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.

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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 con-
trolled 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 suit-
able 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 ex-
pression 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 effecti-
vely 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 par-
ticular 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
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. The function of each AR effector depends on the precise nature of the acid stress. 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, 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. 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. 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 and Lon-mediated degradation of the central activator protein GadE. 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 and growth into stationary phase. 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. Two intermediate transcription factors, YdeO and the AFI-encoded GadE, are transcriptionally activated by the presumably phosphorylated response regulator EvgA. YdeO can also activate gadE transcription forming a coherent feed-forward loop (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. GadE directly activates its own expression while repressing production of YdeO. 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. 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. The homologous transcription factors

![Fig. 1.](image-url)
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\textsuperscript{16} but have also been reported to have a role during low pH response.\textsuperscript{20,29} In addition, GadW is under direct control of the PhoPQ two-component system,\textsuperscript{26} and it has been shown that EvgAS can cross-talk to PhoPQ via the connector protein encoded by \textit{b1500} (also known as \textit{safA}\textsuperscript{26}), which resides directly upstream of and in the same operon as YdeO.\textsuperscript{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 \textit{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,\textsuperscript{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 \textit{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\textsuperscript{34,35} to measure low pH response dynamics of a set AR promoters fused to the \textit{Photobacterium 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 (\textit{b1500-ydeOp}, \textit{gadEp} and \textit{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 \textit{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.\textsuperscript{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 \textit{pKs} values of histidine 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 \textit{in vivo}**

Bacterial Lux fusions are widely used to monitor transcriptional kinetics,\textsuperscript{3,6,39} yet details of the turnover of reporter product (enzymatic light production) \textit{in vivo} are not yet well understood.\textsuperscript{30,41} 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 (\textit{gadBC} and a non-acid responsive control, \textit{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 \textit{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 \textit{rpoS} mutant background (data not shown), which we found resulted in a change in the kinetics of \textit{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 \textit{gfp-mut2} reporter\textsuperscript{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.\textsuperscript{43,44}
We compared the activities of the $b_{1500} - ydeO$, $gadE$, 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 $b_{1500} - ydeO$ 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 $b_{1500}p$ 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,
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 GadE24 (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 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 egfA 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 overexpressed24 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 egfA 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

**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 Mg2+ 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
Mutation of b1500 is complicated by the fact that mutation of b1500 is likely to have polar effects on ydeO expression. 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. However, loss of YdeO alone caused a significant increase in mgtA activity (Fig. 6b, 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.

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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. 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
relative to a similar circuit lacking the feedback, if the normalised steady-state outputs are compared.\textsuperscript{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. \textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.
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 \textit{gadE} and \textit{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 \textit{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 \textit{gadE} and \textit{gadB} promoter activity. This latter condition could effectively mimic the PhoPQ-mediated activation of the \textit{gadE} and \textit{gadB} promoter that has been observed under low Mg\textsuperscript{2+} inducing conditions. 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 (b1500p and ydePp) is activated by EvgA only; group 2 (mgtAp, phoPp and gadWp) is activated by EvgA, B1500, and PhoP; group 3 (gadEp, gadYP, and slpp) is activated by EvgA and YdeO; and group 4 (hdeAp, hdeDp, gadAp, and gadBp) is activated by EvgA, YdeO, and GadE. A final group, group 5, showed no significant changes in any of the mutant backgrounds; these were gadXp and csgAp, 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 hdeDp and hdeAp 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 (mgtAp, gadWp, and phoPp; 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 hdeAp 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. 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) 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 temporal resolution and with excellent reproducibility, making the future use of such data for testing temporal resolution and with excellent reproducibility, making the future use of such data for testing temporal resolution and with excellent reproducibility, making the future use of such data for testing temporal resolution and with excellent reproducibility, making the future use of such data for testing temporal resolution and with excellent reproducibility, making the future use of such data for testing temporal resolution and with excellent reproducibility, making the future use of such data for testing temporal resolution and with excellent reproducibility, making the future use of such data for testing temporal resolution and with excellent reproducibility, making the future use of such data for testing temporal resolution and with excellent reproducibility, making the future use of such data for testing temporal resolution.

