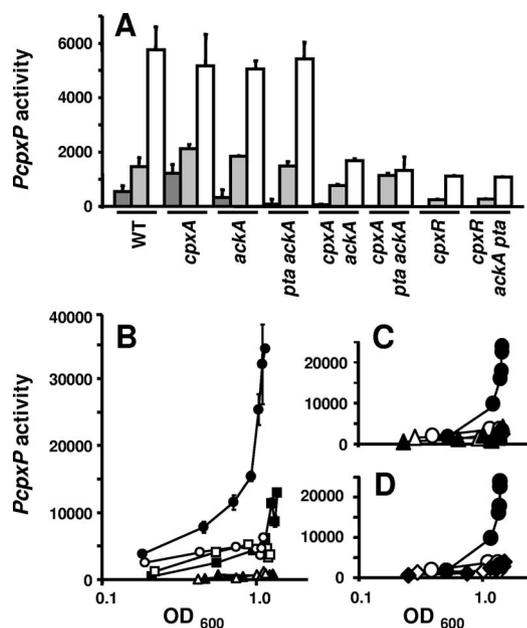


FIG. 5. Cpx response to pyruvate depends on the Pta-AckA pathway. (A) λ *PcpXP* lysogens of strains with the indicated genotypes were grown with aeration at 37°C in TBKpH7 and harvested at regular intervals, and the growth (OD₆₀₀) and β -galactosidase activity were monitored. The values were determined when the OD₆₀₀ of each strain reached 0.17 to 0.18 (dark gray bars), 0.34 to 0.35 (light gray bars), and 1.2 to 1.3 (open bars). (B) β -Galactosidase activities of λ *PcpXP* lysogens of WT cells (squares) and mutants defective for either *cpxA* (circles) or *cpxR* (triangles) in the absence (open symbols) or presence (filled symbols) of 0.8% pyruvate plotted as a function of OD₆₀₀. The values are the means \pm standard deviations of triplicate independent cultures. (C) β -Galactosidase activities of λ *PcpXP* lysogens of the *cpxA* mutant (circles) and the isogenic *cpxA pta ackA* mutant (triangles) in the absence (open symbols) or presence (filled symbols) of 0.8% pyruvate plotted against OD₆₀₀. The values are the means of triplicate independent cultures. The standard deviations are smaller than the symbols. (D) β -Galactosidase activities of λ *PcpXP* lysogens of the *cpxA* mutant (circles) and the isogenic *cpxA ackA* mutant (diamonds) in the absence (open symbols) or presence (filled symbols) of 0.8% pyruvate plotted against OD₆₀₀. The values are the means of triplicate independent cultures. The standard deviations are smaller than the symbols.

mutants. Thus, growth-dependent *PcpXP* activity depends in large part on both CpxA and the Pta-AckA pathway. Each of these factors appears to compensate for the loss of the other, an observation that is consistent with the hypothesis that they have the same function, namely, to activate CpxR during growth. Intriguingly, the *cpxR* and *cpxR ackA pta* mutants both exhibited low-level *PcpXP* activity that increased throughout growth. This observation suggests that there is an unidentified transcription factor that appears to activate *cpxP* transcription independent of CpxR.

Cpx response to excess carbon requires the Pta-AckA pathway. It has been reported that exposure to exogenous glucose induces a CpxR-dependent response that depends on the Pta-AckA pathway (9, 10). However, the medium used to derive this conclusion was not buffered. Since we found that buffering has a significant effect on *PcpXP* activity, we revisited this conclusion.

Exposure to pyruvate (a three-carbon glycolytic intermediate) results in greater accumulation of intracellular acetyl-P

than exposure to glucose (a six-carbon compound) (24a, 26). Thus, we began by exposing cells to pyruvate. To compare responses to the two compounds, we standardized the concentration to the total number of carbons. Because the authors of the previous reports exposed cells to 0.4% glucose (9, 10), we exposed cells to 0.8% sodium pyruvate.

