The glutamate-dependent acid resistance system in *Escherichia coli*: essential and dual role of the His–Asp phosphorelay RcsCDB/AF

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The RcsCDB signal transduction system is an atypical His–Asp phosphorelay. Notably, the response regulator RcsB can be activated either by phosphorylation through the RcsCD pathway or by an accessory cofactor RcsA. Although conserved in *Enterobacteriaceae*, the role of this system in adaptation to environmental stress conditions is largely unknown. This study reveals that the response regulator RcsB is essential to glutamate-dependent acid resistance, a condition pertinent to the lifestyle of *Escherichia coli*. The requirement for RcsB is independent of its activation by either the RcsCD or the RcsA pathway. The basal activity of RcsB appears to be necessary and sufficient for acid resistance. The sensitivity of the *rscB* strain to low pH is correlated to a strong reduction of the expression of the glutamate decarboxylase genes, *gadA* and *gadB*, during the stationary phase of growth. This effect on *gadA/B* expression is not mediated by the general stress sigma factor RpoS, but does require a functional *gadE* allele and the previously identified GadE box. Therefore activation of *gadAB* expression and acid resistance absolutely requires both GadE and RcsB. In contrast, an increase in RcsB activity through the activation of the RcsCD phosphorelay or the RcsA pathway or through overproduction of the protein leads to general repression of the expression of the *gad* genes and a corresponding reduction in acid resistance.

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INTRODUCTION

The RcsCDB system belongs to the minority of complex His–Asp phosphorelays that use more than one phosphorylation step to transduce signals (Zhang & Shi, 2005). Following an intramolecular phosphorylation step, the sensor RcsC transfers the phosphoryl group to its cognate response regulator RcsB, via a histidine-containing phosphotransmitter (Hpt) domain protein, RcsD/YojN (Takeda *et al.*, 2001). The Rcs system is activated by alterations of the envelope (for reviews see Majdalani & Gottesman, 2005; Francez-Charlot *et al.*, 2005a). Most of the signalling to RcsC is mediated by the outer membrane lipoprotein RcsF with a few exceptions such as the overproduction of the membrane-associated chaperone-like protein DjlA (Castani-Cornet *et al.*, 2006). RcsB can be activated independently of the RcsFCD phosphorelay by interacting with an accessory cofactor, RcsA. This activation pathway allows the regulation of a subset of RcsB targets (see Majdalani & Gottesman, 2005; Francez-Charlot *et al.*, 2005a).

Transcriptome analyses indicated that up to 2.5 % of the *Escherichia coli* genome might be regulated by the Rcs system (Ferrires & Clarke, 2003; Hagiwara *et al.*, 2003; our unpublished results). Approximately half of the putative targets have no defined function. The vast majority of the remaining targets are involved in envelope composition or trafficking. The system is conserved in members of the *Enterobacteriaceae*, including animal and plant pathogens (see Huang *et al.*, 2006). It has been shown to be involved in pathogenesis (Dominguez-Bernal *et al.*, 2004; Mouslim *et al.*, 2004; Garcia-Calderon *et al.*, 2005; Detweiler *et al.*, 2003; Bereswill & Geider, 1997; Tobe *et al.*, 2005) and in the development of biofilms (Ferrires & Clarke, 2003). Although reported to be essential for recovery from chlorpromazine-induced stress (Conter *et al.*, 2002), no role of the Rcs system in adaptation to natural environmental stress has been described yet.

E. coli, while preferring neutral pH for growth, is able to resist the extreme acidic conditions that it might encounter, for instance, while passing through the stomach. Three acid resistance (AR) systems have been identified in this organism: the AR1 system, which relies on the general stress sigma factor RpoS, the arginine-dependent acid resistance system (AR3) and the glutamate-dependent

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Abbreviation: GABA, γ -aminobutyric acid.

acid resistance (AR2) system, the latter being the most effective. Highlighting the importance of the AR2 system is the plethora of regulators involved in its regulation, the relative importance of each regulator depending on environmental pH and growth phase. These regulators include: two phosphorelays, EvgAS (Masuda & Church, 2002) and TorRS (Bordi *et al.*, 2003), the stationary phase σ^S -factor (De Biase *et al.*, 1999), the global regulators Crp (Castanié-Cornet & Foster, 2001) and H-NS (Hommais *et al.*, 2001), three AraC-like regulators, GadW (Ma *et al.*, 2002), GadX (Shin *et al.*, 2001) and YdeO (Masuda & Church, 2003), and the Era-like GTPase TrmE (Gong *et al.*, 2004). Central to the AR2 system is the activity of the LuxR-like transcription regulator GadE, as most of the regulators listed target *gadE* expression. The purpose of this complex regulatory network is the regulation of the glutamate decarboxylase genes *gadA* and *gadB* and the glutamate: γ -aminobutyric acid (GABA) antiporter gene *gadC*. The combined activity of these three enzymes increases the intracellular pH to levels compatible with *E. coli* survival (see Foster, 2004 for a review).

