

# Repression of *Escherichia coli* PhoP-PhoQ Signaling by Acetate Reveals a Regulatory Role for Acetyl Coenzyme A

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**The PhoP-PhoQ two-component system regulates the transcription of numerous genes in response to changes in extracellular divalent cation concentration and pH. Here we demonstrate that the *Escherichia coli* PhoP-PhoQ two-component system also responds to acetate. Signaling by the *E. coli* PhoP-PhoQ system was repressed during growth in acetate ( $\geq 25$  mM) in a PhoQ-dependent manner. The periplasmic sensor domain of PhoQ was not required for acetate to repress signaling. Acetate-mediated repression of the PhoP-PhoQ system was not related to changes in the intracellular concentration of acetate metabolites such as acetyl-phosphate or acetyladenylate. Genetic analysis of acetate metabolism pathways suggested that a perturbation of acetyl coenzyme A turnover was the cause of decreased PhoP-PhoQ signaling during growth in acetate. Consistent with this hypothesis, intracellular acetyl coenzyme A levels rose during growth in the presence of exogenous acetate. Acetyl coenzyme A inhibited the autokinase activity of PhoQ *in vitro*, suggesting that the *in vivo* repressing effect may be due to a direct inhibition mechanism.**

The PhoP-PhoQ two-component signal transduction system regulates  $Mg^{2+}$  homeostasis and/or virulence properties in numerous gram-negative bacteria (22). The system consists of a transmembrane histidine kinase (PhoQ) and a cytoplasmic response regulator (PhoP). The PhoP-PhoQ system has been extensively characterized in *Salmonella enterica* serovar Typhimurium, where it regulates the transcription of numerous genes in response to changes in the concentration of divalent cations such as  $Mg^{2+}$  and  $Ca^{2+}$  (21, 45). Limiting concentrations of extracellular divalent cations activate the system, resulting in the net phosphorylation of PhoP by PhoQ (21, 45). Signal propagation occurs through autophosphorylation of PhoQ on a conserved histidine in its cytoplasmic domain, followed by transfer of the phosphoryl group to a conserved aspartate in PhoP (11, 37, 43). Phosphorylated PhoP interacts with the promoters of PhoP-PhoQ-regulated genes and activates or represses transcription (36, 45, 51). Among the genes whose transcription phosphorylated PhoP activates is *mgta*, a gene encoding a high-affinity  $Mg^{2+}$  transporter (27, 45, 51). The periplasmic domains of PhoQ homologs from *S. enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Escherichia coli* have been shown to interact with  $Mg^{2+}$  (20, 21, 30, 48). This interaction is thought to mediate a conformational change in PhoQ that regulates the enzymatic activities of its cytoplasmic domain. Instead of phosphorylating PhoP,  $Mg^{2+}$ -bound PhoQ promotes the dephosphorylation of PhoP (11, 37, 43). In addition to sensing divalent cation concentration, the PhoP-PhoQ two-component system has also been shown to regulate gene expression in response to pH changes (4). However, the mechanism for the pH response remains unclear.

In this work, we add acetate to the list of extracellular signals that are sensed by the PhoP-PhoQ two-component system.

Acetate represses the PhoP-PhoQ two-component system in a PhoQ-dependent manner. We demonstrate that repression of signaling does not involve interactions between acetate and the periplasmic sensor domain of PhoQ. Instead, repression of signaling occurs through a perturbation of intracellular metabolism—the turnover of acetyl coenzyme A (acetyl-CoA). Finally, we demonstrate that acetyl-CoA acts as a noncompetitive inhibitor of the PhoQ autokinase reaction *in vitro*. Taken together, these findings support a model in which an extracellular signal (acetate) represses two-component signaling by influencing the concentration of an intracellular metabolite (acetyl-CoA) that inhibits histidine kinase autophosphorylation.

## MATERIALS AND METHODS

**Chemicals and media.** Na<sup>+</sup> acetate, NaCl, and Na<sup>+</sup> formate were obtained from Sigma (St. Louis, Mo.) and were highly pure (Sigma-Ultra) in order to minimize contamination of N medium with  $Mg^{2+}$ . Acetyl-CoA was obtained from Sigma. N medium used in this study was identical to the N medium described elsewhere (39) with the exception that 0.4% glucose and 0.1% Casamino Acids were used. Antibiotics were used at the following concentrations: tetracycline, 10  $\mu$ g/ml; kanamycin, 25  $\mu$ g/ml; spectinomycin, 100  $\mu$ g/ml; and ampicillin, 100  $\mu$ g/ml.

**Bacterial strains.** All strains used in this study are derivatives of *E. coli* K-12 and are listed in Table 1. BW13711, BW16462, BW16463, and BW16545 were kindly provided by B. Wanner (19, 34). AJW803 was kindly provided by A. Wolfe (29). Transductions with P1<sub>vir</sub> were used to transfer *ackA200 zej223::Tn10*, *pta-200 zej223::Tn10*,  $\Delta$ (*pta-ack-hisQ-hisP*) *zej223::Tn10*, and  $\Delta$ *acs:kan-1* into CSH26. Transductions were performed as described elsewhere (35). Transductants carrying an *ackA200*, *pta-200*, or  $\Delta$ (*pta-ack-hisQ-hisP*) mutation were isolated by selecting for tetracycline resistance and screening for poor growth on Luria-Bertani (LB) plates. The presence of the *ackA200*, *pta-200*, and  $\Delta$ (*pta-ack-hisQ-hisP*) mutations was confirmed by streaking the transductants on M63 plates supplemented with either low (2.5 mM) or high (50 mM) concentrations of acetate as the sole carbon source. As reported by Kumari et al. (29), strains harboring mutations in the Ack-Pta pathway grew poorly on high-acetate plates but lacked a growth defect on the low-acetate plates. Transductants carrying the  $\Delta$ *acs:kan-1* mutation were isolated by selecting for kanamycin resistance. The  $\Delta$ *acs:kan-1* mutation was also confirmed by growing the *acs* mutant on M63 plates supplemented with low (2.5 mM) or high (50 mM) concentrations of acetate as the sole carbon source. As expected (29), the *acs* mutant grew poorly on the low-acetate plates but lacked a growth defect on the high-acetate plates.

