Investigation of *in vivo* cross-talk between key two-component systems of *Escherichia coli*

Daniël T. Verhamme, Jos C. Arents, Pieter W. Postma, Wim Crielaard and Klaas J. Hellingwerf

Author for correspondence: Klaas J. Hellingwerf. Tel: +31 20 5257055. Fax: +31 20 5257056.
e-mail: K.Hellingwerf@chem.uva.nl

Swammerdam Institute for Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands

Intracellular signal transfer in bacteria is dominated by phosphoryl transfer between conserved transmitter and receiver domains in regulatory proteins of so-called two-component systems. *Escherichia coli* contains 30 such systems, which allow it to modulate gene expression, enzyme activity and the direction of flagellar rotation. The authors have investigated whether, and to what extent, these separate systems form (an) interacting network(s) *in vivo*, focussing on interactions between four major systems, involved in the responses to the availability of phosphorylated sugars (Uhp), phosphate (Pho), nitrogen (Ntr) and oxygen (Arc). Significant cross-talk was not detectable in wild-type cells. Decreasing expression levels of succinate dehydrogenase (reporting Arc activation), upon activation of the Pho system, appeared to be independent of signalling through PhoR. Cross-talk towards NtrC did occur, however, in a *ntrB* deletion strain, upon joint activation of Pho, Ntr and Uhp. UhpT expression was demonstrated when cells were grown on pyruvate, through non-cognate phosphorylation of UhpA by acetyl phosphate.

Keywords: Uhp, Pho, Ntr, Arc, non-cognate phosphoryl transfer

INTRODUCTION

Regulatory systems based on the two-component paradigm dominate the signalling machinery of bacteria, to allow them to adapt to their environment. Each system uses transient phosphorylation of sensory and regulatory proteins at a specific histidine or aspartate residue for signal transfer, and thus forms a pathway for phosphoryl transfer. *Escherichia coli* possesses around 30 of these pathways (Mizuno, 1997), some of which diverge, while others converge. An intriguing issue is whether cross-talk interconnects these pathways to form a (neural) network (Hellingwerf *et al.*, 1995, 1998). Cross-talk is defined as the (modulation of) transfer of phosphoryl groups from a sensory kinase to a 'non-cognate' response regulator.

This cross-talk issue was first discussed 13 years ago (Ninfa *et al.*, 1988; Stock *et al.*, 1989) when the abundance of two-component systems in bacteria, together with their high sequence conservation in the interaction domains of sensors and regulators, became evident. Since then several examples of cross-talk *in vitro* (e.g. Fisher *et al.*, 1995; Igo *et al.*, 1989; Ninfa *et al.*, 1988; Yaku *et al.*, 1997) have been reported, whereas

only a few examples in *E. coli* have been reported of the occurrence of cross-talk *in vivo* (Kim *et al.*, 1996; Matsubara & Mizuno, 1999; McCleary *et al.*, 1993; Ninfa *et al.*, 1988; Silva *et al.*, 1998; Wanner, 1992). Cross-talk is distinct from cross-regulation; in cross-regulation one kinase phosphorylates more than one response regulator, as seen in chemotaxis (Hess *et al.*, 1988), nitrate regulation (Rabin & Stewart, 1993) and possibly between metabolic control and phosphate regulation (Wanner, 1992, 1995). Likewise, different kinases could act upon one particular response regulator.

Here we report our cross-talk studies in *E. coli*. An *in vivo* approach was used, in which one or more systems was induced and the subsequent response, if any, was monitored in another system. We examined four two-component systems, UhpABC, PhoBR, NtrBC and ArcAB (Fig. 1). Each of these systems plays a central role in metabolism in *E. coli*. The Uhp system controls the expression of the transport protein UhpT, which enables the cell to acquire phosphorylated sugars from its environment that can be used as carbon and/or energy sources (Kadner, 1995). The Pho system controls expression of the Pho regulon in response to phosphate

Fig. 1. Overview of all possible cross-talk interactions between the four two-component systems selected for this study. The possible roles of non-cognate sensor kinases (X, Y and Z) and small-molecule phosphoryl donors like acetyl phosphate (Ac~P) in response-regulator phosphorylation are also depicted. Further details are given in the text.

limitation, allowing exploitation of alternative sources of phosphate (Wanner, 1995). The Ntr system controls expression of the *glnALG* operon and other nitrogen-regulated genes, in response to decreased availability of ammonia (Magasanik, 1996). The Arc system controls the expression of numerous operons involved in (an-) aerobic catabolism (Lynch & Lin, 1996), in order to maintain respiratory homeostasis. In each of these

signalling pathways the phosphorylated response regulator functions as a transcriptional modulator. *In vivo* activation of Uhp, Ntr and Arc can be monitored via the expression of their target genes, using *lacZ* chromosomal fusion strains. Pho activation can be monitored via the activity of its natural target gene (*phoA*), encoding alkaline phosphatase.

With regard to the above central two-component systems in *E. coli*, our data indicate that the *specificity* of the communication between domains involved in phosphoryl transfer in one pathway is much more important than the *connectivity* between pathways. In other words, non-cognate phosphoryl flow is of a sufficient magnitude to measurably switch on one of these systems only when cells have been mutated in the cognate kinase.