This work shows that the basal activity of the response regulator RcsB is essential for glutamate-dependent acid resistance and reveals an intriguing relationship between the activities of GadE and RcsB. In contrast, stimulation of RcsB activity through either the RcsFCD phosphorelay pathway or the RcsA pathway lowers the acid resistance. The opposing effects of RcsB on acid resistance are both mediated through the regulation of the *gadA* and *gadBC* operons.

METHODS

Bacterial strains, plasmids and bacteriophages. Strains, plasmids and bacteriophages used in this study are described in Table 1. The λ *gad* transcriptional fusions were constructed in the cloning vector pRS550, rescued in the phage λ RS45 and installed as a single copy on the chromosome by lysogenization to create strains SK1593 (*gadW-lacZ*), SK2306 (*gadA_{wt}-lacZ*) and SK2321 (*gadA_M-lacZ*) (Simons *et al.*, 1987). Cloning fragments were generated by PCR and cloned in the *Bam*HI and *Eco*RI sites of pRS550. The *gadW* moiety in the λ *gadW* construct extended from -400 to +30 relative to the ATG of the *gadW* gene. Limits for λ *gadA_{wt/M}* are shown in Fig. 3(a). The *gadA*, *gadB*, *gadX* and *gadE* fusions were described by Castanié-Cornet & Foster (2001) and Ma *et al.* (2002, 2003); the limits for *gadA* and *gadB* are shown in Fig. 3(a). The Δ *rscA::cat*, Δ *rscC::cat* and Δ *rscF::cat* mutations were constructed as described by Datsenko & Wanner (2000). The whole ORFs were deleted and replaced by the chloramphenicol resistance (*cat*) gene. The constructs were checked by PCR and P1 transduced into the appropriate strains.

Glutamate-dependent acid resistance assay. This assay was described by Castanié-Cornet *et al.* (1999). Cells were grown overnight at 37 °C in Luria-Bertani rich medium supplemented with 0.4 % glucose (LBG), then diluted 1000-fold in pre-warmed, pH 2.5, M9 medium salts containing 0.4 % glucose, supplemented or not with 1.5 mM glutamate. After 0, 2 and 4 h, serial dilutions of the cultures were spotted on regular Luria-Bertani broth (LB) plates. After overnight growth at 37 °C, survival rates were determined as

the ratio between c.f.u. ml⁻¹ at 2 or 4 h and c.f.u. ml⁻¹ at 0 h. For each assay, control experiments (acid sensitive) were performed by challenging at pH 2.5 without glutamate (data not shown).

β -Galactosidase assay. Cells were grown in LBG at 37 °C. Unless specified, overnight cultures were diluted 1000-fold and grown for 5–10 generations, prior to addition of 500 μ M IPTG. The cultures were sampled at different intervals for assay of β -galactosidase activities (Miller, 1992). For the experiment presented in Table 3, overnight cultures were diluted 1:20 000 in fresh LBG and cultured at 37 °C with agitation until OD₆₀₀ reached 0.3; they were then diluted 1:20 in LBG with or without 0.5 mM IPTG and after 2 h (OD₆₀₀ ~ 0.5), β -galactosidase activities were measured.

RESULTS

The response regulator RcsB is essential for glutamate-dependent acid resistance

A possible relation between the Rcs phosphorelay and acid resistance emerged from the observation that *rscA*, the gene encoding the accessory cofactor of the response regulator RcsB, was directly regulated by GadE (Hommais *et al.*, 2004). As GadE is central to glutamate-dependent acid resistance, we investigated a possible role of the Rcs system in resistance to low pH. As shown in Fig. 1(a), the survival rate of an *rscB* mutant was three orders of magnitude lower than that of the wild-type strain after 2 h of acid challenge and no surviving cells (<0.001 %) were detected after 4 h, indicating that a functional *rscB* allele is required for glutamate-dependent acid resistance. The implication of RcsB in the AR2 system is specific since no effect of the *rscB* mutation was observed in acid resistance assays involving the AR1 and AR3 systems (data not shown).

