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NOTE

Evidence Against the Physiological Role of Acetyl Phosphate in the Phosphorylation of the ArcA Response Regulator in *Escherichia coli*

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The Arc two-component signal transduction system of *Escherichia coli* comprises the ArcB sensor kinase and the ArcA response regulator. Under anoxic growth conditions, ArcB autophosphorylates and transphosphorylates ArcA, which, in turn, represses or activates its target operons. ArcA has been shown to be able to autophosphorylate *in vitro* at the expense of acetyl-P. Here, the *in vivo* effect of acetyl phosphate on the redox signal transduction by the Arc system was assessed. Our results indicate that acetyl phosphate can modulate the expression of ArcA-P target genes only in the absence of ArcB. Therefore, the acetyl phosphate dependent ArcA phosphorylation route does not seem to play a significant role under physiological conditions.

Keywords*: acetyl phosphate, Arc two-component signal transduction, ArcA response regulator, *E. coli

The ArcB/A (anoxic redox control) two-component signal transduction system of facultative anaerobic bacteria regulates the expression of numerous genes depending on the redox conditions of growth (Fig. 1) (Georgellis *et al.*, 2001b; Malpica *et al.*, 2006; Jung *et al.*, 2008). The ArcB/A system of *Escherichia coli* comprises ArcB as the membrane-bound sensor kinase and ArcA as the cognate response regulator and regulates more than 40 operons (Liu and De Wulf, 2004; Malpica *et al.*, 2006). Under anoxic growth conditions, ArcB undergoes autophosphorylation at His292, a process shown to be enhanced by certain fermentative metabolites such as *D*-lactate, pyruvate, acetate, and NADH. The phosphoryl group is then sequentially relayed to Asp576 and His717, and finally to Asp54 of ArcA (Georgellis *et al.*, 1997; Georgellis *et al.*, 1999; Kwon *et al.*, 2000b; Kwon *et al.*, 2003; Rodriguez *et al.*, 2004).

Phosphorylated ArcA, in turn, represses the expression of many genes involved in respiratory metabolism and activates other genes encoding proteins involved in fermentative metabolism (Malpica *et al.*, 2006). During aerobiosis, the quinone electron carriers that accumulate in their oxidized form in the cytosolic membrane inhibit the autophosphorylation of ArcB (Georgellis *et al.*, 2001a). The quinones provide the oxidizing power for intermolecular disulfide bond formation by two cytosol-located, redox-active, cysteine residues of ArcB (Malpica *et al.*, 2004). Under oxidizing conditions, ArcB also catalyzes the dephosphorylation of ArcA-P via an Asp54-P → His717-P → Asp576-P → Pi reverse phosphorelay (Georgellis *et al.*, 1998; Pena-Sandoval *et al.*, 2005).

In vitro, ArcA can catalyze its own phosphorylation in the presence of low molecular weight phosphor-donors such as acetyl phosphate and carbamoyl phosphate (Drapal and Sawers, 1995; Lynch and Lin, 1996). It has been reported that numerous response regulators such as AlgR (Deretic *et al.*, 1992), CheY (Lukat *et al.*, 1992), FixJ (Reyrat *et al.*, 1993), NarL (Schröder *et al.*, 1994), NtrC (Feng *et al.*, 1992), OmpR (Head *et al.*, 1998), PhoB (Hiratsu *et al.*, 1995), PhoP (Chamnongpol and Groisman, 2000), RssB (Bouché *et al.*, 1998), and VanR (Holman *et al.*, 1994) could be phosphorylated *in vitro* by acetyl phosphate (Stock *et al.*, 1995). These observations raised the question of whether acetyl phosphate serves uniformly as a global integrative signal for two-component systems (Wanner, 1992). Furthermore, it has been suggested that the cellular concentration of acetyl phosphate can vary widely under different metabolic conditions, reflecting the nutritional status of cells (Prüß and Wolfe, 1994).