METHODS

Bacterial strains. The *Escherichia coli* K-12 strains used in this study are listed in Table 1. Strains were kindly provided by several laboratories. P1-mediated transduction was performed as described by Arber (1958). The *glnL* deletion in strain PPA699 was obtained in two steps. First, a *glnA::Tn5-132* mutation was introduced into NCM3286, via selection for tetracycline resistance; this resulted in strain PPA687. The *glnL2001* deletion was subsequently introduced into PPA687 via selection for glutamine prototrophy (*glnA*⁺, i.e. recovery of the ability to grow on NH₄⁺ as the sole nitrogen source). In addition to selection for the linked (Tn10) tetracycline resistance, strains PPA683 and PPA703 were selected for their loss of Uhp function, i.e. no glucose-6-phosphate-induced

Table 1. *E. coli* strains used in this study and their relevant characteristics

Strains	Relevant genotype	Reference or construction
NCM3286	$\Phi(glnA-lacZ[Km])$	S. Kustu*
BW12863	<i>glnA::Tn5-132</i>	Feng <i>et al.</i> (1992)
PPA687	$\Phi(glnA-lacZ[Km])$ <i>glnA::Tn5-132</i>	NCM3286 $\times$ BW12863 (P1)
RB9132	<i>glnL2001</i>	Bueno <i>et al.</i> (1985)
PPA699	$\Phi(glnA-lacZ[Km])$ <i>glnL2001</i>	PPA687 $\times$ RB9132 (P1)
RK1231	$\Delta uhp(A15-189)$	Webber & Kadner (1995)
CAG18499	<i>zid-501::Tn10</i>	Singer <i>et al.</i> (1989)
PPA683	$\Delta uhp(A15-189)$ <i>zid-501::Tn10</i>	RK1231 $\times$ CAG18499 (P1)
PPA703	$\Phi(glnA-lacZ[Km])$ <i>glnL2001</i> $\Delta uhp(A15-189)$ <i>zid-501::Tn10</i>	PPA699 $\times$ PPA683 (P1)
RK5115	$\Phi(uhpT-lacZ)$ $\Delta uhpT$	Shattuck-Eidens & Kadner (1981)
RK1307	$\Delta uhp(B60-C437)$ $\Phi(uhpT-lacZ[Km])$	Webber & Kadner (1995)
OG903	$\Phi(sdh-lacZ)$	Matsushika & Mizuno (1998)
DAC903	<i>arcB::Cm</i> $\Phi(sdh-lacZ)$	Matsushika & Mizuno (1998)
ASA12	$\Phi('cydA-lacZ[Km])$	S. Alexeeva†
PPA718	<i>arcB::Cm</i> $\Phi('cydA-lacZ[Km])$	ASA12 $\times$ DAC903 (P1)
PPA738	<i>phoR69 z::Tn10</i>	Yamada <i>et al.</i> (1989)‡
PPA744	<i>arcB::Cm</i> $\Phi(sdh-lacZ)$ <i>phoR69 z::Tn10</i>	DAC903 $\times$ PPA738 (P1)
PPA747	$\Phi(sdh-lacZ)$ <i>phoR69 z::Tn10</i>	OG903 $\times$ PPA738 (P1)

* University of California, Berkeley, CA, USA.

† Fnr box in *cydA* P1-promoter impaired by a 5 bp substitution (Alexeeva, 2000). Swammerdam Institute for Life Sciences.

‡ Constitutive PhoR kinase phenotype.

growth was observed with 0.2% fructose 6-phosphate as the carbon source (relative to growth in the presence of 0.2% glucose, on MOPS minimal medium agar plates). The *phoR* mutation was introduced into strains OG903 and DAC903 by co-transduction with a linked transposon (Tn10) and identified by alkaline phosphatase activity, independent of phosphate limitation.

Growth conditions and activation of signalling through the two-component systems. Cells were grown in MOPS minimal medium (Neidhardt *et al.*, 1974) containing 0.5% (v/v) carbon source, 14 mM NH_4Cl and 14 mM L-glutamine as nitrogen sources, and 2 mM K_2HPO_4 as a phosphate source. Exponentially growing (aerobic, 37 °C) cells were harvested by centrifugation (3000 g) and distributed, to a final OD_{600} of ~ 0.25 , into: (1) the same fresh medium (no activation); (2) fresh medium without NH_4Cl (Ntr activation); (3) fresh medium with 20 μM instead of 2 mM K_2HPO_4 (Pho activation); and (4) fresh medium with 20 μM K_2HPO_4 and without NH_4Cl (Pho and Ntr activation). When required, glucose 6-phosphate (Boehringer Mannheim) was added to activate the Uhp system. Samples were taken at the indicated time-intervals (Figs 2–7) up to a maximum of 150 min after the initiation of one of these activation protocols.

Reporter assays. β -Galactosidase and alkaline phosphatase activities were assayed as described (Michaelis *et al.*, 1983; Miller, 1972), with minor modifications. Hydrolysis of ONPG, was assayed in cells permeabilized with SDS/chloroform in Z buffer (Miller, 1972) and hydrolysis of *p*-nitrophenyl phosphate (*p*NPP), the substrate for alkaline phosphatase, was assayed in cells permeabilized with SDS/chloroform in 1 M Tris/HCl, pH 8.2. The β -galactosidase reaction was stopped by the addition of 1 M Na_2CO_3 ; the alkaline phosphatase reaction was stopped by the addition of 1 M KH_2PO_4 . Enzyme activities [Miller or alkaline phosphatase (AP) units], normalized for cell density (OD_{600}), were calculated using the equation $[(\text{OD}_{420} \times 1000)/(\text{reaction time (min)} \times \text{culture volume (ml)} \times \text{OD}_{600})]$. Measurements were performed on culture samples obtained from at least two independent induction experiments, with a reproducible outcome. Typical examples are shown.