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 supplemented with 1.5% w/v bacto agar; pH 7) was used for standard cloning procedures. M9-cas [42.3 mM Na2HPO4, 1 mM KH2PO4, 8.56 mM NaCl, 18.7 mM NH4Cl, 2 mM MgSO4, 0.1 mM CaCl2, 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 and 10 mM 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 PI phage transduction were done as described previously. 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source and/or reference</th>
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<tbody>
<tr>
<td>DH5a</td>
<td>E. coli DH5a (F−, recA1, endA1, hsdR17(k−, m−), supE44, λ−, thi−1, gyrA96, relA1)</td>
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<td>MG1655</td>
<td>E. coli K-12 MG1655 (F−,lambda−, thi−, rph−5, rph−1)</td>
<td>Ref. 56</td>
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<td>ΔgadE</td>
<td>MG1655 gadE::FRT</td>
<td>MG1655 gadE::FRT</td>
</tr>
<tr>
<td>ΔgadXW</td>
<td>MG1655 gadX::FRT</td>
<td>MG1655 gadX::FRT</td>
</tr>
<tr>
<td>ΔydeO</td>
<td>MG1655 ydeO::FRT</td>
<td>MG1655 ydeO::FRT</td>
</tr>
<tr>
<td>ΔydeOΔgadS</td>
<td>MG1655 ydeO::FRT</td>
<td>MG1655 ydeO::FRT</td>
</tr>
<tr>
<td>ΔevgA</td>
<td>MG1655 evgA::FRT</td>
<td>MG1655 evgA::FRT</td>
</tr>
<tr>
<td>ΔgadS</td>
<td>MG1655 gadS::CmR</td>
<td>MG1655 gadS::CmR</td>
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<td>ΔphoP</td>
<td>MG1655 phoP::CmR</td>
<td>MG1655 phoP::CmR</td>
</tr>
<tr>
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<td>MG1655 ydeO::FRT phoP::CmR</td>
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<td>MG1655 evgA::FRT</td>
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<td>MG1655 gadS::CmR</td>
<td>MG1655 gadS::CmR</td>
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<tr>
<td>Δb1500</td>
<td>MG1655 b1500::CmR</td>
<td>MG1655 b1500::CmR</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>pCS26-pac</td>
<td>P. luminescens LuxCDABE reporter vector; KmR pZ derivative; pSC101 low copy replicon</td>
<td>Mike Surette, Ref. 32</td>
</tr>
<tr>
<td>pLUX</td>
<td>pCS26 derivative; STOP codons, ribosome binding site, and Ncol restriction site switch; parent plasmid for pLUX series</td>
<td>This study</td>
</tr>
</tbody>
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from NEB or fermentas. Ligation were done using Quick Stick ligase (Bioline). Gene replacement mutagenesis was done as described previously. 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, using primers to remove an Ncol site within the Km gene. Plasmids were digested by digesting with Ncol to confirm the loss of the Ncol cut site relative to the parent construct. A second round of site-directed mutagenesis resulted in the incorporation of an Ncol site (CCATGG) over the ATG of LuxC. A double-stranded oligo with the sequence GATATGTTTAGGAGATTAC was ligated between the BamHI and Ncol sites to introduce three stop codons and a ribosome binding site downstream of the promoter cloning site. A second oligo with the sequence TCGAGGGCGGG was ligated between the Xhol 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 DNA using High-Fidelity DNA polymerase (Phusion). Promoter fragments were purified, Xhol-BamHI digested, and ligated into a BamHI-Xhol digest of pLUX. Transformants were screened by colony PCR and the insert region sequenced.

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**Table 2. Promoter probe plasmids used in this study**

<table>
<thead>
<tr>
<th>Promoter fragment</th>
<th>Name</th>
<th>Start</th>
<th>End</th>
<th>Plasmid name</th>
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<tbody>
<tr>
<td>arsp</td>
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<td>54</td>
<td>pLUXarsp</td>
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<td>–335</td>
<td>119</td>
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<td>eggAp</td>
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<td>gadBp</td>
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<tr>
<td>gadEp</td>
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<tr>
<td>gadEp1.1</td>
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<td>141</td>
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<td>gadXp</td>
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<td>pLUXgadXp</td>
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<tr>
<td>gadYp</td>
<td>–326</td>
<td>69</td>
<td>pLUXgadYp</td>
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<tr>
<td>hdeDp</td>
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<td>pLUXhdeDp</td>
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<td>237</td>
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<tr>
<td>mgdAp</td>
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<td>phoPp</td>
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<td>ydePp</td>
<td>–405</td>
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<td>pLUXydePp</td>
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</tbody>
</table>

* Promoter fragments are PCR-amplified regions of the gene in the promoter fragment name.

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**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 OD600 (optical density at 600 nm) of 0.005, and grown in a shaking water bath to an OD600 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 in an automated plate reader [Flurosokan 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 used 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 Fluorosokan 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 the same strain was grown in parallel in three adjacent wells to protect bacteria Mini Kit (QiaGen, Crawley, UK). The TURBO DNA-free 1000 Spectrophotometer (Labtech Int., Ringmer, UK). A μl of culture was removed 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 of 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^−ΔC_T method, since the same input amount of cDNA was used in all reactions. 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^−ΔC_T was used to convert these values to a linear scale. Finally, at each time point, the mean ±1 standard deviation of 2^−ΔC_T 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 were 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 function in the gplots package within R. The resulting Fig. 7c represents this heat map clustering both mutants and promoters.

Acknowledgements

We are grateful to Michael Surette and Uri Alon for provision of promoter probe plasmids and to Dov Stekel, Francesco Falciani, and Steve Busby for useful discussions. N.A.B., M.D.J., and P.A. acknowledge support for studentships from the Biotechnology and Biological Sciences Research Council.

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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2. Kalir, S., McClure, J., Pabbaraju, K., Southward, C., Ronen, M., Leibler, S. et al. (2001). Ordering genes in a...