It has been shown by transcriptome analysis that acetyl-phosphate affects the expression of about 100 genes (Wolfe *et al.*, 2003), but most of the evidence for direct phosphorylation of response regulators by using acetyl phosphate was obtained *in vitro* (Wolfe, 2005). In addition, it has been suggested that during carbon-starvation, ArcA could be cross-activated by a sensor component other than ArcB or by a

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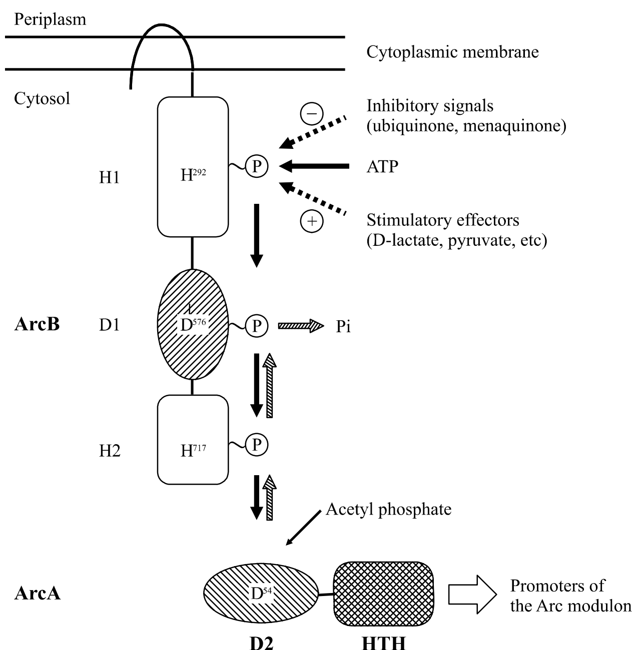

Fig. 1. Signal transduction by the Arc two-component system. The ArcB sensor kinase is depicted as a membrane associated protein having three cytosolic domains: H1, the orthodox or primary transmitter domain; D1, the receiver domain; and H2, the secondary transmitter domain. The ArcA response regulator is depicted as a two-domain protein: D2, the secondary receiver domain and HTH, the helix-turn-helix domain. Thick solid arrows indicate phosphorylation reactions; crosshatched arrows are dephosphorylation reactions; dotted arrows are signals which modulate the ArcB autophosphorylation activity negatively or positively (Georgellis *et al.*, 1997; Georgellis *et al.*, 1999; Kwon *et al.*, 2000b; Kwon *et al.*, 2003; Rodriguez *et al.*, 2004).

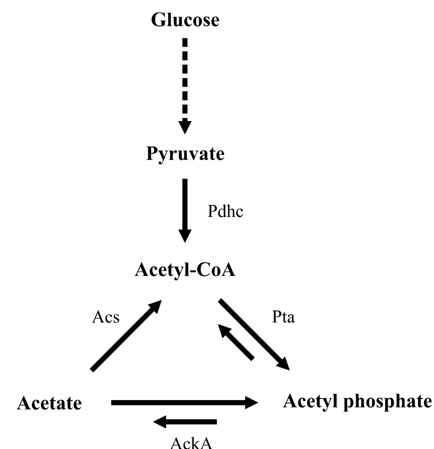

Fig. 2. Routes for acetyl phosphate formation under aerobic conditions. Acetyl phosphate is a central metabolite which can be derived reversibly from acetate and ATP by the action of a specific kinase (AckA) or from acetyl-CoA and phosphate by the action of phosphotransacetylase (Pta) (Wolfe, 2005). Acetyl-CoA can arise from acetate, CoA, and ATP (at the expense of a pyrophosphate release) by the action of acetyl-CoA synthetase (Acs) or from pyruvate and CoA by an oxidative decarboxylation catalyzed by a dehydrogenase complex (Pdhc).

low molecular weight phosphorylated compound whose level increases under starvation conditions (Nyström *et al.*, 1996). However, no studies demonstrating a role for acetyl phosphate in ArcA phosphorylation under physiological conditions have been reported.

In this study, we investigated the acetyl phosphate dependent ArcA phosphorylation *in vivo*. The expressions of the ArcA-P activated Φ(*cydA'-lacZ*) and the ArcA-P repressed Φ(*lldP'-lacZ*) reporter operons (Kwon *et al.*, 2000a) were analyzed in various *E. coli* mutant strains having alterations in the Arc signal transduction system or acetyl phosphate metabolism (Table 1).