RESULTS

At face value there is ample opportunity for cross-talk between the four selected two-component systems (see Fig. 1). In each reporter strain used (Table 1), we could monitor the responses from two different signal transduction pathways: alkaline phosphatase activity, the product of *phoA*, for Pho output, and β -galactosidase activity for any one of the other three systems. Rather than measuring the physiological reporter of Ntr, glutamine synthetase (the product of *glnA*), we also chose to use a *glnA-lacZ* reporter strain for this system. The choice of *uhpT-lacZ* to monitor Uhp activation was straightforward. To study Arc activation, we made use of a *sdh-lacZ*, as well as a *cydA-lacZ* reporter strain: the *sdhCDAB* operon (encoding succinate dehydrogenase) is repressed, while *cydAB* (encoding cytochrome *d* oxidase) expression is stimulated, by ArcA $\sim$ P (Lynch & Lin, 1996). In the *in vivo* experiments, at least one regulatory system was induced (see Methods), and the elicited responses (i.e. expression of the reporter genes) were subsequently recorded. Conditions that

Fig. 2. Activation of the Pho system. Time course of alkaline phosphatase (AP) activity in a wild-type strain (NCM3286) grown on succinate. Growth of cells, activation of the relevant two-component system(s), sampling and calculation of alkaline phosphatase activity were carried out as described in Methods. $\blacklozenge$, No activation; $\blacksquare$, Pho activation; $\square$, Ntr activation; $\circ$, Pho and Ntr activation; $*$, Pho and Uhp activation; $\triangle$, Ntr and Uhp activation; $\bullet$, Pho, Ntr and Uhp activation.

activated Arc (i.e. a limited rate of oxygen supply; Alexeeva, 2000) were not included in our analyses. Furthermore, the activated system and the system in which a co-response was measured, was either wild-type or had a deletion in the gene encoding its sensor kinase or response regulator.

Cross-talk towards PhoB?

In the wild-type background the Pho response was switched on upon phosphate limitation (Fig. 2). No Pho response was observed when only Ntr was activated, and Ntr activation suppressed Pho induction when the two systems were simultaneously induced. This latter effect was even stronger in a $\Delta ntrB$ background (data not shown). Uhp activation delayed the Pho response: addition of glucose 6-phosphate, which is a substrate for alkaline phosphatase, leads to the formation of inorganic phosphate. Simultaneous activation of Pho, Ntr and Uhp strongly suppressed the Pho response. We did not address the possibility of cross-talk to PhoB in a $\Delta phoR$ strain. In such a background the Pho response is switched on constitutively, due to cross-regulation from CreC (Amemura *et al.*, 1990). In addition, acetyl phosphate contributes to PhoB phosphorylation during growth on pyruvate (Kim *et al.*, 1996; Wanner & Wilmes-Riesenberg, 1992).

Cross-talk towards UhpA?

Expression of the sugar-phosphate transporter UhpT is only induced upon the presence of extracellular glucose 6-phosphate. This was evident in the *uhpT-lacZ* reporter strain, RK5115, upon addition of glucose 6-

Fig. 3. Activation of the Uhp system. Time course of β -galactosidase activity of a *uhpT-lacZ* reporter in (a) a wild-type strain (RK5115) grown on succinate, and (b) a $\Delta uhpBC$ strain (RK1307) grown on pyruvate ($\blacklozenge$) or succinate (all other symbols). Twenty-five micromolar glucose 6-phosphate was used for Uhp activation and 50 μ M phosphate for Pho activation in (a). Growth of cells, activation of the relevant two-component system(s), sampling and calculation of β -galactosidase activity were carried out as described in Methods. $\blacklozenge$, $\diamond$, No activation; $\blacksquare$, Pho activation; $\square$, Ntr activation; $\times$, Uhp activation; $\circ$, Pho and Ntr activation; $\bullet$, Pho, Ntr and Uhp activation.

phosphate, during growth on succinate (Fig. 3a). Pho activation (i.e. phosphate limitation) leads to hydrolysis of the inducing signal (as described above). Clearly, in a wild-type background, no Uhp induction was observed without the addition of its activating signal. Similarly, in a Uhp-sensor deletion strain, RK1307 ($\Delta uhpBC$ *uhpT-lacZ*), grown on succinate, Uhp induction could not be measured (Fig. 3b). When RK1307 was grown on pyruvate as the carbon source, however, *uhpT-lacZ* expression was switched on in the absence of extracellular glucose 6-phosphate (Fig. 3b). Growth on pyruvate, which is to a large extent metabolized via the Pta-AckA (phosphotransacetylase-acetate kinase) pathway (Holms, 1996), results in high intracellular levels of acetyl phosphate. This metabolite readily phosphorylates several response regulators *in vitro* and *in vivo*

Fig. 4. Activation of the Ntr system. Time course of β -galactosidase activity of a *glnA-lacZ* reporter in: (a) a wild-type strain (NCM3286) grown on succinate; (b) a $\Delta ntrB$ strain (PPA699) grown on succinate; and (c) a $\Delta ntrB$ strain (PPA699) grown on pyruvate. Growth of cells, activation of the relevant two-component system(s), sampling and calculation of β -galactosidase activity were carried out as described in Methods. $\blacklozenge$, $\blacksquare$, Pho activation; $\square$, Ntr activation; $\times$, Uhp activation; $\circ$, Pho and Ntr activation; $*$, Pho and Uhp activation; $\triangle$, Ntr and Uhp activation; $\bullet$, Pho, Ntr and Uhp activation.

Fig. 5. Activation of the Arc system, using *cydA-lacZ* as a reporter. Time course of β -galactosidase activity in (a) wild-type strain ASA12, and (b) $\Delta arcB$ strain PPA718. Cells were grown on pyruvate ($\blacklozenge$) or succinate (all other symbols) as the carbon source. Growth of cells, activation of the relevant two-component system(s), sampling and calculation of β -galactosidase activity were carried out as described in Methods. $\blacklozenge$, $\lozenge$, No activation; $\blacksquare$, Pho activation; $\square$, Ntr activation; $\times$, Uhp activation.