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TABLE 1. *E. coli* strains

Strain	Relevant genotype	Construction or reference
CSH26	<i>ara</i> $\Delta(lac-pro)$ <i>thi</i>	35
CSH26 $\Delta$ Q	<i>ara</i> $\Delta(lac-pro)$ <i>thi</i> <i>phoQ</i>	48
BW13711	$\Delta lacX74$ <i>rpoS</i> (Am)	34
BW16462	$\Delta lacX74$ <i>rpoS</i> (Am) <i>pta-200</i> <i>zej-223::Tn10</i>	19
BW16463	$\Delta lacX74$ <i>rpoS</i> (Am) $\Delta(pta ackA hisQ hisP)$ <i>zej-223::Tn10</i>	19
BW16545	$\Delta lacX74$ <i>rpoS</i> (Am) <i>ackA200</i> <i>zej-223::Tn10</i>	19
AJW803	CP875 ( $\Delta acs::Km-1$ )	29
CP875	$\Delta lacX74$ <i>thi-1</i> <i>thr-1</i> (Am) <i>leuB6</i> <i>metF159</i> (Am) <i>rpsL136</i> $\Delta lacY$	29
CDW1	CSH26 <i>pta-200</i> <i>zej-223::Tn10</i>	CSH26 $\times$ BW16462 (P1)
CDW2	CSH26 $\Delta(pta ackA hisQ hisP)$ <i>zej-223::Tn10</i>	CSH26 $\times$ BW16463 (P1)
CDW3	CSH26 <i>ackA200</i> <i>zej-223::Tn10</i>	CSH26 $\times$ BW16545 (P1)
CDW4	CSH26 ( $\Delta acs::Km-1$ )	CSH26 $\times$ AJW803 (P1)

**$\beta$ -Galactosidase assays.** Overnight cultures were grown in N medium supplemented with 0.5 mM Mg<sup>2+</sup> at 37°C for 18 to 22 h, diluted 100-fold into N medium lacking Mg<sup>2+</sup>, and grown to an optical density at 600 nm of 0.2 to 0.4.  $\beta$ -Galactosidase activity was measured as described by Miller (35). In some experiments, overnight cultures were diluted 100-fold into N medium supplemented with Na · acetate, NaCl, or Na · formate. In other experiments, overnight cultures were diluted 100-fold into N medium containing 0.4% glucuronate, 0.4% pyruvate, 0.4% glycerol, or 0.4% fumarate in place of glucose as the carbon source.

**Plasmids.** pLPQ2 and pNL3 have been described elsewhere (48). Briefly, pNL3 is a pBR322-derived reporter plasmid in which the PhoP-activated *phoN* promoter region of *S. enterica* serovar Typhimurium is fused to the *lacZ* structural gene to generate a transcriptional fusion. pLPQ2 is a low-copy-number pSC101-derived plasmid in which the *phoP-phoQ* operon of *E. coli* is driven by the lacUV5 promoter. pLPQ $\Delta$ SD2 (43) is a derivative of pLPQ2 in which an in-frame deletion in *phoQ* fuses codons 50 to 182, effectively eliminating the coding region for the periplasmic sensor domain. pNL2 (30) is a low-copy-number pSC101-derived plasmid that carries the *phoN-lacZ* transcriptional fusion. pAED4QTR (32) is an expression vector for the PhoQ cytoplasmic domain in which a fragment of *phoQ* encoding the cytoplasmic domain (codons 219 to 486) was fused to an initiating ATG downstream of the T7  $\phi$ 10 promoter and ribosome binding site of pAED4 (18).

**Protein purification.** The purification of the PhoQ cytoplasmic domain (QTR) has been described previously (32). Briefly, BL21 harboring pAED4QTR was induced to express the PhoQ cytoplasmic domain by addition of 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). Cells were harvested and lysed by sonication. Cell debris was removed by centrifugation. QTR was purified by ammonium sulfate precipitation followed by MonoQ anion exchange chromatography and Superdex 75 gel filtration chromatography. The cytoplasmic domain was judged to be more than 95% pure by gel electrophoresis and Coomassie blue staining.

**Autokinase assays.** Autokinase assays were performed as described previously (32) with a few modifications. All reactions were performed at 22°C in 10 mM Tris-HCl (pH 8.0)–25 mM KCl–0.4 mM MgCl<sub>2</sub>. Nonlabeled ATP (10, 30, 90, and 180  $\mu$ M) and trace amounts of [ $\gamma$ -<sup>32</sup>P]ATP (0.0067, 0.02, 0.06, and 0.12  $\mu$ Ci/ $\mu$ l) were incubated with 1  $\mu$ M purified PhoQ cytoplasmic domain and increasing amounts of acetyl-CoA (0, 1.25, 1.75, and 2.5 mM). Aliquots were removed at various times and stopped by the addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. The samples were then subjected to gel electrophoresis, and phosphorylated protein was quantitated by phosphorimaging by using a Molecular Dynamics 445 SI PhosphorImager. The rate of protein phosphorylation was calculated for each ATP concentration and plotted versus rate/[ATP] (Eadie Hofstee plot).  $K_m$  (negative slope) and  $V_{max}$  ( $y$  axis intercept) were derived from the plots, and  $k_{cat}$  was calculated as  $V_{max}/[enzyme]$ .