We grew WT cells and *cpxA* mutants at 37°C in pyruvate-supplemented TBKpH7, harvested cells at regular intervals, and monitored their growth (data not shown) and *PcpXP* activity (Fig. 4). Although WT cells responded to the presence of pyruvate, the total activity was less than one-half that of WT cells grown in unbuffered pyruvate-supplemented TBK. In contrast, *cpxA* mutants responded strongly when they were exposed to pyruvate regardless of buffering. As reported previously for glucose (9), the *cpxA* mutant response to pyruvate was stronger than the response of its WT parent. Thus, in this context, CpxA inhibited the response to pyruvate, a behavior consistent with its reputed phospho-CpxR phosphatase activity (18, 42). Furthermore, we propose that CpxR can become activated in a CpxA-independent manner.

To determine if the CpxA-independent response to pyruvate requires the Pta-AckA pathway, we compared the behaviors in the presence and absence of pyruvate (Fig. 5B). WT cells responded moderately (about threefold) to pyruvate and only upon entry into stationary phase. In contrast, the *cpxA* mutant cells responded early and dramatically; their *PcpXP* activity increased about ninefold from exponential growth to entry into stationary phase. Thus, the inhibitory effect of CpxA appears to maintain low *PcpXP* activity throughout exponential growth and to limit the response upon entry into stationary phase, at least in medium buffered at pH 7.0. Conversely, cells lacking either AckA alone (*ackA*) and both AckA and Pta (*ackA pta*) did not respond to pyruvate (data not shown). This supports the hypothesis that the response to pyruvate requires the Pta-AckA pathway.

CpxA-independent response does not require acetyl-P. To activate *PcpXP* activity in the absence of its cognate SK, CpxR must have an alternative source of phosphoryl groups. Since the pyruvate response requires the Pta-AckA pathway, a prime candidate is the pathway intermediate acetyl-P, which has been shown to act through at least one other 2CST RR (RcsB) to influence genes involved in flagellation and encapsulation (19). Furthermore, CpxR can use acetyl-P as a phosphoryl donor in vitro (37, 42), and it has been proposed that acetyl-P is responsible for the CpxA-independent glucose response (4, 9, 10). To test this hypothesis, we monitored the *PcpXP* response in the *cpxA ackA* and *cpxA pta ackA* mutant strains. If acetyl-P were the phosphoryl donor for CpxA-independent activity, then one would predict that the *cpxA ackA* mutant would exhibit more activity than the *cpxA* mutant, while the *cpxA ackA pta* mutant would exhibit less. Instead, the two mutants exhibited virtually identical weak responses (Fig. 5C and D) that were barely greater than the response exhibited by cells that lacked CpxR (Fig. 5B). Since *cpxA ackA* colonies are mucoid on LBKpH7 agar at room temperature, while the *cpxA ackA pta* colonies are not (data not shown), and since mucoidy under such conditions is highly diagnostic for elevated acetyl-P levels (19; data not shown), this suggests that the lack of CpxA does not affect the acetyl-P pool and that the CpxR-dependent, CpxA-independent response to pyruvate does not depend on acetyl-P.

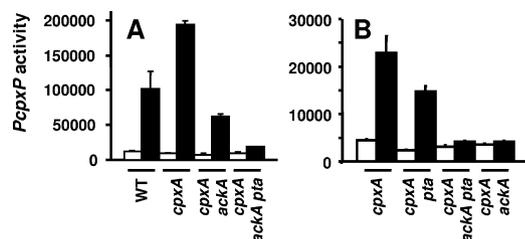


FIG. 6. Cpx response to glucose depends on the Pta-AckA pathway. (A) λ *PcpXP* lysogens of WT cells (strain PAD282) and the isogenic *cpxA* (strain PAD348), *cpxA ackA* (strain AJW2794), *cpxA pta* (strain AJW2970), and *cpxR* (PAD292) mutants were grown with aeration at 37°C in TBKph7 in the absence (open bars) or presence (filled bars) of 0.4% glucose and harvested at regular intervals, and the growth and β -galactosidase activity were monitored. Only the peak values are shown. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations. (B) λ *PcpXP* lysogens of the isogenic *cpxA* (strain PAD348), *cpxA pta* (strain AJW2964), *cpxA pta ackA* (strain AJW2970), and *cpxA ackA* (strain AJW2794) mutants were grown with aeration at 37°C in TBKph7 in the absence (open bars) or presence (filled bars) of 15 mM potassium acetate and harvested at regular intervals, and the growth and β -galactosidase activity were monitored. Only the peak values are shown. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.