(McCleary & Stock, 1994; McCleary *et al.*, 1993). Fig. 3 shows that it is also an efficient phosphoryl donor for UhpA *in vivo*. The level of transcription of *uhpT* was dependent on the growth phase of the cells (data not shown), because the intracellular acetyl phosphate concentration is growth-phase dependent (McCleary & Stock, 1994; Pruss & Wolfe, 1994). In wild-type cells, the effect of growth on pyruvate on *uhpT-lacZ* expression was not observed. Under those conditions UhpB phosphatase activity hydrolyses $P \sim UhpA$ (this activity has also been demonstrated *in vitro*; Verhamme *et al.*, 2001).

Cross-talk towards NtrC?

Nitrogen signalling, elicited by changing the nitrogen source from ammonia plus glutamine to only glutamine, induces *glnA* transcription, via the NtrBC system. This Ntr response was clearly observable in wild-type strain

Fig. 6. Activation of the Arc system, using *sdh-lacZ* as a reporter. Time course of β -galactosidase activity in (a) wild-type strain OG903, and (b) $\Delta arcB$ strain DAC903. Cells were grown on pyruvate ($\blacklozenge$) or succinate (all other symbols) as the carbon source. Growth of cells, activation of the relevant two-component system(s), sampling and calculation of β -galactosidase activity were carried out as described in Methods. $\blacklozenge$, $\lozenge$, No activation; $\blacksquare$, Pho activation; $\square$, Ntr activation; $\times$, Uhp activation.

NCM3286 (*glnA-lacZ*), grown on succinate (Fig. 4a). No response from the Ntr system could be monitored in this strain when either only Uhp (via addition of glucose 6-phosphate) or Pho (via phosphate limitation) was activated. Pho activation actually resulted in a suppression of *glnA* transcription when Ntr and Pho were jointly activated. When three systems (i.e. Ntr, Pho and Uhp) were simultaneously activated, the inhibiting effect of Pho activation on the Ntr response was relieved, due to the presence of glucose 6-phosphate, as described above. Thus, cross-talk from Uhp and Pho to NtrC was not observed with this reporter system in a wild-type background.

We next analysed *glnA* transcription in a $\Delta ntrB$ (*glnL*) background (strain PPA699) to eliminate phosphatase activity of NtrB towards $P \sim NtrC$. In this strain, nitrogen limitation can no longer be sensed, and therefore one would predict that glutamine synthetase

Fig. 7. Effect of phosphate limitation on *sdh* transcription (a, b). Time course of alkaline phosphatase (AP) activity in (a) a wild-type Arc background with either a wild-type PhoR kinase (OG903) or a PhoR with constitutive kinase activity (PhoR*; strain PPA747), and (b) a Δ arcB background with either a wild-type PhoR (DAC903) or a constitutive kinase PhoR* (PPA744). (c, d) Time course of *sdh-lacZ* reporter activity in (c) a wild-type Arc background with either a wild-type PhoR kinase (OG903) or a constitutive kinase PhoR* (PPA747), and (d) a Δ arcB background with either a wild-type PhoR (DAC903) or a constitutive kinase PhoR* (PPA744). Cells were grown on succinate as the carbon source. Growth of cells, activation of the relevant two-component system(s), sampling and calculation of alkaline phosphatase activity were carried out as described in Methods. $\blacklozenge$, No activation; $\blacksquare$, Pho activation; $\diamond$, PhoR*, no activation; $\square$, PhoR*, Pho activation.

expression (as reflected in *glnA-lacZ* levels) would maximally reach the basal, non-induced level, as measured in a wild-type background. A nitrogen response, however, could still be observed in this strain (Fig. 4b) when three systems were activated simultaneously. Co-activation of two systems resulted in a slightly elevated *glnA* transcription level (as compared to the level when no, or only a single, system was activated), but its statistical significance was difficult to assess.

In a 'double knock-out' strain, PPA703 (Δ ntrB, Δ uhpA), the absence of UhpA, in the presence of activated UhpB, did not result in a further increase of *glnA* transcription (i.e. due to enhanced cross-talk from UhpB to NtrC; data not shown).

The use of pyruvate as a carbon source resulted in constitutively high glutamine synthetase expression levels in a Δ ntrB background (PPA699; Fig. 4c). Growth on pyruvate leads to an intracellular concentration of acetyl phosphate, as described above. So, NtrC can also be rapidly phosphorylated by acetyl phosphate, in the absence of NtrB (see also Feng *et al.*, 1992). Uhp activation did not affect the Ntr response, when cells were grown on pyruvate, in a Δ ntrB background (Fig. 4c), nor in a Δ ntrB Δ uhpA background (i.e. strain PPA703, data not shown). Pho, Ntr and Uhp co-activation in PPA699 (Δ ntrB) again resulted in an additive effect on the Ntr response (Fig. 4c), superimposed upon the stimulation by pyruvate.

Cross-talk towards ArcA?

Transcription of *cydAB* is both activated by ArcA ~ P when the oxygen supply is limiting (Alexeeva, 2000; Tseng *et al.*, 1996) and regulated by Fnr (Cotter *et al.*, 1990).

For this reason we used a modified *cydAB* promoter with an inactivated Fnr box (Alexeeva, 2000) in a *cydA-lacZ* reporter strain. Activation of Pho, Ntr or Uhp, under aerobic conditions, did not result in an additive effect on *cydA-lacZ* expression in either the wild-type (Fig. 5a), or the Δ arcB (Fig. 5b) background. Also no 'pyruvate effect' (i.e. increased *cydAB* transcription upon growth on pyruvate) was observed in the absence of ArcB.