It has been reported acetyl phosphate accumulates in *pta* mutants when the growth medium is supplemented with acetate (Wanner, 1992) (Fig. 2). Moreover, acetate leads to a greater increase of acetyl phosphate in *pta acs* double mutants, which cannot convert acetate to acetyl-CoA (Wolfe, 2005; Klein *et al.*, 2007). We therefore examined *cydA'-lacZ* and *lldP'-lacZ* fusion expression in *acs* and *pta* null mutants when grown in the presence and absence of acetate. To minimize effects of ArcB on phosphorylation of ArcA, the reporter strains were cultured under aerobic conditions (Rodriguez *et al.*, 2004). Under these conditions, supplementation with acetate had no effect on the level of ArcA-P, as indicated by the lack of an effect on expression of these reporter fusions (Fig. 3). In contrast, the acetate supplementation resulted in a 2-fold increase of the Φ(*cydA'-lacZ*) fusion and a 2-fold decrease of the Φ(*lldP'-lacZ*) fusion in an *arcB* null mutant. This effect of acetate was abolished in an *ackA* or *arcA* mutant, indicating that acetate is converted to acetyl phosphate that in turn leads to phosphorylation of ArcA. These results show that while acetyl phosphate can donate its phosphoryl group to ArcA *in vivo*, ArcB interferes with accumulation of ArcA-P from acetyl phosphate. Whether ArcB inhibits phosphorylation of ArcA by acetyl phosphate or results in dephosphorylation of ArcA-P under these conditions is unknown.

It is known that acetyl phosphate can influence the expression and *in vivo* half life of RpoS, the alternative sigma factor σ^S, which is a negative regulator of *arcA* transcription. Also, the absence of RpoS results in a 3-fold increase in basal aerobic expression of λΦ(*cydA'-lacZ*), supporting a negative regulatory role for RpoS in *arcA* transcription (Lindqvist *et al.*, 2000). In addition, a study involving *Salmonella typhimurium* showed that a deletion of *rpoS* led to a 1.5-fold increase in the transcription of *arcA* during aerobic growth (Sevcik *et al.*, 2001), which by extrapolation would lead to an increase in *cydAB* expression. However, acetyl-phosphate has been shown to act as a phosphoryl donor for the response regulator RssB, which in turn leads to proteolysis of RpoS (Bouché *et al.*, 1998). Therefore, we tested whether the accumulation of acetyl phosphate decreases the levels of RpoS, which in turn may induce ArcA expression with a consequent activation of *cydA* expression and a repression of *lldP* expression. To this end, the effect of acetate was tested on the expression of the reporter operons in an *arcB rpoS* double mutant. The absence of RpoS did not influence the acetate effect, suggesting that acetyl phosphate

Table 1. *E. coli* K-12 strains, phage, and plasmids used in this study

Strain, phage, or plasmid	Origin	Relevant genotype or characteristics	Reference or source
Strains			
MC4100		F⁻ *araD139 Δ(argF-lac)U169 rpsL50 1 deoC1 relA1 thiA ptsF25 flbB530 rbsR*	Kwon *et al.* (2000a)
CP724	MC4100	*ackA::*Tn*phoA'-9*	Shin and Park (1995)
ZK1000	MC4100	*rpoS::*Kan^r	Bohannon *et al.* (1991)
DY330	W3110	*ΔlacU169 gal490 λcl857 Δ(cro-bioA)*	Yu *et al.* (2000)
ECL5001	MC4100	λΦ(*cydA'-lacZ*)	Kwon *et al.* (2000a)
ECL5002	MC4100	λΦ(*lldP'-lacZ*)	Kwon *et al.* (2000a)
ECL5053	MC4100	λΦ(*cydA'-lacZ*) *arcB*	This study
ECL5054	MC4100	λΦ(*lldP'-lacZ*) *arcB*	This study
ECL5223	MC4100	*arcA::*Kan^r	This study
ECL5300	DY330	*pta::*Amp^r	This study
ECL5304	ECL5053	λΦ(*cydA'-lacZ*) *arcB pta::*Amp^r	This study
ECL5305	ECL5054	λΦ(*lldP'-lacZ*) *arcB pta::*Amp^r	This study
ECL5306	DY330	*acs::*Tet^r	This study
ECL5307	ECL5001	λΦ(*cydA'-lacZ*) *pta::*Amp^r *acs::*Tet^r	This study
ECL5308	ECL5002	λΦ(*lldP'-lacZ*) *pta::*Amp^r *acs::*Tet^r	This study
ECL5309	ECL5053	λΦ(*cydA'-lacZ*) *arcB pta::*Amp^r *acs::*Tet^r	This study
ECL5310	ECL5054	λΦ(*lldP'-lacZ*) *arcB pta::*Amp^r *acs::*Tet^r	This study
ECL5313	ECL5053	λΦ(*cydA'-lacZ*) *arcB pta::*Amp^r *acs::*Tet^r *arcA::*Kan^r	This study
ECL5314	ECL5054	λΦ(*lldP'-lacZ*) *arcB pta::*Amp^r *acs::*Tet^r *arcA::*Kan^r	This study
ECL5317	ECL5053	λΦ(*cydA'-lacZ*) *arcB pta::*Amp^r *acs::*Tet^r *rpoS::*Kan^r	This study
ECL5318	ECL5054	λΦ(*lldP'-lacZ*) *arcB pta::*Amp^r *acs::*Tet^r *rpoS::*Kan^r	This study
ECL5321	ECL5053	λΦ(*cydA'-lacZ*) *arcB pta::*Amp^r *acs::*Tet^r *ackA::*Tn*phoA'-9*	This study
ECL5322	ECL5054	λΦ(*lldP'-lacZ*) *arcB pta::*Amp^r *acs::*Tet^r *ackA::*Tn*phoA'-9*	This study
ECL5323	ECL5053	λΦ(*cydA'-lacZ*) *arcB arcA::*Kan^r	This study
ECL5324	ECL5054	λΦ(*lldP'-lacZ*) *arcB arcA::*Kan^r	This study
ECL5336	ECL5001	λΦ(*cydA'-lacZ*) *pta::*Tet^r*::ackA*	This study
$P1_{vir}$			Laboratory stock
Plasmids			
pI53		*ΔarcB*	Kwon *et al.* (2000a)
pKO3			Link *et al.* (1997)
pDB	pKO3	*ΔarcB* in pKO3	This study