This response, however, does require an intact Pta-AckA pathway.

Since exposure to pyruvate did not elicit an acetyl-P-dependent response, we asked if the same was true for exposure to glucose. Thus, we repeated the experiments described above, but this time we exposed the strains to 0.4% glucose (Fig. 6A). The results were almost identical to those obtained with pyruvate. WT cells responded to glucose, the *cpxA* mutants responded more strongly, and, as reported previously, the CpxA-independent response depended on the presence of the Pta-AckA pathway; cells that lacked CpxA, Pta, and AckA exhibited almost no response (9). We propose that the response to excess carbon requires an intact Pta-AckA pathway but neither CpxA nor acetyl-P.

However, it is possible that acetyl-P can play a role under certain circumstances. There was a distinct difference between the response to glucose and the response to pyruvate. In contrast to the lack of a response to pyruvate, the *cpxA ackA* mutant responded to glucose, albeit rather weakly. To determine if this response was due to phosphotransfer from acetyl-P to CpxR, we took advantage of the fact that *pta* mutants retain a functional AckA. Since AckA converts acetate to acetyl-P, we could set acetyl-P levels by controlling the amount of acetate added to the medium (24a). Therefore, we grew strains lacking CpxA and either AckA or Pta or both in TBKph7 or TBKph7 supplemented with 15 mM potassium acetate. Both the *cpxA* and *cpxA pta* mutants responded by exhibiting significant *PcpXP* activity, while the *cpxA ackA* and *cpxA ackA pta* mutants did not exhibit significant *PcpXP* activity (Fig. 6B). We therefore propose that the role of the Pta-AckA pathway in mediating the response to glucose (and, by extension, the response to pyruvate) does not require that it generate ATP or excrete acetate. We further infer that acetyl-P can indeed donate its phosphoryl group to CpxR. Finally, we propose that the observed response of the *cpxA ackA* mutant to glucose (Fig. 6A)

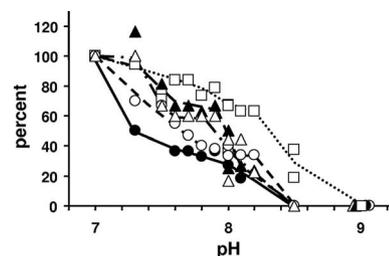


FIG. 7. Mutants defective for the Pta-AckA pathway are sensitive to alkaline pH. λ *PcpXP* lysogens of WT cells (strain PAD282) (\square) and the isogenic *cpxA* (strain PAD348) (\circ), *ackA pta* (strain AJW2791) (\triangle), *cpxA ackA pta* (strain AJW2920) (\blacktriangle), and *cpxR* (PAD292) (\bullet) mutants were grown with aeration at 37°C in LBKph7 overnight, serially diluted in buffer, plated onto LBK plates buffered at the indicated pHs, and incubated overnight. The colony diameters of 100 colonies were determined, and the mean diameter was calculated for each strain at each pH. The mean diameter on pH 7.0 plates was arbitrarily defined as 100%. The data shown are a compilation of data from several independent experiments. The standard deviations are smaller than the symbols.

was a hybrid: the dominant effect was reduced activity due to general disruption of the Pta-AckA pathway, and the minor effect was partial compensation due to phosphotransfer from acetyl-P to CpxR.