Activation of the Arc system represses transcription of *sdhCDAB* (Shen & Gunsalus, 1997). In a wild-type strain, as well as in a Δ arcB strain, grown aerobically, a small additive effect on the Arc reporter (i.e. decreasing *sdh-lacZ* expression levels) was monitored in the first hour after induction of Uhp (i.e. until glucose 6-phosphate was exhausted; Fig. 6a, b). A smaller (and delayed) effect was observed upon activation of Ntr and a rather pronounced effect (especially in the Δ arcB strain) was observed upon activation of Pho (Fig. 6a, b). The Δ arcB strain showed higher (initial) Sdh levels than the wild-type strain, implying a regulatory role for Arc under aerobic conditions, as has been observed by Park *et al.* (1995). Growth of the wild-type strain on pyruvate led to an increase in *sdh-lacZ* expression (~300 Miller units) when compared to cells grown on succinate. In the Δ arcB strain this expression level (Fig. 6a, b) was the same for cells grown on succinate and pyruvate. So although there was an effect of pyruvate on the Arc reporter, it was presumably not mediated through phosphorylation of ArcA by acetyl phosphate.

To further investigate whether the additive effect of Pho activation on *sdh* expression was due to cross-talk from PhoR to ArcA we replaced the wild-type PhoR with a constitutively active PhoR* kinase (Yamada *et al.*, 1989). This resulted in a constitutively activated Pho kinase in

strains PPA744 and PPA747. Indeed, alkaline phosphatase was constitutively expressed at high levels in these strains (Fig. 7a, b). Therefore, if cross-talk occurs from PhoR to ArcA, *sdh-lacZ* expression should be reduced independently of the activation of the Pho system. However, this was not observed (Fig. 7c, d): expression of the *sdh-lacZ* reporter only decreased when phosphate was limiting (i.e. upon Pho activation). Therefore, the effect of Pho activation on *sdh* transcription must be a metabolic effect, not mediated by phosphoryl transfer from PhoR.

DISCUSSION

In this study we analysed the interactions between four major two-component regulatory systems in *E. coli*, to investigate to what extent cross-talk affects their responses *in vivo* (Fig. 1). This is relevant because considerable cross-talk between these systems would provide this bacterial signal transduction network with neural-network-like characteristics, including properties like associative memory and learning behaviour (Hellingwerf *et al.*, 1995).

We subjected *E. coli* to selected signalling conditions and employed reporter constructs to monitor the response of each system separately. Using this *in vivo* approach we did observe cross-talk from Pho and Uhp towards NtrC, in a background in which *ntrB* had been deleted (Fig. 4; see below). However, with this procedure, we did not detect cross-talk between these systems in a wild-type background. When the Pho and Uhp system were simultaneously activated they were indirectly mutually inhibitory. This may be true for every system co-activated with the Pho system, because of the impact that the latter system has on the energy status (ATP:ADP ratio) of the cell (Wanner, 1995). This may also be the explanation for the mutual effects of activation of Ntr and Pho.

Although we detected repression of an Arc reporter (*sdh-lacZ*), clearly by activation of Pho and weakly by activation of Uhp and Ntr, these effects were presumably metabolic rather than via cross-talk (Fig. 6), as was shown in more detail for the effect of phosphate limitation (Fig. 7). Sdh operates after succinyl-CoA synthetase (*sucCD*) in the tricarboxylic acid cycle. This latter enzyme uses phosphate as a substrate to generate ATP (Wanner, 1995). Expression of *sucCD* is controlled from the *sdh* promoter (Park *et al.*, 1997). So decreasing *sucCD* expression, due to substrate (i.e. phosphate) limitation, may be accompanied by decreasing *sdh* expression.

The ArcB and PhoR transmitter, as well as the ArcA and PhoB receiver domains, belong to the same subfamily of two-component systems (Parkinson & Kofoed, 1992). Apparently, even this additional sequence relatedness does not result in detectable cross-talk between these systems *in vivo*. This is consistent with the absence of cross-talk *in vitro*, between PhoR and ArcA (Verhamme *et al.*, data not shown) and between ArcB and PhoB (Georgellis *et al.*, 1999).

To avoid cognate phosphatase activity, which might obscure cross-talk towards its specific response regulator, we used kinase-deletion strains. Indeed, it was observed in the $\Delta ntrB$ background that *glnA-lacZ* expression was constitutive during growth on pyruvate (Fig. 4c), because of the activation of NtrC by acetyl phosphate in the absence of NtrB phosphatase activity (Feng *et al.*, 1992). This made it very difficult to resolve whether, with growth on pyruvate, there was any additional, positive effect of activation of signalling through joint Pho, Ntr and Uhp induction. Whether or not this supplemental response is caused by cross-talk must be further investigated. Also the phenomenon that *glnA* transcription is elevated upon nitrogen limitation and the co-activation of Uhp and Pho in a $\Delta ntrB$ background, during growth on succinate (Fig. 4b), should be addressed in future studies. Nitrogen regulated, NtrC-controlled *glnA* transcription, independent of NtrB, has been described before (Backman *et al.*, 1983; McFarland *et al.*, 1981). Small amounts of acetyl phosphate in cells grown on succinate could result in limited activation of NtrC (see also Feng *et al.* 1992), but this does not explain the positive effect of activation of Uhp plus Pho on *glnA* transcription. Furthermore, because phosphorylated NtrC is absolutely required for *glnA* transcription (Ninfa & Magasanik, 1986), cross-talk from PhoR and UhpB may be the explanation for this observation.

