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Novel Aspects of the Acid Response Network of *E. coli* K-12 Are Revealed by a Study of Transcriptional Dynamics

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Understanding gene regulation and its adaptive significance requires not only a detailed knowledge of individual molecular interactions that give rise to changes in gene expression but also an overview of complete genetic networks and the ways in which components within them interact. Increasingly, such studies are being done using luminescent or fluorescent reporter proteins that enable monitoring of gene expression dynamics in real time, particularly during changes in expression. We show here that such an approach is valid for dissecting the responses of the AR2 or GAD network of *Escherichia coli* K-12 to changes in pH, which is one of the most complex networks known in *E. coli*. In addition to confirming several regulatory interactions that have been revealed by previous studies, this approach has identified new components in this system that lead to complex dynamics of gene expression following a drop in pH, including an auto-regulatory loop involving the YdeO activator protein and novel roles for the PhoP protein.

Keywords: acid resistance; *Escherichia coli*; transcription dynamics; bacterial luciferase; regulatory networks

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Introduction

A complete understanding of the regulation of gene expression in any organism requires an understanding not only of which genes are controlled by which regulators but also of the dynamics of change in gene expression in response to shifting experimental conditions. Although this point has been appreciated for many years,[1] it is only relatively recently, with the advent of suitable *in vivo* tools for quantifying changes in gene expression in or close to real time, that the investigation of such dynamics over large numbers of genes has become feasible. There is increasing evidence that subtle differences in timing of expression of different genes within a network can indeed have adaptive significance.[2–6]

Studies on the dynamics of gene expression that occur in organisms in response to stress have great potential for modeling, since stresses are effectively perturbations, the effects of which can be used to build and test models of network connections. This in turn can lead to a deeper understanding of the roles of the various components with networks and how they may integrate information about the state of the cell from different sources, as exemplified recently.[7] Dynamic models of networks are most rigorously tested using expression data with high temporal resolution. Despite this, there is a paucity of such research on networks of stress response genes in the current literature.

We are interested in the diverse ways in which *Escherichia coli* (both as a laboratory strain and as commensal and pathogenic isolates) responds to different stresses and the details of the network architectures underlying these responses. One particular stress that *E. coli* encounters on a regular basis is low pH, since as an enteric organism *E. coli* has to pass through the stomach en route to the

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Abbreviations used: AR, acid resistance; RT-qPCR, quantitative reverse transcription polymerase chain reaction; AFI, acid fitness island; LB, lysogeny broth; CFU, colony-forming unit; FRT, flippase recombination target.

intestine. It is now known that *E. coli* possesses a number of effectors that enable it to survive otherwise lethal doses of inorganic and organic acid stress (termed acid resistance or AR), many of which are not present in closely related bacteria such as *Salmonella* spp.[8] The function of each AR effector depends on the precise nature of the acid stress.[9–13] For example, two isomeric glutamate decarboxylases encoded by *gadA* and *gadB* and a glutamate-γ-aminobutyric acid antiporter *gadC* contribute to AR only when glutamate is available in the extracellular environment,[10] a phenomenon termed glutamate-dependent AR or AR2, whereas the periplasmic chaperone encoded by *hdeA* only appears to contribute to AR in dense cell populations.[11,14] Several effectors reside within a 15-kb genomic patch termed the "acid fitness island" (AFI) and, along with the non-proximal *gadBC* locus, are co-regulated under diverse situations.[15–18] In *E. coli* K-12, these effectors are subject to tight transcriptional control and are expressed at low levels during rapid growth at neutral pH, due in part to global H-NS-mediated repression[15,19] and Lon-mediated degradation of the central activator protein GadE.[20] For induction of this latter set of genes, distinct regulatory networks operate depending on the inducing conditions, which include a shift to sub-lethal pH[21] and growth into stationary phase.[19,22]

A drop from pH 7 to sub-lethal pH 5.5 induces a number of AR effectors via the two-component system EvgA/EvgS, which is encoded outside of the AFI.[23,24] Two intermediate transcription factors, YdeO and the AFI-encoded GadE, are transcriptionally activated by the presumably phosphorylated response regulator EvgA.[18,24–26] YdeO can also activate *gadE* transcription forming a coherent feed-forward loop[18] (Fig. 1a). However, it is not clear what purpose this regulation serves *in vivo* since several studies have shown that EvgA can activate the *gadE* promoter in a *ydeO* mutant.[24,26] GadE directly activates its own expression while repressing production of YdeO.[24] Many structural genes within the AFI, as well as the *gadBC* locus, are activated directly by GadE, although effectors such as *slp* appear not to be under GadE control.[15,18,27] The contribution of the alternate general stress and stationary phase sigma factor RpoS may also have a role under low pH inducing conditions, since GadE positive feedback can be further supported by its presence.[28] The homologous transcription factors

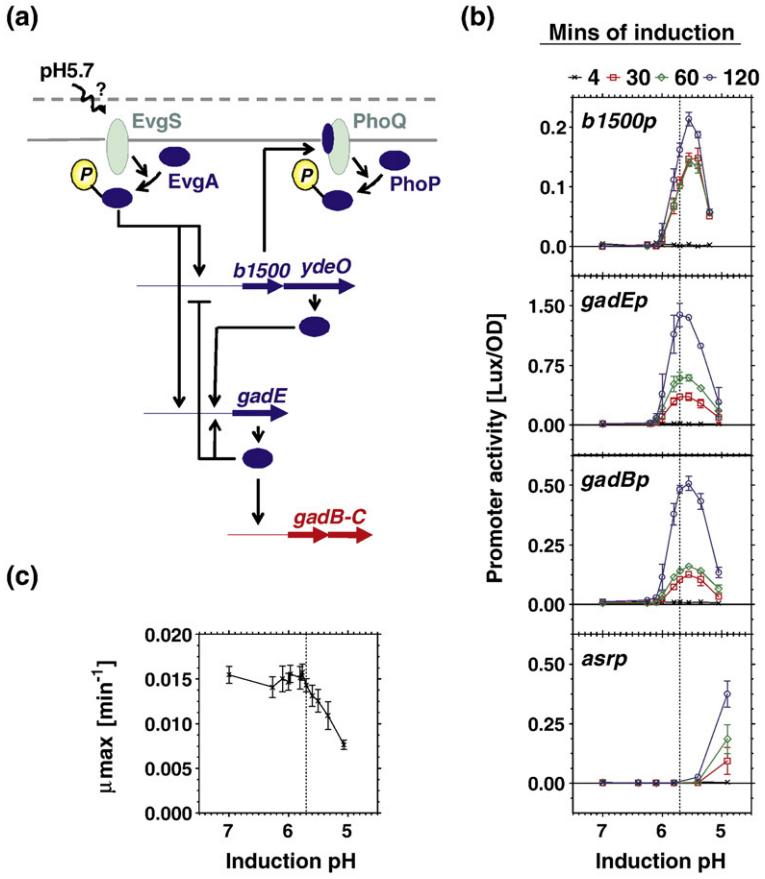

Fig. 1. The pH range of activation of genes downstream of EvgAS. (a) Cartoon summary of previously reported regulatory events downstream of EvgAS (see the text for details). A broken and a continuous grey horizontal line represent the outer and inner cell membrane, respectively. Thick red and blue horizontal arrows are genes encoding effector proteins and regulators, respectively, green ovals are two-component sensor kinases, the small blue oval is B1500, larger blue ovals are transcription factors, black lines with arrowheads and blocked ends represent positive and negative regulatory interactions, respectively, and a yellow circle containing the letter P represents a phosphate group attached as a consequence of two-component system activation. (b) Transcriptional fusions of the *b1500-ydeO*, *gadE*, *gadBC*, and *asr* gene promoters were constructed in the pLUX vector, and the response of each of these promoters to a shift from pH 7 to pH values ranging between 6.3 and 4.9 (as well as a mock induction at pH 7) was assayed in an *E. coli* MG1655 wild-type genetic background. For each induction pH (*x*-axis), the Lux/OD values (*y*-axis) determined at 4, 30, 60, and 120 min after induction (black crosses, red squares, green diamonds, and blue circles, respectively) are plotted. Each experiment was repeated twice. A broken line is drawn at $x=5.7$ as a guide. (c) For each induction pH used in (b), the maximum growth rates (μ min^{-1}) of the cultures have been plotted. In all cases, the culture growth rate was maximal shortly after induction and gradually decreased as cultures approached stationary phase (data not shown). The data are based on six growth curves at each pH value.

GadX and GadW, along with the small regulatory RNA GadY, collaborate with RpoS to relieve H-NS-mediated repression of the system when cultures enter into stationary phase[16] but have also been reported to have a role during low pH response.[20,29] In addition, GadW is under direct control of the PhoPQ two-component system,[30] and it has been shown that EvgAS can cross-talk to PhoPQ via the connector protein encoded by *b1500* (also known as *safA*[26]), which resides directly upstream of and in the same operon as YdeO.[18,31]

As a first step to building a mathematical description of this complex network, we wished to assess whether a dynamic approach to monitoring gene expression within the network was both feasible and valid and in particular whether such an approach would reveal any novel aspects of the network that had not been detected to date. We focused our attention on the dynamics of the response to a sub-lethal low pH shock incurred during cell growth. This condition is likely to be a relevant *in vivo* stimulus for this crucial set of AR effectors, and the highly interconnected regulatory network thus far characterised suggests that this system will show a complex response. To date, no paper has described or analysed the dynamics of the response of this circuit.

Libraries of promoter fusions to reporters such as luciferase or green fluorescent protein are well suited for studies of gene expression dynamics. High-throughput experiments can be run at low cost,[32,33] enabling gene expression to be assessed in close to real time over a large parameter space, both by varying growth conditions and by conducting studies in a range of different mutant backgrounds. We describe here the use of bacterial luciferase (Lux) as a reporter to dissect the detailed dynamics of several key components of the AR2 network in *E. coli* K-12 (MG1655) and show that it does indeed enable a more detailed description of the network, revealing both rapid and delayed aspects to the response, and enabling different aspects of the kinetics to be pinpointed to key regulators, a number of which have not previously been described.