**Determination of intracellular acetyl-CoA.** Cultures (800 ml) were grown to an optical density at 600 nm of 0.320 (cell density,  $4 \times 10^8$  CFU/ml) in N medium

or N medium supplemented with 75 mM Na · acetate. Cells were harvested by centrifuging at  $8,000 \times g$  for 10 min. Harvested cells were resuspended in 0.8 ml of 10 mM sodium phosphate (pH 7.5)–10 mM MgCl<sub>2</sub>–1 mM EDTA. Cell extracts were prepared by perchloric acid extraction (42). Ice-cold HClO<sub>4</sub> was added to a final concentration of 0.5 M, and the cell suspension was incubated for 40 min on ice. The suspension was centrifuged for 5 min at  $2,800 \times g$ , and 1.2 ml of the supernatant liquid was recovered and neutralized with 0.35 ml of saturated KHCO<sub>3</sub>. A 0.48-ml sample of this neutralized extract was assayed for acetyl-CoA by using a coupled enzyme assay involving the enzymes malate dehydrogenase and citrate synthase (42), and acetyl-CoA was measured indirectly by monitoring the reduction of NAD<sup>+</sup> to NADH. The reaction mixture contained 150 mM Tris-HCl (pH 8.0), 10 mM malic acid, 3 mM MgCl<sub>2</sub>, 7.5 mM NAD, and 10  $\mu$ g of malate dehydrogenase (porcine heart mitochondria, 1,460 U/mg). The reaction mixture was incubated at 30°C for 30 min, and an initial optical density at 340 nm was recorded. Twenty micrograms of citrate synthase (porcine heart, 132 U/mg) was added, the reaction was incubated for an additional 45 min at 30°C, and a final optical density at 340 nm was recorded. The acetyl-CoA concentration in the 0.48 ml of neutralized perchloric acid extract was determined by subtracting the initial optical density reading at 340 nm from the final optical density reading at 340 nm. This value was then compared to a standard curve prepared by using known concentrations of acetyl-CoA (50, 100, and 250  $\mu$ M). Intracellular acetyl-CoA concentration was calculated by using a measured cell density of  $4 \times 10^8$  CFU/ml and an approximated cell volume of  $1 \times 10^{-12}$  liter/cell (26).

## RESULTS

**Exogenous acetate represses PhoP-PhoQ signaling.** The influence of extracellular acetate on PhoP-PhoQ signaling was assessed by monitoring the expression of the PhoP-regulated *phoN::lacZ* transcriptional fusion (30). *E. coli* cells carrying *phoN::lacZ* on a low-copy-number plasmid were grown in N medium without Mg<sup>2+</sup> and with increasing amounts of Na · acetate. As shown in Fig. 1a, reporter expression decreased when Na · acetate was added to the medium. This decrease in PhoP-PhoQ signaling was specific for the acetate anion, since the addition of equivalent amounts of NaCl or Na · formate had a negligible effect on  $\beta$ -galactosidase levels. In addition, K · acetate caused repression of PhoP-PhoQ signaling that was identical to the repression observed with Na · acetate (data not shown). The decrease in PhoP-PhoQ signaling was not the result of pH effects, because the pH of the medium was always 7.4 in these experiments due to the presence of 100 mM Tris-HCl (pH 7.4). Experiments with *S. enterica* serovar Typhimurium showed that acetate repressed PhoP-PhoQ signaling in that organism as well and that the effect was not limited to the *phoN* promoter (data not shown).

The role of PhoQ in acetate-mediated repression of PhoP-PhoQ signaling was determined by monitoring reporter gene expression in a *phoQ* null mutant. Instead of repressing PhoP-PhoQ signaling, Na · acetate caused roughly a fivefold stimulation of reporter expression, while NaCl or Na · formate again had little effect on signaling (Fig. 1b). Reporter expression in the *phoQ* null mutant was low due to the absence of PhoQ-mediated phosphorylation of PhoP. These results indicate that PhoQ is required for acetate-mediated repression of PhoP-PhoQ signaling.

Since PhoQ was required for acetate to repress PhoP-PhoQ signaling, it was possible that acetate exerted its effect through a direct interaction with PhoQ's periplasmic sensor domain. To test this possibility, acetate-mediated repression was assessed in a *phoQ* null mutant complemented with a plasmid expressing either wild-type *phoQ* or a sensor domain deletion mutant of *phoQ* (43). As shown in Fig. 2, repression of signaling was still observed in the sensor domain deletion mutant, indicating