The contribution of RcsA to acid resistance was then tested using a strain in which *rscA* was deleted. No effect of this mutation was observed (Fig. 1a), indicating that the requirement for RcsB in acid resistance is not mediated by RcsA and that under these experimental conditions, the regulation of *rscA* by GadE is not pertinent. We also tested the contribution of the RcsB phosphorylation pathway (RcsFCD). As shown in Fig. 1(a), the absence of any of the genes of the phosphorelay did not affect acid resistance. Therefore, either a third RcsB activation pathway is involved or only basal RcsB activity is required for resistance to low pH.

The basal RcsB activity is required for glutamate-dependent acid resistance

To evaluate the RcsB activity under these experimental conditions, the expression of two targets of RcsB, the *cps* and *bdm* genes, was measured using *lacZ* transcriptional fusions with their promoters. Regulation of the *cps* genes by RcsB is modulated by the accessory factor RcsA (Stout *et al.*, 1991) whereas that of the *bdm* gene is not (Francez-Charlot *et al.*, 2005b). Each fusion was installed into the chromosome of the strains tested as a single copy (Simons *et al.*, 1987). The β -galactosidase activity of the fusions was monitored after overnight growth in glucose-supplemented LB broth. As

Table 1. Bacterial strains, plasmids and phages used in this study

Relevant genotype		Comment(s) or reference
Strains		
MG1655	F ⁻ λ^- <i>rph-1</i>	Bachmann (1996)
CF6343	MG1655 Δ <i>lacIZ</i> (<i>Mlu</i> I)	Parental to all SK/MPC strains
EF832	K-12 <i>lac::cat trpDC::putPA1303-Km-gadB::lacZ</i> (-203 to +788)	Castanié-Cornet & Foster (2001)
EF833	K-12 <i>lac::cat trpDC::putPA1303-Km-gadA::lacZ</i> (-165 to +788)	Ma <i>et al.</i> (2002)
EF1106	K-12 Δ <i>gadE::Kn/pGadE</i>	Ma <i>et al.</i> (2003)
EF1334	K-12 <i>lac::cat gadX-lacZ</i>	J. W. Foster*
EF1357	K-12 <i>lac::cat trpDC::putPA1303-Km-gadE-lacZ</i> (-804 to +331)	Ma <i>et al.</i> (2004)
DH339	Δ <i>rcsD::Kan</i>	Majdalani <i>et al.</i> (2005)
RH90	MC4100 <i>rpoS-359::Tn10</i>	Lange & Hengge-Aronis (1991)
SK2012	Δ <i>rcsA::cat</i>	This study
SK1898	Δ <i>rcsB::cat</i>	Francez-Charlot <i>et al.</i> (2005b)
SK1769	Δ <i>rcsC::cat</i>	This study
SK1938	Φ (<i>wza-lacZ</i>)	Davalos-Garcia <i>et al.</i> (2001)
SK1940	Φ (<i>bdm-lacZ</i>)	Francez-Charlot <i>et al.</i> (2005b)
SK1941	Δ <i>rcsB::cat</i> Φ (<i>wza-lacZ</i>)	SK1938 + P1(SK1898)
SK1943	Δ <i>rcsB::cat</i> Φ (<i>bdm-lacZ</i>)	SK1940 + P1(SK1898)
SK1593	Φ (<i>gadW-lacZ</i>)	This study
SK2306	Φ (<i>gadA_{wt}-lacZ</i>) (-71 to +1)	This study
SK2321	Φ (<i>gadA_M-lacZ</i>) (-71 to +1)	This study
MPC104	Δ <i>rcsD::Kn</i>	CF6343 + P1(DH339)
MPC173	Δ <i>rcsF::cat</i>	This study
MPC295	<i>trpDC::putPA1303-Km-gadA::lacZ</i> (-165 to +788)	CF6343 + P1(EF833)
MPC297	<i>trpDC::putPA1303-Km-gadB::lacZ</i> (-203 to +788)	CF6343 + P1(EF832)
MPC299	<i>trpDC::putPA1303-Km-gadE::lacZ</i> (-804 to +331)	CF6343 + P1(EF1357)