Results and Discussion

Genes downstream of EvgA are optimally activated between pH 5.7 and 5.5

We modified a micro-plate reader-based assay[34,35] to measure low pH response dynamics of a set AR promoters fused to the *Photorhabdus luminescens lux* operon on a low copy plasmid (Table 2; see Materials and Methods for details). We first determined how promoters at different levels in the regulatory hierarchy downstream of EvgAS (*b1500-ydeOp*, *gadEp* and *gadBp*; Fig. 1a) responded to a range of pH values. Each promoter was activated over a similar range of pH values, starting at pH 6, peaking in activity between pH 5.7 and 5.4, and then sharply declining in activity at the lower pH values tested (Fig. 1b). The consistent decline in the magnitude of response at lower pH could have been due to the drop in growth rate observed at lower pH values (Fig. 1c). However, an equivalent *lux* fusion to the promoter of the *asr* gene (a gene of unknown function, which is strongly activated at low pH) was strongly activated only around pH 5 and below (Fig. 1b), consistent with the pH range for induction of this gene reported previously.[36–38] This showed that Lux reporter function was not inhibited by slow growth rate. Thus, the data show a distinct range and clear optimum for pH activation of genes downstream of EvgAS. For the remainder of this work, pH 5.7 was used as the pH to study the regulatory dynamics of the network, since this gave close-to-maximal expression for all the fusions and was well within the buffering capacity of the media used. We note that 5.7 is close to one of the pK_a values of histidyl residues, which raises the possibility that the EvgS sensor kinase, or some other component of the response network, is activated by protonation of one or more histidine residues.

The bacterial Lux system can report accurate high temporal resolution promoter kinetics *in vivo*

Bacterial Lux fusions are widely used to monitor transcriptional kinetics,[3,6,39] yet details of the turnover of reporter product (enzymatic light production) *in vivo* are not yet well understood.[40,41] For the purpose of this study, it was important to determine how well the levels of luciferase activity arising from a plasmid-borne promoter fusion correlated with levels of transcription from the same promoter at its endogenous chromosomal location. For two of the Lux fusions (*gadBC* and a non-acid responsive control, *csrA*), we measured relative levels of endogenous RNA production (in the absence of a reporter plasmid) by quantitative reverse transcription polymerase chain reaction (RT-qPCR) at several time points after induction. We found good correlation at all time points with Lux activity, despite differences in the levels of fold induction for the *gadB* promoter, which likely relate to different sensitivities to very low levels of expression between the two techniques (Fig. 2a). A similar correlation was seen for both genes in an *rpoS* mutant background (data not shown), which we found resulted in a change in the kinetics of *gadB* promoter activity relative to the wild-type background (Fig. 3a, xvi). From these data, we infer that the Lux system has a relatively high and constant rate of turnover in the cell over the assay period and is well suited to analysing transcriptional kinetics in this context. We also used the stable *gfp-mut2* reporter[42] to measure activities of a number of promoters and found relatively good correlation to Lux/OD and to mRNA levels when the first derivative of GFP accumulation was calculated, as done elsewhere[34,43]

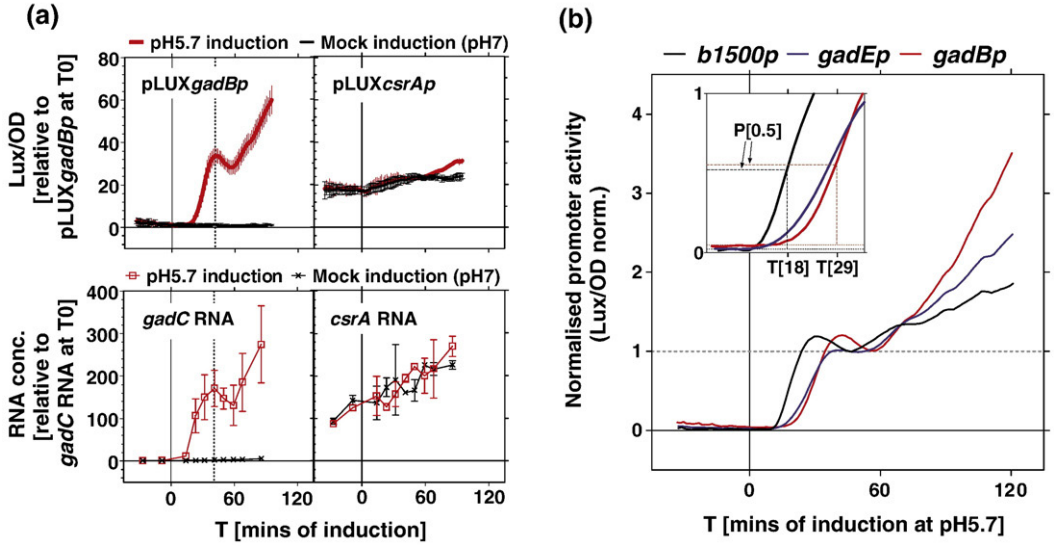

Fig. 2. The reporter output from lux fusions closely tracks native RNA levels and reveals sequential activation of the *b1500*, *gadE*, and *gadB* promoters. (a) Validation of the use of plasmid-based Lux transcriptional fusions as reporters of promoter activity. The *gadB* and *csrA* promoters were assayed using a plasmid-based Lux reporter fusion (top panels; Lux/OD) and by directly measuring relative native *gadC* and *csrA* native mRNA levels in the absence of the reporter plasmid, by RT-qPCR (bottom panels). The units given in each case represent fold induction relative to *gadBC* promoter or *gadC* mRNA level at T0, respectively. Each experiment was repeated 4 times. (b) Responses to a pH 7 to pH 5.7 down-shift of the *b1500-ydeO*, *gadE*, and *gadBC* promoters (black, blue, and red lines, respectively) were measured in *E. coli* MG1655 using the relevant Lux fusion plasmid. For comparison in the same plot, Lux/OD levels of each promoter are normalised to 1 at the time point where activity finishes decreasing after the initial rise observed in each case (at T46 for *b1500p*, T51 for *gadEp*, and T56 for *gadBp*). Normalised values are denoted [Lux/OD norm]. A grey broken line is drawn at [Lux/OD norm]=1 as a guide. Each promoter was assayed 4 times. Inset: Enlarged figure of first 40 min after pH shift of *b1500* (black continuous line), *gadE* (blue continuous line), and *gadB* (red continuous line) promoter activities. The light blue and red upper broken horizontal lines indicate P[0.5] for the *b1500* and *gadB* promoters, respectively, defined as the halfway point between non-induced activity (lower broken horizontal line of corresponding colour) and a normalised level of 1. The time point at P[0.5] for each promoter is indicated under the *x*-axis.

(data not shown); however, the sensitivity of this system was poorer due to background fluorescence from the growth medium.

We compared the activities of the *b1500-ydeO*, *gadE*, and *gadBC* promoters, measured at 80-s intervals up to 2 h after induction (Fig. 2b). The promoters showed similar induction kinetics, with each undergoing a surge in activity leading to a transient peak, which was more pronounced at the *b1500-ydeO* and *gadB* promoters. This was followed by a period of slower increase, which was greater at the *gadE* and *gadB* promoters. To compare the kinetics of the above three promoters, which varied in absolute values of activity, we normalised the data such that the point in time after the initial surge in activity where activity fell to a minimum for each promoter was made equal to 1. Based on this normalisation, a clear difference in the timing of induction of the promoters can be observed, with approximately 10 min separating induction at *b1500-ydeOp* and *gadBp*, while the *gadE* promoter was reproducibly induced between these two (Fig. 2b, inset). This order of induction is consistent with YdeO first inducing the *gadE* promoter and then GadE in turn activating the *gadB* promoter.[18,27]

A transcriptional cascade from EvgA to YdeO to GadE to GadB must be completed to enable a rapid increase in glutamate-dependent AR

To start to assign the regulators responsible for the kinetics seen in Fig. 2b, we re-assayed this set of promoters, in addition to a similarly constructed *evgAS*–Lux promoter fusion (Table 2), in strain derivatives in which the key regulators EvgS, EvgA, YdeO, GadE, and RpoS had been removed by mutation.

As expected, low pH activation of *ydeO*, *gadE*, and *gadB* transcription was at all time points dependent on the presence of EvgA (Fig. 3a, ii–iv) and EvgS (data not shown), whereas *evgAS* transcription was mildly repressed in the presence of EvgA (Fig. 3a, i) and EvgS (data not shown). EvgA auto-activation has been reported,[18] although this was only observed following overexpression of EvgA under neutral pH conditions.[18] The data presented here agree with a model whereby transcriptional induction of this two-component system does not occur.[24] The fact that both EvgA and EvgS are required for downstream promoter induction suggests that EvgS-mediated phosphorylation of EvgA occurs under low pH conditions,