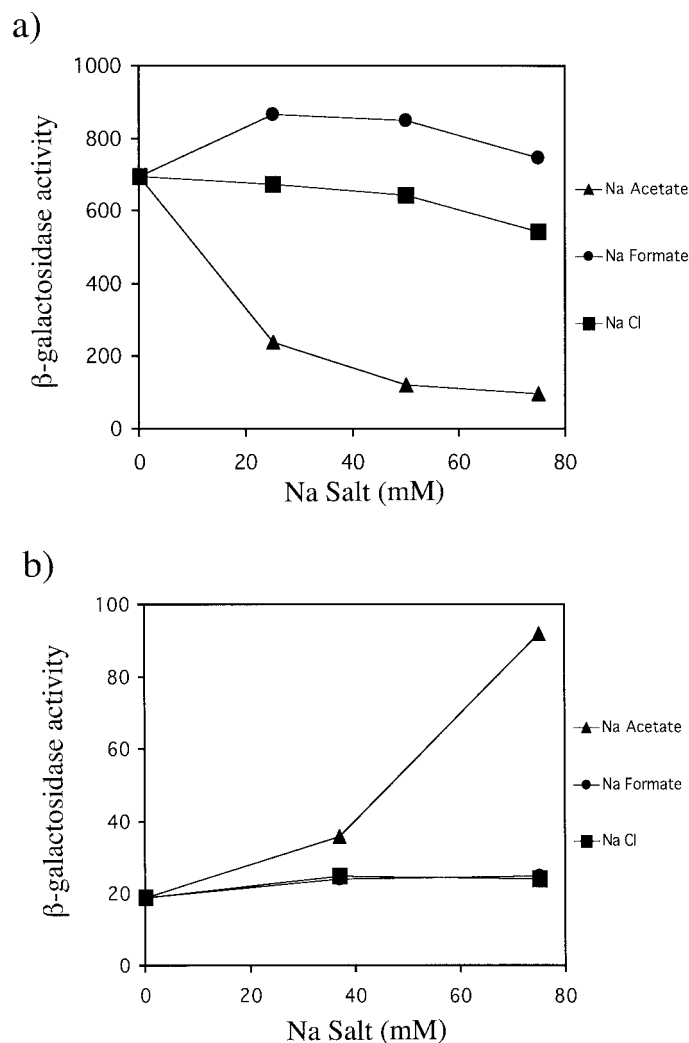


FIG. 1. (a) In vivo PhoP-PhoQ signaling responses to increasing concentrations of Na · acetate, Na · formate, and NaCl. *E. coli* strain CSH26 carrying the pNL2 reporter plasmid was assayed for  $\beta$ -galactosidase activity following growth in N medium supplemented with 0, 25, 50, and 75 mM Na salts. (b) PhoQ dependence of acetate-mediated repression of PhoP-PhoQ signaling. *E. coli* strain CSH26 $\Delta$ Q carrying the pNL3 reporter plasmid was assayed for  $\beta$ -galactosidase activity following growth in N medium supplemented with 0, 37.5, and 75 mM Na salts. The pNL3 reporter plasmid was used rather than pNL2 because its higher copy number enhanced the low expression levels that are observed in a *phoQ* mutant. The values are means calculated from at least three independent experiments for each strain. The standard deviations were less than 20% of the mean in all cases.

that acetate influences PhoQ activity via a sensor domain-independent mechanism. Presumably, the sensor domain deletion mutant of PhoQ supported less signaling than wild-type PhoQ because the periplasmic sensor domain is necessary to form PhoQ's fully active signaling conformation (43). In the absence of the sensor domain, PhoQ must adopt a partly active conformation that is still sensitive to acetate.

**The acetate kinase-phosphotransacetylase (Ack-Pta) pathway is required for normal PhoP-PhoQ signaling.** Acetate has been shown to modulate the properties of several other two-component signal transduction pathways (28, 33, 50). In most cases, acetate exerts its effect on these signaling pathways by influencing the intracellular concentration of its metabolized intermediates—acetyl-adenylate, acetyl-phosphate, and acetyl-CoA. Protonated acetate enters the cytoplasm by diffusing across the inner membrane, where it can be converted to acetyl-CoA through two distinct pathways (Fig. 3) (10). In one

pathway, acetyl-CoA synthetase (Acs) converts acetate to acetyl-CoA through a mechanism that involves an acetyl-adenylate intermediate (10, 29). Acs and acetyl-adenylate have been implicated in the modulation of chemotaxis signaling properties through acetylation of the response regulator CheY (1–3, 50). In the second pathway, acetate kinase (Ack) catalyzes the reversible conversion of acetate to acetyl-phosphate, and phosphotransacetylase (Pta) catalyzes the reversible conversion of acetyl-phosphate to acetyl-CoA (10). Acetyl-phosphate has been shown to phosphorylate numerous response regulator proteins in vitro (19, 31, 33) and has been shown to influence several two-component signal transduction pathways in vivo (1, 9, 16, 19, 24, 33, 44, 49). In both pathways, acetate is metabolized to acetyl-CoA, which then feeds directly into the tricarboxylic acid (TCA) cycle or serves as a precursor for lipid biosynthesis (13).

To determine if either of the two acetate metabolism path-

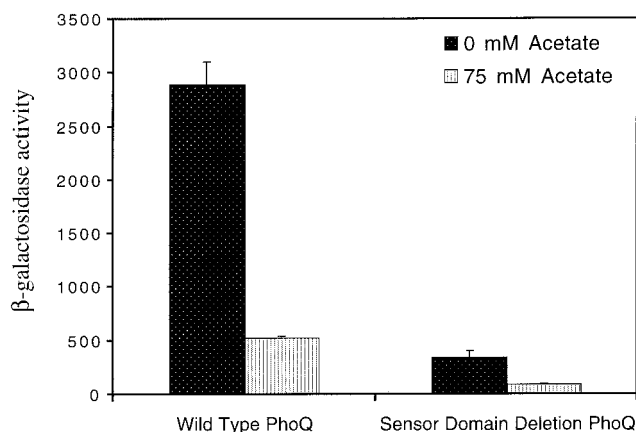


FIG. 2. In vivo PhoP-PhoQ signaling responses to acetate in a *phoQ* mutant of *E. coli* complemented with a plasmid expressing either wild-type PhoQ or a sensor domain deletion mutant of PhoQ. CSH26 $\Delta$ Q/*F'lacI*<sup>9</sup>/pNL3/pLPQ2 (wild-type PhoQ) and CSH26 $\Delta$ Q/*F'lacI*<sup>9</sup>/pNL3/pLPQ $\Delta$ SD2 (sensor domain deletion PhoQ) were assayed for  $\beta$ -galactosidase activity following growth in N medium supplemented with 0 or 75 mM Na $\cdot$ acetate. The values are means calculated from at least three independent experiments for each strain. Error bars denote one standard deviation.

ways were responsible for repression of PhoP-PhoQ signaling by acetate, mutants lacking each of the pathways were assessed for effects on PhoP-PhoQ signaling. As shown in Fig. 4, the phenotype of an *ack-pta* mutant was unlike that of the wild type in that reporter expression was low in this strain regardless of the presence or absence of exogenous acetate, whereas the phenotype of an *acs* mutant was indistinguishable from that of the wild type. Since the reduction in PhoP-PhoQ signaling caused by eliminating the Ack-Pta pathway appeared to mimic the effect of growing the wild-type strain in the presence of high concentrations of exogenous acetate, we focused our attention on the molecular functions of the Ack-Pta pathway and the physiological consequences of its disruption.