UhpT was constitutively expressed in a $\Delta uhpBC$ strain when the latter was grown on pyruvate (Fig. 3b). This is a new example of response regulator activation by acetyl phosphate *in vivo*. This effect of growth on pyruvate is not a general phenomenon, as can be concluded from the results obtained with the Arc reporter strains in a $\Delta arcB$ background (Fig. 5 and 6). Several additional response regulators are activated by acetyl phosphate in *E. coli in vivo* (Bouché *et al.*, 1998; Danese *et al.*, 1995; Dorel *et al.*, 1999; Feng *et al.*, 1992; Heyde *et al.*, 2000; Matsubara & Mizuno, 1999; McCleary & Stock, 1994; Pruss & Wolfe, 1994; Wanner & Wilmes-Riesenberg, 1992). Significantly, only for RssB (an 'orphan' regulator involved in σ^S proteolysis: Bouché *et al.*, 1998) and for OmpR, during repression of flagellar synthesis (Pruss, 1998; Pruss & Wolfe, 1994), has this activation been shown to occur in a wild-type background for the relevant sensor kinase. The intracellular concentration of acetyl phosphate is strongly dependent on the metabolic state of the cell, as well as on growth phase, carbon source, pH and temperature (Heyde *et al.*, 2000; Matsubara & Mizuno, 1999; McCleary & Stock, 1994; Pruss & Wolfe, 1994). Therefore, the concentration of cytoplasmic acetyl phosphate can be regarded as a physiologically very relevant signal, feeding into the signal transduction systems of *E. coli*.

Considering which other two-component systems in *E. coli* potentially may be involved in physiologically relevant cross-talk, the CpxRA system is a candidate, because of its essential role in the extensive stress-response network. However, it has been reported that a 'kinase-on' CpxA mutant did not measurably activate

non-cognate response regulators (De Wulf & Lin, 2000). In contrast, it was reported recently (Matsubara *et al.*, 2000) that cross-talk can take place from ArcB towards OmpR. Via protein profile analysis (porin expression) and *in vitro* phosphorylation measurements, and with reporter assays, the ArcB-mediated and OmpR-dependent control of OmpC and OmpF expression, exclusively under anaerobic conditions, was demonstrated. This interaction appeared to be dependent on a functional histidine phosphoryl-transfer (HPT) domain of the sensor kinase ArcB and thus reinforces the suggestion that signalling domains within multi-component phosphorelay systems (Appleby *et al.*, 1996; Yaku *et al.*, 1997) are potential participants in physiological cross-talk. However, in contrast, no significant cross-talk was detected between the HPT domains of BvgS and EvgS and the non-cognate response regulators BvgA and EvgA, either *in vitro* or *in vivo* (Beier *et al.*, 1995; Perraud *et al.*, 1998, 1999).

To show the subtle effects of cross-talk, existing under various conditions, the DNA microarray method may become a powerful tool in future experiments, provided that it will allow sufficiently quantitative analysis. In addition, the demonstration of the underlying change in the phosphorylation level of the relevant response regulators *in vivo* remains crucial for the absolute proof of the occurrence of cross-talk. However, reliable methods to monitor this phosphorylation level are not yet available. To resolve the underlying specificity in biomolecular recognition between sensor kinases and (non-cognate) response regulators *in vivo*, the application of two-hybrid analyses may become of crucial importance.

ACKNOWLEDGEMENTS

We thank S. Alexeeva, R. J. Kadner, S. Kustu, T. Mizuno, A. J. Ninfa and B. L. Wanner for making strains available. H. V. Westerhoff, J. Tommassen and S. M. Hoffer are gratefully acknowledged for stimulating discussions.

This work was supported by the Netherlands Organization for Scientific Research (NWO), through the division of Earth and Life Sciences (Gebied ALW).