Mutants defective for the Pta-AckA pathway are sensitive to alkaline pH. Pta-AckA pathway mutants exhibit weak Cpx signaling, which protects cells against alkaline pH (9). We reasoned, therefore, that the Pta-AckA pathway protects cells against exposure to alkaline pH. We grew cells at 37°C in LBKph7 to mid-exponential phase (OD_{600} , ~ 0.4) or early stationary phase, serially diluted the cultures, plated the dilutions onto LBK plates buffered at various pHs from pH 7 to 9, incubated the plates overnight, and calculated the mean diameter of the colonies (Fig. 7). Whereas WT cells were the most resistant to alkaline pH, *cpxR* mutant cells were the least resistant. In contrast, the other mutants (*cpxA*, *ackA pta*, *cpxA ackA pta*, *ackA*, and *cpxA ackA* mutants) (data not shown) exhibited intermediate resistance. This behavior is consistent with the hypothesis that CpxA and the Pta-AckA pathway each contribute to the activation of CpxR.

DISCUSSION

Summary. As part of our long-term effort to map the impact of acetyl-P on the network of *E. coli* 2CST pathways, we discovered that its pathway (Pta-AckA) has a significant effect on CpxR-dependent signaling even when the cognate SK (CpxA) is present and functional. Although we showed that the activation of CpxR can correlate with the intracellular concentration of acetyl-P, the observed CpxA-independent responses depended less on the availability of acetyl-P and more on the status of its pathway. Although the nature of this CpxA- and acetyl-P-independent activation of CpxR remains unknown, we believe that the implications of our findings are broad based and highly significant. First, we clearly illustrated the complexity of the Cpx pathway and demonstrated that it is necessary to control for multiple pathway stimuli and to perform a complete epistasis analysis. Second, we provided further genetic evidence that the SK CpxA can function as a phosphatase and

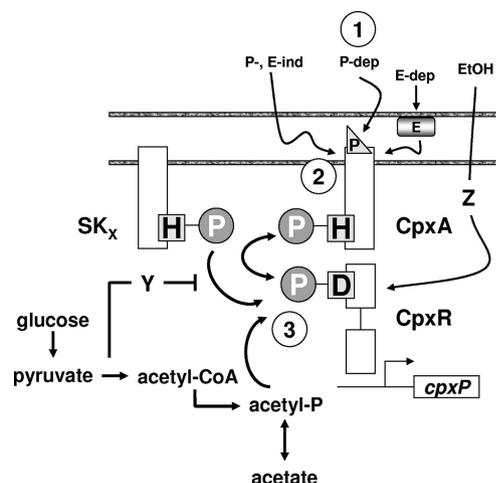


FIG. 8. Cpx pathway integrates environmental information at several levels. 1, CpxP integrates signals resulting from misfolded proteins; 2, the periplasmic domain of CpxA integrates CpxP-dependent (P-dep), NlpE-dependent (E-dep), and CpxP- and NlpE-independent (P-, E-ind) signals; 3, by receiving phosphoryl groups from several donors, CpxR integrates CpxA-dependent signals and CpxA-independent signals. The CpxA-independent signals include the undefined CpxR-dependent response to ethanol (EtOH) and signals that emanate through acetyl-P and a proposed noncognate SK (SK_x). The activity of this SK depends on the status of the Pta-AckA pathway. Disruption of this pathway permits accumulation of a molecule, Y, which directly or indirectly inhibits the transfer of phosphoryl groups from SK_x to CpxR. The mediator of the ethanol response, Z, in theory could be SK_x .

propose that this activity may be the default status (i.e., in the absence of a strong stress signal, such as alkaline pH). Other examples have been described (19). Third, we convincingly demonstrated that the Cpx pathway receives signals associated with growth and central metabolism and that these signals are linked to the Pta-AckA pathway. Finally, we showed that this linkage between a central metabolic pathway and a signal transduction pathway influences the ability of cells to survive at an alkaline pH. Each of these issues is discussed in greater detail below.