#### Perturbation of acetyl-CoA turnover is responsible for reduced PhoP-PhoQ signaling during growth in acetate and in

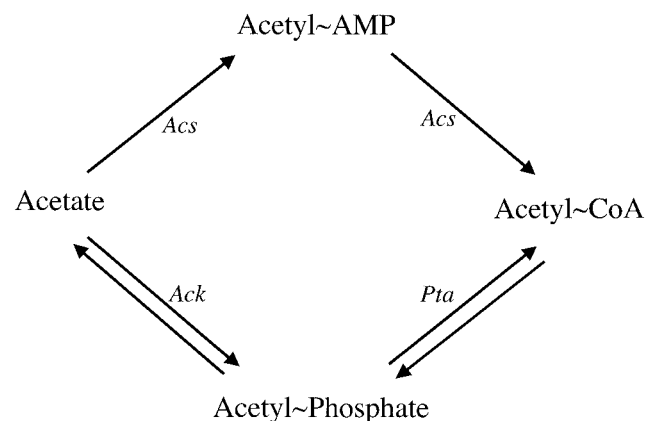


FIG. 3. Acetate metabolism pathways in *E. coli*. Abbreviations: Acs, acetyl coenzyme A synthetase; Ack, acetate kinase; Pta, phosphotransacetylase; acetyl~Amp, acetyladenylate; acetyl~CoA, acetyl coenzyme A.

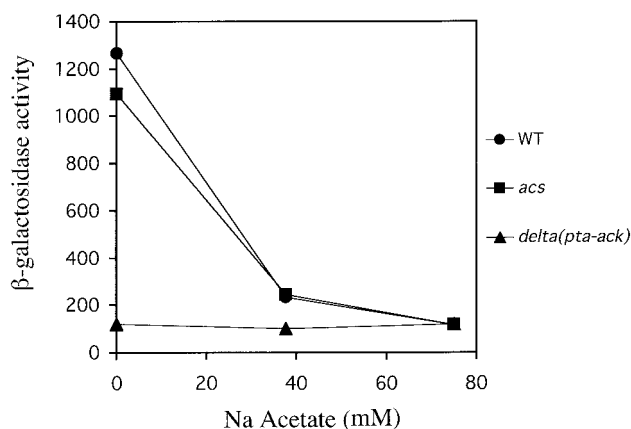


FIG. 4. In vivo responses of wild-type *E. coli* and *acs* and *ack-pta* mutants to increasing concentrations of Na $\cdot$ acetate. *E. coli* strains CSH26 (wild type), CDW2 (*ack-pta*), and CDW4 (*acs*) carrying the pNL2 reporter plasmid were assayed for  $\beta$ -galactosidase activity following growth in N medium supplemented with 0, 37.5, and 75 mM Na $\cdot$ acetate. The values are means calculated from three independent experiments for each strain. The standard deviations were less than 20% of the mean in all cases.

***ack-pta* mutants.** The most obvious difference between acetate uptake mediated by Acs and uptake through the Ack-Pta pathway is the intermediate compound in the conversion of acetate to acetyl-CoA (acetyladenylate versus acetyl-phosphate) (Fig. 3). Since acetyl-phosphate can play a role in other two-component systems by directly phosphorylating the response regulator (19, 24, 33, 49) and is a unique intermediate in the Ack-Pta pathway, it seemed possible that the *ack-pta* mutant phenotype might be due to reduced intracellular levels of acetyl-phosphate in this mutant. To test this possibility, we examined PhoP-PhoQ signaling in *ack* and *pta* single mutants, as well as in the *ack-pta* mutant, during growth in N medium without acetate. When *E. coli* is grown in glucose (as is the case in the N medium used here), more acetyl-CoA is produced than can be utilized by the TCA cycle. The excess acetyl-CoA is converted to acetate via the Ack-Pta pathway (12, 25). Knocking out *ack* prevents the conversion of acetyl-phosphate to acetate. As a consequence, acetyl-phosphate levels rise (42). Therefore, if acetyl-phosphate contributes to PhoP-PhoQ signaling, an *ack* mutant should exhibit elevated signaling. Knocking out *pta* prevents acetyl-CoA's conversion to acetyl-phosphate. Therefore, acetyl-phosphate levels are low in a *pta* mutant (42), and a *pta* mutant should exhibit reduced signaling similar to that of the *ack-pta* mutant. As shown in Fig. 5, reporter expression was similarly low in all three mutant strains relative to wild-type levels, indicating that the reduced PhoP-PhoQ signaling is not the result of altered intracellular acetyl-phosphate levels.

An alternative hypothesis to explain why mutants lacking a functional Ack-Pta pathway displayed reduced PhoP-PhoQ signaling (Fig. 4 and 5) is that disruption of the Ack-Pta pathway prevents proper acetyl-CoA turnover and that PhoP-PhoQ signaling is sensitive to the disrupted turnover. When *ack*, *pta*, and *ack-pta* mutants are grown in glucose, the excess acetyl-CoA that is produced is not properly turned over because the enzymes required to catalyze the conversion of acetyl-CoA to



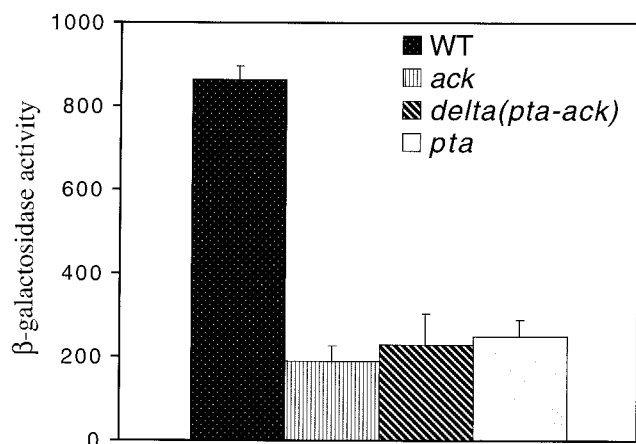


FIG. 5. In vivo PhoP-PhoQ signaling responses of wild-type *E. coli* and *ack*, *ack-pta*, and *pta* mutants. *E. coli* strains CSH26 (wild type), CDW1 (*pta*), CDW2 (*ack-pta*), and CDW3 (*ack*) carrying the pNL2 reporter plasmid were assayed for  $\beta$ -galactosidase activity following growth in N medium. The values are means calculated from at least three independent experiments for each strain. Error bars denote one standard deviation.

acetate are absent. This alternative hypothesis can also explain why growth of wild-type *E. coli* in N medium supplemented with acetate represses PhoP-PhoQ signaling (Fig. 4). During growth in glucose and acetate, acetyl-CoA turnover is disrupted because the acetate represses Pta (28).