REFERENCES

- Alexeeva, S. (2000). *Molecular physiology of responses to oxygen in Escherichia coli*. PhD thesis, University of Amsterdam.
- Amemura, M., Makino, K., Shinagawa, H. & Nakata, A. (1990). Cross talk to the phosphate regulon of *Escherichia coli* by PhoM protein: PhoM is a histidine protein kinase and catalyzes phosphorylation of PhoB and PhoM-open reading frame 2. *J Bacteriol* **172**, 6300–6307.
- Appleby, J. L., Parkinson, J. S. & Bourret, R. B. (1996). Signal transduction via the multi-step phosphorelay: not necessarily a road less traveled. *Cell* **86**, 845–848.
- Arber, W. (1958). Transduction of chromosomal genes and episomes in *Escherichia coli*. *Virology* **11**, 273–288.
- Backman, K. C., Chen, Y. M., Ueno-Nishio, S. & Magasanik, B. (1983). The product of *glnL* is not essential for regulation of bacterial nitrogen assimilation. *J Bacteriol* **154**, 516–519.
- Beier, D., Schwarz, B., Fuchs, T. M. & Gross, R. (1995). *In vivo* characterization of the unorthodox BvgS two-component sensor protein of *Bordetella pertussis*. *J Mol Biol* **248**, 596–610.
- Bouché, S., Klauck, E., Fischer, D., Lucassen, M., Jung, K. & Hengge-Aronis, R. (1998). Regulation of RssB-dependent proteolysis in *Escherichia coli*: a role for acetyl phosphate in a response regulator-controlled process. *Mol Microbiol* **27**, 787–795.
- Bueno, R., Pahel, G. & Magasanik, B. (1985). Role of *glnB* and *glnD* gene products in regulation of the *glnALG* operon of *Escherichia coli*. *J Bacteriol* **164**, 816–822.
- Cotter, P. A., Chepuri, V., Gennis, R. B. & Gunsalus, R. P. (1990). Cytochrome *o* (*cyoABCDE*) and *d* (*cydAB*) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH, and the *fur* gene product. *J Bacteriol* **172**, 6333–6338.
- Danese, P. N., Snyder, W. B., Cosma, C. L., Davis, L. J. & Silhavy, T. J. (1995). The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. *Genes Dev* **9**, 387–398.
- De Wulf, P. & Lin, E. C. (2000). Cpx two-component signal transduction in *Escherichia coli*: excessive CpxR-P levels underlie CpxA⁺ phenotypes. *J Bacteriol* **182**, 1423–1426.
- Dorel, C., Vidal, O., Prigent-Combaret, C., Vallet, I. & Lejeune, P. (1999). Involvement of the Cpx signal transduction pathway of *E. coli* in biofilm formation. *FEMS Microbiol Lett* **178**, 169–175.
- Feng, J., Atkinson, M. R., McCleary, W., Stock, J. B., Wanner, B. L. & Ninfa, A. J. (1992). Role of phosphorylated metabolic intermediates in the regulation of glutamine synthetase synthesis in *Escherichia coli*. *J Bacteriol* **174**, 6061–6070.
- Fisher, S. L., Jiang, W., Wanner, B. L. & Walsh, C. T. (1995). Cross-talk between the histidine protein kinase VanS and the response regulator PhoB. Characterization and identification of a VanS domain that inhibits activation of PhoB. *J Biol Chem* **270**, 23143–23149.
- Georgellis, D., Kwon, O. & Lin, E. C. (1999). Amplification of signaling activity of the Arc two-component system of *Escherichia coli* by anaerobic metabolites. An *in vitro* study with different protein modules. *J Biol Chem* **274**, 35950–35954.
- Hellingwerf, K. J., Postma, P. W., Tommassen, J. & Westerhoff, H. V. (1995). Signal transduction in bacteria: phospho-neural network(s) in *Escherichia coli*? *FEMS Microbiol Rev* **16**, 309–321.
- Hellingwerf, K. J., Crielard, W. C., Joost Teixeira de Mattos, M., Hoff, W. D., Kort, R., Verhamme, D. T. & Avignone-Rossa, C. (1998). Current topics in signal transduction in bacteria. *Antonie Leeuwenhoek* **74**, 211–227.
- Hess, J. F., Oosawa, K., Kaplan, N. & Simon, M. I. (1988). Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis. *Cell* **53**, 79–87.
- Heyde, M., Laloi, P. & Portalier, R. (2000). Involvement of carbon source and acetyl phosphate in the external-pH-dependent expression of porin genes in *Escherichia coli*. *J Bacteriol* **182**, 198–202.
- Holms, H. (1996). Flux analysis and control of the central metabolic pathways in *Escherichia coli*. *FEMS Microbiol Rev* **19**, 85–116.
- Igo, M. M., Ninfa, A. J. & Silhavy, T. J. (1989). A bacterial environmental sensor that functions as a protein kinase and stimulates transcriptional activation. *Genes Dev* **3**, 598–605.
- Kadner, R. J. (1995). Expression of the Uhp sugar-phosphate transport system of *Escherichia coli*. In *Two-Component Signal Transduction*, pp. 263–274. Edited by J. A. Hoch & T. J. Silhavy. Washington, DC: American Society for Microbiology.