MPC301	<i>trpDC::putPA1303-Km-gadX-lacZ</i>	CF6343 + P1(EF1334)
MPC303	Δ <i>rcsB::cat trpDC::putPA1303-Km-gadA::lacZ</i>	SK1898 + P1(EF833)
MPC305	Δ <i>rcsB::cat trpDC::putPA1303-Km-gadB::lacZ</i>	SK1898 + P1(EF832)
MPC307	Δ <i>rcsB::cat trpDC::putPA1303-Km-gadE::lacZ</i>	SK1898 + P1(EF1357)
MPC309	Δ <i>rcsB::cat trpDC::putPA1303-Km-gadX::lacZ</i>	SK1898 + P1(EF1334)
MPC321	<i>rpoS::Tn10 trpDC::putPA1303-Km-gadA::lacZ</i>	MPC295 + P1(RH90)
MPC322	Δ <i>rcsB::cat rpoS::Tn10 trpDC::putPA1303-Km-gadA::lacZ</i>	MPC303 + P1(RH90)
MPC323	<i>rpoS::Tn10 trpDC::putPA1303-Km-gadB::lacZ</i>	MPC297 + P1(RH90)
MPC324	Δ <i>rcsB::cat rpoS::Tn10 trpDC::putPA1303-Km-gadB::lacZ</i>	MPC305 + P1(RH90)
MPC344	Δ <i>rcsB::cat gadW-lacZ</i>	SK1593 + P1(SK1898)
MPC325	Δ <i>gadE::Kn</i>	CF6343 + P1(EF1106)
MPC327	Δ <i>rcsB::cat</i> Δ <i>gadE::Kn</i>	SK1898 + P1(EF1106)
Plasmids		
pHRcSB	pFAB1, Φ (<i>PlacUV5-rcsB</i>) in p15A vector, Kn ^R Sp ^R	Carballes <i>et al.</i> (1999)
pHRcSA	Φ (<i>placUV5-rcsA</i>) in p15A vector Kn ^R Sp ^R	Davalos-Garcia <i>et al.</i> (2001)
pRS550	ColE1 derived cloning vector, Ap ^R Kn ^R	Simons <i>et al.</i> (1987)
pGadE	pQEg <i>gadE</i> (plasmid expressing <i>gadE</i> under the control of the IPTG-regulated phage T5 promoter), Ap ^R	Ma <i>et al.</i> (2003); Masuda & Church (2003)
pPSG958	pTRC99A-6His <i>Ptac-djlA</i>	Clarke <i>et al.</i> (1997)
Phages		
λ RS45	λ ^{imm21} to rescue cloned fragments from pRS550	Simons <i>et al.</i> (1987)
λ bdm1	Φ (<i>bdm-lacZ</i>) fusion in λ RS45, Kn ^R	Francez-Charlot <i>et al.</i> (2005b)
λ cps	Φ (<i>wza-lacZ</i>) fusion in λ RS45, Kn ^R	Davalos-Garcia <i>et al.</i> (2001)
λ gadA _{wt} and λ gadA _M	Φ (<i>gadA-lacZ</i>) fusion in λ RS45, Kn ^R	This study

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shown in Fig. 2a, when the Rcs phosphorelay was activated by expression of *djlA* from the pSG958 plasmid, strong induction of the expression of the *cps-lacZ* was observed and this effect was strictly dependent on having a functional *rcsB* allele. A similar induction was also observed when RcsB activity was increased through the overproduction of RcsB from the pHRcsB plasmid. In contrast, in the absence of *djlA*- or *rcsB*-expressing plasmids, the activities in the wild-type and *rcsB* backgrounds for the *cps-lacZ* fusion were low (51 and 38 units, respectively) and not significantly different ($P > 0.05$), indicating that in the wild-type strain under those growth conditions there was no significant induction of RcsB activity. The expression of the *bdm-lacZ* fusion was also stimulated by expressing either *djlA* or *rcsB* from plasmids, although to a lesser extent with *djlA* (Fig. 2a). In contrast, without these plasmids the expression of the fusion remained low, and the observation that this expression was twofold lower in the *rcsB* than in the wild-type backgrounds (20 and 40 units, respectively) has to be interpreted as the