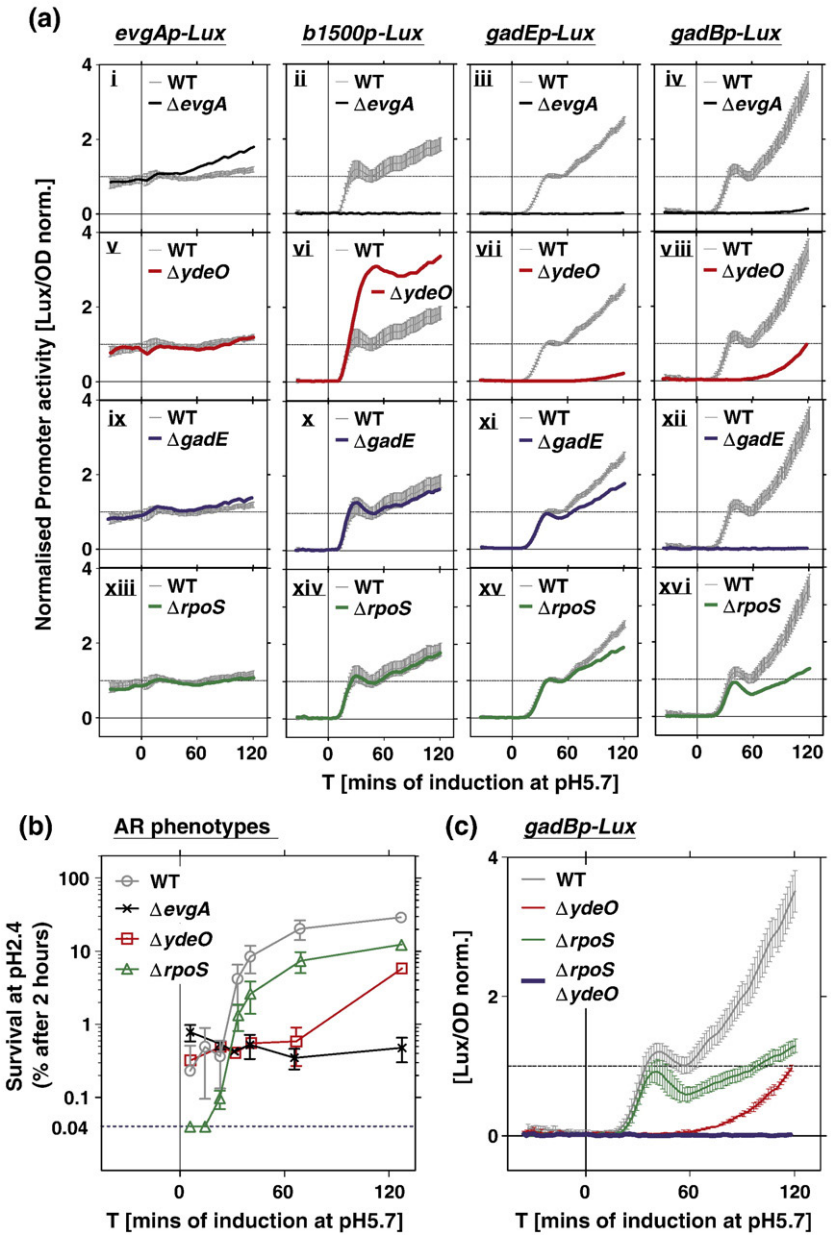

Fig. 3. Regulation influencing observed promoter kinetics within the EvgAS-dependent network. (a) Response dynamics to a shift from pH 7 to pH 5.7 of the *evgAS*, *b1500-ydeO*, *gadE*, and *gadBC* promoters assayed in derivatives of *E. coli* MG1655 mutated singly for *evgA*, *ydeO*, *gadE*, and *rpoS* measured using the relevant Lux fusion plasmid (Table 2). Data from the same mutant background are presented in rows, and data from the same promoter are presented in columns, with the promoter name above the column. In each plot, activity of the promoter in the mutant background is compared to the activity of the same promoter in the wild-type background (thin grey lines with error bars; wild-type data other than that for *evgAp* are reproduced from Fig. 2). Normalised data are presented for ease of data cross-comparison (refer to legend to Fig. 2 for details; in the case of *evgAp*, normalisation was done such that activity in the wild-type background equalled 1 at T60). Each promoter was assayed 4 times in wild-type background and between 1 and 5 times in each mutant background. (b) AR, defined as survival in M9-E pH 2.4 for 2 h, compared between wild type (grey circles), Δ*evgA* (black crosses), Δ*ydeO* (red squares), and Δ*rpoS* (green triangles) strains of *E. coli* MG1655 at different time points following pH induction (shift from pH 7 to pH 5.7). Error bars represent the standard deviation of the mean of 3 to 5 repeated experiments. A blue broken line marks the limit of detection of this assay at 0.04% survival. (c) Activity of the *gadB* promoter in a *ydeO rpoS* double mutant (thick blue line) assayed as in (a). The data are compared to activity in single *ydeO* (red) and *rpoS* (green) mutants in the same plot.

leading to transcriptional activation of downstream promoters. The mild negative feedback reported here may help to balance the levels of phosphorylated EvgA.

We then investigated the contribution of YdeO to the dynamics of a previously characterised feed-forward loop motif involving EvgA, YdeO, and GadE[24] (Fig. 1a). Importantly, the *gadE* and *gadB* promoters were also entirely dependent on YdeO during the initial phases of induction (Fig. 3a, vii–viii), showing that information from EvgAS flows via YdeO to initiate *gadE* promoter activity. As expected, GadE in turn was required for *gadB* promoter induction (Fig. 3a, xii). These data are consistent with the temporal succession of promoter induction observed above (Fig. 2b). It was reported elsewhere that the YdeO section of the cascade can be bypassed.[24,26] Our data show that this is true (see later time points in Fig. 3a, vii–viii) but that EvgA-mediated induction of *gadE* and *gadB* in the absence of YdeO is severely delayed. Furthermore, data to be presented below will suggest that EvgA alone may in fact have little effect on *gadE* promoter activity, as additional regulatory interactions linked to EvgAS are required for residual induction of the *gadE* promoter in the absence of YdeO.

We confirmed the role of YdeO by studying the induction of the AR2 phenotype. At different time points following induction at pH 5.7, wild-type MG1655 and *evgA* and *ydeO* mutants were

transferred to very low pH (pH 2.4) in the presence of glutamate, and survival after 2 h was quantified. The resistance quantified under these conditions was dependent on *gadC* and the presence of glutamate in the challenge media (data not shown), confirming it to be a consequence of AR2. Following pH 5.7 induction, a sharp increase in AR2 was observed in the wild-type strain and this depended on the regulation conferred by EvgA for up to 2 h after induction (Fig. 3b), in agreement with earlier findings that also showed a key role for *evgA* in mounting AR2 in low pH shifted log-phase but not stationary-phase cells.[24] The initial increase in AR2 was also entirely dependent on YdeO, but at later time points post-induction, an increase in AR2 occurred in the absence of *ydeO* (Fig. 3b), consistent with our findings concerning the kinetics of *gadB* promoter regulation (Figs. 2 and 3a). Interestingly, we found that EvgA-dependent AR was not as heavily dependent on YdeO or GadC during challenge at pH 2.7, showing that other EvgA-regulated AR effectors, not dependent on YdeO, have a role in protection at this less severe pH (data not shown). We speculate that a likely candidate for this resistance is the EvgAS-induced gene *ydeP*, which encodes a putative oxido-reductase previously shown to confer AR when overexpressed[18] and has a crucial role in glutamate-independent AR in *Shigella flexneri*.[44]

YdeO-mediated negative feedback partially controls the induction surge at the *b1500-ydeO* promoter

We reasoned that the initial surge followed by a slower increase in promoter activation seen for the *ydeO*, *gadE*, and *gadB* promoters could be due to negative feedback at the top of the hierarchy. In agreement with this hypothesis, it is shown in Fig. 3a, vi, that removal of YdeO by mutation leads to a greater than 2-fold increase in transcription of the *b1500-ydeO* promoter following induction. Previously, it was shown that a mutation in *gadE* caused elevated levels of *b1500-ydeO* transcript during long-term growth at pH 5.5.[24] However, under the present conditions, YdeO-mediated feedback did not require GadE, as no effect of a *gadE* mutation on *b1500* promoter activity was found (Fig. 3a, x). This novel YdeO-mediated repression of *b1500-ydeO* promoter activity occurs within 10 min of induction of YdeO synthesis and contributes substantially in setting the steady-state level of activity observed at this promoter. However, from the shape of these curves, another component is also likely to be involved in regulating *b1500-ydeO* promoter activity, as although activity is higher in the *ydeO* mutant background, a distinct peak is still observed. This point is discussed further below. We speculate that GadE-mediated repression of the *b1500-ydeO* promoter may only occur during long-term acid adaptation (>2 h after shift) since the data here show that it is not relevant in dictating early induction kinetics.

GadE positive feedback constitutes a delayed element of the low pH response and requires the presence of the sigma factor RpoS

Data presented in Fig. 2b showed that after the initial surge in activation discussed above, the *gadE* and *gadB* promoters underwent a second increase in activity that was more pronounced than that observed at the *b1500* promoter. We speculated that this difference could be due to GadE auto-regulation, which has been previously reported.[24,45] By measuring the activation kinetics of the *gadE* promoter in a strain lacking GadE, we found that GadE auto-activation did partially account for the differential kinetics (Fig. 3a, xi). This shows that GadE has a very different contribution to *gadE* promoter activation kinetics compared with YdeO, operating on a slower time scale to produce a more gradual change in promoter activity over time.

Auto-regulation of the *gadE* promoter has been linked to the presence of RpoS.[28] Interestingly, we found that during low pH induction of the *gadE* promoter, an *rpoS* mutation had a very similar effect to a *gadE* mutation (Fig. 3a, xv). While we did not directly investigate the impact of *gadE* auto-regulation on downstream GadE-dependent promoter activities, we did find that an *rpoS* mutation led to a delayed decrease in *gadB* promoter activity (Fig. 3a, xvi). This delayed regulation of the *gadB* promoter could be partially accounted for by decreased GadE production in this condition, in addition to any direct regulation by RpoS (Fig. 3a, xvi). Together, the results suggest a mechanistic cooperativity between GadE and RpoS in this component of the response, and indeed when we combined the two mutations to create a double *gadE rpoS* knock out, *gadE* promoter was unchanged relative to the *gadE* single mutant (data not shown).

At the phenotypic level, an *rpoS* mutant displayed a similar rapid induction of AR2 as the wild-type strain, which was in stark contrast to the situation in an *evgA* or a *ydeO* mutant (Fig. 3b). However, AR2 was reproducibly lower in the *rpoS* strain at later time points after induction, reflecting the changes seen in *gadB* promoter activity in an *rpoS* background (Fig. 3a, xvi). These data confirm the physiological relevance of the time-dependent input of YdeO and RpoS in regulating the *gadE* and, in turn, *gadBC* promoters. The lower level of initial survival observed in an *rpoS* background (Fig. 3b) correlates with a low level of RpoS-dependent *gadE* and *gadB* promoter activity, which is observed in un-induced wild-type cultures (data not shown). RpoS levels can be induced by low pH, although this depends on the genetic background and precise growth conditions.[46,47] Further studies are required to establish whether RpoS levels are induced by low pH shift in the present context or if the higher magnitude of RpoS-dependent regulation at low pH reflects synergy with additional pH-induced regulatory partners.