- Kim, S. K., Wilmes-Riesenberg, M. R. & Wanner, B. L. (1996). Involvement of the sensor kinase EnvZ in the *in vivo* activation of the response-regulator PhoB by acetyl phosphate. *Mol Microbiol* **22**, 135–147.
- Lynch, A. S. & Lin, E. C. C. (1996). Responses to molecular oxygen. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1526–1538. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- McCleary, W. R. & Stock, J. B. (1994). Acetyl phosphate and the activation of two-component response regulators. *J Biol Chem* **269**, 31567–31572.
- McCleary, W. R., Stock, J. B. & Ninfa, A. J. (1993). Is acetyl phosphate a global signal in *Escherichia coli*? *J Bacteriol* **175**, 2793–2798.
- McFarland, N., McCarter, L., Artz, S. & Kustu, S. (1981). Nitrogen regulatory locus *glnR* of enteric bacteria is composed of cistrons *ntrB* and *ntrC*: identification of their protein products. *Proc Natl Acad Sci USA* **78**, 2135–2139.
- Magasanik, B. (1996). Regulation of nitrogen utilization. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1344–1356. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Matsubara, M. & Mizuno, T. (1999). EnvZ-independent phosphotransfer signaling pathway of the OmpR-mediated osmoregulatory expression of OmpC and OmpF in *Escherichia coli*. *Biosci Biotechnol Biochem* **63**, 408–414.
- Matsubara, M., Kitaoka, S. I., Takeda, S. I. & Mizuno, T. (2000). Tuning of the porin expression under anaerobic growth conditions by His-to-Asp cross-phosphorelay through both the EnvZ-osmosensor and ArcB-anaerosensor in *Escherichia coli*. *Genes Cells* **5**, 555–569.
- Matsushika, A. & Mizuno, T. (1998). A dual-signaling mechanism mediated by the ArcB hybrid sensor kinase containing the histidine-containing phosphotransfer domain in *Escherichia coli*. *J Bacteriol* **180**, 3973–3977.
- Michaelis, S., Inouye, H., Oliver, D. & Beckwith, J. (1983). Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. *J Bacteriol* **154**, 366–374.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mizuno, T. (1997). Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli*. *DNA Res* **4**, 161–168.
- Neidhardt, F. C., Bloch, P. L. & Smith, D. F. (1974). Culture medium for enterobacteria. *J Bacteriol* **119**, 736–747.
- Ninfa, A. J. & Magasanik, B. (1986). Covalent modification of the *glnG* product, NRI, by the *glnL* product, NRII, regulates the transcription of the *glnALG* operon in *Escherichia coli*. *Proc Natl Acad Sci USA* **83**, 5909–5913.
- Ninfa, A. J., Ninfa, E. G., Lupas, A. N., Stock, A., Magasanik, B. & Stock, J. (1988). Crosstalk between bacterial chemotaxis signal transduction proteins and regulators of transcription of the Ntr regulon: evidence that nitrogen assimilation and chemotaxis are controlled by a common phosphotransfer mechanism. *Proc Natl Acad Sci USA* **85**, 5492–5496.
- Park, S. J., Tseng, C. P. & Gunsalus, R. P. (1995). Regulation of succinate dehydrogenase (*sdhCDAB*) operon expression in *Escherichia coli* in response to carbon supply and anaerobiosis: role of ArcA and Fnr. *Mol Microbiol* **15**, 473–482.
- Park, S. J., Chao, G. & Gunsalus, R. P. (1997). Aerobic regulation of the *sucABCD* genes of *Escherichia coli*, which encode alpha-ketoglutarate dehydrogenase and succinyl coenzyme A synthetase: roles of ArcA, Fnr, and the upstream *sdhCDAB* promoter. *J Bacteriol* **179**, 4138–4142.
- Parkinson, J. S. & Kofoed, E. C. (1992). Communication modules in bacterial signaling proteins. *Annu Rev Genet* **26**, 71–112.
- Perraud, A. L., Kimmel, B., Weiss, V. & Gross, R. (1998). Specificity of the BvgAS and EvgAS phosphorelay is mediated by the C-terminal HPT domains of the sensor proteins. *Mol Microbiol* **27**, 875–887.
- Perraud, A. L., Weiss, V. & Gross, R. (1999). Signalling pathways in two-component phosphorelay systems. *Trends Microbiol* **7**, 115–120.
- Pruss, B. M. (1998). Acetyl phosphate and the phosphorylation of OmpR are involved in the regulation of the cell division rate in *Escherichia coli*. *Arch Microbiol* **170**, 141–146.
- Pruss, B. M. & Wolfe, A. J. (1994). Regulation of acetyl phosphate synthesis and degradation, and the control of flagellar expression in *Escherichia coli*. *Mol Microbiol* **12**, 973–984.
- Rabin, R. S. & Stewart, V. (1993). Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in *Escherichia coli* K-12. *J Bacteriol* **175**, 3259–3268.
- Shattuck-Eidens, D. M. & Kadner, R. J. (1981). Exogenous induction of the *Escherichia coli* hexose phosphate transport system defined by *uhp-lac* operon fusions. *J Bacteriol* **148**, 203–209.
- Shen, J. & Gunsalus, R. P. (1997). Role of multiple ArcA recognition sites in anaerobic regulation of succinate dehydrogenase (*sdhCDAB*) gene expression in *Escherichia coli*. *Mol Microbiol* **26**, 223–236.
- Silva, J. C., Haldimann, A., Prahalad, M. K., Walsh, C. T. & Wanner, B. L. (1998). *In vivo* characterization of the type A and B vancomycin-resistant enterococci (VRE) VanRS two-component systems in *Escherichia coli*: a nonpathogenic model for studying the VRE signal transduction pathways. *Proc Natl Acad Sci USA* **95**, 11951–11956.
- Singer, M., Baker, T. A., Schnitzler, G. & 7 other authors (1989). A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol Rev* **53**, 1–24.
- Stock, J. B., Ninfa, A. J. & Stock, A. M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol Rev* **53**, 450–490.
- Tseng, C. P., Albrecht, J. & Gunsalus, R. P. (1996). Effect of microaerophilic cell growth conditions on expression of the aerobic (*cyoABCDE* and *cydAB*) and anaerobic (*narGHJI*, *frdABCD*, and *dmsABC*) respiratory pathway genes in *Escherichia coli*. *J Bacteriol* **178**, 1094–1098.
- Verhamme, D. T., Arents, J. C., Postma, P. W., Crielaard, W. & Hellingwerf, K. J. (2001). Glucose-6-phosphate-dependent phosphoryl flow through the Uhp two-component regulatory system. *Microbiology* **147**, 3345–3352.
- Wanner, B. L. (1992). Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria? *J Bacteriol* **174**, 2053–2058.
- Wanner, B. L. (1995). Signal transduction and cross regulation in the *Escherichia coli* phosphate regulon by PhoR, CreC and acetyl phosphate. In *Two-component Signal Transduction*, pp. 203–221. Edited by J. A. Hoch & T. J. Silhavy. Washington, DC: American Society for Microbiology.
- Wanner, B. L. & Wilmes-Riesenberg, M. R. (1992). Involvement of phosphotransacetylase, acetate kinase, and acetyl phosphate synthesis in control of the phosphate regulon in *Escherichia coli*. *J Bacteriol* **174**, 2124–2130.

Webber, C. A. & Kadner, R. J. (1995). Action of receiver and activator modules of UhpA in transcriptional control of the *Escherichia coli* sugar phosphate transport system. *Mol Microbiol* **15**, 883–893.

Yaku, H., Kato, M., Hakoshima, T., Tsuzuki, M. & Mizuno, T. (1997). Interaction between the CheY response regulator and the histidine-containing phosphotransfer (HPT) domain of the ArcB sensory kinase in *Escherichia coli*. *FEBS Lett* **408**, 337–340.

Yamada, M., Makino, K., Amemura, M., Shinagawa, H. & Nakata, A. (1989). Regulation of the phosphate regulon of *Escherichia coli*: analysis of mutant *phoB* and *phoR* genes causing different phenotypes. *J Bacteriol* **171**, 5601–5606.

.....
Received 23 July 2001; revised 13 September 2001; accepted
27 September 2001.