Fig. 1. Glutamate-dependent acid resistance tests. The means $\pm$ SD of at least three independent assays are presented. Overnight cultures in LBG medium were diluted 1000-fold in M9 medium (pH 2.5) supplemented with 1.5 mM glutamate then spotted on LB plates after 0, 2 and 4 h. Percentage survival is calculated as the ratio between c.f.u. ml⁻¹ remaining after 2 h (light grey bars) or 4 h (dark grey bars) and c.f.u. ml⁻¹ at time zero. (a) Effect of *rcs* mutations on glutamate-dependent acid resistance. Strains tested were CF6343 (wild-type), SK1898 (*rcsB*), SK1769 (*rcsC*), MPC173 (*rcsF*), MPC104 (*rcsD*) and SK2012 (*rcsA*). (b) GadE and RcsB are both required for glutamate-dependant acid resistance. Overnight cultures in LBG medium with (black bars) or without (grey bars) 0.5 mM IPTG, were acid challenged. Percentage survival is calculated as the ratio between c.f.u. ml⁻¹ remaining after 2 h and c.f.u. ml⁻¹ at time zero. Strains tested and relevant genotypes are indicated below the graph. The presence in the strain of plasmids expressing either RcsB (pHRcsB) or GadE (pGadE) is indicated below the graph. (c) Activation of RcsB negatively affects glutamate-dependant acid resistance. The wild-type strain, CF6343, with or without pHRcsB, pPSG958 or pHRcsA, was cultured in LBG with (+) or without (-) 0.5 mM IPTG.

contribution of RcsB to the constitutive expression of *bdm* in the wild type strain. Altogether, these results suggest that the basal RcsB activity, i.e. the activity in uninduced growth conditions, is necessary and sufficient for development of resistance to low pH.

RcsB is required for the activation of *gadA* and *gadBC* during the stationary phase of growth

The glutamate-dependent acid resistance AR2 system relies on the glutamate decarboxylase activity encoded by either *gadA* or *gadB* and on the glutamate:GABA antiporter encoded by *gadC*. The decarboxylation of glutamate leads to the production of CO₂ and GABA, which are then expelled from the cytoplasm. The reaction, by consuming H⁺ ions, results in the elevation of the intracellular pH to a level compatible with cell metabolism (see Foster, 2004). Since the *gadA* and *gadBC* operons play a crucial role in this process, we tested the expression of chromosome-inserted *gadA*- and *gadB*-*lacZ* fusions in the *rcsB* background. Table 2 and Fig. 2(b) show that the transcription of both operons is dramatically reduced at the stationary phase of growth in the *rcsB* mutant. This effect was complemented by a plasmid expressing a functional *rcsB* allele through the leaky activity of the *plac_{UV5}* promoter (*rcsB*/pHRcsB strain in Table 2). These results indicate that the basal RcsB activity positively regulates *gadA* and *gadBC* transcription. Therefore the essential role of RcsB in acid resistance is probably the maintenance of an appropriate level of GadA and GadBC.

The data in Table 2 also show that the increase in *gadA* expression during the stationary phase of growth is still observed in the *rpoS* background: the magnitude of the effect was in the same order as that in the wild-type background (46-fold and 100-fold in early stationary phase, and 88-fold

Fig. 2. (a) Expression of *cps-lacZ* and *bdm-lacZ* fusions after overnight growth in LBG. The means $\pm$ SD of at least three independent β -galactosidase assays are presented. Strains tested for *cps* fusions were SK1938 (wild-type) and SK1941 (*rcsB*), and for *bdm* fusions SK1940 (wild-type) and SK1943 (*rcsB*). These strains did or did not contain *rcsB*- or *djlA*-expressing plasmids (pHRcsB or pPSG958 respectively) as indicated below the graph. (b) Effect of *rcsB* mutation on *gadA*, *gadB*, *gadE*, *gadX* and *gadW* transcription. β -Galactosidase activities were measured after overnight growth in LBG. The means $\pm$ SD of at least three independent assays are presented. In both panels, wild-type background is indicated by open bars and *rcsB* background by grey bars.

and 172-fold in late stationary phase, respectively). Similar results were obtained with the *gadB* fusion (Table 2). Therefore the dramatic activation of *gadAB* expression is only modestly mediated by RpoS. We note that this increase during the transition from exponential to late stationary phase was significantly reduced in the *rpoS rcsB* double mutant (sixfold) but not totally abolished. This result indicates that, under these conditions, other factors contribute to the regulation of the *gad* genes.