We hypothesised that the residual induction of *gadB* and *gadE* promoter activity in the absence of

YdeO was due to GadE and RpoS. As shown in Fig. 3c, RpoS (and presumably also GadE) did indeed regulate this delayed activation of the *gadB* promoter. We also found this to be the case at the *gadE* promoter (data not shown). This suggested that RpoS-mediated activation of these promoters in a wild-type cell is at least partially independent of YdeO. By constructing and assaying a truncated variant of the *gadE* promoter termed *gadEp*.1 (Fig. 4a), we also found that whereas the YdeO-induced *gadE* promoter surge relies heavily upon regions upstream of −360 bp from the translational start site, GadE- and RpoS-mediated regulation requires only the proximal *gadE* promoter region (Fig. 4b and c). Together, these data show that despite co-reliance on EvgAS, there is a degree of mechanistic independence between the regulatory elements governing rapid and delayed responses to low pH at the *gadE* promoter, which corroborates and extends recent findings.[28]

In summary, the above data show that through EvgAS, a strong response to a pH 5.7 shift is initially transmitted through the circuit via YdeO, which we show here negatively auto-regulates its own production to dampen this response. After these events, the general stress sigma factor RpoS becomes involved, contributing to a positive auto-regulatory loop at the *gadE* promoter and driving further activation of the *gadB* promoter. Taken together, the data in Figs. 3 and 4 clearly demonstrate that EvgA alone is not sufficient to trigger low pH activation of the *gadE* or *gadB* promoter for up to 2 h after pH shift.

A novel role for PhoP in the AR2 network

We next turned our attention to the observation that negative auto-regulation of *ydeO* did not seem to fully account for the initial peak in the activity of the *b1500-ydeO*, *gadE*, and *gadB* promoters. To search for the missing link, we considered what additional regulators might be part of the network. We assessed whether a recently described activation cross-talk from EvgAS to PhoPQ via B1500 is active under low pH inducing conditions, as predicted previously.[31] Indeed, the PhoP-dependent *mgtA* gene promoter was activated in response to a gradient of pH changes with the same dose–response curve as was seen for the *b1500-ydeO*, *gadE*, and *gadB* promoters (Fig. 5a). All these experiments were done under concentrations of Mg^{2+} close to 1 mM, where the PhoPQ system would normally be inactive.[48] The low pH induction of the *mgtA* promoter was abolished by deletion of *evgA*, *evgS*, *b1500*, or *phoP* (Fig. 5b, i–iii; data not shown for *evgS* mutant). The interpretation of the

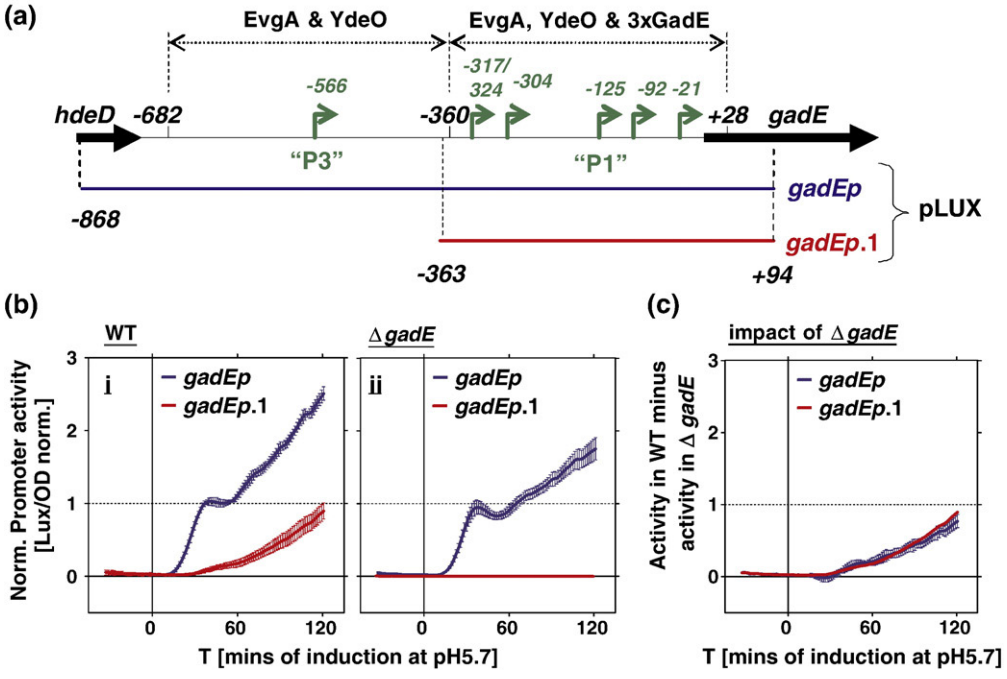

Fig. 4. Integration of GadE/RpoS-independent and GadE/RpoS-dependent response dynamics at the *gadE* promoter. (a) Cartoon of *gadE* promoter region. Start sites identified in previous publications[15,24,26,28,29] are indicated with green arrows. (b) Response to a shift from pH 7 to pH 5.7 of *gadEp*.1 variant fused to Lux and assayed (i) in wild-type MG1655 and (ii) in a *gadE* mutant derivative of MG1655. In each case, the activity has been compared in the plot to that of the full-length *gadEp* variant. (c) The effect of the presence of *gadE* on the activity of *gadEp* and *gadEp*.1 is inferred by subtracting the activity obtained for each fusion in the *gadE* mutant from the activity obtained from the same fusion in wild-type background. Note that the concentration of GadE in the cell is expected to be the same whether assaying the full-length or half-length *gadE* promoter, since gadE production relies on the chromosomal promoter region that is unmodified in each case.

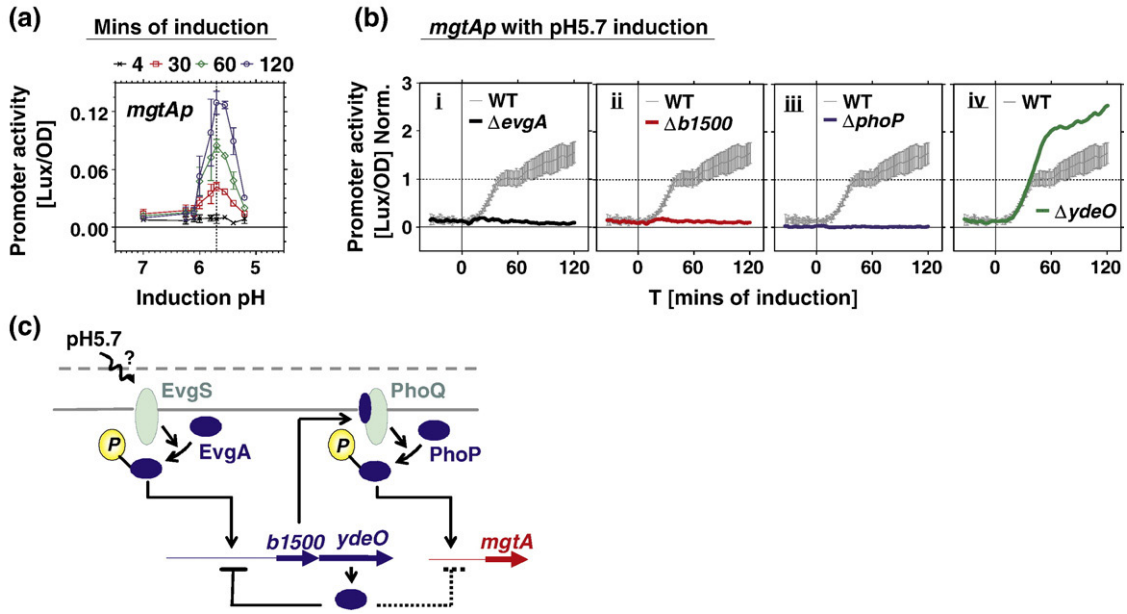

Fig. 5. Shift from pH 7 to pH 5.7 activates the EvgAS–B1500–PhoPQ connector pathway. (a) A plasmid-based Lux fusion of the *mgtA* promoter (pLUXmgtAp; Table 2) was constructed and assayed at a range of different pH shift values as described in the legend to Fig. 1a. (b) Response dynamics of *mgtA* promoter (pLUX*mgtAp*) to a shift from pH 7 to pH 5.7 in Δ*evgA* (i), Δ*b1500* (ii), Δ*phoP* (iii), or Δ*ydeO* (iv) derivatives of *E. coli* MG1655, compared to wild-type activity (grey line with error bars in each plot). (c) Cartoon model of regulatory interactions occurring within the connector pathway following a shift of culture pH from 7 to 5.7. See Fig. 1a for details.

b1500 mutation is complicated by the fact that mutation of *b1500* is likely to have polar effects on *ydeO* expression.[26] However, loss of YdeO alone caused a significant increase in *mgtA* activity (Fig. 5b, iv). Although this could be due to direct repression by YdeO at the *mgtA* promoter, it could also be explained by our novel finding that YdeO negatively regulates *b1500* and *ydeO* expression. The increase in B1500 production in a *ydeO* mutant could lead to more PhoP phosphorylation by PhoQ, which subsequently enhances transcription of *mgtAp*. Altogether, the data strongly supported PhoPQ activation by EvgAS during adaptation at pH 5.7, via the small connector protein B1500.