Luria-Bertani (LB) broth and LB agar (15 g/L) were used for routine growth of bacterial strains. Ampicillin, tetracycline, kanamycin, and chloramphenicol were provided at final concentrations of 50, 12, 40, and 20 μg/ml, respectively. $P1_{vir}$ transduction was employed to transfer mutant alleles from a donor strain to a recipient strain as described previously (Kwon *et al.*, 2000a).

modulates the activity of ArcA response regulator through direct phosphorylation (Fig. 3).

It was previously reported that the intracellular acetyl phosphate level is about 10-fold higher in cells grown aerobically with pyruvate as the sole carbon and energy source than in cells grown on glycerol (McCleary and Stock, 1994). This provided another means to evaluate the physiological role of acetyl phosphate dependent phosphorylation of ArcA. We found no significant difference in the expression of the reporter fusions in wild-type *arcB*$^+$ cells during growth on pyruvate or glycerol (Fig. 4). In contrast, in an *arcB* mutant, expression of the Φ(*cydA'-lacZ*) was 4-fold higher and the expression of Φ(*lldP'-lacZ*) was 3-fold lower during growth on pyruvate than during growth on glycerol. These results indicate that acetyl phosphate can modulate the activity of ArcA *in vivo* only in the absence of its cognate sensor kinase ArcB. To confirm that the signaling molecule acetyl phosphate acts through the ArcA response regulator, reporter expression was analyzed in *pta* and *arcA* mutants during growth on pyruvate or glycerol. Under these conditions, activation of Φ(*cydA'-lacZ*) and repression of Φ(*lldP'-lacZ*) on pyruvate was eliminated, confirming that the elevated levels of acetyl phosphate in pyruvate-grown cells were responsible for phosphorylation of ArcA in the absence of ArcB.

Although ArcA is capable of catalyzing its own phosphorylation *in vitro* using acetyl phosphate as a donor, there was no evidence that this significantly modulates the ArcA-P level in the presence of ArcB under several experimental conditions. In this respect, our results are consistent with the findings from several other two-component systems. Activation of PhoB by acetyl phosphate was evident in mutants lacking both the sensor kinase proteins PhoR and CreC, but not in the wild-type (Wanner and Wilmes-Riesenberg, 1992). Also, autophosphorylation of the response regulator NtrC or CheY by acetyl phosphate was shown to occur only in cells lacking the cognate sensor kinase NtrB or CheA, respectively (Feng *et al.*, 1992; Dailey and Berg, 1993).