RcsB-dependent regulation of *gadABC* is not mediated by the regulation of *gadEWX* genes

The expression of *gadA* and *gadBC* is regulated by a complex network involving notably the GadE, GadW and GadX regulators (Ma *et al.*, 2003; see Foster, 2004). It was conceivable that the RcsB effect was mediated by one of these regulators. In order to test this possibility, the activity

of chromosomal *lacZ* fusions to the promoters of *gadE*, *gadW* and *gadX* was monitored. As shown in Fig. 2(b), whereas the activation of *gadA* and *gadB* is lost in the *rcsB* background, no effect of this mutation on the expression of *gadE*- and *gadX-lacZ* was seen. A 25 % reduction of *gadW-lacZ* fusion expression was observed in the *rcsB* strain, leaving the possibility that RcsB might activate *gadAB* expression through *gadW*. This is however unlikely, as GadW was reported to be a negative regulator of *gadAB* expression (Ma *et al.*, 2002, 2003; Tucker *et al.*, 2003). In addition, a *gadW* mutant was reported to resist acid as well as a wild-type strain (Tucker *et al.*, 2003). Altogether these results indicate that the regulation of *gadABC* by RcsB is not mediated through the modulation of the transcription of *gadE*, *gadX* or *gadW*.

Both RcsB and GadE are required for acid resistance

Although GadE, GadX and GadW are together involved in glutamate-dependent acid resistance, the requirement for GadX or GadW can be overcome by overproduction of GadE, whereas the opposite is not true. In addition, the acid induction of *gadABC* expression is primarily due to the acid induction of *gadE* transcription (Ma *et al.*, 2003). Therefore GadE appears to be the key player in acid resistance. Strengthening the central role of GadE, Fig. 1(b) shows that the low survival rate of the *gadE* mutant cannot be overcome by the production of RcsB from plasmid pHRcsB. More importantly, the acid sensitivity of the *rcsB* mutant cannot be suppressed by overproduction of GadE from a plasmid-borne gene. Therefore both GadE and RcsB are absolutely required for glutamate-dependent acid resistance. As expected, the *rcsB gadE* double mutant was sensitive to acid. This sensitivity can only be suppressed when both regulators are overproduced (Fig. 1b).

Both RcsB and GadE are required for activation of *gadABC* expression

The expression of *gadE* from pGadE strongly stimulates the transcription of *gadA* and *gadBC* operons in the wild-type background, as expected from previous reports (Table 3a, MPC295 and 297). The GadE-dependent regulation requires a 20 bp GadE box located upstream of the *gadA/B* promoters (Castanié-Cornet & Foster, 2001, Fig. 3a). As expected, *gadA*- and *gadB-lacZ* fusions lacking sequences upstream of the GadE box were still regulated by GadE (Table 3b, SK2306), whereas the same constructs in which a single substitution was introduced into the GadE box displayed dramatically lower activity and no longer responded to GadE overproduction (*gadA-lacZ* fusion in Table 3b, SK2321). Interestingly, expression of the wild-type *gadA/B-lacZ* fusions was also lower in the *rcsB* background and was also unaffected by the overproduction of GadE (Table 3a, MPC303 and 305). Therefore activation of *gadA/BC* expression by GadE requires both the GadE box and a functional *rcsB* allele. This result agrees with the

Fig. 3. *gadA* and *gadB* regulatory region. (a) Sequences of the *gadAB* regulatory regions and limits of the *gad-lacZ* fusions are shown. The single substitution from G to C in the 20 nucleotide GadE box is indicated. (b) Alignment of the sequence of the GadE box to that of the RcsAB box of the *rcsA* regulatory region (Wehland & Bernhard, 2000; our unpublished results). Conserved bases are boxed.

absence of suppression of the sensitivity of the *rcsB* mutant to acid by the overproduction of GadE.