We speculated that PhoP could be the missing link involved in a negative feedback loop to the *b1500-ydeO* promoter. To test this hypothesis, we measured the activities of the *b1500-ydeO*, *gadE*, and *gadB* promoters in the *phoP* mutant background. The activities of these promoters rose to higher levels in a *phoP* background (Fig. 6a, ii–iv), consistent with negative feedback of PhoP on the *b1500-ydeO* promoter in addition to the novel YdeO loop identified above. By contrast, no change in the activity of the *evgAS* promoter was seen, as predicted as this lies upstream of *b1500-ydeO* in the regulatory hierarchy (Fig. 6a, i). We were able to identify a consensus PhoP box-like sequence[49] in the *b1500-ydeO* promoter, suggesting that the regulation at this level was direct (Fig. 6b). To test this, we created three different mutants in the sequence with the best match to the PhoP box and analysed acid-induced expression from all of them. In all cases, expression was raised compared to that seen in the wild-type background, although not to the level seen in the *phoP* mutant, consistent with there being a direct interaction of PhoP at this site, which is weakened in the three mutants (Fig. S1).

YdeO and PhoP negatively feedback to the *b1500-ydeO* and *ydeP* promoters causing a transient surge in transcriptional activation

To investigate the relative contribution of the YdeO and PhoP regulators in controlling the induction surge at the *b1500-ydeO* promoter, we constructed a *ydeO phoP* double mutant and analysed *b1500-ydeO* promoter activity in this background. As shown in Fig. 6c, YdeO and PhoP can repress this promoter separately, since the promoter activity is higher in the double mutant than in either single mutant. However, the complex interlocking nature of the circuit means that it is not simple to deduce whether there is any cooperativity in their interactions from the current results (see Fig. 6e). The promoter of *ydeP*, which lies immediately upstream of *b1500-ydeO*, may also contribute to transcription of *b1500* and *ydeO*.[18] Interestingly, a transcriptional fusion of this promoter was found to share the regulatory inputs of the *b1500-ydeO* promoter, and we also identified a potential PhoP binding site in this promoter region (Fig. S2; see also Fig. 7 below). The results show that the kinetics of promoter activation within the *ydeP–ydeO* region is influenced by two levels of feedback, which are at least partially independent. It has been previously shown that incorporating negative feedback into a synthetic circuit introduces a response acceleration

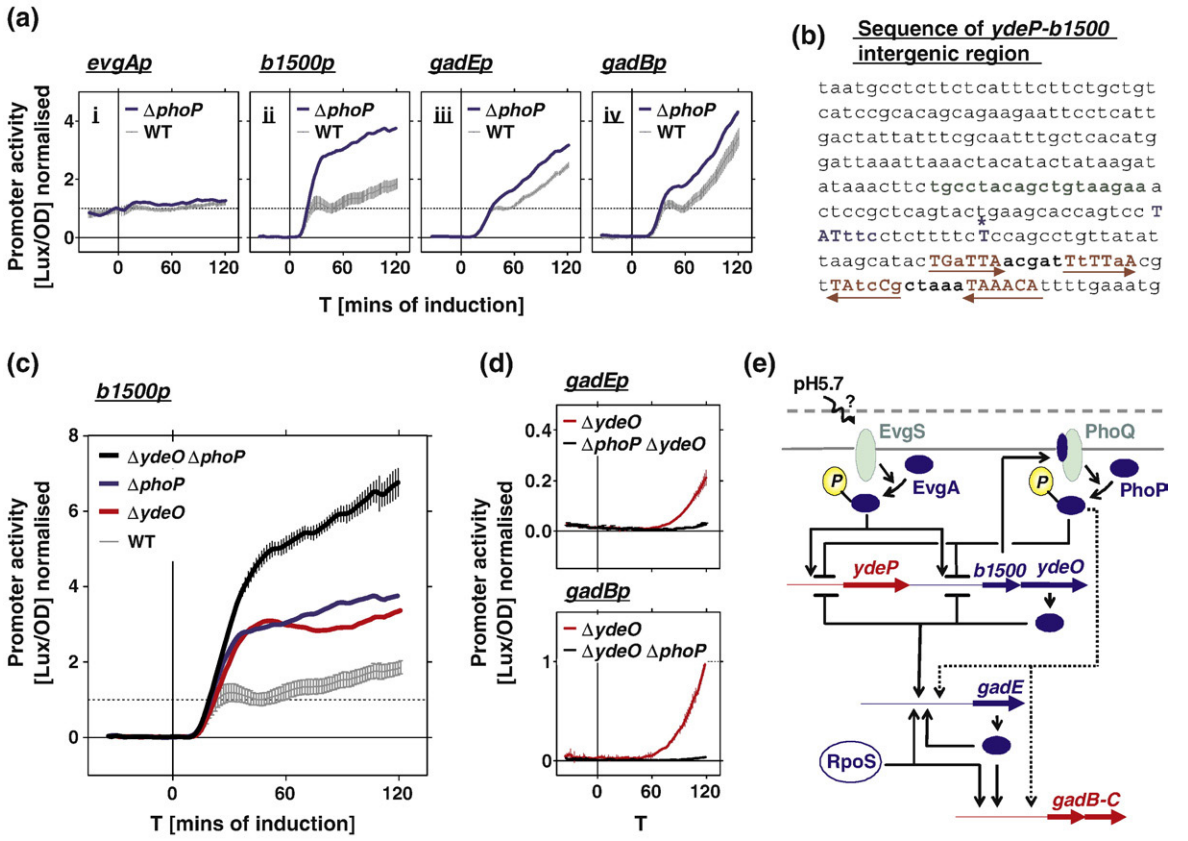

Fig. 6. Both PhoP and YdeO contribute to negative feedback at the *b1500* promoter, generating surge-like kinetics. (a) Response dynamics to a shift from pH 7 to pH 5.7 of the *evgAS* (i), *b1500-ydeO* (ii), *gadE* (iii), and *gadBC* (iv) promoters in a *phoP* mutant derivative of *E. coli* MG1655 (Table 2). Data are presented as in Fig. 3, with experiments in Δ*phoP* repeated between 2 and 5 times. (b) The sequence of the *ydeP-b1500* intergenic region. Blue boldface letters mark a major transcript start site (base "T" below asterisk) and inferred −10 sequence, with consensus bases capitalised.[26] Green boldface letters mark the EvgA consensus site.[18] Red boldface letters mark putative PhoP binding site based on the consensus (T/G) GTTTA–5n–(T/G)GTTTA[49] (red arrows under letters mark direct repeat). (c) Response dynamics to a shift from pH 7 to pH 5.7 of the *b1500-ydeO* promoter (pLUX*b1500p*) in a *phoP ydeO* double-mutant derivative of *E. coli* MG1655 (black line; Table 1) compared to single-mutant derivatives and wild type. The experiment was repeated 5 times in each background. (d) As (c) for *gadE* (top panel) and *gadB* (bottom panel) promoters. Experiments were repeated twice. (e) Cartoon model of proposed regulatory interactions occurring within the EvgAS-dependent transcriptional circuit following from a shift of culture pH from 7 to 5.7. See Fig. 1a for details. Black broken arrows represent regulation occurring when the system is assayed in a *ydeO* mutant.

relative to a similar circuit lacking the feedback, if the normalised steady-state outputs are compared.[50] A similar argument can be made in the present context, since there is a considerably higher output of the *b1500-ydeO* promoter in the absence of feedback. Thus, without auto-regulation, a weaker promoter would be required to achieve an equivalent absolute steady-state output, and it would do so at a considerable delay (data not shown; see also Ref. 50). Thus, the feedback may have evolved to ensure a fast and efficient increase in the levels of YdeO and B1500, which reside at the top of the regulatory hierarchy. It is of note that approximately 50% of transcriptional repressors auto-regulate their production in *E. coli*.[50] One such carefully studied example occurs in the SOS response in *E. coli*, where auto-regulation of the master regulator LexA has been associated with buffering the system output against fluctuations in the input signals.[35] The non-quantifiable output of the *b1500-ydeO* promoter in the absence of induction, regardless of feedback status, makes this issue hard to assess in the present context. However, we did find that removal of feedback control by mutation of *ydeO* and *phoP* did not have a strong effect on the sensitivity of the promoter to different induction pH values (data not shown).

The negative effects of PhoP at the *b1500-ydeO* promoter might be responsible for an increase in activity at the *gadE* and *gadB* promoters in a *phoP* background, although we have not directly tested this hypothesis. We noted that a putative PhoP binding site has also been identified in the *gadE* promoter, although this was proposed as an activating site, not repression as here.[30] In order to shed light on whether PhoP could regulate the *gadE* and *gadB* promoters independently of YdeO, we measured the activities of the *gadE* and *gadB* promoters in the *ydeO phoP* double-mutant background and compared the activities obtained to

those in a *ydeO* single mutant, where *gadE* and *gadB* promoter activity was induced after a delay in an EvgA- and RpoS-dependent fashion (Fig. 3a, vii–viii; Fig. 3c). Surprisingly, we found that PhoP was also required for activation of *gadE* and *gadB* promoters in the absence of YdeO (Fig. 6d). Thus, in the absence of YdeO, at least two additional transcription factors, namely, RpoS and PhoP, must be present for the delayed EvgAS-dependent activation of the *gadE* and *gadB* promoters.