Cpx signal integration. On the basis of our observations and previously published reports, we propose that the Cpx pathway integrates signals at three distinct levels (Fig. 8). (i) In the periplasm, CpxP integrates stimuli predicted to cause misfolding of envelope proteins (for reviews, see references 15, 16, 43, and 48). (ii) At the cytoplasmic membrane, the periplasmic domain of CpxA integrates information concerning the status of the periplasmic chaperone CpxP, signals that depend on the lipoprotein NlpE, and stimuli that depend on neither Cpx nor NlpE (7, 14, 36, 44, 45, 66). (iii) Finally, in the cytoplasm, CpxR integrates these CpxA-dependent signals with those that are independent of CpxA (e.g., entry into stationary phase and exposure to excess carbon [9; this paper]).

CpxA phosphatase-kinase switch. In vitro, CpxA possesses autokinase, phosphotransfer, and phospho-CpxR phosphatase activities (18, 42). The existence of point mutations in the CpxA gene (the so-called CpxA* alleles) that lead to constitutively activated CpxR-dependent targets (e.g., *PcpxP* and *PdegP* [10, 12, 37, 42, 45]) has been interpreted in terms of a mutant protein defective in phosphatase activity and suggests

that the WT protein exhibits phosphatase activity in vivo. The observations that, compared to WT cells, *cpxA* null mutants can exhibit substantially greater *PcpxP* activity (4, 8–10, 35; this paper) and can express elevated steady-state levels of CpxR (P. DiGiuseppe-Champion and T. Silhavy, personal communication) provide further support. If this hypothesis is correct, then the two activities must exist in an equilibrium that can be shifted in response to specific stimuli. The stimuli associated with the extracytoplasmic environment (Fig. 8) clearly favor the autokinase activity (7, 18). We now propose that signals associated with the intracellular environment (e.g., entry into stationary phase and assimilation of excess pyruvate or glucose) favor the phosphatase activity. We further posit that the default status of CpxA is to function as a phosphatase and that envelope stresses shift the equilibrium toward the kinase activity.

Nature of growth-dependent Cpx activation. It has been reported previously that entry into stationary phase generates a CpxA-independent response (11, 14). The nature of the response, however, has remained elusive. The response of cells grown in glucose minimal phosphate-buffered medium depends in part on σ^S (11), while the response of cells grown in LB does not (14). Like LB, TBK is based on tryptone. Furthermore, σ^S is not fully functional this early in the transition to stationary phase, at least during growth in LB (60). Finally, deletion of both CpxA and the Pta-AckA pathway reduced growth-dependent *PcpxP* activity to levels that resembled those of *cpxR* mutants (Fig. 5A). We therefore propose that *PcpxP* became activated in response to both CpxA-dependent and Pta-AckA-dependent stimuli and that each pathway can compensate for the other. This raises an intriguing question. Why, during normal growth in a tryptone-based broth, would Cpx signaling respond to both envelope-associated and growth-associated cues? We propose that such growth is stressful and that cells monitor both their periplasmic and cytoplasmic compartments for signs of growth-associated stress. Indeed, other workers have postulated that normal growth generates CpxP substrates and, hence, a CpxA-dependent response (23).

Pta-AckA connection. Contrary to previous reports (4, 9, 10), acetyl-P is unlikely to be the primary contribution of the Pta-AckA pathway to Cpx signaling, at least not under the conditions tested. This conclusion is supported by the observation that the *cpxA* null mutant expresses more CpxR than its WT parent expresses (DiGiuseppe-Champion and Silhavy, personal communication). Instead, CpxA-independent activation must be a consequence of a general disruption in the Pta-AckA pathway. The resultant decrease in *PcpxP* activity could be due to the loss of a pathway product that activates Cpx signaling. Alternatively, it could result from accumulation of a pathway precursor that inhibits signaling.