Activation of the RcsCDB phosphorelay represses *gadABC* genes and lowers acid resistance

If RcsB positively contributes to the basal level of *gadABC*, one might expect that increased activity of RcsB would stimulate the expression of those genes. However, unexpectedly,

expression of *gadA/B-lacZ* fusions in strains overproducing RcsB from plasmid pHRcsB, after addition of IPTG (0.5 mM), was dramatically lower than in the wild-type control sample, 11.2-fold and 11.6-fold for *gadA-lacZ* and *gadB-lacZ* fusions, respectively (Table 2). In addition, expression of *gadE*, *gadW* and *gadX* fusions was also negatively affected by the overproduction of RcsB, although to a minor extent (data not shown). Therefore the activation of RcsB has a general negative effect on the expression of the *gad* genes. Accordingly, under those conditions, the

Table 2. Effect of *rcsB* and *rpoS* on *gadA* and *gadBC* transcription

gadA and *gadB* transcriptional fusions in wild-type (WT) (MPC295 and MPC297), in *rcsB* (MPC303 and MPC305), in *rpoS* (MPC321 and MPC323) and in *rpoS rcsB* background (MPC322 and MPC324) were used (see Table 1). Cultures were grown in LBG medium. Exponential phase, $OD_{600} \sim 0.5$; early stationary phase, $OD_{600} \sim 2.5$; late stationary phase, after overnight growth. Experiments were done in triplicate; the means expressed in Miller units are presented. ND, Not determined.

Growth phase	<i>gadA-lacZ</i> fusion					<i>gadB-lacZ</i> fusion				
	wt	<i>rcsB</i>	<i>rcsB</i> + pHRcsB	<i>rpoS</i>	<i>rpoS rcsB</i>	wt	<i>rcsB</i>	<i>rcsB</i> + pHRcsB	<i>rpoS</i>	<i>rpoS rcsB</i>
Exponential	47	17	39*	43	14	24	25	28*	49	23
Early stationary	4657	88	2247*	1969	49	1777	139	1637*	1031	68
Late stationary	8187	172	6950*	3806	87	4119	270	3394*	2112	105
Late stationary 0.5 mM IPTG	ND	ND	730†	ND	ND	ND	ND	354†	ND	ND

*Expression of *rcsB* from the leaky activity of *lacUV5p* promoter in pHRcsB.

†Expression of *rcsB* after induction of the *lacUV5p* promoter in pHRcsB with 0.5 mM IPTG.

Table 3. RcsB (a) and the GadE box (b) are required for the regulation of *gadAB* by GadE

See Table 1 for description of the strains. Experiments were done in triplicate (a) or duplicate (b) and the means expressed in Miller units are presented with standard deviations. In (b) a plasmid expressing *gadE* from the IPTG-regulated phage T5 promoter was used.

(a)	MPC295 <i>gadA-lacZ</i> (-165 to +788) + pGad	MPC303 <i>gadA-lacZ</i> (-165 to +788) $\Delta rcsB::Cm$ + pGadE	MPC297 <i>gadB-lacZ</i> (-203 to +788) + pGadE	MPC305 <i>gadB-lacZ</i> (-203 to +788) $\Delta rcsB::Cm$ + pGadE
-IPTG	74 $\pm$ 1	27 $\pm$ 1	87 $\pm$ 1	33 $\pm$ 1
+IPTG	7999 $\pm$ 305	12 $\pm$ 0	2845 $\pm$ 32	33 $\pm$ 1
(b)	SK2306 $\lambda gadA_{wt}-lacZ$ (-71 to +1) + pGadE		SK2321 $\lambda gadA_M-lacZ$ (-71 to +1) + pGadE	
-IPTG	71 $\pm$ 14		37 $\pm$ 3	
+IPTG	526 $\pm$ 25		30 $\pm$ 4	

glutamate-dependent acid resistance of a wild-type strain decreased by 1.5 log following 4 hours of acid challenge (Fig. 1c, dark grey bars). Because this effect could be misleading due to the overproduction of RcsB beyond a physiological range, the same test was performed by activating the RcsCDB phosphorelay through the expression of *djlA*: an even stronger reduction of acid resistance was observed (Fig. 1c, dark grey bars). We therefore conclude that the activation of RcsB has a negative effect on acid resistance. Overproduction of the RcsB accessory cofactor RcsA also represses the expression of *gadA/B*, although modestly (approximately twofold; data not shown). Accordingly, under those conditions, the resistance of the strain to acid was slightly lower than that of the wild-type (50 % versus 100 %, Fig. 1c).