The result could imply that PhoP has different regulatory functions at different times following induction at pH 5.7. However, we found that in the absence of YdeO, PhoP-dependent *mgtA* promoter activity increased, suggesting an increase in the levels of active PhoP compared to levels in the wild

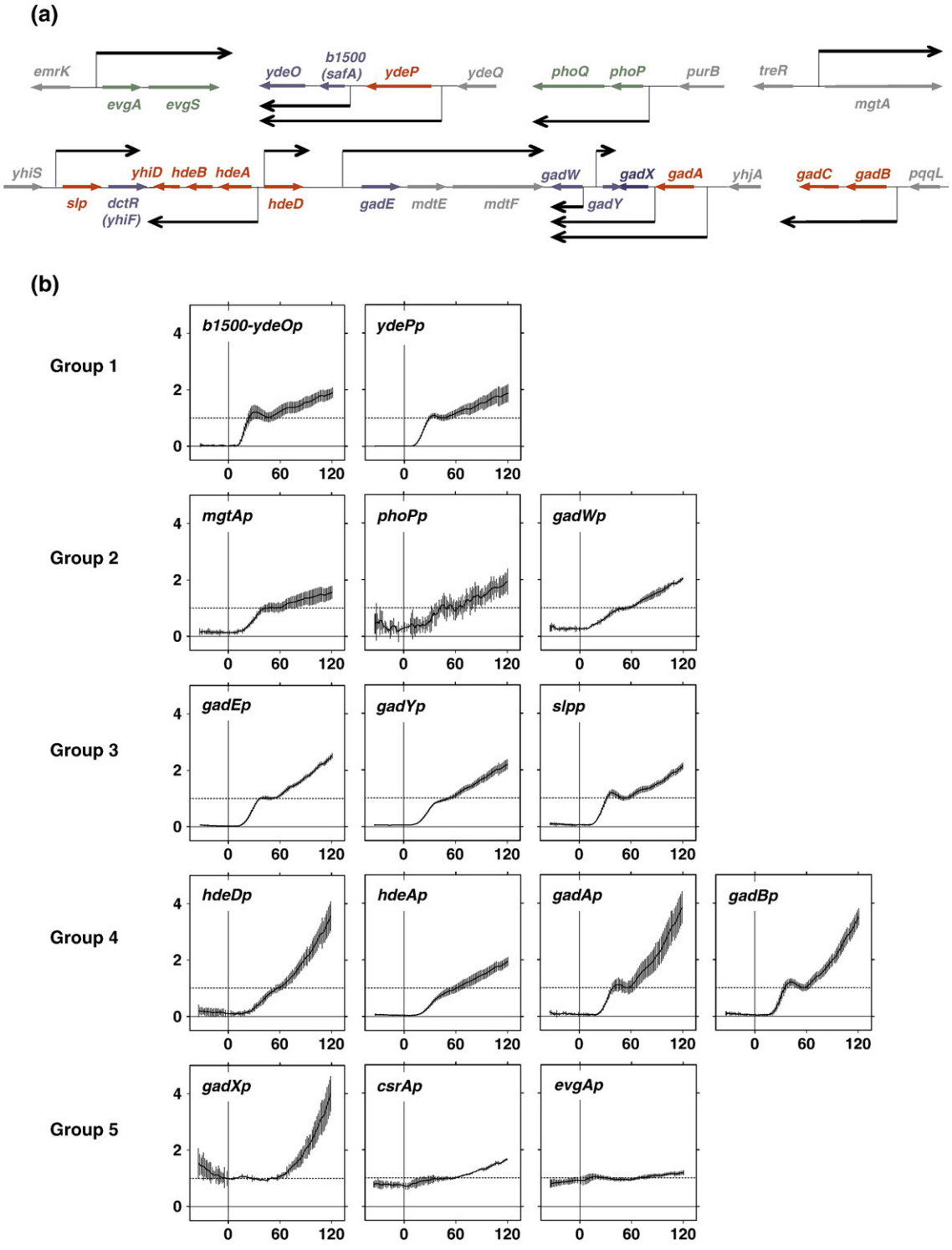

Fig. 7 (*legend on next page*)

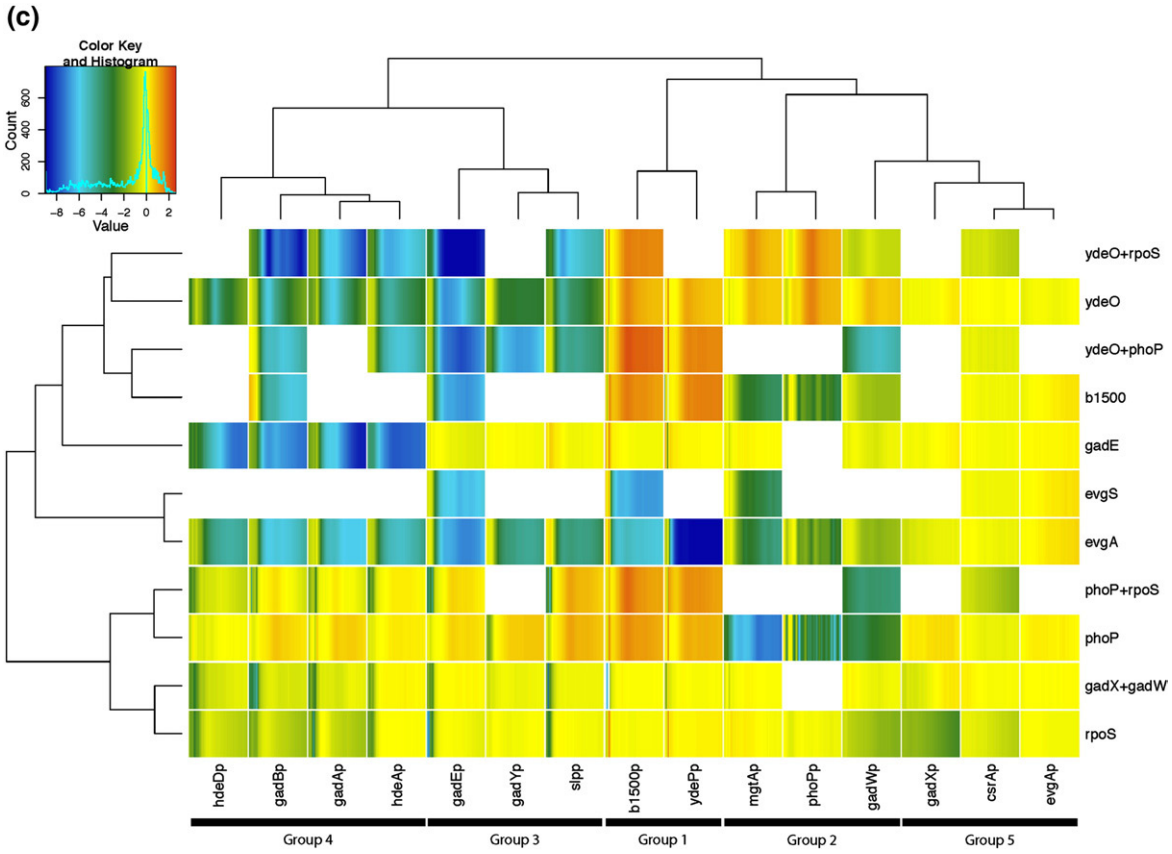

Fig. 7. Global effects on promoter dynamics of regulatory interactions studied in this article. (a) Major transcripts driven by promoters analysed in (b) and (c) are denoted by thick horizontal black arrows. Red horizontal and blue horizontal arrows are genes encoding AR effector and regulatory proteins, respectively. (b) Normalised response dynamics to a shift from pH 7 to pH 5.7 of the promoter regions indicated in (a) in wild-type MG1655 assayed using the relevant Lux promoter fusion constructs (Table 2). For cross-comparison of different promoters, data were normalised according to criteria in the legend to Fig. 2. Where no induction was observed, activity at T60 was made equal to 1. Promoters are named according to the first gene in the operon they control and have been grouped according to regulatory factors required for activation during the first hour after shift to pH 5.7, as deduced from (c) below: group 1: EvgA; group 2: EvgA, B1500, and PhoP; group 3: EvgA and YdeO; group 4: EvgA, YdeO, and GadE; and group 5: no activation observed during this period. (c) Heat map of Log2 fold differences over time between wild-type background and a range of relevant regulator mutant backgrounds (rows) of promoters assayed in (b) (columns). Activity in a mutant background that is higher than that seen in wild type is indicated by red colouration (the regulator exerts net negative influence on the promoter during low pH induction), whereas green to blue colouration indicates weakly and strongly less activity than in wild-type background, respectively (the regulator exerts weak or strong net positive influence on the promoter). Cluster analysis was done as described in Materials and Methods, and promoter groups as shown in (b) are indicated below the promoter names.

type (Fig. 5b, iv). Thus, the two situations are not directly comparable, and it is far from clear that PhoP is involved in activation of the *gadE* and *gadB* promoters in the presence of YdeO, as is the case for RpoS.

In summary, the results show that within this regulatory circuit, the dominant direction of regulation by PhoP depends on the genetic background. In a wild-type cell, the presence of PhoP results in a lower level of promoter activation produced by an early EvgAS-induced surge, most likely through a direct repression at the *b1500-ydeO* promoter. In the absence of YdeO, however, the presence of PhoP contributes, along with RpoS and GadE, to delayed and gradual induction in *gadE* and *gadB* promoter activity. This latter condition could effectively mimic the PhoPQ-mediated activation of the *gadE* and *gadB* promoter that has been observed under low Mg^{2+} inducing conditions.[30] A summary of all of the above findings is presented in Fig. 6e.

Global analysis of transcriptional kinetics within the EvgA-dependent low pH response network

Finally, we assessed the way in which the dynamic functions of this regulatory network influence the activities of additional promoters within the AFI that control the production of other AR effectors and additional regulatory proteins that could affect other aspects of cellular physiology. All the promoters analysed are shown in Fig. 7a. We

analysed both the dynamic behaviour and contributing regulatory interactions at these promoters to see whether the novel interactions described above at the *b1500*, *gadE*, *mgtA*, and *gadB* promoters had more widespread effects. Firstly, promoter dynamics were assessed in a wild-type MG1655 background prior to and following shift from pH 7 to 5.7 (Fig. 7b). The different promoter–Lux fusions were then analysed under the same conditions in a panel of relevant regulator mutant backgrounds, and the data from this analysis were condensed into a heat map (Fig. 7c). We included in the mutant backgrounds analysed a double mutant of the homologous GadX and GadW regulators, since these reside within the AFI, and, as shown below, the promoters of these genes were subject to low pH induction within the EvgAS-dependent regulatory network. This analysis enabled us to group the promoters according to which of the regulators we examined was required for their activation in the first hour after acid induction. Group 1 (*b1500*p and *ydeP*p) is activated by EvgA only; group 2 (*mgtA*p, *phoP*p and *gadW*p) is activated by EvgA, B1500, and PhoP; group 3 (*gadE*p, *gadY*p, and *slp*p) is activated by EvgA and YdeO; and group 4 (*hdeA*p, *hdeD*p, *gadA*p, and *gadB*p) is activated by EvgA, YdeO, and GadE. A final group, group 5, showed no significant changes in any of the mutant backgrounds; these were *gadX*p and *evgA*p, plus the control promoter *csrAp*.