It is unlikely that CpxR-dependent *PcpxP* activity is induced by a direct product of the Pta-AckA pathway, which produces acetate, ATP, and CoA (Fig. 1B). Figure 6B shows that acetate can induce *PcpxP* activity in the absence of CpxA but not in the absence of both CpxA and the Pta-AckA pathway. If acetate mediated the excess carbon source response, then exogenous acetate should have compensated for the lack of the Pta-AckA pathway. Since it did not, acetate must be removed from consideration. The results shown in Fig. 6B also eliminate ATP and CoA from contention, because the Pta-AckA-dependent

response to acetate should require both ATP and CoA, while the response to excess carbon source should generate it (Fig. 1B). It is hard to comprehend how these opposite consequences could result in the same behavior.

Disruption of the Pta-AckA pathway also can have indirect effects. *E. coli* produces limiting amounts of CoA. Therefore, cells must recycle it to maintain glycolytic flux, and loss of the Pta-AckA pathway removes their “favored” option, at least at neutral pH. An alternative might be for cells to convert the acetyl-CoA to ethanol (for a review, see reference 63). Preliminary experiments indicated that ethanol can activate *PcpXP* in a CpxR-dependent manner. Furthermore, they showed that the effect is independent of both CpxA and the Pta-AckA pathway. However, the level of activity elicited by ethanol is too low to explain the much larger response to excess carbon source (B. Lima and A. J. Wolfe, unpublished data).

Having eliminated the most obvious direct and indirect products of the Pta-AckA pathway as activators of CpxA-independent signaling, we propose that the normal function of the Pta-AckA pathway maintains some inhibitory substance (Y) at a low concentration (Fig. 8). The identity of Y remains unknown; however, some clues exist. In many respects, mutants that lack AckA or both Pta and AckA behave similarly. In batch culture, they excrete little or no acetate, and they limit their production of ethanol. Instead, they both excrete pyruvate, lactate, and glutamate, a behavior that might be explained by the observation that these mutants exhibit increased expression and activity of key glycolytic and TCA cycle enzymes (for a review, see reference 63). Surprisingly, they do not increase expression of their CoA biosynthetic machinery (64), nor do they alter their acetyl-CoA pool relative to that of their WT parent (26). The increased expression and activity of both glycolytic and TCA cycle enzymes and the altered excretion profile suggest that cells attempt to compensate for the loss of the Pta-AckA pathway (64). Perhaps the inhibitory substance Y is a consequence of this compensation.

Whatever its nature, this inhibitory substance must act upon a CpxR that can be phosphorylated without the assistance of either CpxA or acetyl-P. The best candidate is a noncognate SK (Fig. 8). One such SK (EnvZ) has been shown to transfer its phosphoryl group to CpxR (53), and it possesses phospho-CpxR phosphatase activity in vitro (65). However, glucose-induced, CpxA-independent repression of the CpxR target gene *ompF* does not require EnvZ (4).

pH sensitivity. Since disruption of the Pta-AckA pathway inhibits Cpx signaling (Fig. 2), it should be no surprise that the mutants are sensitive to alkaline pH (Fig. 7). This relationship appears to be physiologically relevant. Exposure to alkaline pH induces Pta expression (55), while mutants that lack an intact Pta-AckA pathway are skewed toward acid resistance (25, 64). Although mutants that lack CpxA, the Pta-AckA pathway, or CpxR cannot grow when they are exposed to pH 8.0, they do not die in unbuffered TBK even though the pH in this environment reaches a potentially lethal level, pH 8.4 (Fig. 2C). We infer that during growth in broth, these mutants have the capacity to develop protection against exposure to alkaline pH. The nature of this process remains unknown.

Conclusion. Cells experience multiple stimuli that impact the phosphorylation status of CpxR. Thus, the CpxR-dependent behavior of WT cells represents an integration of all these

stimuli, and efforts to dissect this (and other) complex pathways must take this into account. To perform successful dissections, the most appropriate tool is a complete and careful epistasis analysis. A good place to start is to reevaluate studies that relied strictly on *ackA pta* mutants to conclude that acetyl-P donates its phosphoryl group to certain 2CST RRs, including NR₁/NtrC (1), OmpR (2, 3, 22, 31), and RssB/SprE (6).

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