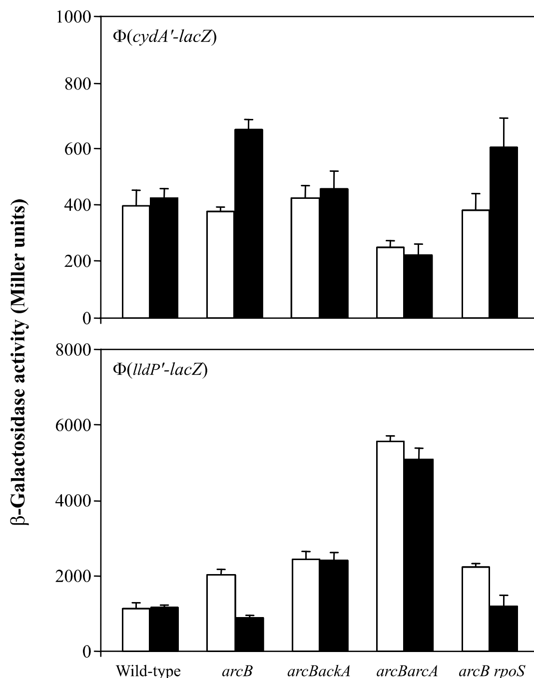

Fig. 3. Effects of acetate on the expression of Φ(*cydA'-lacZ*) or Φ(*lldP'-lacZ*). The Φ(*cydA'-lacZ*) or Φ(*lldP'-lacZ*) reporter strains were constructed in an *acs* and *pta* null mutant to avoid effects of endogenous acetyl phosphate formation. For assaying the expression of Φ(*cydA'-lacZ*), the strains were grown in 5 ml 0.1 M MOPS (morpholinepropanesulfonate) buffered Luria-Bertani (LB) broth (pH 7.6) containing 20 mM D-xylose with or without addition of 10 mM sodium acetate. For assaying the expression of Φ(*lldP'-lacZ*), the strains were grown in the above medium supplemented with 20 mM lithium L-lactate as an inducer (Kwon *et al.*, 2000a). Cultures were incubated aerobically at 37°C in 300-ml baffled flasks on a shaker rotated at 300 rpm until a density of OD_{600} of 0.3~0.5 was reached. ß-Galactosidase activity was assayed and expressed in Miller units (Miller, 1992). Open bars: grown without the addition of acetate. Solid bars: grown with the addition of acetate. The height of each bar represents the average value of five separate experiments. Strains bearing Φ(*cydA'-lacZ*): wild-type, ECL5307; *arcB*, ECL5309; *arcB ackA*, ECL5321; *arcB arcA*, ECL 5313; and *arcB rpoS*, ECL5317. Strains bearing Φ(*lldP'-lacZ*): wild-type, ECL5308; *arcB*, ECL5310; *arcB ackA*, ECL5322; *arcB arcA*, ECL5314; and *arcB rpoS*, ECL5318.

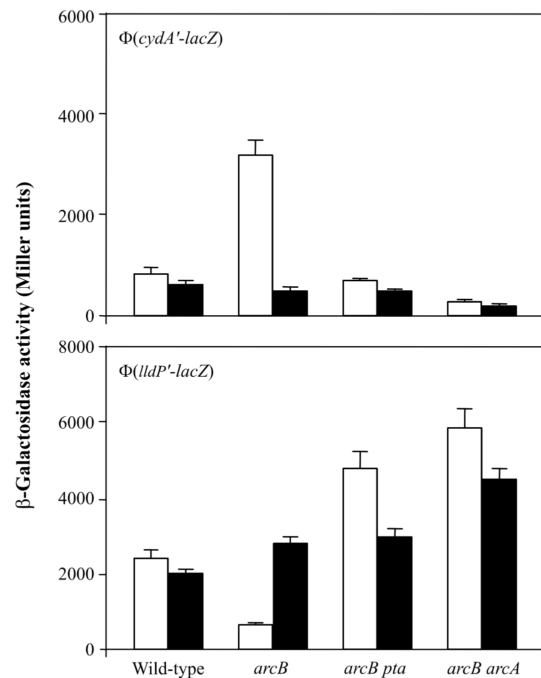

Fig. 4. Φ(*cydA'-lacZ*) and Φ(*lldP'-lacZ*) expressions in bacterial strains grown using pyruvate versus glycerol as a carbon source. Cells were grown in 5 ml defined medium (1 mM KH_2PO_4, 40 mM KCl, 34 mM NaCl, 20 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4$, 0.3 mM $MgSO_4$, 1 μM $ZnCl_2$, 10 μM $CaCl_2$, and 0.1 M MOPS, at final pH 7.6) with either 0.4% sodium pyruvate or 0.4% glycerol as the sole carbon and energy source. The Φ(*lldP'-lacZ*)-bearing strains were grown in media supplemented with 20 mM lithium L-lactate as an inducer (Kwon *et al.*, 2000a). For other experimental conditions, see legend of Fig. 3. Open bars: grown with pyruvate supplementation. Hatched bars: grown with glycerol supplementation. The height of each bar represents the average value of five separate experiments. Strains bearing Φ(*cydA'-lacZ*): wild-type, ECL5001; *arcB*, ECL5053; *arcB pta*, ECL5304; and *arcB arcA*, ECL5323. Strain bearing Φ(*lldP'-lacZ*): wild-type, ECL5002; *arcB*, ECL5054; *arcB pta*, ECL5305; and *arcB arcA*, ECL5324.