We observed that early kinetics of most YdeO-activated promoters (groups 3 and 4) were at least partially shaped by PhoP-mediated repression. Among those indirectly YdeO activated via GadE (group 4), the *hdeD* and *hdeA* promoters appeared not to be sensitive to this feedback. It remains to be determined why these promoters should differ in this respect to the *gadB* and *gadA* promoters. In addition, early kinetics of all three PhoP-activated promoters (*mgtA*, *gadW*, and *phoP*; group 3) was shaped by YdeO-mediated repression. These findings are consistent with the feedback regulation at the *b1500-ydeO* promoter identified in this study being influential at downstream promoters, although at this stage, we cannot conclude whether these effects are direct or indirect. The data nonetheless demonstrate how both YdeO and PhoP each contribute globally to both activation and fine-tuning repression within this transcriptional network.

We also observed that later kinetics at a number of promoters was strongly shaped by the presence of RpoS during low pH induction, most strikingly at the *gadX* promoter, which lacked an early surge of activation altogether. Among *gadE*-regulated promoters (group 4), only *hdeA* was not affected in an *rpoS* mutant at low pH, suggesting that some of the effects of RpoS below the level of GadE were direct. Indeed, the *gadB* and *gadA* promoters have hallmarks of RpoS-regulated promoters.[19,51] RpoS functioned as an activator in both the presence and absence of either PhoP or YdeO, in some cases, having a quantifiable effect only in the absence of YdeO-mediated activation (e.g., at *slpp* and *hdeAp*). The data suggest that RpoS does not absolutely depend on YdeO or PhoP to activate transcription at those promoters that are sensitive to its presence. By contrast, GadE-regulated promoters lose sensitivity to RpoS in the absence of *gadE*. This observation supports a conclusion that the observed response kinetics can be considered by the presence or absence of distinct and partially independent regulatory phases, as discussed above (Figs. 3 and 4).

As had already been observed at the *gadE* and *gadB* promoters, promoters usually subject to strong YdeO-dependent induction (groups 3 and 4) displayed a delayed and gradual increase in activity in the absence of YdeO. This activity was in all cases abolished in either a *ydeO rpoS* or a *ydeO phoP* double mutant, extending the findings above concerning the *gadE* and *gadB* promoters to other co-regulated promoters, not all of which are downstream of GadE (e.g., *slp* and *gadY*). Intriguingly, the normally PhoP-activated *gadW* promoter showed a similar delayed and dampened response in a *phoP* background. Surprisingly, this was lost in a *ydeO phoP* double mutant, showing that YdeO has a positive influence on this promoter in the absence of *phoP*, despite the fact that a single *ydeO* mutation leads to greater activity at this promoter. In this particular case, the divergently transcribed *gadY* promoter is dependent on YdeO for low pH activation, and *gadY* promoter activity is increased substantially in a *phoP* mutant. In future studies, more subtle alterations within the network, such as mutation of cis-acting sites, will clarify which direct effects are important during a normal low pH response. We note from the global analysis that promoters generally behaved similarly in *b1500* and *ydeO phoP* mutant backgrounds, strongly suggesting that our B1500 mutation did indeed have strong polar effects on YdeO expression.

Interestingly, a *gadXW* double mutation did not affect kinetics at a single promoter tested despite substantial *gadW* and *gadY* (a small RNA that stabilises the *gadX* transcript[16,52]) promoter induction in the wild-type background. We found that when induced at pH 5.7, cells mutated in *gadXW* were able to increase resistance to the same level as wild-type cells when challenged at pH ranging from 2.7 to 2 (data not shown), although a low level of basal resistance in un-induced cultures did require *gadXW* (data not shown). It is not clear whether these regulators affect a different aspect of cell physiology in this condition or if the pH 5.7 induction observed here has no functional relevance.

As a control in each assay, promoter–mutant combinations were also analysed in cultures maintained at pH 7 throughout the assay period. This provided an indication of which regulators contributed to basal transcription across the network. We found, not surprisingly, that the regulatory organisation differed substantially from that observed at low pH. GadE, RpoS, GadXW, and PhoP were each involved in regulating a low level of activity at

specific promoters within the network at neutral pH (data not shown). In some cases, the control of basal regulation showed unexpected features. For example, we found that basal transcription of *gadY* relies on the presence of PhoP (data not shown), whereas the net effect of PhoP is a repression of *gadY* transcription during low pH induction (Fig. 7b). Finally, the data set confirmed that all the effects of EvgA and YdeO (and B1500) were specific to pH 5.7 inducing conditions.

Concluding remarks

In conclusion, the use of promoter–*lux* fusions has enabled the analysis of the dynamics of many genes in the AR2 network at high resolution following a shift in pH. Validation with RT-qPCR shows that the readout from *lux* (and *gfp*) fusions closely tracks actual levels of mRNA. This semi-quantitative analysis has enabled us to unpick the fine details of temporal succession in a regulatory cascade in the system, to uncover novel feedback loops in the system that control the magnitude of an initial surge of EvgAS-dependent transcription at the *b1500-ydeO* promoter, to monitor the regulatory cross-talk with the general stress response sigma factor RpoS, and to identify complex time-resolved patterns of regulation for different genes within the network. Our findings emphasise the importance of measuring the kinetics of induction in order to correctly understand coordinated cellular responses to stress. Using the Lux reporter, we acquired data at high temporal resolution and with excellent reproducibility, making the future use of such data for testing of dynamic models of the whole network eminently feasible.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and parent plasmid vectors used in this study are listed in Table 1. The full series of promoter probe plasmid constructs used is presented in Table 2. A plasmid map of the reporter vector pLUX is presented in Fig. S3.

Growth media

E. coli was grown at 37 °C, unless otherwise stated. Lysogeny broth (LB; 1% w/v tryptone, 0.5% w/v yeast extract, and 1% w/v salt; pH 7) or LB Agar (LB supplanted with 1.5% w/v bacto agar; pH 7) was used for standard cloning procedures. M9-cas [42.3 mM Na_2HPO_4, 1 mM KH_2PO_4, 8.56 mM NaCl, 18.7 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.03 mM thiamine hydrochloride, 0.4% (w/v) D-glucose, 0.2% (w/v) cas-amino acids, 100 mM Mops, and 100 mM 4-morpholineethanesulfonic acid hydrate; adjusted to pH 7 with KOH and cold filtered] was used for reporter assays, phenotype assays, and RT-qPCR assays. For extreme acid challenge in the presence of glutamate, cultures were diluted into M9-E, which is M9-cas containing 0.5 mM glutamic acid in place of cas-amino acids. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 25 μg/ml.

General molecular biology techniques

Preparation and transformation of chemically competent and electro-competent cells and P1 phage transduction were done as described previously.[53] Unless stated, chemicals were purchased from Becton Dickinson or Sigma-Aldrich. Restriction enzymes were purchased

Table 1. Bacterial strains and parent plasmids used in this study

Strain	Relevant genotype	Source and/or reference
DH5α	*E. coli* DH5α (F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ-, *thi*-1, *gyrA*96, *relA*1)	Invitrogen, Paisley, UK
MG1655	*E. coli* K-12 MG1655 (F^-, lambda$^-$, *ilvG*, *rfb*-50, *rph*-1)	Ref. 56
Δ*rpoS*	MG1655 rpoS: :FRT	MG1655 rpoS: :Km^R, Ref. 57
Δ*gadXW*	MG1655 gadX: :FRT gadW: :Cm^R	This study
Δ*gadE*	MG1655 gadE: :FRT	This study (by P1 transduction from MC4100 gadE: :Km^R, Ref. 20)
Δ*ydeO*	MG1655 ydeO: :FRT	This study (by P1 transduction from BW25113 ydeO: :Km^R, Ref. 58)
Δ*ydeO*Δ*rpoS*	MG1655 ydeO: :FRT rpoS: :Cm^R	This study
Δ*evgA*	MG1655 evgA: :FRT	This study (by P1 transduction from BW25113 evgA: :Km^R, Ref. 58)
Δ*evgS*	MG1655 evgS: :Cm^R	This study
Δ*phoP*	MG1655 phoP: :Cm^R	This study
Δ*ydeO*Δ*phoP*	MG1655 ydeO: :FRT phoP: :Cm^R	This study
Δ*rpoS*Δ*phoP*	MG1655 rpoS: :FRT phoP: :Cm^R	This study
Δ*rcsB*	MG1655 rcsB: :Cm^R	This study
Δ*gadC*	MG1655 gadC: :Cm^R	This study
Δ*b1500*	MG1655 b1500: :Cm^R	This study

Plasmid	Description	Source
pCS26-pac	*P. luminescens* LuxCDABE reporter vector; Km^R pZ derivative; pSC101 low copy replicon	Mike Surette, Ref. 32
pLUX	pCS26 derivative; STOP codons, ribosome binding site, and NcoI restriction site switch; parent plasmid for pLUX series	This study

Table 2. Promoter probe plasmids used in this study

Promoter fragment[a]			Plasmid name
Name	Start[b]	End[b]	
asrp	−568	54	pLUXasrp
b1500p	−335	119	pLUXb1500p
csrAp	−390	136	pLUXcsrAp
evgAp	−600	125	pLUXevgAp
gadAp	−288	273	pLUXgadAp
gadBp	−553	273	pLUXgadBp
gadEp	−868	94	pLUXgadEp
gadEp.1	−363	94	pLUX*gadEp*.1
gadWp	−446	141	pLUXgadWp
gadXp	−514	114	pLUXgadXp
gadYp	−326	69	pLUXgadYp
hdeAp	−323	69	pLUXhdeAp
hdeDp	−363	237	pLUXhdeDp
mgtAp	−446	98	pLUXmgtAp
phoPp	−288	162	pLUXphoPp
slpp	−314	70	pLUXslpp
ydePp	−405	63	pLUXydePp