A disruption of acetyl-CoA turnover should cause an increase in the intracellular concentration of acetyl-CoA (12, 17). To test this prediction, intracellular acetyl-CoA levels were measured in an *ack-pta* mutant grown in N medium, the wild-type strain grown in N medium, and the wild-type strain grown in N medium supplemented with exogenous acetate. Intracellular acetyl-CoA levels during growth on glucose have been measured at 324 (47) to 600  $\mu$ M (46), depending upon the method chosen for the measurement. Using a coupled enzyme assay (see Materials and Methods), we measured an intracellular acetyl-CoA concentration of  $571 \pm 44 \mu$ M for the wild-type strain grown in N medium. In agreement with the hypothesis, elevated intracellular acetyl-CoA levels were measured in the wild-type strain supplemented with 75 mM acetate ( $739 \pm 30 \mu$ M) and in the *ack-pta* mutant grown in N medium ( $1,049 \pm 80 \mu$ M).

To further test the hypothesis that the inhibition of acetyl-CoA turnover was responsible for decreased PhoP-PhoQ signaling, reporter expression was assayed in a variety of carbon sources, including some that result in the production of excess acetyl-CoA and some that do not. Like glucose, carbon and energy sources such as glucuronate and pyruvate are efficiently taken up and glycolysed by *E. coli*, resulting in the production of an excess of acetyl-CoA that is normally turned over by the Ack-Pta pathway (25). Conversely, glycerol is inefficiently taken up and metabolized such that excess acetyl-CoA is not produced when glycerol is used as a primary carbon and energy source (25). Also, TCA cycle intermediates such as fumarate are metabolized directly into the TCA cycle such that acetyl-CoA levels remain low when fumarate is used as a primary carbon and energy source (25). As was the case for cells grown with glucose as the primary carbon and energy source, mutants

lacking a functional Ack-Pta pathway displayed reduced PhoP-PhoQ signaling when they were grown in glucuronate and pyruvate (Fig. 6a). However, PhoP-PhoQ signaling in mutants lacking a functional Ack-Pta pathway was not affected when the cells were grown with glycerol or fumarate as the primary carbon and energy sources (Fig. 6b). We also noted differences in reporter expression in the wild-type strain when grown in the various carbon and energy sources, indicating that there are additional specific effects of these carbon and energy sources on signaling. Nevertheless, these results clearly show that disruption of the Ack-Pta pathway affects PhoP-PhoQ signaling only when the Ack-Pta pathway is required to ensure proper turnover of excess acetyl-CoA.

**Acetyl-CoA inhibits PhoQ autokinase activity in vitro.** Our in vivo results indicated that growth conditions which led to elevated levels of intracellular acetyl-CoA through a perturbation of acetyl-CoA turnover also resulted in repression of PhoP-PhoQ signaling in *E. coli*. To test whether acetyl-CoA directly affected PhoQ activity, the autokinase reaction of the purified cytoplasmic domain of PhoQ (QTR) was assayed in the presence of increasing amounts of acetyl-CoA. As shown in Fig. 7, acetyl-CoA began to inhibit autokinase activity at concentrations of  $\geq 1.25$  mM. Analysis of the mechanism of inhibition revealed that acetyl-CoA caused a marked decrease in the  $k_{cat}$  for the autokinase reaction, but it did not influence the  $K_m$  for ATP (Table 2). Therefore, acetyl-CoA acted as a non-competitive inhibitor of the PhoQ autokinase activity. L-Glutamate and pyruvate, two compounds whose excretion is elevated in cells lacking a functional Ack-Pta pathway (12), were also tested for their effects on the autokinase activity of QTR. Neither compound appreciably influenced PhoQ autokinase activity (Fig. 7).

## DISCUSSION

The results presented here show that the *E. coli* PhoP-PhoQ system can be repressed by extracellular acetate in addition to previously described responses to extracellular divalent cations (21, 27, 45) and pH (4). Repression by acetate is PhoQ dependent, but unlike the case with divalent cations, acetate does not appear to affect signaling through interactions with the extracellular sensor domain. The mechanism for PhoP-PhoQ response to external pH is currently unknown.

Acetate is normally excreted both as a product of fermentation by anaerobically growing cells (8) and as a by-product of excess acetyl-CoA that is produced by cells growing aerobically in a rich environment (25). Acetate is produced and excreted in the latter case through conversion of acetyl-CoA via acetyl-phosphate by the Ack-Pta pathway (10). The excreted acetate is then reused as the preferred carbon and energy source is depleted. Acetate is reused primarily via acetyl-CoA synthetase to produce acetyl-CoA (10, 29). In batch culture with glucose as the sole carbon and energy source, acetate is first produced and excreted, and as the glucose begins to become depleted, cometabolism of glucose and acetate occurs until both sources are exhausted simultaneously (6). These conditions presumably mimic the situation for free-living *E. coli* growing in a rich environment and should lead to a situation where PhoP-PhoQ signaling is repressed (glucose plus acetate). *E. coli* residing in the mammalian intestine also encoun-