DISCUSSION

Although conserved in enterobacterial species and involved in the regulation of potentially 2.5 % of the genome of *E. coli*, clues as to the role of the Rcs system in adaptation to the environment are limited. To elucidate these roles we took advantage of known information on the regulation of the Rcs system. The report that RcsB accessory factor gene *rcsA* was positively regulated by GadE, a key transcription regulator for the glutamate-dependent acid resistance AR2 system (Hommais *et al.*, 2004), led us to investigate a potential role of the Rcs system in acid resistance. This study has brought to light the opposing dual roles of the Rcs system in acid resistance: the basal activity of the response regulator RcsB (due to the unphosphorylated form of the protein, to the basal pool of phosphorylated forms, or to a combination of both) is absolutely required for the glutamate-dependent acid resistance system, whereas the activation of RcsB, either through the RcsCD phosphorelay or through RcsA, reduces the cell's potential to resist low pH. Both activities are mediated by the activation and repression of the expression of the glutamate decarboxylase isoenzyme genes *gadA/gadB* and the glutamate:GABA antiporter gene *gadC*. Enterobacteria such as *E. coli* have to cope with low pH in the gastrointestinal tract. To the best

of our knowledge, this is the first report of an essential role for the Rcs system in adaptation to a pertinent environmental condition.

The stationary phase and the acid induction of *gadA/B* expression are primarily due to the increase in *gadE* expression under both conditions. Accordingly, a *gadE* mutant is sensitive to low pH (Ma *et al.*, 2003; Weber *et al.*, 2005; this study). This work shows that the activity of GadE in acid resistance is absolutely dependent on a functional *rcsB* allele. Since the acid sensitivity of either *gadE* or *rcsB* mutants cannot be cross-suppressed by plasmids expressing either *rcsB* or *gadE*, it is likely that GadE and RcsB act at the same step in the pathway leading to acid resistance. Acid resistance is correlated with the ability of both regulators to activate the expression of the *gadA* and *gadBC* operons during the stationary phase of growth. The activation of *gadA/B* expression by GadE requires a GadE-binding box upstream of the promoter (Castanié-Cornet & Foster, 2001; Ma *et al.*, 2003). Deletion and mutation analyses of *gadA/BC* regulatory regions did not reveal a motif that would specifically affect RcsB-dependent activation without affecting that of GadE. This result could suggest that GadE- and RcsB-activating motifs are intimately overlapping, preventing their dissection by mutations. The observation of similarities at the sequence level between the left part of the *gadA/gadB* GadE boxes and the RcsAB box in the regulatory region of *rcsA* (Fig. 3b) might support this hypothesis. Although the functional significance of this sequence similarity needs to be experimentally tested, the observation that the GadE regulon (Hommais *et al.*, 2004) and the RcsB regulon do not significantly overlap (Ferrières & Clarke, 2003; Hagiwara *et al.*, 2003) nevertheless indicates that GadE does not regulate all RcsB-dependent promoters and vice versa.

This study shows that only the basal RcsB activity is essential to acid resistance. The form of the protein that contributes to this basal activity is an open issue. In *Salmonella enterica* serovar Typhimurium, the response regulator OmpR, independently of its cognate kinase EnvZ, is required for

the stationary-phase acid tolerance response. However, OmpR still needs to be activated, probably by receiving a phosphoryl group from acetyl phosphate (Bang *et al.*, 2000). Evidence exists that RcsB can also be activated through phosphorylation by acetyl phosphate, notably when the phosphatase activity of the sensor RcsC is missing (Fredericks *et al.*, 2006; Majdalani *et al.*, 2005; our unpublished data). However, in the *ackA pta* strain, where there is no production of acetyl phosphate, or in the *rscC ack pta* triple mutant, acid resistance is maintained (data not shown). If the basal RcsB activity depends on the phosphorylated form of the protein, then a third phosphorylation pathway (parallel to those of RcsC and acetyl phosphate) must exist as already suggested by Majdalani *et al.* (2005) and Fredericks *et al.* (2006). Alternatively, the unphosphorylated form of RcsB might have a regulatory activity on its own.

The increased activity of RcsB, either by the activation of the RcsCD phosphorelay or by the overproduction of the accessory cofactor RcsA, diminishes resistance to acid. This effect is through a general negative regulation of the expression of the *gad* genes. While still an open issue, the role of this negative regulation might be to prevent costly runaway expression of the *gad* genes, or to shut off the response, once the acid stress is over. This could be crucial for the bacteria, for instance, once the gastric acid barrier is passed, in order to efficiently settle into the less acidic intestinal environment. Any of these hypotheses might then explain the reported activation of *rscA* expression by GadE that leads to the activation of RcsB activity, and consequently, via a feedback loop, to the repression of the *gad* genes.

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