[a] Promoter fragments are PCR-amplified regions of *E. coli MG1655* genomic DNA expected to contain a promoter.
[b] The start and end coordinates are relative to the ATG of the gene in the promoter fragment name.

from NEB or fermentas. Ligations were done using Quick Stick ligase (Bioline). Gene replacement mutagenesis was done as described previously.[54] Site-directed mutagenesis on plasmid DNA template was done using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Cambridge, UK) according to the manufacturer's instructions.

pLUX construction

Site-directed mutagenesis was done on pCS26-pac,[32] using primers to remove an NcoI site within the Km^R open reading frame without altering the amino acid sequence of the Km^R gene. Plasmids were screened by digesting with NcoI to confirm the loss of the NcoI cut site relative to the parent construct. A second round of site-directed mutagenesis resulted in the incorporation of an NcoI site (CCATGG) over the ATG of LuxC. A double-stranded oligo with the sequence GATCCTCTAGTTAGTTAGTAAGGAGTTTAC was ligated between the BamHI and NcoI sites to introduce three stop codons and a ribosome binding site downstream of the promoter cloning site. A second oligo with the sequence TCGAGCCCGGGG was ligated between the XhoI and BamHI cloning sites to generate pLUX (see Fig. S3). The manipulated regions of this plasmid were sequenced at each stage.

Reporter plasmid construction

Reporter plasmids (Table 2) were made by firstly amplifying promoter regions from MG1655 genomic DNA using High-Fidelity DNA polymerase (Phusion™, NEB). Promoter regions were defined as the full intergenic region and an additional 50 to 300 bp into each flanking open reading frame as described previously[34] (see Table 2 for coordinates of each promoter relative to ATG of the gene of interest). Promoter fragments were purified, XhoI-BamHI digested, and ligated into a BamHI-XhoI digest of pLUX. Transformants were screened by colony PCR and the insert region sequenced.

Lux reporter pH down-shift assay

A single colony of a strain freshly transformed with one of the constructs in Table 2 was grown 16–18 h in M9-cas. This stationary phase culture was used to inoculate a fresh 25-ml sterile conical flask containing 10-ml pre-warmed M9cas, to a starting OD_{600} (optical density at 600 nm) of 0.005, and grown in a shaking water bath to an OD_{600} of 0.12 (log phase). At this point, culture was transferred to a micro-titre plate (96-well micro-titre, white walled, see-through bottoms, pre-warmed to 37 °C; Fisher Scientific, Loughborough, UK) for continued growth and to start monitoring reporter activity measurement in an automated plate reader [Fluoroskan Ascent, Thermo Scientific, Basingstoke, UK]. Each well contained 280 µl culture. Adjacent wells were left between cultures bearing different reporter constructs to minimise luminescent cross-talk, and the outer wells were not used to avoid edge effects. During growth in the plate reader, culture plates were shaken and measured on an 80-s protocol [40 s shake, read luminescence (standard PMT voltage; 1 s integration per well; 40 s total per plate], which was repeated on a loop. OD measurements were taken during a short 20-s pause in this loop at 17-min intervals (1 OD read for 12 Lux reads) in an adjacent plate reader (Multiskan MS Thermo Scientific, Basingstoke, UK). Cultures were grown for approximately 35 min to acclimatise to the change in growth conditions and for pre-induction reporter analysis (first 24 reads time points). After this period, the plate was briefly removed from the reader in order to pH down-shift cultures from pH 7 to pH 5.7. This was achieved by pipetting cultures into a second pre-warmed micro-titre plate containing a small volume of hydrochloric acid (240 µl of culture pipetted into 40 µl 0.75 M HCl). Where desired, different end pH values were achieved by varying the ratio of HCl to water in this 40 µl (as done in Fig. 1a). In every experiment, the same culture was subject to a "mock induction" whereby pH was kept at pH 7 by transferring the culture into an equivalent volume of sterile distilled water. Three technical replicates of a culture at each pH (5.7 and 7) were run on a plate as a standard consistency check. Multi-channel pipettes were used throughout this procedure to facilitate the high-throughput approach. The induction procedure took 2.5 min. We checked that a culture gave similar results when grown at different positions on a plate (data not shown).

Data analysis

Lux measurement values from the plate at each time point pre- and post-induction were exported from the Fluoroskan Ascent and re-arranged in Excel to create a time course of reads for each well. The time of each measurement recorded automatically in the fluoroscan was adjusted so that time zero was precisely the point of acidification for each culture. OD measurement values were then exported from the Multiskan MS, arranged into a time course of OD reads from each well, and a blank, defined as the average OD value from the wells containing fresh M9, was subtracted from each culture OD measurement. OD values at each of the time points at which Lux was read were then interpolated, using the statistical software package R to iteratively converge upon the best-fit parameters of a logistical growth curve equation based on the raw OD reads. The Lux reads were then divided by the equivalent OD reads (Lux/OD) to approximate Lux activity per unit cell mass for each well. The Lux/OD

values of the three technical replicate wells of each culture at each pH value were averaged and plotted against time to generate a Lux activity profile. The standard deviations of the technical replicates were generally very small and are not plotted in any figure. Data plotted for a given reporter strain in the figures of this article typically represent the average of two or more full experimental repeats carried out on separate machine runs, with error bars reflecting the ±1 standard deviation of the mean.

Extreme acid challenge assay

For comparison of data, cultures were manipulated precisely as described above (Lux reporter pH down-shift assay), with the exception that strains under assay did not harbour reporter vectors. At several time points during induction (see Fig. 3b), 20 μl of culture was removed from a well and serially diluted (10-fold dilution; six times) in M9-E media at pH 2.4 and, in parallel, at pH 7. All dilutions were plated onto LB agar at time zero (immediately after serial dilution) and after 2 h of incubation at 37 °C (2 h acid challenge). After overnight growth at 37 °C, colony-forming units (CFU) were counted and CFU/ml was calculated from these counts. Percentage survival was quantified as follows: [(CFU/ml pH 2.4 after 2 h challenge)/(CFU/ml pH 7 time zero)×100]. CFUs that could be counted from the first dilution (10^{-1}) were discarded from all analysis since these were challenged in media with a slightly raised pH (due to the dilution) and were also challenged at a high density of between 10^7 and 10^8 ml^{-1} and thus could be influenced by density-dependent resistance, as described previously.[11] Therefore, the limit of detection of the assay was defined as a single CFU being present in the 10^2 dilution at the first time point. Over several experiments, this approximated to a survival of 0.04% relative to the number of cells prior to challenge.

Relative quantification of target mRNA by two-step RT-qPCR

Cultures were manipulated precisely as described above (Lux reporter pH down-shift assay), with the exception that strains under assay did not harbour reporter vectors. At several time points during induction (see Fig. 2a), RNA was extracted from 690 μl of culture, using an RNeasy Protect Bacteria Mini Kit (QIAgen, Crawley, UK). The same strain was grown in parallel in three adjacent wells to obtain this volume, and 230 μl culture from each of these wells was pooled together at each pH value. The concentration of RNA in each sample was normalised to the same value after quantification on A NanoDrop™ ND-1000 Spectrophotometer (Labtech Int., Ringmer, UK). A TURBO DNA-free™ kit (Applied Biosystems, Warrington, UK) was used to remove low-level contaminating genomic DNA in the RNA preparations. Following DNase digestion, the samples were re-quantified using the Nanodrop and each sample was diluted to 40 ng/μl using RNase-free water. RNA (400 ng) was converted to cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Random hexamers were used to prime this reaction. For consistency of conversion, the same thermal cycler was used throughout the study. Custom TaqMan Gene Expression Assays (Applied Biosystems) were used in combination with TaqMan Gene Expression Master Mix (Applied Biosystems) according to the manufacturer's recommended guidelines to detect and quantify specific amplification of target cDNA (*gadC* and *csrA*) within total cDNA samples in RT-qPCR reactions (sequences of probes and primers used can be supplied on request); each set was checked against the *E. coli* K-12 genome using BLASTn (National Center for Biotechnology Information) prior to assay. Primer and probe combinations were designed and synthesised by Applied Biosystems to ensure 100% effective PCR amplification of target cDNA during thermal cycling when used at final concentrations of 250 nm probe and 900 nM primer (user bulletin).

Data were analysed according to the $2_T^{-\Delta C'}$ method, since the same input amount of cDNA was used in all reactions.[55] At time −8 min (pre-induction reference time point), C_T values of a target RNA were averaged from repeated experiments. This calibrator C_T value was subtracted from the C_T values in each experimental repeat at subsequent time points (ΔC_T). The formula $2_T^{-\Delta C'}$ was used to convert these values to a linear scale. Finally, at each time point, the mean ±1 standard deviation of $2_T^{-\Delta C'}$ values of repeated experiments were calculated.

Cluster analysis of expression profiles

In order to visualize the expression profiles as a function of time, we first fitted a polynomial line through the observed data points using smooth splines in the statistical programming language R (reference). Inference of data from 0 to 120 min in 1-min intervals was then performed from each of the fitted lines. The resulting data were then used as an input to a principal component analysis, which calculated principal components for each promoter, summarizing the variance of each mutant. The first principal component, which, by definition, summarizes most of the variance in the data set, was then used as an input to a hierarchical clustering method using Euclidean distance. This resulted in a hierarchical clustering of the promoters. The data were then sorted to reflect the clustering. The mutants were then clustered as part of the implemented clustering algorithm of the heat map[2] function in the gplots package within R. The resulting Fig. 7c represents this heat map clustering both mutants and promoters.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.06.054

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