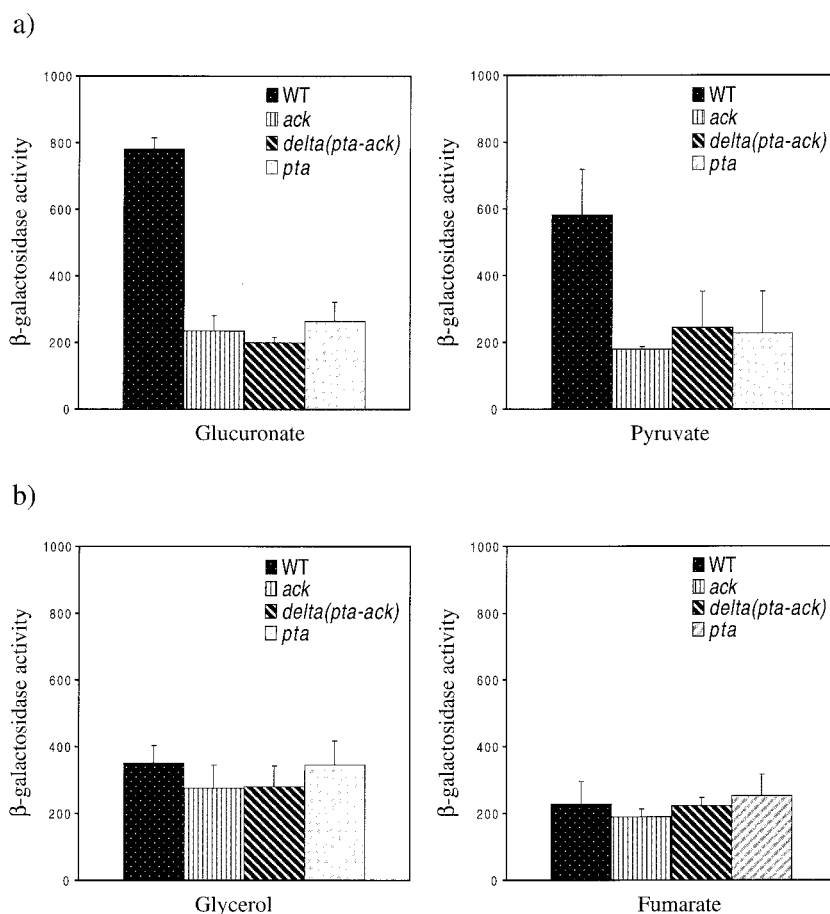


FIG. 6. (a) In vivo PhoP-PhoQ signaling responses of wild-type *E. coli* and *ack*, *ack-pta*, and *pta* mutants grown with 0.4% glucuronate (left panel) and 0.4% pyruvate (right panel) as the primary carbon and energy source. *E. coli* strains CSH26 (wild type), CDW1 (*pta*), CDW2 (*ack-pta*), and CDW3 (*ack*) carrying the pNL2 reporter plasmid were assayed for  $\beta$ -galactosidase activity following growth on the indicated carbon source. (b) In vivo PhoP-PhoQ signaling responses of wild-type *E. coli* and *ack*, *ack-pta*, and *pta* mutants grown with 0.4% glycerol (left panel) and 0.4% fumarate (right panel) as the primary carbon and energy source. *E. coli* strains CSH26 (wild type), CDW1 (*pta*), CDW2 (*ack-pta*), and CDW3 (*ack*) carrying the pNL2 reporter plasmid were assayed for  $\beta$ -galactosidase activity following growth on the indicated carbon sources. The values are means calculated from at least three independent experiments for each strain. Error bars denote one standard deviation.

ters a variety of carbon and energy sources as well as high levels of acetate (up to 75 mM) (15).

The studies presented here indicate that the Ack-Pta pathway is required for maximal PhoP-PhoQ signaling levels observed in wild-type *E. coli*. Furthermore, the requirement re-

lates to the function of this pathway in converting excess acetyl-CoA to acetate, since mutations in the pathway affect signaling only when bacteria are grown in carbon and energy sources that are efficiently taken up and converted to acetyl-CoA. This finding offers a mechanism to explain the repression of PhoP-

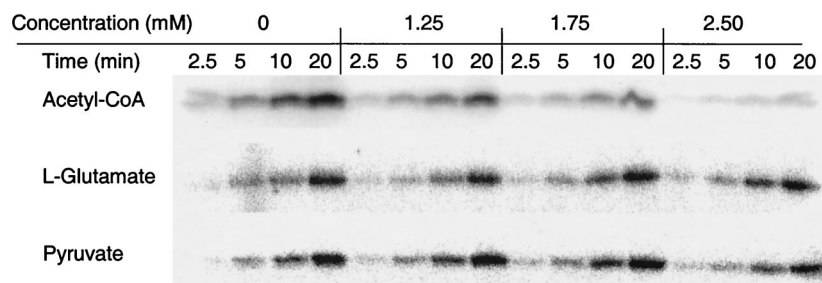


FIG. 7. Autokinase activity of the PhoQ cytoplasmic domain in the presence of increasing concentrations of acetyl-CoA, L-glutamate, and pyruvate. Purified PhoQ cytoplasmic domain was incubated in a solution of 10 mM Tris-HCl (pH 8.0), 25 mM KCl, 0.4 mM MgCl<sub>2</sub>, 90  $\mu$ M ATP, and 0.06  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP/ $\mu$ l at 22°C with increasing amounts of acetyl-CoA, L-glutamate, and pyruvate. Aliquots were removed at the indicated times and stopped by the addition of SDS-PAGE loading buffer. All samples were subjected to SDS-10% PAGE, and the radiolabeled protein bands were visualized by phosphorimaging.

TABLE 2. Kinetic parameters for autophosphorylation of the PhoQ cytoplasmic domain in the presence of increasing amounts of acetyl-CoA<sup>a</sup>

Acetyl-CoA (mM)	$K_m$ ( $\mu$ M)	$k_{cat}$ ( $\text{min}^{-1}$ )	$k_{cat}/K_m$ ( $\text{M}^{-1} \text{min}^{-1}$ )
0	55 $\pm$ 14	0.0026 $\pm$ 0.0011	47 $\pm$ 18
1.25	52 $\pm$ 4	0.0018 $\pm$ 0.0008	34 $\pm$ 15
1.75	49 $\pm$ 17	0.0008 $\pm$ 0.0003	17 $\pm$ 0.2
2.50	58 $\pm$ 19	0.0002 $\pm$ 0.0001	4.0 $\pm$ 3.0

<sup>a</sup> Autokinase assays were performed at 22°C in 10 mM Tris-HCl (pH 8.0)–25 mM KCl–0.4 mM MgCl<sub>2</sub>. Kinetic parameters were derived as described in Materials and Methods. Mean values and standard deviations were calculated from at least three independent experiments.