Yet, results from several other experiments confound a simple interpretation of acetyl phosphate-dependent activation of ArcA. A recent report has shown that PhoB phosphorylation by acetyl phosphate in the *phoR creC* double mutant is dependent on the noncognate sensor kinase EnvZ (Kim *et al.*, 1996). In *S. enterica* a PhoP* response regulator mutant responsive to Mg^{2+} and showing enhanced acetyl phosphate-dependent autophosphorylation activity was isolated from a *phoQ* mutant. Yet, this route of autophosphorylation (Mg^{2+}-independent) was suppressed in the presence of PhoQ (Chamnongpol and Groisman, 2000). An involvement of acetyl phosphate as a phosphoryl donor was demonstrated in the EnvZ/OmpR system in the presence of EnvZ (Heyde *et al.*, 2000). In this case, an increase in the level of acetyl phosphate was correlated with the level of phosphorylated OmpR, as shown by increased synthesis of OmpC and decreased synthesis of OmpF (Matsubara and Mizuno, 1999).

There are several reasons why *in vivo* acetyl phosphate dependent phosphorylation of the response regulator was difficult to demonstrate. First, the cognate kinase is likely to have a relatively high V_{max} and low K_m for the phosphorylation of the response regulator, thereby tending to obscure the acetyl phosphate pathway even if it is operative. Second, the absence of the sensor kinase also abolishes the specific phosphatase, a likely condition that would allow the accumulation of a significant level of the phosphorylated response regulator via the acetyl phosphate route (Fredericks *et al.*, 2006). Third, it has been pointed out that the importance of the acetyl phosphate-dependent phosphorylation of PhoB is especially questionable because of the K_m for the phosphodonor is 7~8 mM (McCleary, 1996).

Until recently, it has been generally accepted that the intracellular concentration of acetyl phosphate rarely exceeds 1 mM (McCleary and Stock, 1994). Yet, the level of Pho

regulon expression dependent upon acetyl phosphate in a *phoR* mutant has been reported to be as high or higher than the level dependent upon PhoR under conditions of P_i limitation (Wanner and Wilmes-Riesenberg, 1992; Kim *et al.*, 1996). However, it has to be mentioned that this differs from what is expected when the actual intracellular concentration of the phosphodonor and the kinetic properties of the protein are taken into account (Prüß and Wolfe, 1994; McCleary, 1996). In fact, it has been reported that the intracellular concentration of acetyl phosphate in wild-type *E. coli* cells reaches at least 3 mM, a concentration sufficient to phosphorylate response regulators (Klein *et al.*, 2007). What is not yet clear is whether the phosphatase activity of the sensor protein increases as its kinase activity diminishes with the attenuation of signaling (Hoch, 1995). In addition to these complicating factors, acetyl phosphate can also regulate RssB-dependent proteolysis of RpoS by phosphorylation of RssB (Bouché *et al.*, 1998), highlighting another potential physiological role of acetyl phosphate *in vivo.* In any event, the steady-state level of the phospho-response regulator appears to depend on the balance between the kinase and the phosphatase activities of the sensor kinase.

In an evolutionary context, it has been suggested that the response regulators dependence on phosphorylation by low molecular weight phosphodonors existed before the evolution of the sensor proteins (Magasanik, 1995). Accordingly, the functional activity of the response regulators might have been initially under the control of compounds such as acetyl phosphate. It is likely that with the emergence of sensor kinases, autophosphorylation of the response regulators became progressively less important to becoming merely a basal level of their original function. Although, in some cases, the role of regulation by acetyl phosphate is no longer demonstrable in the physiological time scale, the importance of sensor kinase-independent phosphorylation in the evolutionary time scale cannot be ruled out without long term competition experiments between strains bearing wild-type and mutant response regulators which have not yet been conducted.

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