PhoQ signaling by exogenous acetate. It has recently been demonstrated that acetate represses Pta (28). Therefore, during growth in a rich carbon and energy source and acetate, repression of Pta should inhibit the Ack-Pta pathway's ability to convert excess acetyl-CoA to acetate. As a consequence, excess acetyl-CoA accumulates. The elevated intracellular acetyl-CoA concentration leads to repression of PhoP-PhoQ signaling in a PhoQ-dependent manner. Consistent with this proposed mechanism, intracellular acetyl-CoA increased during growth of both a wild-type strain in N medium supplemented with exogenous acetate and an *ack-pta* mutant grown in N medium.

Our *in vitro* studies show that PhoQ's autokinase activity is inhibited by acetyl-CoA at concentrations of  $\geq 1.25$  mM, suggesting that *in vivo* repression of PhoP-PhoQ signaling may be the result of direct inhibition of the PhoQ autokinase reaction by excess acetyl-CoA. The inhibition of PhoQ autokinase activity by acetyl-CoA was observed with the purified PhoQ cytoplasmic domain. Therefore, the *in vitro* studies are consistent with *in vivo* results showing that acetate-mediated repression is PhoQ dependent and periplasmic sensor domain independent. Glutamate and pyruvate, compounds which are excreted at elevated levels in cells lacking a functional Ack-Pta pathway (12), did not appreciably influence PhoQ's autokinase activity. Although malonyl-CoA and CoA also inhibited PhoQ autokinase activity at concentrations of  $\geq 1.25$  mM (data not shown), they are not likely to affect PhoQ *in vivo*, as their intracellular concentrations are not seen to exceed roughly 100  $\mu$ M (47). Nevertheless, we cannot rule out the possibility that the repression of PhoP-PhoQ signaling observed during growth in acetate, or by inactivation of the Ack-Pta pathway, is an indirect consequence of elevated intracellular acetyl-CoA concentration. Fatty acyl CoA derivatives whose intracellular concentrations could also conceivably increase under growth conditions in which acetyl-CoA levels are elevated might also contribute to the inhibition of PhoQ autokinase activity. The local concentrations of these CoA species might be higher near the membrane-cytosol interface where the PhoQ cytoplasmic domain is localized.

Why is the PhoP-PhoQ system responsive to acetate and acetyl-CoA? Perhaps the PhoP-PhoQ system is sensitive to these compounds in order to coordinate the expression of genes controlled by PhoP-PhoQ signaling with the ecological niches in which acetate is abundant or with the physiological processes in which acetyl-CoA is involved. Although little is known about the PhoP-PhoQ regulon in *E. coli*, it has been extensively characterized in *S. enterica* serovar Typhimurium.

In that organism, PhoP-PhoQ regulates genes involved in magnesium import, lipopolysaccharide modification, epithelial invasion, and peptidoglycan remodeling (22). The epithelial invasion genes *prgHIJK* are repressed by phosphorylated PhoP (5, 40). It is possible that the high concentrations of acetate found in the intestine (15) along with divalent cations facilitate the derepression of these genes and the invasion of the epithelium. The PhoP-PhoQ-regulated gene *pagP* is involved in lipopolysaccharide modification through the biosynthesis of hepta-acylated lipid A containing palmitate (7, 23). The phospholipids that PagP utilizes to modify lipid A are derived from acetyl-CoA precursors (14), suggesting a potential physiological link between PhoP-PhoQ function and acetyl-CoA levels.

Given its central role in cellular metabolism, it is tempting to speculate that acetyl-CoA may play a role as a barometer of some aspect(s) of cellular physiology to serve as a global regulator of other histidine kinases in addition to PhoQ. The notion that a perturbation of acetyl-CoA turnover can influence signal transduction has recently been proposed for the signaling pathway that controls the turnover of the general stress sigma factor RpoS (28). The turnover of RpoS is up-regulated in *E. coli* by phosphorylation of a response regulator, RssB (SprE) (38, 41), to which a partner histidine kinase has not been identified. An *ack-pta* deletion leads to reduced RpoS turnover under growth conditions in which turnover should be very rapid (9). Originally, it was proposed that acetyl-phosphate contributed to RssB phosphorylation *in vivo*. Stabilization of RpoS in the *ack-pta* mutant stemmed from the loss of RssB phosphorylation resulting from the decrease in intracellular acetyl phosphate (9). However, it has recently been noted that the RpoS regulon is activated by growing wild-type *E. coli* in LB supplemented with 50 mM acetate (28). Growth in LB supplemented with 50 mM acetate should increase intracellular acetyl-phosphate. If the activation of the RpoS regulon during growth in acetate reflects a reduction in RpoS turnover, then the acetate-mediated activation of the RpoS regulon is inconsistent with the hypothesis that acetyl phosphate contributes to RssB phosphorylation. Instead, RpoS may be stabilized as a result of excess acetyl-CoA (28). We speculate that the stabilization of RpoS by excess acetyl-CoA occurs due to a decrease in RssB phosphorylation by an unidentified histidine kinase that is sensitive to acetyl-CoA, analogous to the case with PhoP-PhoQ. Kirkpatrick et al. have suggested that accumulation of extracellular acetate in rich media and the ensuing increase in acetyl-CoA levels may be an indicator of high cell density and the onset of stationary phase (28). Perhaps it is advantageous to modulate the activity of some two-component systems as the cell enters stationary phase under these conditions to conserve energy. Given the findings reported here, it may be fruitful to examine the extent to which perturbation of acetyl-CoA levels can influence other two-component systems.

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